Transcriptional Control of the \textit{pref-1} Gene in 3T3-L1 Adipocyte Differentiation

SEQUENCE REQUIREMENT FOR DIFFERENTIATION-DEPENDENT SUPPRESSION\textsuperscript{*}

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Preadipocyte factor-1 (Pref-1) is a transmembrane epidermal growth factor-like domain-containing protein highly expressed in 3T3-L1 preadipocytes, but is undetectable in mature fat cells; this down-regulation is required for adipocyte differentiation. We show here that \textit{pref-1} transcription is markedly suppressed during adipose conversion and results in decreased Pref-1 RNA levels. Using 3T3-L1 cells stably transfected with Pref-1 5'-deletion constructs truncated at −6000, −2100, −1300, −692, −300, −235, −193, −183, −170, −93, and −45 base pairs, we determined that the −183 to −170 region is responsible for the suppression of the \textit{pref-1} gene during adipogenesis. This is distinct from the −93 to −45 sequence important for \textit{pref-1} promoter activity in preadipocytes. The placement of a 40-base pair −193 to −154 \textit{pref-1} sequence containing the putative SAD (suppression in adipocyte differentiation) element upstream of the SV40 promoter decreased promoter activity by 85\% upon adipocyte differentiation, compared with 40\% observed with the SV40 promoter alone. The SAD element is therefore sufficient for adipocyte differentiation-dependent down-regulation of a heterologous promoter. A DNA-protein complex was observed when the −193 to −174 sequence was used with 3T3-L1 nuclear extracts in gel mobility shift assays. Competition with oligonucleotides harboring base substitution mutations identified a core sequence of −183AAAGA\textsuperscript{−179} as crucial for DNA-protein complex formation. UV cross-linking predicts that an −63-kDa protein specifically binds the SAD element.

Differentiation of fibroblastic preadipocytes to mature lipid-filled adipocytes involves an extensive array of changes in gene expression. The 3T3-L1 preadipocyte cell line is a highly faithful in vitro model system in which to delineate mechanisms governing differentiation-dependent adipocyte gene expression and consequently adipose cell and tissue development (1–3). Although the expression levels of several hundred genes are dramatically altered during this process, the majority of these remain unidentified (4). The underlying molecular mechanisms governing transcriptional regulation during adipocyte differentiation have been addressed for only a handful of these genes. To date, these studies have centered on activation, as opposed to repression, of gene transcription. In this regard, detailed studies of the adipocyte fatty acid-binding protein gene \textit{aP2} have identified a role for c-Fos/c-Jun heterodimer binding to an AP-1 site and involvement of the CCAAT enhancer-binding protein (C/EBP)\textsuperscript{1} in \textit{aP2} gene activation during adipocyte conversion (5, 6). Studies on \textit{aP2} have also determined that binding of the nuclear receptor PPAR\textgamma as a heterodimer with the retinoid X receptor directs adipocyte-specific gene expression (7, 8). Overall, these findings lead to the conclusion that PPAR\textgamma and C/EBPs function cooperatively to transactivate adipocyte genes and to bring about adipocyte differentiation (9–12). It has only recently become evident that suppression of gene expression in differentiation is of equal importance as activation, permitting the specialization of cells for new functions. Whether similar families of transcription factors, or novel molecules and molecular mechanisms, function in the repression of genes during adipocyte differentiation is currently unknown. Defining these mechanisms will allow a thorough integration of the regulatory events and signals that govern the complex array of changes in gene expression during adipogenesis and those that may control the differentiation of preadipocytes to adipocytes \textit{in vivo}.

Preadipocyte factor-1 (Pref-1) is a novel transmembrane EGF repeat domain-containing protein of 385 amino acids identified in 3T3-L1 preadipocytes that functions in the maintenance of the preadipose state (13–15). Only a few genes have been identified that are down-regulated during adipose conversion. During \textit{in vitro} differentiation of 3T3-L1 cells, \textit{pref-1} is highly expressed in preadipocytes, but is completely absent in mature fat cells (13). \textit{pref-1} therefore provides a unique opportunity to address the differentiation-dependent down-regulation of preadipocyte genes during adipocyte differentiation. Studies to date point to Pref-1 as a unique inhibitor of the adipose conversion process in either a permissive or instructive manner. Adipose conversion was severely inhibited in stably transfected 3T3-L1 cells that constitutively expressed Pref-1 (13). Addition of the Pref-1 ectodomain, expressed as a glutathione S-transferase fusion protein in \textit{Escherichia coli}, was also effective in inhibiting adipose conversion when added to culture media during 3T3-L1 differentiation (16). The presence of Pref-1 inhibited expression of both PPAR\textgamma and C/EBPα, the regulatory molecules that transactivate adipocyte genes and lead to adipogenesis (16). These observations are consistent with the concept that the down-regulation of \textit{pref-1} is a prerequisite for C/EBPα and PPAR\textgamma expression and subsequent adipocyte differentiation. Furthermore, Pref-1 belongs to that

