Pref-1 is an epidermal growth factor-like domain-containing transmembrane protein that is cleaved to generate a soluble factor. It is abundant in 3T3-L1 preadipocytes but absent in mature adipocytes. Constitutive expression of pref-1 or the addition of its ectodomain inhibits adipogenesis. We find that the pref-1 gene is an early target of dexamethasone, a component of the dexamethasone/methylisobutylxanthine differentiation mixture used routinely for adipoconversion. The time course of the decrease in pref-1 mRNA by dexamethasone reflected the pref-1 mRNA half-life determined by actinomycin D treatment. Nuclear run-on assays showed that dexamethasone attenuates pref-1 transcription. We demonstrate a correlation between pref-1 down-regulation and adipoconversion by varying the time period and concentration of dexamethasone. Increasing the dexamethasone treatment from 2 to 4 days resulted in a time-dependent pref-1 down-regulation and increased differentiation as measured by adipocyte marker mRNAs. The dexamethasone concentration between 1 and 10 nM showed a dose-dependent decrease in pref-1 mRNA and an enhancement of adipogenesis. To test the hypothesis that dexamethasone initiation of adipoconversion may be via down-regulation of pref-1, we lowered endogenous pref-1 mRNA levels by stably transfecting 3T3-L1 preadipocytes with antisense pref-1. At 1 μM, antisense cells had enhanced adipoconversion; a similar degree of differentiation occurred with 2 nM dexamethasone, a concentration that does not support differentiation of control 3T3-L1 cells. We conclude that dexamethasone-mediated repression of pref-1 contributes to the mechanisms whereby glucocorticoids promote adipogenesis.

Preadipocyte factor-1 (pref-1) is an epidermal growth factor (EGF) repeat domain-containing transmembrane protein with an anti-adipogenic function (1–4). Pref-1 is highly expressed in 3T3-L1 preadipocytes and is totally absent after their differentiation to mature adipocytes. Interfering with this normal repression of pref-1 during adipogenesis by constitutive expression of pref-1 in 3T3-L1 preadipocytes or by the addition of soluble pref-1 ectodomain markedly decreases adipoconversion (1–4); pref-1 expression is also abolished during the adipose conversion of primary rat preadipocytes in cultures, and their differentiation is inhibited by the pref-1 ectodomain (5). Taken together, these data, and the detection of soluble pref-1 in circulation (6), support a functional role for pref-1 in adipocyte differentiation in vivo. In this regard, pref-1 mRNA levels were recently shown to be elevated by adipose-specific expression of SREBP-1c in a transgenic mouse model of congenital generalized lipodystrophy, a condition characterized by poorly developed white and brown adipose tissue (7). Pref-1 belongs to that class of proteins that can act as either transmembrane or soluble molecules; membrane-associated pref-1 is cleaved at two sites in the extracellular domain, thereby extending its potential range of function (4). Therefore, pref-1 is hypothesized to exert its inhibitory function by mediating cell communication or interaction with an as yet unidentified receptor protein via its EGF repeats. In addition to full-length pref-1 (pref-1A), three alternately spliced transcripts with deletions in the juxtamembrane region are present in 3T3-L1 preadipocytes. Although all four transcripts generate transmembrane pref-1, two of these undergo processing to a soluble form of pref-1 corresponding to their respective complete ectodomains. The mode of pref-1 inhibitory function, juxtacrine or paracrine, may therefore depend on the specific alternate pref-1 transcript expressed. 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 (8–10), suggests that transmembrane and/or soluble pref-1 may function by interaction of its EGF-like domains with EGF-like domains of the putative pref-1 receptor present on the cell surface to maintain the preadipose phenotype.

A combination of the synthetic glucocorticoid dexamethasone and the phosphodiesterase inhibitor methylisobutylxanthine (MIX), first employed 20 years ago by Rubin and co-workers (11), is the standard protocol for in vitro differentiation of 3T3-L1 preadipocytes. Adipocyte differentiation of 3T3-L1 cells is routinely induced by a 2-day treatment of confluent preadipocytes with 1 μM dexamethasone and 0.5 mM MIX in the presence of fetal calf serum. 3–5 days after dexamethasone/MIX removal, the majority of cells attain an adipocyte phenotype. During the dexamethasone/MIX hormonal induction phase cells briefly express c-fos, c-jun, and c-myc; decrease surface area; and undergo postconfluent mitoses, subsequent clonal expansion, and growth arrest; however no overt adipose conversion occurs (12, 13). Because the continued presence of dexamethasone/MIX is not needed for the initial appearance of lipid droplets nor for maintenance of the adipocyte phenotype, these agents have been hypothesized to initiate and/or potentiate differentiation signals. Although the molecular targets of dexamethasone/MIX remain largely unknown, the basic transcriptional machinery mediating adipocyte differentiation involves the nuclear hormone receptor PPARγ and the C/EBP family of transcription factors (14, 15). These transcription
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The crude membrane fraction was recovered by centrifugation at 13,000 \( \times g \) 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, clarified by brief spinning in a microcentrifuge, and protein content determined (Bio-Rad). The indicated amount of protein was loaded per lane in SDS-PAGE and electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore) using 10 mM CAPS, 10% methanol transfer buffer. For immunodetection of proteins, membranes were blocked for 1 h at room temperature in 1 x NET (145 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100 and 50 mM Tris-HCl, 7.4) followed by incubation for 1 h at room temperature with primary antisera. For blocking experiments, the pref-1 antisera was preincubated with 30 µg of the noted proteins for 1 h before the addition of the primary antisera. Detection of the antigen-antibody complex was accomplished via goat anti-rabbit IgG-herosedar peroxidase conjugate (Bio-Rad), and the peroxidase conjugate was developed with 0.015% H2O2, 16% methanol, 8.3 mM Tris-HCl, pH 7.4, and 0.05% w/v 4-chloro-1-naphthol. For enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) detection, processing was essentially as above except that 5% bovine serum albumin was used for the blocking step, and product visualization was according to the manufacturer’s instructions.

