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Published in:
Molecular and Cellular Biology

DOI:
[10.1128/MCB.22.22.8015-8025.2002](https://doi.org/10.1128/MCB.22.22.8015-8025.2002)

2002

[Link to publication](#)

Citation for published version (APA):
Åkerblad, P., Lind, U., Liberg, D., Bamberg, K., & Sigvardsson, M. (2002). Early B-cell factor (o/e-1) is a promoter of adipogenesis and involved in control of genes important for terminal adipocyte differentiation. *Molecular and Cellular Biology*, 22(22), 8015-8025. <https://doi.org/10.1128/MCB.22.22.8015-8025.2002>

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PO Box 117
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+46 46-222 00 00

Early B-Cell Factor (O/E-1) Is a Promoter of Adipogenesis and Involved in Control of Genes Important for Terminal Adipocyte Differentiation

Peter Åkerblad,^{1*} Ulrika Lind,¹ David Liberg,² Krister Bamberg,¹ and Mikael Sigvardsson²

Department of Molecular Biology, AstraZeneca R & D Mölndal, SE-431 83 Mölndal,¹ and Department for Stem Cell Biology, Lund University, SE-221 84 Lund,² Sweden

Received 6 February 2002/Returned for modification 14 May 2002/Accepted 19 August 2002

Olf-1/early B-cell factor (O/E-1) is a transcription factor important for B-lymphocyte and neuronal gene regulation. Here we report that all three known O/E genes (O/E-1, -2, and -3) are expressed in mouse adipose tissue and are upregulated during adipocyte differentiation. Forced expression of O/E-1 in either the preadipocyte cell line 3T3-L1 or mouse embryonic fibroblasts augmented adipogenesis, and constitutive expression of O/E-1 in uncommitted NIH 3T3 fibroblasts led to initiation of adipocyte differentiation. Furthermore, a dominant negative form of O/E-1 partially suppressed 3T3-L1 adipogenesis, indicating that expression from endogenous O/E target genes is required for 3T3-L1 terminal differentiation. Thus, our data point to the importance of O/E target genes for adipocyte differentiation and suggest a novel role for O/E-1 as an initiator and stimulator of adipogenesis.

Mesodermal stem cells differentiate into various cell types, including myocytes and adipocytes. Immortalized murine preadipocyte cell lines such as 3T3-L1 and 3T3-F442A have been used extensively to study the molecular mechanisms controlling adipocyte development. These preadipocytes can be induced by hormonal stimulation to differentiate into adipocytes that resemble cells found in white adipose tissue. By using 3T3-L1 cells as a model system for adipocyte development, it has been possible to dissect this process into distinct stages with coordinated expression of specific genes (6, 32, 33). These include transcription factors such as peroxisome proliferator-activated receptor γ (PPAR γ), sterol regulatory binding protein 1 (SREBP1)/adipocyte determination and differentiation factor 1, and CCAAT/enhancer binding proteins (C/EBPs), all of which participate in the control of genes important for lipid metabolism and have the ability to stimulate adipogenesis (22, 25, 37, 42, 43). Other transcription factors are likely to contribute to adipogenesis, and we have focused on the role of Olf-1/early B-cell factor (O/E-1), a protein known to be expressed in adipose tissue (17). This protein is a helix-loop-helix transcription factor shown to be important for the control of B-lymphocyte-specific genes and for the transcriptional regulation of genes in olfactory receptor neurons (1, 2, 17, 35, 39). O/E-1 binds DNA response elements as a homo- or heterodimer via a unique zinc coordination motif (18). In mice, the O/E family of transcription factors contains at least three highly related members, O/E-1, -2, and -3 (40), and the O/E proteins are highly conserved in such diverse species as *Caenorhabditis elegans* (31), *Drosophila melanogaster* (7), *Xenopus laevis* (10), zebra fish (3), chicken

(29), and human (16). O/E-1 is abundantly expressed in B-lymphoid cells, neurons, and adipose tissue, while O/E-2 and -3 expression is more restricted to neuronal compartments (15, 17, 40). Mice devoid of O/E-1 by homologous disruption of the gene show a complete lack of B lymphocytes (26) and a defect in embryonic striatum (14), implying a crucial role for this protein during the development of B lymphocytes and certain neuronal cell types. These mice are also reported to be smaller than their littermate controls, but the reason for this phenotype is unknown (26). O/E-1 has been implicated to be involved in the regulation of the *Glut4* gene in fat cells (9), but apart from this, the role of O/E proteins in adipogenesis is unknown.