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1 The abbreviations used are: C/EBP, CCAAT enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; Pref-1, preadipocyte factor-1; EGF, epidermal growth factor; MOPS, 3-(N-morpholino)propanesulfonic acid; bp, base pair(s); MIX, 1-methyl-3-isobutylxanthine.
Transcriptional Suppression of pref-1 during Adipogenesis

class of proteins that can act as either transmembrane or soluble molecules. We recently reported that membrane-associated Pref-1 is cleaved at two sites in the extracellular domain, thereby extending its potential range of function (16). Recent studies revealed that pref-1 expression is also abolished during the adipose conversion of primary cultures of rat preadipocytes and that their differentiation is inhibited by the Pref-1 ectodomain (17). Taken together, these data and the detection of soluble Pref-1 in circulation (18, 19) suggests a possible role for Pref-1 in adipocyte differentiation in vivo. The inhibitory role identified for Pref-1 in the adipose conversion process indicates that detailed understanding of the mechanisms governing pref-1 regulation will lend insights to general mechanisms of control of differentiation and serve to link transcriptional and cell-surface events. The presence of EGF-like domains in the Pref-1 ectodomain, a protein motif demonstrated to mediate protein-protein interaction to control cell growth and differentiation in a variety of biological settings (20–22), suggests that transmembrane and/or soluble Pref-1 may function by interaction of its EGF-like domains with EGF-like domains present in cell-surface or extracellular matrix molecules to maintain the preadipose phenotype.

We have begun characterization of the molecular details underlying pref-1 down-regulation during adipocyte differentiation. These studies will afford clarification of the specific mechanisms governing the expression of this unique adipoinhibitory molecule and may also form a framework for addressing common mechanisms employed in the differentiation-dependent down-regulation of gene expression during adipocyte differentiation. In this report, we show that pref-1 expression is suppressed during adipose conversion at the transcriptional level. We identify the DNA sequence element involved in this differentiation-dependent down-regulation and characterize nuclear protein binding to this element. Here, we report that a distinct negative element that we map to −183 to −170 is responsible for the differentiation-dependent suppression of the pref-1 gene in adipocytes and that this element is distinct from sequences between −93 and −43 involved in the basal promoter activity. Within this sequence, a core element of −183AAAGA−170 is involved in the formation of a specific DNA-protein complex.

MATERIALS AND METHODS

Cell Culture—3T3-L1 preadipocytes (1, 2) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cultures for transfection were inoculated at a density of 1–2 × 10^5 cells/100-mm dish. Adipocyte differentiation was induced by treating confluent cultures with 0.5 mM 1-methyl-3-isobutylxanthine and 1 μM dexamethasone for 2 days, and adipocytes were harvested after maintenance in drug-free medium for 3–5 days (3).

RNA Preparation and Northern Blot Analysis—Cell monolayers were washed twice with phosphate-buffered saline, and total RNA was prepared using TRIzol (Life Technologies, Inc.) extraction. RNA was electrophoresed on 1% formaldehyde-agarose gels in 2.2 mM formaldehyde, 20 mM MOPS buffer, and 1 mM EDTA, stained with ethidium bromide, and transferred to Hybond N (Amerham Pharmacia Biotech). After UV cross-linking, filters were incubated at 42 °C for at least 4 h in 50% formamide, 5× SSC, 5× Denhardt’s solution, 0.5% SDS, and 50 μg/ml herring sperm DNA. Following prehybridization, filters were hybridized under identical conditions to 32P-labeled random-primed cDNA probes for at least 16 h. Post-hybridization washes were for 30 min at room temperature in 1× SSC and 1% SDS and then in 0.1× SSC and 0.1% SDS for 1 h at 65 °C. After exposure to x-ray film with an intensifying screen at −80 °C, autoradiograms were scanned using an imaging densitometer (GS670, Bio-Rad) and analyzed with Molecular Analyst (Bio-Rad) or Alpha Innotech digital imaging software.

Transcription Run-on Assays—3T3-L1 preadipocytes and adipocytes differentiated according to the procedure described above were harvested, and nuclei were isolated according to Zechner et al. (23) and resuspended in 50 mM Tris (pH 7.9), 5 mM MgCl2, 0.5 mM μ-mercaptoethanol, and 40% glycerol. Run-on transcription was carried out at 25 °C for 45 min in a reaction mixture containing 105 nuclei and 100 μCi of [32P]UTP (3000 Ci/mmol) in a final volume of 0.5 ml. Labeled RNA was isolated and hybridized to 5 μg of denatured plasmids fixed on nitrocellulose as described previously (24).