RNA Preparation and Northern Blot Analysis—Cell monolayers were washed twice with phosphate-buffered saline, and total RNA was prepared by guanidine isothiocyanate/cesium chloride ultracentrifugation or TRIZol (Life Technologies, Inc.) extraction methods. RNA was electrophoresed in 1% formaldehyde-agarose gels in 2.2 M formaldehyde, 20 mM MOPS, 1 mM EDTA, stained with ethidium bromide, and transferred to Hybond N (Amersham Corporation). 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. After prehybridization, filters were hybridized under identical conditions to 32P-labeled random primed cDNA probes for at least 16 h or with strand-specific in vitro transcribed probes using the MaxiScript kit (Ambion). Posthybridization washes were for 30 min at room temperature in 1 × SSC, 1% SDS and then in 0.1 × SSC, 0.1% SDS at 65 °C for 1 h. After exposure to x-ray film with 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.

Ribonuclease Protection Assays—A template for the production of antisense RNA probe was prepared by insertion of a restriction fragment from bases 132 through 1312 of the pref-1 cDNA sequence into the EcoRI site of pcDNA1 (Invitrogen) followed by linearization with Smal at base 970 of the pref-1 sequence. The Smal restriction site occurs in the region that is present in the pref-1A form of the transcript but is deleted in the alternate forms of the transcript, pref-1B, pref-1C, and pref-1D. Because pref-1C and pref-1D differ at the 5’-end but have the same 3’-end of their respective deletions, this probe will not distinguish the two forms of the transcript. A 32P-labeled antisense riboprobe of 397 base pairs was generated by use of SP6 RNA polymerase (Promega); this includes 53 base pairs of vector sequence and 342 base pairs of pref-1 cDNA sequence. The probe was purified by elution from 6% polyacrylamide gel and employed in a ribonuclease protection assay utilizing the RPAII kit (Ambion) according to the manufacturer’s recommendations. Products were analyzed on 6% sequencing gels and fragment lengths determined by comparison with DNA sequencing ladder.

Isolation of Nuclei and Transcription Run-on Assays—3T3-L1 cells were washed three times with phosphate-buffered saline and scraped in lysis buffer containing 1% Nonidet P-40, 0.32 mM sucrose, 3 mM MgCl2, 5 mM HEPES, pH 6.9, and 0.5 mM β-mercaptoethanol. Nuclei collected by centrifugation were washed once by centrifugation in lysis buffer without Nonidet P-40 and stored in liquid nitrogen in 50 mM Tris-HCl, pH 7.9, 5 mM MgCl2, 0.5 mM β-mercaptoethanol, and 40% glycerol. Run-on transcription was carried out at 30 °C for 45 min in a reaction mixture containing 5 x 107 nuclei and 125 pCi of [methy-3H]UTP in a final volume of 0.5 ml. Labeled RNA was extracted with phenol and SDS at 65 °C, ethanol-precipitated, and subjected to a Sephase G-50 spin column. 5 µg of each plasmid DNA was denatured by treating with 0.3 x NaOH for 30 min at 4 °C and applied to nitrocellulose filters using a slot-blot apparatus. Hybridization was carried out with 5 x 107 cpm in 10 mM Tris-HCl, pH 7.4, 0.2% SDS, and 10 mM EDTA at 65 °C for 72 h. The filters were washed twice in 0.1% SDS, 2 x SSC at 22 °C and in 0.1% SDS, 0.1 x SSSC at 60 °C.

Experimental Procedures

Plasmid Construction—The pref-1 antisense construct, pAS-pref-1, was prepared by polymerase chain reaction amplification of the pref-1 coding sequence using the complete pref-1 cDNA sequence in pcDNA (Invitrogen) as a template. The primers were designed to add HindIII sites to both ends of the amplified product. The HindIII-digested polymerase chain reaction fragment was inserted into HindIII sites of the pcDNA3.1 (Invitrogen) vector, which includes a neomycin-selectable marker. Orientation was determined by multiple restriction mapping.

Cell Culture and Transfection—3T3-L1 preadipocytes were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum. For standard induction of adipocyte differentiation, confluent 3T3-L1 preadipocytes in fetal calf serum-containing medium were treated with 0.5 mM MIX and 1 µM dexamethasone. For evaluation of test agents, the medium was changed 1 day before the experiments, and confluent 3T3-L1 cells were treated for the indicated times and concentrations. For antisense studies the pAS-pref-1 plasmid construct was transfected via the calcium phosphate coprecipitation method, and stable clones were established by selection in 400 µg/ml G418 for 3 weeks. Clones were expanded and analyzed for the level of endogenous pref-1. For comparison of differentiation, 1 x 106 cells were plated in quadruplicate in six-well plates, and 7 days after differentiation RNA was harvested and subjected to Northern analysis.