We here report that all three known mouse O/E family members are expressed in mouse adipose tissue as well as in 3T3-L1 preadipocytes and that the expression level is increased upon terminal differentiation into lipid-containing fat cells. O/E-1 was also able to augment adipogenesis of 3T3-L1 and mouse embryonic fibroblasts (MEF), which are cells with adipogenic potential. Furthermore, O/E-1 could initiate differentiation of NIH 3T3 cells that are not assigned to the adipocyte lineage. Finally, we report that a dominant negative form of the O/E-1 protein impaired the development of preadipocytes into mature lipid-containing cells. These findings define O/E-1 as a potent stimulator of adipogenesis and indicate that this factor participates in the control of genes crucial for the terminal differentiation of adipocytes.

MATERIALS AND METHODS

Plasmid constructs. The retroviruses were based on the pBabepuro vector, which allows for ectopic expression of either mouse PPAR γ 2 (a kind gift from Karsten Kristiansen), a human O/E-1 (16), or O/E-1-Engrailed. The Engrailed fusion protein was constructed by an in-frame fusion of an O/E-1 protein truncated by the introduction of an *Xba*I site at amino acid 430 (34) and the repressor domain (amino acids 1 to 298) of the *Drosophila* Engrailed protein (a kind gift from Sally Johnson). The Engrailed fusion protein was also cloned into a cDNA3 expression vector (Invitrogen Life Technologies AB, Lidingö, Sweden) for use in transient transfections. The reporter genes contained either the mouse *B29*

* Corresponding author. Mailing address: Department of Molecular Biology, AstraZeneca R & D Mölndal, SE-431 83 Mölndal, Sweden. Phone: 46 317 064 493. Fax: 46 317 763 852. E-mail: peter.akerblad@astrazeneca.com.

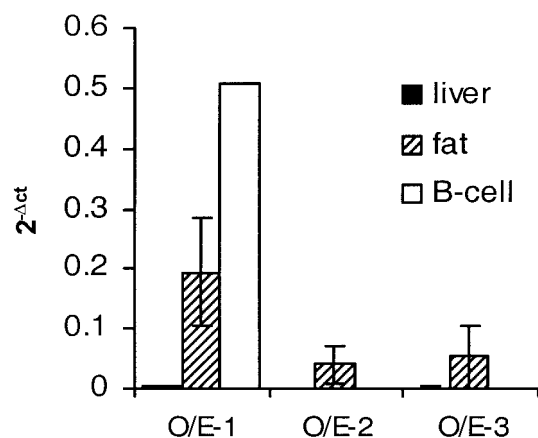


FIG. 1. O/E-1, -2, and -3 mRNAs are expressed in mouse adipose tissue. Total RNA was extracted from the visceral adipose tissues and livers from five animals and from the 70Z/3 B-lymphoid cell line. After cDNA synthesis with DNase-treated RNA samples, transcript levels of O/E-1, -2, and -3 were analyzed by quantitative real-time PCR.

promoter (−152 to +1) (1), the mouse *mb-1* promoter (−126 to +26), or a cytomegalovirus (CMV) promoter cloned 5' of the luciferase gene in a pGL3 reporter vector (Promega, Scandinavian Diagnostic Services AB, Falkenberg, Sweden).

Retrovirus production and infection. Phoenix retroviral packaging cells were transfected with pBabepuro retrovirus vectors in 100-mm-diameter dishes at 70% confluence, and virus supernatants were harvested after 48 h. Target cells were infected in 100- or 60-mm-diameter dishes at 50% confluence with 1 volume of virus supernatant diluted in 1 volume of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% (vol/vol) calf serum, after which Polybrene (Sigma Sweden AB, Stockholm, Sweden) was added at a final concentration of 6 μ g/ml. The medium was replenished 8 to 12 h following infection. Twenty-four hours after infection the cells were split 1:3 to 100-mm-diameter dishes and selected with 2 μ g of puromycin (Sigma) per ml for 72 to 96 h. Dishes with selected cells were trypsinized, pooled, and replated on 60-mm-diameter dishes for differentiation.

Cell culture and differentiation. 3T3-L1 and NIH 3T3 cells were propagated in DMEM containing 10% (vol/vol) calf serum at 37°C in a humidified atmosphere of 10% CO₂. Medium was changed every second day in all experiments. 3T3-L1 cells were induced to differentiate 2 days postconfluence (day 0) by using DMEM with 10% (vol/vol) fetal calf serum supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 μ M dexamethasone (Sigma), and 1 μ g of insulin (Roche Diagnostics Scandinavia AB, Bromma, Sweden) per ml (MDI) for 2 days and 1 μ g of insulin per ml alone for 2 additional days. From day 4, 3T3-L1 cells were cultured in DMEM with 10% fetal calf serum. For the dexamethasone and darglitazone differentiation protocol, 3T3-L1 cells were induced 2 days postconfluence in DMEM with 10% fetal calf serum supplemented with 1 μ M dexamethasone and 0.5 μ M darglitazone for 2 days and then with 0.5 μ M darglitazone alone throughout the experiment. NIH 3T3 cells were induced to differentiate 2 days postconfluence by using DMEM with 10% (vol/vol) fetal calf serum supplemented with MDI and, where indicated, 0.5 μ M darglitazone or similar amounts of a dimethyl sulfoxide (DMSO) vehicle for 2 days and with 1 μ g of insulin per ml and 0.5 μ M darglitazone for 2 additional days. From day 4, NIH 3T3 cells were cultured in DMEM with 10% fetal calf serum supplemented with 0.5 μ M darglitazone or the DMSO vehicle. Embryonic fibroblasts from CBA \times C57BL/6 F₁ mice (MEF) were maintained in DMEM with 10% (vol/vol) fetal calf serum, and differentiation was induced 2 days postconfluence as described in reference 20. Where indicated, 0.5 μ M darglitazone or a similar amount of the DMSO vehicle was added to the MEF cultures at the time of induction of differentiation and was included throughout the experiment.