Plasmid Co-Transfections— pref-1−2100LUC plasmid was generated by ligating an AluI restriction fragment, containing sequences from −2100 to +110 of the murine pref-1 gene, into the BglII cloning site of the promoterless lucerase reporter plasmid pGL2-Basic (Promega, Madison, WI). This construct served as the basis for the generation of reporter constructs containing various 5′-deletions of the pref-1 5′-flanking sequence. The deletion constructs −45LUC, −93LUC, −193LUC, and −2100LUC were made using convenient restriction sites in the −2100LUC plasmid and each extended through +110 at the 3′-end. For −45LUC, the −2100LUC plasmid was digested with SacI at −45 in the pref-1 promoter and with SmaI, which cuts 5′ to the pref-1 insert in the multicloning site of −2100LUC. The restriction fragment corresponding to the plasmid backbone and the adjoining −45 to +110 of the pref-1 promoter sequence was purified, blunt-ended, and self-ligated to yield −45LUC. Likewise, to generate −193LUC, −2100LUC was cut with KpnI at −193 in the pref-1 promoter sequence and in the plasmid multicloning site. −170LUC was prepared by digestion of −2100LUC with XhoI at −170 and in the plasmid multicloning sites, and similarly, −93LUC was generated by digestion of −2100LUC with BsaI. −2100LUC was cut at the PvuII site at −1300 in the pref-1 promoter and with SmaI in the plasmid multicloning site, blunt-ended, and self-ligated to create −1300LUC.

For the generation of Pref-1/LUC constructs extending farther 5′ than −2100, all but −175 of the pref-1 5′-flanking region was removed from the −2100LUC construct by digestion at the NolI site at −175 in the pref-1 promoter sequence and at the MluI site in the plasmid multicloning site. An −6000 bp pref-1 genomic fragment with MluI-NolI ends was ligated into this plasmid backbone. −6000LUC was used as the starting point to generate the −4000LUC and −3000LUC constructs by cutting −6000LUC in the multicloning site with MluI and with HindIII or BglIII, respectively, followed by re-ligation of the plasmid backbone and adjacent pref-1 5′-flanking sequences. The −183LUC, −300LUC, and −692LUC constructs were made using the promoterless pGL2-Basic vector and polymerase chain reaction fragments generated using primers containing a 5′-XhoI and a 3′-SacI cloning site; each of these constructs extended to +25 in the pref-1 sequence. The heterologous Pref-1/SV40LUC construct, SAD/SV40LUC, was created using oligonucleotides containing one copy of the −193 to −154 pref-1 sequence. The oligonucleotides were annealed to their complementary strand and ligated into the SmaI site of the plasmid backbone. The insert sequences were verified by dyelex sequencing using the Sequenase® kit (25).

Stable and Transient Transfections—For stable transfection, exponentially growing 3T3-L1 cells were transfected with 20 μg of the indicated Pref-1/LUC fusion construct plasmids and 5 μg of SV2neo plasmid DNA/100-mm dish via the calcium phosphate/DNA coprecipitation method (26). Cells were selected for 3 weeks in 400 μg/ml G418. Transient transfections were used to transiently express constructs extended farther 5′ from −2100 in three separate experiments. Luciferase and β-Galactosidase Assays—Cells were lysed in 200 μl of a buffer containing 1% Triton X-100, 25 mM glycylglycine (pH 7.8), 15 mM MgSO4, 4 mM EGTA, and 1 mM dithiothreitol. Cell extracts were prepared by a 5-min centrifugation at 16,000 × g. Luciferase activity was assayed according to a previously reported procedure (28) using a luminometer (Berthold, Nashua, NH). The reaction mixture contained 25 mM glycylglycine (pH 7.8), 15 mM MgSO4, 4 mM EGTA, 15 mM potassium phosphate, 1 mM dithiothreitol, and 2 mM ATP. β-Galactosidase activity was assayed spectrophotometrically as described previously (29). In transient transfections, luciferase activity was normalized to β-galactosidase activity to adjust for transfection efficiency. In stable transfections, the data were normalized by total lucase activity in each cell extracts. Protein concentration was determined by the Coomassie dye binding assay (Bio-Rad) using bovine serum albumin as a standard (30).
Mutant oligonucleotides of Pref-1 SADa containing randomly altered bases as listed below are underlined and were used for competition in electrophoretic mobility shift assay studies: Pref-1 SAD (193 to 154), 5'-CGGTCGTCGAGGTTTCTG-3'; Pref-1 SADa (193 to 174), 5'-CGGTCGTCGAGGTTTCTG-3'; Pref-1 SAD (193 to 174) mutations (underlined), MUT1: 5'-AAACGGGGCTAGAGTGCG-3', MUT2: 5'-CGCCTAACCCTGCGACGGCCGCCTGCG-3', and MUT4: 5'-CGTCTAGCCAAAGAGTGCG-3'.