Western Blot Analysis—Cell monolayers of 3T3-L1 preadipocytes were washed twice with phosphate-buffered saline and scraped into phosphate-buffered saline containing 2 mM phenylmethylsulfonyl fluoride. The cell suspension was subjected to three freeze-thaw cycles, and...
**RESULTS**

**Dexamethasone, a Key Component of the Adipogenic Mixture, Markedly Decreases Pref-1 Protein Levels in 3T3-L1 Preadipocytes**—Standard in vitro differentiation of 3T3-L1 preadipocytes involves induction of differentiation by treatment of confluent preadipocytes with dexamethasone/MIX for 48 h in the presence of fetal calf serum. This results in differentiated adipocytes 3–5 days later. The exact mechanism whereby dexamethasone/MIX promotes adipocyte differentiation is not known, and no overt differentiation to the adipocyte phenotype occurs during the dexamethasone/MIX treatment phase. A potential point at which these inducing agents exert their effects may involve down-regulation of the adipogenic inhibitor factor pref-1. To begin to address if these agents affect pref-1 protein levels in 3T3-L1 preadipocytes, antiserum was prepared against a TrpE/pref-1 fusion protein. Fig. IA demonstrates that in Western analysis of crude 3T3-L1 preadipocyte membrane preparations, the pref-1 antibody detects at least seven discrete pref-1 protein bands of 45–60 kDa. We have reported that this heterogeneity likely arises from multiple pref-1 transcripts and products were visualized by color detection as described under "Experimental Procedures." The bracket indicates pref-1 protein species.

Western analysis of pref-1 protein levels after the standard 2-day incubation of confluent 3T3-L1 preadipocytes with 1 \( \mu \)M dexamethasone and 0.5 mM MIX in combination or individually is shown in Fig. 1, B and C. Cells treated with dexamethasone/MIX for 48 h had a drastic reduction in all forms of the pref-1 protein compared with untreated control cells (Fig. 1B, bracket). No differential effect was noted; dexamethasone/MIX decreased all forms of the protein in a manner proportional to their initial levels in control cells. The degree of reduction in pref-1 protein by dexamethasone was comparable to that of dexamethasone/MIX, indicating that the decreased pref-1 protein content was primarily attributable to the effects of dexamethasone (Fig. 1C, bracket). We observed previously that in pref-1-transfected COS cells, the pref-1 protein half-life is less than 7 h (4). The decrease in pref-1 protein content we observe with dexamethasone treatment of 3T3-L1 preadipocytes is consistent with this relatively short half-life. The pattern of pref-1 protein species, with the disappearance of the higher molecular mass proteins in MIX-treated cells, resembles that for pref-1 protein in 3T3-L1 cells when N-linked glycosylation is blocked by tunicamycin (4), indicating that MIX could possibly alter the glycosylation profile and subsequently influence protein half-life.

**Dexamethasone Decreases All Forms of the Pref-1 Transcript**—Given the striking decrease in pref-1 protein levels by dexamethasone, we determined if this reflected changes in the pref-1 mRNA level by conducting northern analyses of pref-1 mRNA after a 48-h incubation of confluent 3T3-L1 preadipocytes with 1 \( \mu \)M dexamethasone, 0.5 mM MIX, or 1 \( \mu \)M dexamethasone and 0.5 mM MIX in combination. Dexamethasone and dexamethasone/MIX treatment affect pref-1 mRNA levels most dramatically. As shown in Fig. 2A, pref-1 mRNA levels in dexamethasone-treated cells decreased to approximately 20% of that of nontreated controls. Furthermore, the reduction of pref-1 mRNA by dexamethasone/MIX in combination is equal to that observed with dexamethasone alone. Decreased pref-1 mRNA content by dexamethasone and dexamethasone/MIX in combination paralleled the effects we observed at the protein level and suggests that dexamethasone is the primary agent down-regulating pref-1 levels when 3T3-L1 cells are subjected to the standard dexamethasone/MIX differentiation protocol. Although not as pronounced as the effects of dexamethasone, MIX decreased pref-1 mRNA levels somewhat. However, as shown in Fig. 1C, MIX treatment resulted in a decrease in only the larger species of the pref-1 protein. Therefore, the Western and Northern analyses we present indicate that pref-1 is reduced drastically by the dexamethasone component of the adipogenic mixture and that this occurs during the dexamethasone/MIX hormonal induction phase, a time period when cells are presumably receiving differentiation signals. Boney et al. (31) have reported that in 3T3-L1 preadipocytes, although neither dexamethasone nor MIX had an effect, decreased pref-1 mRNA levels were noted only with the complete differentiation mixture of dexamethasone/MIX plus insulin. These studies probably reflect gene expression during adipocyte differentiation but not the immediate effect of glucocorticoids on pref-1 expression.