Oil Red O staining. Culture dishes were washed twice in phosphate-buffered saline and fixed for 30 min in phosphate-buffered saline containing 4% formaldehyde. After a single wash in water, cells were stained with Oil Red O for 30 min. After the staining, dishes were washed twice in water and photographed. Oil Red O was prepared by diluting a stock solution (0.5 g of Oil Red O [Sigma] in 100 ml of isopropanol) with water (3:2), followed by filtration.

Real-time PCR analysis. Total RNA was isolated with RNA STAT-60 (BioSite AB, Täby, Sweden) according to the manufacturer's instructions. DNAs were removed from RNA preparations (DNA-free kit; Intermidica AB, Stockholm, Sweden), and first-strand synthesis was performed with random primers (Superscript first-strand synthesis system for reverse transcription-PCR; Invitrogen). Quantitation of mRNA was performed by a quantitative, real-time-PCR approach with the ready-to-use SYBR Green PCR core reagent kit based on the TaqMan technology (Applied Biosystems, Stockholm, Sweden). The threshold cycles (Ct) for the endogenous control (36B4) and genes of interest were determined and relative RNA levels were calculated by the comparative-Ct method where Δ Ct is the Ct of the gene of interest minus the Ct of 36B4. The Δ Ct values were used to calculate $2^{-\Delta$ Ct. All targets and the 36B4 control were amplified with similar PCR efficiencies. Real-time-PCR experiments were performed in triplicate.

The following oligonucleotides were used in real-time PCR analysis: mEBF1 fw, 5'-AGGTTGGATTCTGCTACGAAAGTT-3'; mEBF1 rev, 5'-TGATTCTCTTAAAAAGG-CCTGA-3'; mEBF2 fw, 5'-AGCACAAAACCTACTTATTCGATGG-3'; mEBF2 rev, 5'-GTCCAACATGGCCGCTTG-3'; mEBF3 fw, 5'-TCGTGAATATGCACCGTTTGTG-3'; mEBF3 rev, 5'-ATGAGTACAGAAAAATGTCTCGAGG-3'; mSREBP1 fw, 5'-GCAGACCCTGGTGAGTGGA-3'; mSREBP1 rev, 5'-GTCGGTGGATGGGAGTTT-3'; mC/EBP α fw, 5'-TCTGCGAGCAGACGACGTC-3'; mC/EBP α rev, 5'-GCCAGGAACCTCGTCGTTGAA-3'; mFABP fw, 5'-TTCGATGAAATCACCGCAGA-3'; mFABP rev, 5'-AGGGCCCCGCCATCT-3'; mGPDH fw, 5'-TGCTAAATGGGAGAGAGCTACA-3'; mGPDH rev, 5'-TGTGTTGGAGAATGCTGTGCA-3'; mPPAR γ 2 fw, 5'-GCATGGTGCCTTCGCTGA-3'; mPPAR γ 2 rev, 5'-TGGCATCTCTGTGTCAACCATG-3'; mPPAR γ 1 fw, 5'-TGAAAGAAGCGGTGAACCACTG-3'; mPPAR γ 1 rev, 5'-TGGCATCTCTGTGTCAACCATG-3'; m36B4 fw, 5'-GAGGAATCAGATGAGGATATGGGA-3'; m36B4 rev, 5'-AAGCAGGCTGACTTGGTTGC-3'; mGlut4 fw, 5'-TCATTGTCCGCATGGGTTT-3'; mGlut4 rev, 5'-CGGCAATAGAAGGAAGACGTA-3'; mAdipsin fw, 5'-CCTTGCAATACGAGGACAAAAG-3'; and mAdipsin rev, 5'-CACACCCCAACGACAC-3'.