Nuclear extracts were prepared as described (31, 32). Complementary strands of oligonucleotides, shown above, were annealed by combining equal amounts of each oligonucleotide in buffer (50 mM Tris, pH 8.0, 10 mM MgCl₂, and 50 mM NaCl), heating to 70 °C for 10 min, and cooling to room temperature. The annealed oligonucleotides were 5'-end-labeled with [32P]ATP using T4 polynucleotide kinase. Gel shift binding reactions were performed at room temperature in 30 μl of 50 mM Tris, 62.5 mM MgCl₂, 0.1 mM EDTA, 14 mM Hepes (pH 7.4), 0.01% Nonidet P-40, 12.5% glycerol, 0.5 mM dithiothreitol, and 2 μl of poly(dI-dC)poly(dI-dC). Each reaction contained 20,000 cpm (0.5–1 ng) of oligonucleotide and the indicated amount of nuclear extracts. For competition experiments, unlabeled competitor DNA was added to the reaction mixture prior to the addition of nuclear extracts. After a 30-min incubation, samples were subjected to 4% nonreducing polyacrylamide gel electrophoresis loading buffer was added, and the mixture was boiled for 5 min and then applied to 10% SDS-polyacrylamide gel. The gel was fixed with 10% acetic acid and 10% methanol, dried, and exposed to x-ray film with an intensifying screen.

**Results**

**Transcriptional Suppression of the pref-1 Gene during Adipocyte Differentiation**—Pref-1 is a novel plasma membrane protein containing six EGF-like repeats in the extracellular domain. We have previously reported that Pref-1 protein and mRNA levels are expressed at a high level in 3T3-L1 preadipocytes, but are not detectable in mature fat cells (13). Constitutive expression of Pref-1 inhibits adipogenesis, demonstrating that the down-regulation of pref-1 is required for preadipocytes to undergo adipose conversion (13). The inverse relationship between Pref-1 mRNA levels and the capacity for adipocyte differentiation is clearly shown in Fig. 1A (upper panel). Preadipocytes at confluence (day 0) expressed easily detectable levels of Pref-1 mRNA, but not molecular markers of early adipocyte differentiation (PPARγ or C/EBPβ) or the terminal differentiation marker adipocyte fatty acid-binding protein. Following adipocyte differentiation (day 6), Pref-1 mRNA was reduced to undetectable levels, whereas the adipocyte-expressed C/EBPα, PPARγ, and fatty acid-binding protein mRNA were readily detected. To more closely address the regulation of the pref-1 gene during adipocyte differentiation, we used Northern analysis to examine the level of Pref-1 mRNA at specific times during the differentiation of 3T3-L1 preadipocytes to mature adipocytes. Fig. 1A (lower panel) shows the level of Pref-1 mRNA detected at confluence and at 1, 2, 3, and 6 days post-induction of adipocyte differentiation. The Pref-1 mRNA level decreased to ~50% of the levels in untreated confluent preadipocytes after 1 day post-induction of differentiation, the earliest time point examined. By 6 days post-induction, when most of the cell population consisted of mature adipocytes, Pref-1 mRNA was not detectable. The Pref-1 mRNA level did not change with prolonged incubation since similar levels were present at confluence (0c) and in preadipocytes not induced to differentiate, but kept in culture 6 days post-confluence (6pc).

To address whether the decrease in Pref-1 mRNA levels during adipocyte differentiation is due to the changes in transcription of the pref-1 gene, we carried out nuclear run-on assays using nuclei isolated from confluent 3T3-L1 preadipocytes and cells differentiated into adipocytes by dexamethasone/MIX treatment. As shown in Fig. 1B, pref-1 gene transcription was easily detectable in preadipocytes and was markedly decreased to undetectable levels in adipocytes. Actin transcription, as previously reported (33), was somewhat decreased during differentiation. Transcription of the stearoyl-CoA desaturase gene, previously shown to be expressed in a differentiation-dependent manner in adipocytes (62), was not detectable in preadipocytes, but increased during adipocyte differentiation. These results clearly demonstrate that the down-regulation of pref-1 expression during adipocyte differentiation is primarily at the transcriptional level.

**5'-Deletion Analysis Determines DNA Sequences Responsible for Suppression of the pref-1 Gene in Adipocytes**—The transcriptional down-regulation of the pref-1 gene determined by run-on assays indicates that DNA sequences within the pref-1 5'-flanking region may contain a cis-element governing the differentiation-dependent suppression of the pref-1 gene in adipocytes. To identify these sequence(s), we transfected 3T3-L1 preadipocytes with a series of luciferase reporter constructs with nested deletions of the pref-1 5'-flanking region. We observed that transfection efficiency was much higher in preadipocytes than in adipocytes; this difference made it difficult to compare promoter activity via transient transfection even after
Transcriptional Suppression of pref-1 during Adipogenesis

We next determined if the down-regulation of pref-1 during adipogenesis occurs via an element that controls pref-1 promoter activity in preadipocytes. In this case, the pref-1 SAD element would overlap with the element required for pref-1 expression in preadipocytes. We used transient transfection of 3T3-L1 preadipocytes employing various 5′-deletion promoter constructs. For comparison purposes, the luciferase activity of −4000LUC was assigned the value of 100% (Fig. 2, inset). Transfection of the −45LUC plasmid produced extremely low levels of promoter activity. On the other hand, −93LUC produced >2 orders of magnitude higher promoter activity, and this high level was maintained through −4000 bp of the pref-1 5′-transcription start site. This indicates that regulatory sequences important for the high level of pref-1 promoter activity in 3T3-L1 preadipocytes are present within −93 to −45 of the transcription start site. Furthermore, these data indicate that the SAD element that governs pref-1 suppression in adipocytes, between −183 and −170 that we defined above, is distinct from the element from −93 to −45 that contributes to pref-1 promoter activity in preadipocytes.