Northern analysis shows dexamethasone-mediated down-regulation of a pref-1 mRNA of 1.7 kilobases in 3T3-L1 preadipocytes. This signal, however, reflects the sum of at least four pref-1 transcripts that we have identified previously by reverse transcriptase-polymerase chain reaction and through the iso-
Confluent 3T3-L1 preadipocytes were cultured for 48 h in the presence of 1 μM dexamethasone and 0.5 mM MIX, or control (no additions). A. Northern analysis. 5 μg of total RNA was hybridized on Northern blots with 32P-labeled pref-1 cDNA probe. 28S and 18S ribosomal RNA from ethidium bromide-stained gels are shown. B. Ribonuclease protection assay. 5 μg of total RNA harvested from 3T3-L1 preadipocytes cultured for 48 h in the presence of 1 μM dexamethasone and 0.5 mM MIX, 1 μM dexamethasone, or control nontreated preadipocytes was subjected to RNase protection assay using a pref-1 probe that distinguishes the indicated form of the transcript.

Pref-1 mRNA Down-regulation by Dexamethasone—To examine the time course of dexamethasone down-regulation of pref-1 mRNA during the critical hormonal induction phase of adipocyte differentiation, RNA was prepared from confluent 3T3-L1 preadipocytes treated with 1 μM dexamethasone for 3, 6, 12, 24, and 48 h and untreated control cells at confluence (time 0) and at 48 h postconfluence (48 h). Upper panel, Northern analysis of 5 μg of total RNA with 32P-labeled pref-1 cDNA probe. Ethidium bromide-stained gel with 28 S and 18 S ribosomal RNA is shown directly below. Lower panel, Northern analysis was conducted on 20 μg of total RNA with 32P-labeled PPARγ or SCID1 cDNA probes. The rightmost lane contains total RNA from differentiated 3T3-L1 adipocytes (Ad) as a positive control for signal detection. B. Determination of pref-1 half-life by actinomycin D treatment. Confluent 3T3-L1 preadipocytes were untreated (-) or treated (+) with 10 μg/ml actinomycin D. RNA was harvested at 0, 15, 24, and 48 h, and 10 μg of total RNA was subjected to Northern blot analysis with 32P-labeled pref-1 cDNA probe. C. Graphical comparison of pref-1 mRNA half-time and time course of dexamethasone down-regulation. The autoradiograms shown in panels A and B were used for densitometric scanning. Values were corrected against the intensity of ethidium bromide staining of ribosomal RNA. Levels of pref-1 mRNA in the presence of actinomycin D are indicated by solid boxes. The hollow boxes indicate values for the pref-1 mRNA levels in the presence of dexamethasone for the indicated times, and the solid circle indicates the value of the pref-1 decrease after a 48-h treatment with dexamethasone and actinomycin D in combination.
was attributable solely to the half-life of pref-1 mRNA, 3T3-L1 preadipocytes were treated with actinomycin D to block RNA polymerase II-dependent gene transcription. Total RNA was isolated at 0 h and after 15, 24, and 48 h of exposure of confluent preadipocytes to 10 μg/ml actinomycin D or from control untreated cells at 48 h. Northern analysis in Fig. 3B indicates that the pref-1 mRNA level decreased steadily throughout the time period analyzed, whereas the level in control cells did not change up to 48 h. Graphical representation of this data determines a t1/2 of approximately 24 h for the pref-1 mRNA (Fig. 3C, solid boxes). The time course of pref-1 mRNA down-regulation during dexamethasone treatment, derived from the Northern blot in Fig. 3A, is represented as hollow boxes in the graph in Fig. 3C. Comparison of the level of pref-1 mRNA over time in actinomycin D and dexamethasone-treated cultures indicates a very similar rate of pref-1 mRNA decrease. A 50% decrease in the pref-1 mRNA level occurred after 24 h of dexamethasone exposure, the same time required for a 50% reduction in the level of pref-1 mRNA during actinomycin D treatment. Treatment with actinomycin D and dexamethasone in combination resulted in a decrease in pref-1 mRNA level to 26% of controls by 48 h (Fig. 3C, solid circle), the same decrease noted with each of these agents alone. These data indicate that the dexamethasone-mediated decrease of pref-1 mRNA is most likely solely transcriptional, with dexamethasone acting to attenuate transcription of the pref-1 gene rapidly and markedly.

Dexamethasone-mediated Repression of Pref-1 Gene Transcription—Actinomycin D treatment determines that the pref-1 mRNA half-life is approximately 24 h. This is the same time required for a 50% decrease in pref-1 mRNA during dexamethasone exposure and strongly suggests that dexamethasone acts to repress transcription of the pref-1 gene. To obtain direct evidence that glucocorticoid down-regulation of pref-1 is at the transcriptional level, nuclear run-on assays were conducted using nuclei isolated from dexamethasone-treated and control untreated 3T3-L1 preadipocytes. Fig. 4 shows a high level of pref-1 gene transcription in 3T3-L1 preadipocytes (control). In contrast, dexamethasone-treated cells have a marked decrease in pref-1 transcription to approximately 20% of that in control cells, indicating negative transcriptional regulation of the pref-1 gene by glucocorticoids. Although nuclear run on assays showed marked effects of dexamethasone on pref-1 transcription, there were no appreciable differences in the transcription of actin or glyceraldehyde phosphate dehydrogenase in dexamethasone-treated and control 3T3-L1 cells, nor did we detect any increase in PPARγ (data not shown). These data indicate that not only is the dexamethasone effect specific for pref-1 gene transcription, but the decrease in pref-1 gene transcription is not the result of adipocyte differentiation.