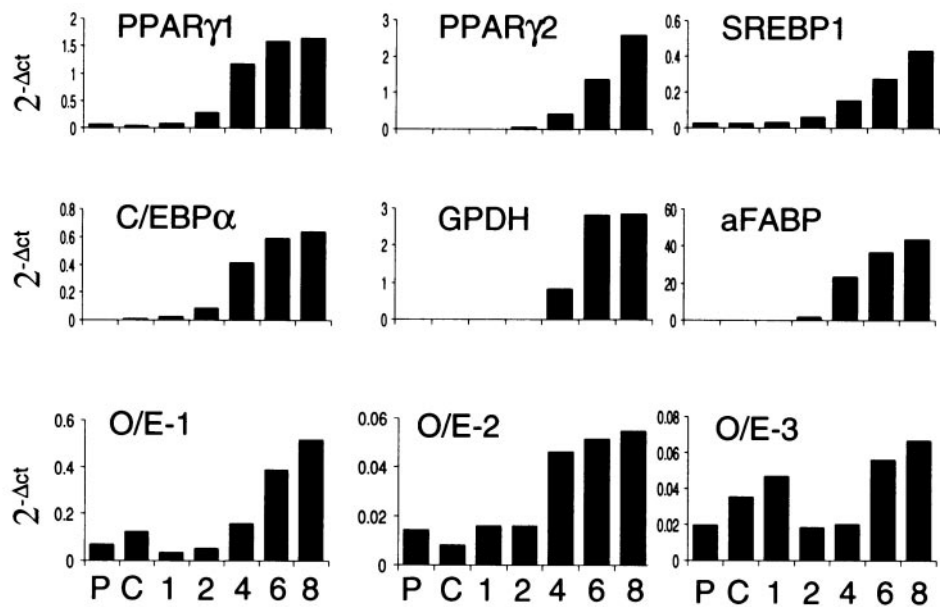
Protein extracts and electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared according to the method of Schreiber et al. as described in reference 1. DNA probes were labeled with [γ -³²P]ATP by incubation with T4 polynucleotide kinase (Roche), annealed, and purified on a 5% polyacrylamide Tris-borate-EDTA gel. Nuclear extract was incubated with labeled probe (20,000 cpm, 3 fmol) for 30 min at room temperature in binding buffer (10 mM HEPES [pH 7.9], 70 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 2.5 mM MgCl₂, 1 mM ZnCl₂) with 0.75 μ g of poly(dI-dC) (Amersham Biosciences, Uppsala, Sweden). DNA competitors were added 10 min before the addition of the DNA probe. The samples were separated on a 6% polyacrylamide-Tris-borate-EDTA gel, which was dried and subjected to autoradiography.

Oligonucleotides used for EMSAs were as follows: mouse mb-1 O/E-1 sense, 5'-GAGAGAGACTCAAGGGAATTGTGG-3'; mouse mb-1 O/E-1 antisense, 5'-CCACAATCCCTTGAGTCTCTCTC-3'; Oct sense, 5'-TTCATTGATTGTCATCGCATGAGACGCTAACATCGTACGTTTC-3'; Oct antisense, 5'-GAACGTACGATGTTAGCGTCTCATGCGATGCAATCAATGAA-3'; human mb-1 O/E-1 sense, 5'-AGCCACCTCTCAGGGGAATTGTGG-3'; human mb-1 O/E-1 antisense, 5'-CCACAATCCCTCTGAGAGGTGGCT-3'; mutated human mb-1 O/E-1 sense, 5'-AGCCACCTCTCAGCCGTTTGTGG-3'; and mutated human mb-1 O/E-1 antisense, 5'-CCACAAAACGGCTGAGAGGTGGCT-3'.

The antibodies used for supershifting were SC15888 (Santa Cruz Biotechnology, Scandinavian Diagnostic Services AB) and an O/E-1-specific rabbit antiserum raised against a polypeptide in the carboxy terminus of O/E-1 (M. Sigvardsson, unpublished data).

Transient transfections and luciferase assays. 70Z/3 pre-B cells were grown at 37°C under 5% CO₂ in RPMI medium supplemented with 7.5% fetal calf serum, 10 mM HEPES, 2 mM pyruvate, 50 μ M 2-mercaptoethanol, and 50 μ g of gentamicin per ml (all purchased from Invitrogen) (complete RPMI medium). The cells were washed twice in Tris-buffered saline (TBS; 140 mM NaCl, 5 mM KCl, 25 mM Tris [pH 7.4], 0.6 mM Na₂HPO₄, 0.5 mM MgCl₂, 0.7 mM CaCl₂). Transfection was performed by incubating 2.5×10^6 cells for 30 min at 20°C in 0.65 ml of DEAE-dextran (0.7 mg/ml in TBS; Amersham Biosciences) containing 2 μ g of the reporter gene construct and 1 μ g of expression vectors. The transfected cells were thereafter washed once in TBS and then cultured in 5 ml of complete RPMI medium in six-well plates for 48 h. Protein extracts were prepared and luciferase assays were performed with the Luciferase Reporter assay system (Promega) and 20% of the total protein extract.