The Putative pref-1 SAD Sequence Confers Adipocyte Differentiation-dependent Suppression on a Heterologous Promoter—To demonstrate a definitive role of the pref-1 SAD sequence in differentiation-dependent down-regulation of gene expression, we tested whether this sequence could function to repress the activity of a heterologous promoter in a differentiation-dependent manner. The SAD region of the pref-1 5′-flanking region, defined by our transfection studies, was inserted in front of SV40LUC, a luciferase reporter plasmid containing the SV40 promoter. Because the deletion studies do not necessarily provide information on the 3′-boundary of the SAD sequence, in constructing SAD/SV40LUC, we used a 40-bp sequence that extended an additional 23 bp 3′ to −170 and therefore encompassed −193 to −154. Cells transfected with the unmodified parental SV40LUC plasmid showed a 40% decrease in luciferase activity upon differentiation to adipocytes (Fig. 3). This decrease in SV40 promoter activity upon differentiation of preadipocytes to adipocytes has been previously observed by other investigators (34). As shown in Fig. 3, in assays done in parallel, the luciferase activity of cells transfected with the SAD/SV40LUC construct further decreased to 85% as the result of their differentiation to adipocytes. These data indicate that the pref-1 5′-flanking sequence from −193 to −154 contains a DNA element capable of adipocyte differentiation-dependent down-regulation that confers adipocyte differentiation-dependent suppression on a heterologous promoter.

Correction for pCMV-β-galactosidase. We therefore chose to employ multiple pools of stable transfectants. In addition, by employing stable pools of many independent clones, the effects of the integration site of the plasmid in cellular DNA could be alleviated. Luciferase activity was measured in the same pool of stably transfected preadipocytes at confluence and following their differentiation to adipocytes by dexamethasone/MIX treatment. Fig. 2 depicts the relative activity of the various Pref-1/LUC reporter constructs in preadipocytes versus adipocytes. A value of 1 indicates an equal level of activity of the Pref-1/LUC reporter construct in preadipocytes and adipocytes, and the indicated negative values are the ratio of activity in preadipocytes/adipocytes. As reported below, the luciferase activity of −45LUC was extremely low in both adipocytes and preadipocytes. The luciferase activity in preadipocytes in comparison to that in adipocytes was similar in cells transfected with the −93LUC and −170LUC constructs. On the other hand, cells transfected with constructs that contain longer pref-1 5′-flanking sequences, including −183LUC, −193LUC, −235LUC, −300LUC, −692LUC, −1300LUC, −2100LUC, and −6000LUC, showed 4-fold higher luciferase activity in preadipocytes as compared with adipocytes. These data indicate that sequences between −183 and −170 include sequence important for Pref-1 suppression in adipocytes, which we designate herein as a SAD element.

We next determined if the down-regulation of pref-1 in adipocytes occurs via an element that controls pref-1 promoter activity in preadipocytes. In this case, the pref-1 SAD element would overlap with the element required for pref-1 expression in preadipocytes. We used transient transfection of 3T3-L1 preadipocytes employing various 5′-deletion promoter constructs. For comparison purposes, the luciferase activity of −4000LUC was assigned the value of 100% (Fig. 2, inset). Transfection of the −45LUC plasmid produced extremely low levels of promoter activity. On the other hand, −93LUC produced >2 orders of magnitude higher promoter activity, and this high level was maintained through −4000 bp of the pref-1 5′-transcription start site. This indicates that regulatory sequences important for the high level of pref-1 promoter activity in 3T3-L1 preadipocytes are present within −93 to −45 of the transcription start site. Furthermore, these data indicate that the SAD element that governs pref-1 suppression in adipocytes, between −183 and −170 that we defined above, is distinct from the element from −93 to −45 that contributes to pref-1 promoter activity in preadipocytes.

Comparison of pref-1 promoter activity in 3T3-L1 preadipocytes and adipocytes identifies a DNA region for differentiation-dependent suppression. 3T3-L1 cells were cotransfected with the indicated series of 5′-deletion Pref-1/LUC and SV2neo plasmids. Pools of G418-resistant cells were used either as preadipocytes at confluence or as adipocytes after differentiation by dexamethasone/MIX treatment. The fold difference in luciferase activity was assigned a positive value when expressed as adipocytes/preadipocytes and a negative value when expressed as preadipocytes/adipocytes. The inset shows the localization of basal promoter activity by transient transfection of 3T3-L1 preadipocytes with Pref-1/LUC fusion constructs. 3T3-L1 preadipocytes were cotransfected with 10 μg of the indicated fusion constructs containing various deletions of the pref-1 promoter region ligated to the luciferase reporter gene and with 1 μg of pCMV-β-galactosidase. Cells were harvested 48 h post-transfection and assayed for luciferase and β-galactosidase activities. The ratio of luciferase to β-galactosidase served as a measure of normalized luciferase activity, which is presented as percent of the −4000LUC construct.