The Concentration Dependence of Dexamethasone-mediated Pref-1 mRNA Down-regulation Correlates with Dexamethasone Efficacy in Adipocyte Differentiation—In the standard dexamethasone/MIX differentiation conditions for 3T3-L1 preadipocytes, dexamethasone is employed at 1 μM. To determine the dose response of pref-1 mRNA to dexamethasone, 3T3-L1 cells were incubated for 48 h with dexamethasone concentrations from 1 nM to 1 μM. Northern analysis shown in Fig. 5A was quantified and is presented graphically in Fig. 5B. At 1 nM dexamethasone, the lowest concentration employed, pref-1 mRNA levels were the same as those of nontreated control cells. Concentrations of 10 nM dexamethasone and higher decreased pref-1 mRNA to approximately 20% of that in untreated controls, identifying from 1 nM to 10 nM as the critical range for dexamethasone-mediated down-regulation of pref-1 mRNA. Within this range, dexamethasone concentrations of 3, 5, and 7 nM decreased pref-1 mRNA levels to 85, 62, and 33% of controls, respectively. Compared with levels in differentiated adipocytes, we observed low but detectable levels of PPARγ mRNA in 3T3-L1 preadipocytes; dexamethasone had no effect on PPARγ mRNA over the range of concentrations tested. The lack of an induction of PPARγ mRNA and the absence of SCD1 mRNA (Fig. 5A, lower panel) indicate that the decrease of pref-1 mRNA we observe is not the result of adipocyte conversion but rather the direct effect of dexamethasone on pref-1
mRNA levels during the critical 48-h hormonal induction phase.

Our results indicate that pref-1 mRNA is markedly down-regulated by the 1 μM dexamethasone concentration employed in the standard protocol for 3T3-L1 differentiation. Furthermore, pref-1 mRNA was decreased to a similar extent at 100 nM and slightly less at 10 nM. We hypothesized that if pref-1 down-regulation by dexamethasone is a mechanism whereby glucocorticoids promote adipogenesis, then this should also be reflected by the effectiveness of these dexamethasone concentrations in supporting adipogenesis. To test the effects of reduced dexamethasone concentrations, confluent preadipocytes were initiated to differentiate by a 48-h exposure to a combination of 0.5 mM MIX and 1 μM dexamethasone or at reduced dexamethasone concentrations of 100 μM, 1 nM, 3 nM, 5 nM, 7 nM, 10 nM, and 100 nM in the presence of 0.5 mM MIX. 5 days after the removal of agents, the extent of adipocyte differentiation was determined by expression of mRNAs for the adipocyte markers PPARγ, SCD1, and aFABP, and is shown in Fig. 6A; these data are plotted in Fig. 6B. Cultures treated with 0.5 mM MIX alone showed nearly no adipose conversion (Fig. 6A, lane 1). Minimal, if any, adipocyte differentiation occurs, as judged by morphology (data not shown) and expression of adipocyte markers, with dexamethasone concentrations of 1 nM and lower. A low but detectable increase in expression of adipocyte markers is observed at 3 nM dexamethasone which increases markedly by 10 nM dexamethasone to, on average, levels of 75% of the maximal, 1 μM, level. Taken with the dose response of pref-1 mRNA to dexamethasone shown in Fig. 5, A and B, with the critical range identified to be between 1 and 10 nM, these data not only reveal that dexamethasone concentrations 100 times lower than the standard 1 μM can significantly decrease pref-1 mRNA levels and support adipocyte differentiation, but they also indicate a strong correlation between dexamethasone-mediated down-regulation of pref-1 and the degree of differentiation of 3T3-L1 preadipocytes in response to dexamethasone concentration.

**Time-dependent Dexamethasone Down-regulation of Pref-1 mRNA Predicts the Degree of Subsequent Adipocyte Differentiation**—Our data show that during the dexamethasone/MIX adipogenic induction phase, pref-1 mRNA levels and protein are markedly reduced. This is consistent with attenuation of pref-1 gene transcription by dexamethasone determined by transcription run-on assay. We identify dexamethasone to be the primary component responsible for pref-1 down-regulation during the early, hormonal induction phase of the adipocyte differentiation process. Furthermore, the observed correlation between the concentration of dexamethasone which down-regulates pref-1 mRNA and that which supports adipocyte differentiation is consistent with the concept that a possible mechanism whereby dexamethasone exerts its adipogenic effects is through down-regulation of pref-1. To address this further we next determined if there is a relationship between the extent of the decrease in pref-1 mRNA at the end of the dexamethasone/MIX treatment phase and the degree of subsequent terminal differentiation to adipocytes. To this end, we modulated pref-1 levels in 3T3-L1 preadipocytes by varying the dexamethasone exposure times. Because dexamethasone alone is not sufficient for differentiation of these cells, the effects of prolonged dexamethasone exposure time were, by necessity, determined in the context of the dexamethasone/MIX differentiation protocol. The standard dexamethasone/MIX protocol involves a 48-h treatment, at which time we have shown the pref-1 mRNA has been reduced by approximately 80%.