A



B

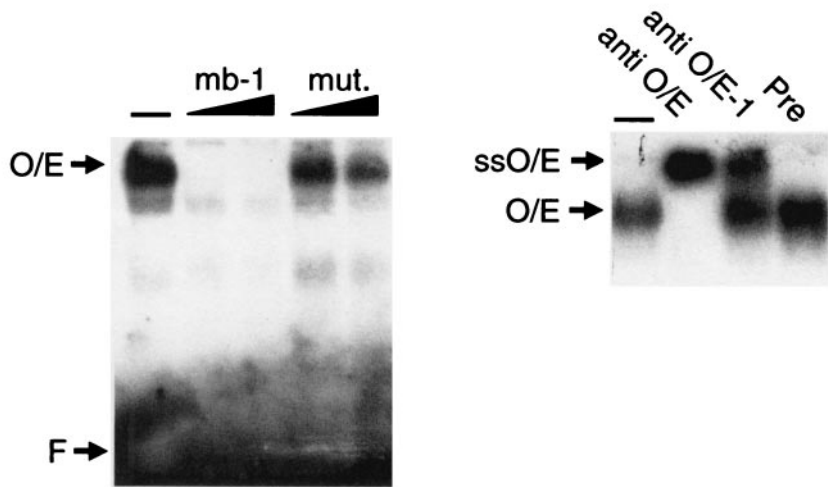


FIG. 2. O/E proteins are present in differentiated 3T3-L1 cells, and O/E mRNA levels are modulated during 3T3-L1 differentiation. (A) 3T3-L1 preadipocytes were induced to differentiate by the addition of MDI 2 days postconfluence. Total RNA was extracted from samples isolated at preconfluence (lanes P), confluence (lanes C), and days 1, 2, 4, 6, and 8 postinduction (lanes 1, 2, 4, 6, and 8, respectively). DNase-treated RNAs were used as templates for cDNA synthesis, and the cDNAs were used to determine the expression levels of O/E-1, -2, and -3; PPARγ1 and -2; SREBP-1; C/EBPα; aFABP; and GPDH by real-time PCR. The panels display the results of one representative experiment out of two. (B) The presence of O/E proteins was analyzed in an EMSA with a mouse *mb-1* O/E-1 probe and extracts from 3T3-L1 cells after 4 days of differentiation with MDI. The left panel shows the results of a competition assay where the shift obtained was competed with an intact (*mb-1*) or mutated (*mut.*) human *mb-1* O/E-1 site. F indicates free probe. The right panel shows the results of a supershift experiment with the same extract and probe as in the left panel but with anti-O/E antibody (interacts with O/E-1, -2, and -3), anti-O/E-1 antibody (O/E-1 specific), or preimmune serum (lane Pre) added to the EMSA. ssO/E, supershifted O/E.