### Fig. 2
Comparison of pref-1 promoter activity in 3T3-L1 preadipocytes and adipocytes identifies a DNA region for differentiation-dependent suppression. 3T3-L1 cells were cotransfected with the indicated series of 5′-deletion Pref-1/LUC and SV2neo plasmids. Pools of G418-resistant cells were used either as preadipocytes at confluence or as adipocytes after differentiation by dexamethasone/MIX treatment. The fold difference in luciferase activity was assigned a positive value when expressed as adipocytes/preadipocytes and a negative value when expressed as preadipocytes/adipocytes. The inset shows the localization of basal promoter activity by transient transfection of 3T3-L1 preadipocytes with Pref-1/LUC fusion constructs. 3T3-L1 preadipocytes were cotransfected with 10 μg of the indicated fusion constructs containing various deletions of the pref-1 promoter region ligated to the luciferase reporter gene and with 1 μg of pCMV-β-galactosidase. Cells were harvested 48 h post-transfection and assayed for luciferase and β-galactosidase activities. The ratio of luciferase to β-galactosidase served as a measure of normalized luciferase activity, which is presented as percent of the −4000LUC construct.

![Graph showing comparison of pref-1 promoter activity in 3T3-L1 preadipocytes and adipocytes](image-url)
dependent down-regulation on a heterologous promoter.

Sequence-specific Binding of Nuclear Factor(s) to the Putative SAD Element—We next employed electrophoretic mobility shift assays to characterize nuclear factor binding to the −193 to −154 sequence that we identified as functionally sufficient for differentiation-dependent suppression. Oligonucleotides corresponding to the pref-1 SAD sequence were end-labeled with 32P and incubated with nuclear extracts prepared from 3T3-L1 preadipocytes. As shown in Fig. 4A (lane 2), formation of a major DNA-protein complex was detected (arrow), and a faster migrating band was also noted (asterisk). This latter complex was not as discrete as the slower migrating band and may be the result of proteolysis. To further define the sequence within this 40-bp SAD element that is required for DNA-protein complex formation, we used either the 20-bp 5′-half (SADA, −193 to −174) or the 20-bp 3′-half (SADb, −173 to −154) of this SAD sequence as competitor oligonucleotide in the gel mobility shift assays shown in Fig. 4A (lanes 4 and 5). Excess SADA oligonucleotides competed as effectively as the full 40-bp SAD sequence (lanes 4 and 3, respectively), whereas SADb oligonucleotides did not affect DNA-protein complex formation (lane 5). The sequence of the 40-bp SAD element and the SADa and SADb sequences are shown in Fig. 4C. The boxed areas indicate a core sequence we subsequently determined to be important in DNA-protein complex formation. Use of SADA directly as a probe in gel shift analysis resulted in the formation of a DNA-protein complex (data not shown) that comigrated with the major slower migrating band observed when the labeled full 40-bp SAD sequence was employed (Fig. 4A, arrow). As shown in Fig. 4B, the migration pattern of the DNA-protein complex and the effects of unlabeled competitor oligonucleotides appeared similar in assays employing either preadipocyte or adipocyte nuclear extracts. The formation of similar complexes with preadipocyte and adipocyte nuclear extracts indicates that transcriptional suppression of pref-1 in adipocytes occurs without changes in DNA binding, possibly via phosphorylation, ligand binding, or non-DNA-binding accessory proteins, as has previously been observed in other genes (35–49). Regardless, our experiments localize those sequences important for DNA-protein complex formation to the 20-bp sequence comprising the 5′-half of the SAD sequence (SADA) and suggest that sequences in the 3′-half (SADb) are dispensable for protein binding to the SAD element. Furthermore, the region of the pref-1 promoter identified by functional assays to be involved in differentiation-dependent suppression in Fig. 2 (located between −183 and −170) coincides with that of the SADA sequence (spanning −193 to −174) that we identified to be important in DNA-protein complex formation.