To address the effects of intermediate or prolonged dexamethasone exposure times on differentiation, 3T3-L1 cells were treated with dexamethasone/MIX for 7, 24, 48, and 96 h. Fig. 7A shows the level of pref-1 mRNA present at each of these dexamethasone/MIX treatment times. The 24-h half-life we determined for pref-1 mRNA predicts that at 96 h pref-1 mRNA levels will be reduced to approximately 6% of initial levels. After the indicated dexamethasone/MIX exposure periods, cultures were shifted to normal growth medium and continued until 6 days after the onset of dexamethasone/MIX exposure. At this time, several days after dexamethasone/MIX had been removed from growth medium, cells were judged for degree of differentiation by morphological criteria including cell shape and lipid accumulation and by the expression of the adipocyte marker mRNAs PPARγ and SCD1(Fig. 7B). Cells exposed to dexamethasone/MIX for 7 h, a time at which a decrease in pref-1 mRNA levels is not readily apparent, showed minimal if any morphological and molecular indications of differentiation at 6 days after the initiation of differentiation. In comparison, lowering pref-1 mRNA levels via longer dexamethasone treatment periods increased the extent of adipocyte differentiation. The most dramatic morphological differences are apparent between 48 and 96 h, and this is reflected by the Northern analysis shown in Fig. 7B. Maximal reduction of pref-1 mRNA by dexamethasone, the 96-h treatment period, resulted in virtually 100% of the cells attaining typical adipocyte morphology with marked lipid accumulation. SCD1 levels rise dramatically with increasing dexamethasone/MIX exposure times and correlate well with overall morphological differentiation. PPARγ levels, apart from the 7-h treatment, are not as indicative of adipocyte differentiation because similar PPARγ mRNA levels are detected as the result of the 24-, 48-, and 96-h dexamethasone/MIX treatment times. Given that dexamethasone may have multiple effects in adipocyte differentiation, it would be premature to conclude that the effects of dexamethasone in adipocyte differentiation are mediated solely through its ability
treatment for 7, 24, 48, and 96 h and subjected to Northern analysis with a 32P-labeled pref-1 cDNA probe. The DEX analysis of pref-1 mRNA. RNA was prepared from 3T3-L1 preadipocytes (0 h) or after exposure to the standard dexamethasone (DEX) probes for SCD1, PPAR. Differentiation was continued through 6 days after the onset of the dexamethasone/MIX treatment. 3T3-L1 preadipocytes were exposed to dexamethasone/MIX for the times indicated below, after which agents were removed. Differentiation was continued through 6 days after the onset of the dexamethasone/MIX treatment. Upper panel, photomicrography of representative fields of live cells after the indicated dexamethasone/MIX exposure periods. Lower panel, Northern analysis with 32P-labeled cDNA probes for SCD1, PPARγ, and ethidium bromide-stained 28 S and 18 S ribosomal RNA. Dexamethasone/MIX exposure times were: 7 h (lane 1), 24 h (lane 2), 48 h (lane 3), and 96 h (lane 4).

to dramatically reduce pref-1 levels. These data, however, clearly indicate that dramatically lowering pref-1 levels, via prolonged exposure to dexamethasone/MIX, greatly optimizes differentiation.

Decreasing Pref-1 Levels by Transfection of Antisense Pref-1 in 3T3-L1 Cells Attenuates Dexamethasone Dependence of Adipocyte Differentiation—Our results indicate that dexamethasone, a key component of the adipogenic differentiation mixture, down-regulates pref-1 mRNA during the hormonal treatment phase. Furthermore, the time course of the decrease in pref-1 mRNA and the transcription run-on assays reveal that transcriptional suppression of the pref-1 gene by dexamethasone occurs immediately upon the addition of dexamethasone. This decrease in pref-1 mRNA is therefore one of the earliest responses known to date to a component of the adipogenic differentiation mixture. Our studies herein indicate that we are able to affect the extent of subsequent adipocyte differentiation by modulating pref-1 mRNA levels by the dexamethasone concentration or treatment time employed. In these studies, however, although we showed that the extent to which dexamethasone lowered pref-1 mRNA levels correlated with adipocyte differentiation, effects of changes in dexamethasone concentration or treatment time, other than those on pref-1, cannot be excluded.