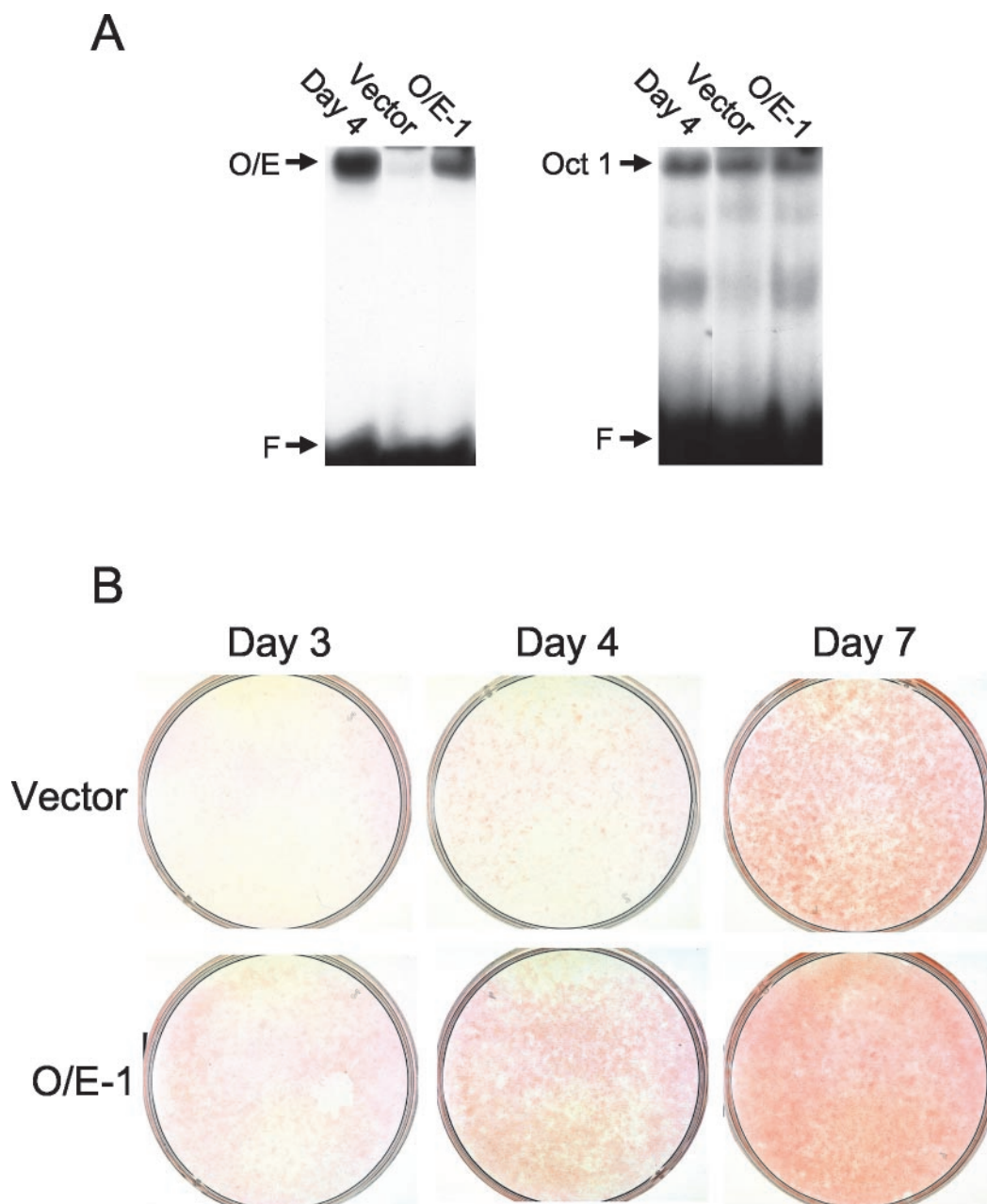


FIG. 3. Ectopic expression of O/E-1 stimulates 3T3-L1 differentiation. (A) The presence of O/E proteins in 3T3-L1 cells after retroviral infection was determined by EMSA. Nuclear extracts were prepared from 3T3-L1 cells infected with retroviruses containing the empty pBabepuro vector (lanes Vector) or with retroviruses harboring the O/E-1 cDNA (lanes O/E-1) before the initiation of differentiation and from untransduced cells after 4 days of differentiation with MDI (lanes Day 4). The *mb-1* O/E-1 probe was used to determine the presence of O/E proteins, and an Oct probe was used as a control for protein content. F indicates free probe. (B) Cells infected with empty retroviruses or viruses containing O/E-1 cDNA were induced to differentiate by the addition of dexamethasone and darglitazone. Three, 4, and 7 days postinduction, culture dishes were stained with Oil Red O to assess adipocyte differentiation. The results of one representative experiment out of three are shown.

RESULTS

The O/E genes are expressed and regulated during adipogenesis. Nuclease protection analysis has shown that O/E-1 mRNA is present in mouse adipose tissue (17), but little is known about the temporal expression pattern during adipocyte differentiation or the presence of other family members in adipocytes. To investigate this issue, we performed quantita-

tive real-time-PCR analysis using oligonucleotides for O/E-1, -2, and -3 and cDNAs derived from mouse adipose tissue and liver and the 70Z/3 B-lymphoid cell line. The results suggested that all three isoforms of O/E are expressed in mouse adipose tissue, although at different relative levels (Fig. 1). As expected, O/E-1 mRNA was the only O/E transcript detected in B-lymphoid cells and none of the family members could be detected in the liver.