DNA Sequences That Function in Adipocyte Differentiation-dependent Transcriptional Suppression Include a SAD Core Sequence, 20-bp3′AAAGA−179, Required for Nuclear Factor Binding—Examination of DNA-protein complexes formed in gel shift assays indicates that protein binding to the SADA sequence located at −193 to −174 likely mediates the adipocyte differentiation-dependent down-regulation we observed in functional assays employing transfection of 3T3-L1 cells. Our experiments indicated that nucleotide sequences present in SADAs are involved in DNA-protein complex formation and in the down-regulation of gene expression during adipocyte differentiation. The transfection studies with −183LUC that are shown in Fig. 2A indicated that extending the pref-1 promoter sequence employed in the luciferase constructs from −170 up to −183 affects the differentiation-dependent down-regulation of the luciferase reporter gene. This region corresponds to the 3′-half of the 20-bp SADA element. To further define which nucleotide sequences within SADAs are required for DNA-protein complex formation, we synthesized four 20-bp oligonucleotides, each of which differed from SADA by the presence of successive mutations of 5-nucleotide blocks. The sequences of these four oligonucleotides and the wild-type SADA sequence are shown in Fig. 5B. Each of these oligonucleotides was used as a competitor in gel shift analysis employing 32P-labeled wild-type SADA sequence as a probe. As shown in Fig. 5A, excess unlabeled oligonucleotides MUT1, MUT2, and MUT4 were each able to effectively compete with the wild-type SADA probe for protein binding. However, the MUT3 oligonucleotide, with mutations at −183 to −179, was a much less effective competitor at both 25- and 50-fold molar excesses. This suggests that the sequence −183AAAGA−179 is involved in nuclear protein binding to the SADA element. In support of this conclusion, mutation of the 2 bases GA at −180 and −179 rendered MUT3 ineffective for DNA-protein complex formation when used as a probe (data not shown). The sequence −183AAAGA−179, which is crucial for protein binding, resides within the −183 to −170 sequence required for the differentiation-dependent suppression determined by transfection assays. This core sequence and 10 flanking nucleotides and its complement were analyzed for possible transcription factor interactions using MatInspector software (50). Top matches to core and matrix similarity include the winged-helix/forkhead family proteins HNF-3β and HNF-2 and the serum response factor as well as potential interactions with various CCAAT box-binding proteins. However, although such analysis may provide some insights regarding various transcription factor families that may possibly interact with the SADA element, a definitive characterization of the DNA-binding protein mediating adipocyte differentiation-dependent suppression necessitates biochemical characterization and/or cloning of the SAD-binding protein.

To begin characterization of the nature of the protein binding to the SADA element, we performed UV cross-linking employing as probe the sequence from −183 to −164 (SADm), which includes the critical SAD core sequence of 20-bp3′AAAGA−179. Complex formation between this 32P-labeled probe and a nuclear extract was conducted under the same conditions employed in gel shift assays. Proteins were then cross-linked to the DNA probe by UV light exposure, and reactions were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 6, multiple cross-linked DNA-protein complexes were observed. However, one complex migrating near a molecular mass of 75 kDa (Fig. 6, arrow) was effectively competed when cross-linking was carried out in the presence of a 50-fold excess of unlabeled oligonucleotide, indicating specificity of this DNA-protein interaction. The SADA oligonucleotide (−193 to −174)

![Diagram](Image)
was also effective in competition. The SADb oligonucleotide, which does not contain the $^{183}$AAAGA–$^{177}$SAD core sequence and which had been shown to be unable to compete in DNA-protein complex formation (Fig. 4), could not compete away the cross-linking of this 75-kDa DNA-protein complex. At present, we do not know the identity of this binding protein. By subtracting the molecular mass of the oligonucleotide used in cross-linking, we predict that a protein of $\sim 63$ kDa may bind to the SAD element and repress pref-1 gene transcription in adipocytes.

**DISCUSSION**

The basic principle underlying differentiation is differential gene expression and includes both positive and negative modes of action. In adipocyte differentiation, as in other studies of eukaryotic gene regulation, the focus has been on the identification and characterization of positive regulatory sequences and their cognate transactivating factors. 3T3-L1 preadipocytes exhibit the properties of fibroblasts during growth, but undergo adipocyte conversion under appropriate cell culture conditions. Many genes coding for enzymes involved in fatty acid and lipid metabolism and transport, such as adipocyte fatty acid-binding protein (aP2), glycerol-3-phosphate dehydrogenase, fatty-acid synthase, and stearoyl-CoA desaturase, are induced during adipocyte differentiation (10–12). Studies to date have identified C/EBPs and PPARγ as the major transactivators of several adipocyte genes and subsequent adipogenesis. C/EBPs is involved in the differentiation-dependent activation of the aP2 (fatty acid-binding protein), stearoyl-CoA desaturase I, and fsp27 (5, 6, 51) genes. PPARγ is involved in the expression of aP2 and probably other adipocyte genes (8, 52). Ectopic expression of C/EBPs and PPARγ induces adipocyte differentiation of fibroblastic cells when maintained in a medium permissive to differentiation (7, 9) and indicates that these transcription factors may play a central role in gene activation during adipose conversion.

In addition to the activation of genes required for adipose cell function, those genes inhibitory to adipogenesis or whose inappropriate expression would be detrimental to adipocyte function must be repressed during adipocyte differentiation. pref-1 is the first gene described whose expression is completely abolished during adipocyte differentiation; it had previously been reported that actin and tubulin mRNA levels decrease by $\sim 50\%$ during adipose conversion (33). The down-regulation of the platelet-derived growth factor receptor during adipocyte differentiation has also recently been reported (53). We have been addressing the mode of action of Pref-1, an adipocyte-inhibitory factor highly expressed in preadipocytes. Our finding that pref-1 expression is extinguished during adipogenesis indicates that this gene provides a unique model system highly suited to study differentiation-dependent repression of gene transcription during adipogenesis. A recent report suggests that the down-regulation of pref-1 may mark an irreversible checkpoint in the differentiation process (54); 3T3-L1 adipocytes subjected to what is termed dedefinition by tumor necrosis factor-α treatment decrease expression of the adipogenic transcription factor PPARγ and other adipocyte genes. In contrast, pref-1 levels remain undetectable, indicating that a highly robust mechanism for transcriptional repression of pref-1 is likely functioning in these cells.