To address specifically if dexamethasone-mediated down-regulation of the anti-adipogenic factor pref-1 is a mechanism whereby glucocorticoids promote adipocyte differentiation we employed antisense pref-1. This thereby allows a reduction in the level of endogenous pref-1 outside of its modulation by dexamethasone. We theorized that if a major functional role of dexamethasone in promoting adipocyte differentiation was via its reduction of pref-1 levels, then reducing these levels independent of dexamethasone treatment by transfection of the antisense pref-1 sequence would substitute for dexamethasone treatment in the promotion of adipocyte differentiation. We transfected 3T3-L1 preadipocytes with a pref-1 antisense expression construct. After selection and expansion of stable clones, approximately 40 clonal lines of 3T3-L1 preadipocytes were screened for a reduction in endogenous pref-1 mRNA level via Northern analysis, and two that had markedly reduced pref-1 mRNA levels were identified. One of these antisense clones was further studied. Endogenous pref-1 mRNA levels of this antisense clone by Northern analysis are shown in Fig. 8A, along with a control clone that had pref-1 mRNA levels similar to those before transfection. At confluence, antisense and control cells were treated with 0.5 mM MIX alone (no dexamethasone) or in combination with 2 nM, 10 nM, or 1 μM dexamethasone. In addition to the standard 1 μM dexamethasone, we chose to test the differentiation capacity of these cells in response to these lower dexamethasone concentrations. This was based on our studies in Fig. 6, showing that although it was not as effective as 1 μM dexamethasone, 10 nM dexamethasone was highly effective in supporting adipocyte differentiation, but 2 nM was not. After subjecting antisense and control cells to the indicated differentiation conditions, the extent of adipocyte conversion was determined by Northern analysis for a panel of adipocyte differentiation markers, fatty acid synthase, SCD1, PPARγ, and aFABP (Fig. 8B), and signals were quantitated and corrected for RNA loading. Whereas the antisense cells showed a similar level of expression of these markers at 2 nM, 10 nM, or 1 μM dexamethasone, control cells exhibited the same dexamethasone dose responsiveness in their conversion to adipocytes as was shown in Fig. 6. SCD1 expression was 70% at 10 nM and 33% at 2 nM compared with SCD1 mRNA levels at 1 μM dexamethasone. A similar dexamethasone dose dependence was noted with PPARγ, fatty acid synthase, and aFABP mRNA levels. In the absence of dexamethasone, but in the presence of 0.5 mM MIX, although control cultures showed no expression of adipocyte marker mRNAs such as SCD1 and aFABP, the antisense clone showed low but significant SCD1 and aFABP mRNA levels (Fig. 8C). Because of the low overall degree of adipose conversion in these cells, the autoradiogram was subjected to prolonged exposure and therefore should not be compared directly with that in Fig. 8B. These data therefore indicate that in the presence of 0.5 mM MIX, the same degree of differentiation of antisense cells occurs in response to 2 nM, 10
nM, and 1 μM dexamethasone, and low but detectable differentiation occurs in the absence of dexamethasone. Furthermore, of the initial 40 clones isolated, the two clones with reduced pref-1 mRNA showed enhanced adipocyte conversion and none of those that did not show decreased pref-1 mRNA levels showed increased differentiation at lowered dexamethasone concentration (data not shown). Fig. 8D shows photomicrographs of live antisense cells after differentiation in the noted dexamethasone concentrations. Adipogenesis and Repression of Pref-1 by Dexamethasone

DISCUSSION

Studies on adipocyte differentiation indicate that signals to differentiate are likely combinatorial and involve growth arrest, proper extracellular matrix environment, transcriptional effectors, and factors in fetal calf serum which may inhibit or potentiate responses of known modulators (24, 25). For 3T3-L1 preadipocytes, signals for the initiation of differentiation are provided by the combined exposure to the hormonal inducing agents dexamethasone and MIX. Two-dimensional gel electro-
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phoresis indicates that levels of more than 100 as yet uncharacterized proteins change during the first 5 h of initiation of differentiation (32). Because pref-1 is an inhibitor of adipogenesis and its down-regulation is integral to 3T3-L1 adipocyte differentiation, we hypothesized that pref-1 levels may be repressed by the differentiation-inducing agents dexamethasone/MIX. We present findings here that the promotion of adipogenesis by dexamethasone is mediated in part by its repression of pref-1. Our observations are the first direct link between a well established agent that induces adipocyte differentiation and pref-1 regulation. We have demonstrated that pref-1 protein and mRNA levels are decreased dramatically by dexamethasone/MIX and that dexamethasone is the primary agent acting to decrease pref-1 expression. Although the transcriptional activation of genes during terminal adipocyte differentiation has received considerable study, pref-1 is one of a few genes identified to be repressed during the dexamethasone/MIX initiation phase. The similar time course of decrease in pref-1 mRNA levels in both dexamethasone-treated and actinomycin D-treated 3T3-L1 preadipocytes indicates that dexamethasone acts to attenuate pref-1 gene transcription immediately; we find by transcription run-on assays that dexamethasone acts to repress pref-1 directly. To our knowledge, this immediate down-regulation of pref-1 gene expression is the earliest response to the dexamethasone component of the differentiation mixture. We find that not only the standard 1 μM concentration but also 10 nM dexamethasone down-regulates pref-1 markedly while initiating effective adipocyte differentiation. Furthermore, 1 nM dexamethasone was not effective at either dexamethasone-mediated down-regulation of pref-1, nor did it support adipocyte differentiation. This indicates a strong correlation between the dexamethasone concentration effective in supporting adipocyte differentiation and dexamethasone-mediated down-regulation of pref-1. In addition, the degree of reduction of pref-1 mRNA during the dexamethasone/MIX exposure period correlates with the extent of adipocyte conversion which occurs during subsequent terminal differentiation. Reducing pref-1 mRNA to nondetectable levels in preadipocytes, via a longer dexamethasone/MIX exposure period, greatly accentuates adipocyte differentiation. Moreover, employing antisense pref-1 to lower endogenous pref-1 mRNA levels partially relieved the dexamethasone requirement for differentiation, indicating that dexamethasone promotes adipogenesis, in part, through transcriptional down-regulation of pref-1.