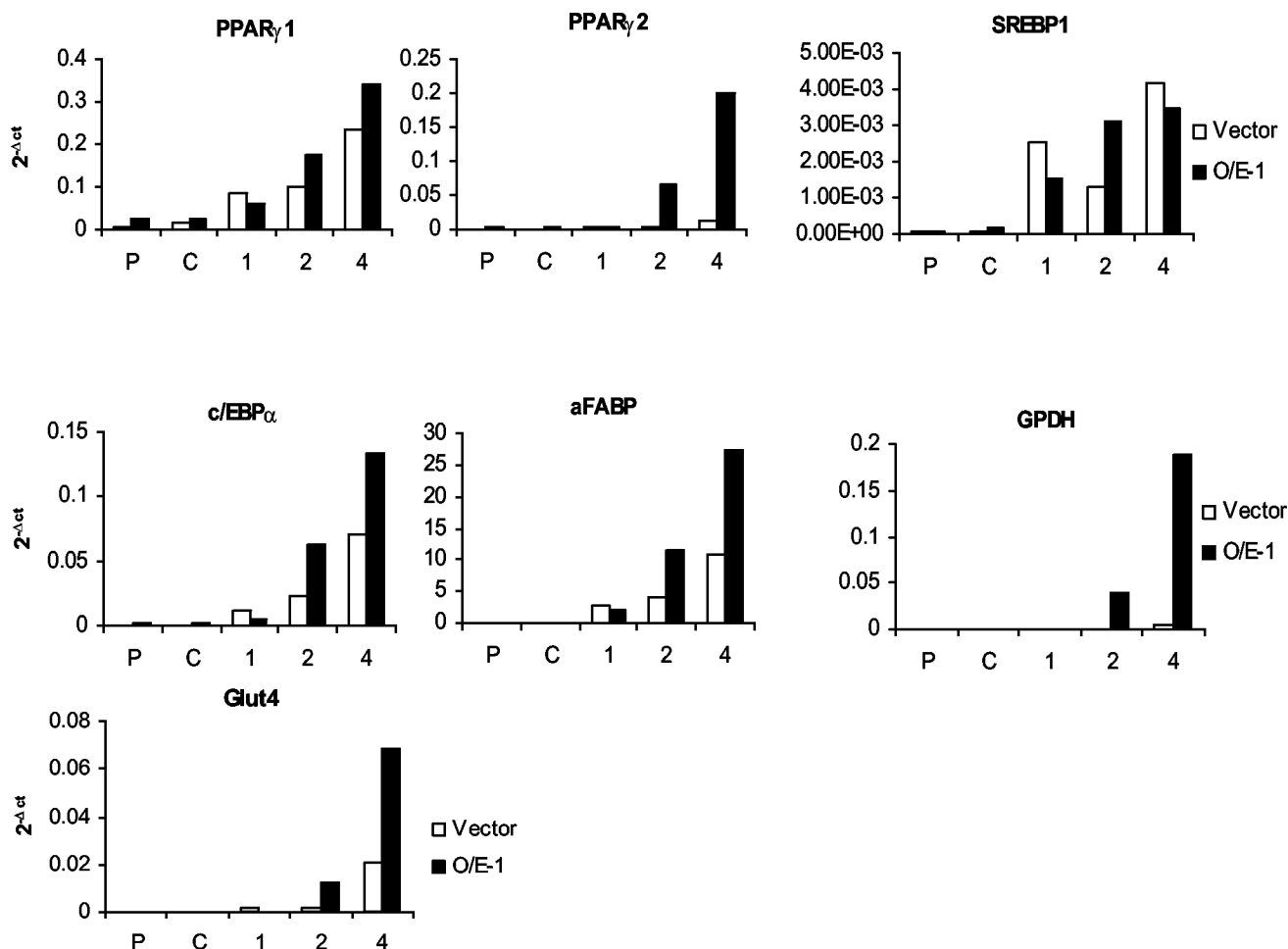


FIG. 4. mRNA levels of adipocyte differentiation markers during normal and O/E-1-stimulated 3T3-L1 differentiation. Cells infected with retroviruses containing empty vector or O/E-1 cDNA were induced to differentiate by the addition of dexamethasone and darglitazone 2 days after confluence. Total RNA was extracted from cultures at preconfluence (lanes P), confluence (lanes C), and days 1, 2, and 4 postinduction (lanes 1, 2, and 4, respectively). cDNAs were synthesized from DNase-treated samples and used in a quantitative real-time PCR. The results of one representative experiment out of four are shown.

To examine the expression profiles of O/E family members during adipocyte differentiation, 3T3-L1 preadipocytes were grown to confluence and stimulated to differentiate to adipocytes 2 days postconfluence. Differentiation was initiated by the addition of the adipogenic inducer MDI for 2 days, followed by insulin only for 2 additional days. mRNA levels for O/E-1, -2 and -3, as well as for known markers of adipocyte differentiation, were then determined by real-time PCR (Fig. 2A). All O/E forms were expressed already in noninduced, preconfluent 3T3-L1 cells, indicating that these factors are present at early stages of adipocyte development. O/E-2 and -3 appeared to be present at lower levels than O/E-1, which is consistent with the expression pattern observed in mouse adipose tissue (Fig. 1). During 3T3-L1 differentiation, O/E-1 was up-regulated approximately eightfold while the other isoforms were induced by a factor of 2 to 3. To compare the expression pattern of the O/Es with that of known markers of adipocyte development, we measured mRNA levels of PPAR γ 1, PPAR γ 2, SREBP1, C/EBP α , adipocyte fatty-acid binding protein (aFABP), and glycerol-3-phosphate dehydrogenase