It is clear that repression has a key role in establishing and refining the precise pattern of spatial and temporal gene expression during cell fate determination. For example, many transcriptional regulators that control pattern formation in early Drosophila embryo development behave as repressors (55, 56). However, in general, considerably less is known about the way in which DNA-binding proteins repress transcription.
Transcriptional Suppression of pref-1 during Adipogenesis

Fig. 5. An AAAGA core sequence mediates nuclear protein binding to the pref-1 SAD element. A, gel mobility shift analysis of the putative pref-1 SADa sequence using block-mutated oligonucleotides as competitors. End-labeled double-stranded oligonucleotides corresponding to the pref-1 SADs sequence from 164 to 174 were incubated with preadipocyte nuclear extracts. The DNA-protein complex is indicated with an arrow. Lane 1, probe only; lane 2, probe incubated with nuclear extracts; lanes 3–16, probe incubated with various excess unlabeled oligonucleotides as indicated. B, sequences of wild-type (WT) and mutant SADa oligonucleotides. The 20-bp wild-type SADs sequence is shown, and the locations of the 5-bp block mutations in the oligonucleotides MUT1, MUT2, MUT3, and MUT4 are underlined. The critical 183AAAGA 179 SAD core sequence is boxed.

As in the paradigm of prokaryotic repression, modes of eukaryotic repression have been found that target a variety of distinct components and stages of the transcription process (57, 58). 1) A negative factor (repressor) may interact with its binding site and dominantly suppress gene expression, perhaps by contacting the basal transcriptional machinery either directly or through co-repressors. A negative factor may affect gene expression indirectly 2) by preventing expression of the gene encoding for a positive factor or 3) by competing with a positive factor for binding to a common or overlapping DNA-binding site. 4) Alternatively, a negative factor may complex with a positive factor and prevent DNA binding or render an activator nonfunctional once bound to DNA. No cis-elements and nuclear factors responsible for repression of preadipocyte genes during adipocyte differentiation have been described, although DNA-binding proteins that appear to repress adipocyte genes in preadipocytes have been reported. C/EBPα undifferentiated protein (CUP), an isofrom of the transcription factor AP-2, acts to maintain the C/EBPα gene in a repressed state prior to adipocyte differentiation (59, 60). In the aP2 promoter, the AE-1 sequence functions as a positive element via interaction with C/EBPβ or acts as a negative element via interaction with AEBP1 to repress aP2 gene expression in preadipocytes (61).

In this report, we demonstrate that pref-1 down-regulation during adipocyte differentiation is due to a decrease in pref-1 gene transcription. The differential expression of the pref-1 gene in preadipocytes and adipocytes is a unique example of cell type-specific expression, with the gene transcriptionally active in preadipocytes and silent in adipocytes. Using a series of fusion constructs containing the 5′-flanking region of the pref-1 gene linked to a luciferase reporter, we have determined that the SAD core element required for differentiation-dependent suppression is contained within −183 to −170. This SAD element is distinct from that between −93 to −45 that is required for pref-1 promoter activity in preadipocytes. The SAD element confers adipocyte differentiation-dependent down-regulation on a heterologous promoter and therefore does not require any other sequences present in the pref-1 5′-flanking region, indicating that the SAD element functions in a context-independent manner. The relative proximity of this SAD element to the transcription start site suggests that protein binding to this site could act directly on the basal transcriptional machinery. Within this 40-bp SAD sequence, the 5′ 20-bp SADα (and specifically a 183AAAGA 179 core sequence) is important in DNA binding and functional down-regulation. Although it is premature to speculate in any detail on the specific DNA-binding protein(s) that may interact with this sequence, we have shown by UV cross-linking the formation of a specific 75-kDa DNA-protein complex, which indicates that a protein with a predicted molecular mass of ~63 kDa binds the SAD element. Unlike suppression of genes that occurs through direct DNA binding of a repressor protein to compete with the binding of activators on the same DNA site, these data indicate that the mechanism of pref-1 suppression is via an element that is separable from those that control pref-1 expression in preadipocytes, possibly by post-translational modification of the existing bound factor or through interaction with a non-DNA-binding co-repressor protein. A complete picture of adipocyte differentiation requires understanding the control and interplay of those genes that are transactivated versus repressed. Identification of the proteins that function through the SAD element is critical to understanding the molecular mechanisms underlying the suppression of gene expression during adipocyte differentiation, and these studies are currently underway.

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