The observation that the transcription factors C/EBPβ and C/EBPδ are induced approximately 5–10-fold during hormonal treatment of 3T3-L1 preadipocytes by MIX and dexamethasone, respectively (26, 27), led to the suggestion that C/EBPβ and C/EBPδ might relay the adipogenic effects of dexamethasone/MIX early in adipocyte differentiation by increasing PPARγ levels. Subsequent functional studies by Farmer and co-workers have addressed this hypothesis in detail (28, 29). Expression of ectopic C/EBPβ in NIH 3T3 cells obviates the MIX requirement for their differentiation. This indicates that C/EBPβ may be a primary effector of MIX action in adipocyte differentiation. However, differentiation of these cells is enhanced dramatically by inclusion of dexamethasone, with a clear dexamethasone dose dependence of PPARγ mRNA observed. To this end it had been proposed that the dexamethasone-mediated induction of C/EBPδ could lead to the formation of C/EBPδ/C/EBPβ heterodimers, which were postulated to be more transcriptionally active than C/EBPβ homodimers. To the contrary, however, C/EBPδ was found to contribute only minimally to functional C/EBP complexes during the initial stages of differentiation of dexamethasone-treated C/EBPβ-expressing NIH 3T3 cells (28). Moreover, dexamethasone treatment was necessary for adipogenic differentiation of NIH 3T3 fibroblasts ectopically expressing high levels of both C/EBPδ and C/EBPβ (28), indicating that dexamethasone must be fulfilling an adipogenic function other than enhancing C/EBPβ levels. Our studies herein clearly show that dexamethasone does not increase expression of PPARγ mRNA in 3T3-L1 preadipocytes. Based on our data here we propose that down-regulation of pref-1 may be a function of dexamethasone in promoting adipogenesis. Additional target genes that relay dexamethasone action during adipocyte differentiation remain to be clarified.

It is of interest that we identify dexamethasone as the major component of the dexamethasone/MIX differentiation mixture that mediates the down-regulation of pref-1 because dexamethasone has been shown to have a physiological role in several other models of adipocyte differentiation. Mesenchymal stem cells with the potential to form the mesodermal cell types of adipose tissue, muscle, bone, and cartilage have a widespread distribution in the connective tissue compartments of many organs and organ systems (33–35). In vitro studies show their differentiation to be dependent upon dexamethasone treatment in a dose- and time-dependent manner, with indications that the formation of adipocytes is particularly related to dexamethasone exposure. In RCJ 3.1 cells, a clonal population derived from 21-day fetal rat calvaria, prolonged dexamethasone exposure favored maintenance of the adipocyte and muscle phenotypes (34). For the D1 bone-marrow derived pluripotent mesenchymal cell line, dexamethasone stimulates their differentiation into adipocytes at the expense of osteoblast differentiation (35).

In contrast to our studies in 3T3-L1 preadipocytes, where treatment with 1 μM dexamethasone for 2 days drastically down-regulates pref-1 mRNA levels to 20% of that in non-treated controls, previous studies on pref-1 in a human neuroblastoma cell line indicate that dexamethasone increases pref-1 mRNA expression in these cells (36). In this study, the low but detectable levels of pref-1 mRNA in neuroblastoma cells increased 8–9-fold upon exposure to 100 μM dexamethasone for 7 days. It is unclear whether the week-long dexamethasone exposure affected the differentiation state of the neuroblastoma cells, which in turn might alter pref-1 mRNA levels. The opposite responses to dexamethasone in these two cell types might be attributable to different experimental conditions; in addition, multiple examples exist for opposite glucocorticoid effects on the regulation of a given gene, depending on the cell or tissue type examined. For example, the phosphoenolpyruvate carboxykinase gene is stimulated by glucocorticoids in the liver and kidney and repressed in adipose tissue (37). The current general model for glucocorticoid action is that transcriptional activation involves binding of the receptor complex to simple glucocorticoid regulatory elements. On the other hand, repression and more complex regulation occur via composite glucocorticoid elements and require action of a glucocorticoid receptor combined with one or more nonreceptor sequence-specific regulators. In the latter case, regulation is dependent upon the presence or absence of accessory DNA-binding proteins in a given cell type. Further studies are needed to identify the elements in the pref-1 promoter responsible for dexamethasone-mediated repression (38).

We do not at present know the cellular pathways that are affected as the consequence of pref-1 down-regulation via dexamethasone, which in turn may mediate adipocyte differentiation. Indications are that permanent exit from the cell cycle may mark the irreversible commitment to adipocyte differentiation, and both PPARγ and C/EBPα have been shown to be involved in growth arrest (39, 40). Studies in 3T3-L1 cells
suggest that dexamethasone could be responsible for establishing the postmitotic growth arrest state that is required for adipoctye differentiation (41). A recent report indicates that tumor necrosis factor-α treatment of mature adipocytes, which causes a modulation of their phenotype wherein they decrease lipid content and take on a fibroblastic appearance, decreases PPARγ levels whereas pref-1 expression remains permanently abolished (42). This argues that pref-1 is not involved in the later, lipogenic stages of terminal differentiation but that its effects are exerted early during the commitment of preadipocytes to the differentiation program, i.e., the dexamethasone/MIX treatment period. This is consistent with our observations here and with our previous findings that 3T3-L1 cells subjected to the dexamethasone/MIX differentiation protocol in the presence of soluble pref-1 have markedly reduced expression of PPARγ. Our data clearly demonstrate that transcriptional repression of pref-1 is an early action of dexamethasone in 3T3-L1 adipocyte differentiation. The fact that pref-1 transcription is blocked by dexamethasone, a component of the standard differentiation mixture, indicates that down-regulation of pref-1 by glucocorticoids may be a mechanism for promoting adipogenesis. In sum, our data support a model whereby exposure of 3T3-L1 cells to dexamethasone/MIX provides initial signals for differentiation and that these include rapid attenuation of pref-1 transcription.

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