(GPDH) by real-time PCR. As shown in Fig. 2A, the O/Es are expressed at an early stage when only low levels of SREBP1 and PPAR γ 1 are detected but before the onset of PPAR γ 2, C/EBP α , aFABP, or GPDH transcription. Thus, adipocytes express O/E-1, -2, and -3 mRNA and the levels of these messages are modulated during terminal adipocyte differentiation. To verify that the expressed mRNA was translated into protein, we performed EMSAs using the mouse *mb-1* promoter O/E-1 site and nuclear extracts from 3T3-L1 cells 4 days after the initiation of differentiation (Fig. 2B). This resulted in one prominent complex that could be competed for by the addition of a cross-reactive site from the human *mb-1* promoter (16) but not by inclusion of a point-mutated site. To further investigate the obtained complex, we included either an antiserum binding all O/E proteins or an O/E-1-specific antiserum. Addition of the former resulted in a complete supershift of the EMSA complex, while the O/E-1 antiserum only partially shifted the complex. This further supports the idea that O/E proteins are expressed and translated into functional protein in adipocytes.

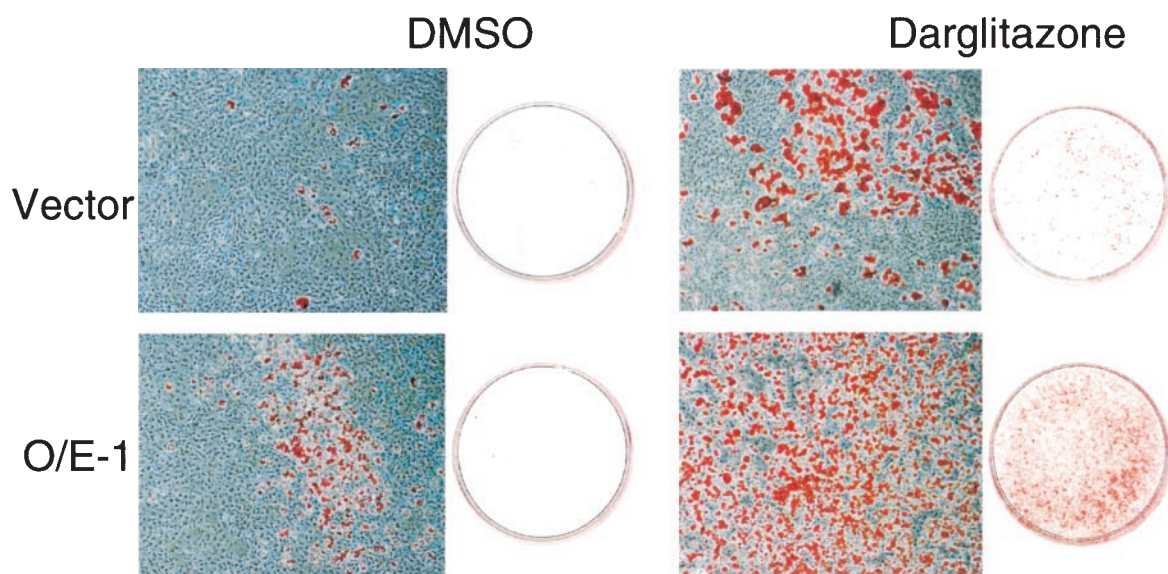


FIG. 5. Ectopic expression of O/E-1 enhances adipocyte differentiation of mouse embryonic fibroblasts. Cells were infected with an empty retrovirus vector or viruses harboring O/E-1 cDNA and were induced to differentiate by the addition of MDI plus darglitazone or the vehicle (DMSO) 2 days postconfluence. Cultures were stained with Oil Red O 10 days postinduction. Petri dishes and micrographs from one representative experiment out of two are shown.

O/E-1 augments adipogenesis of preadipocytes. A feature of many transcription factors with key roles in adipogenesis is their ability to stimulate terminal adipocyte differentiation of preadipocytes (33). To investigate whether O/E-1 possessed this ability, we infected 3T3-L1 cells with retrovirus vector alone or with retroviruses carrying the cDNA encoding O/E-1. Infection resulted in a significant increase of O/E-1 binding activity in EMSAs using the *mb-1* promoter binding site (19), while the levels of Oct protein remained constant (Fig. 3A). The levels of DNA binding activity obtained were comparable to those observed in 3T3-L1 cells 4 days after the induction of differentiation. After selection, cells were stimulated to differentiate to adipocytes, as assessed by Oil Red O staining, at a low rate with dexamethasone alone (data not shown) or in combination with the PPAR γ agonist darglitazone (Fig. 3B). Under these attenuated-differentiation conditions, O/E-1-infected cells differentiated more efficiently than vector-infected cells, suggesting that O/E-1 has a potential to stimulate terminal adipocyte differentiation. To verify this effect, we analyzed the mRNA levels of markers of adipocyte development by real-time PCR during differentiation (Fig. 4). At early stages of differentiation, we observed no significant differences in levels of gene expression between vector- and O/E-1-infected cultures. However, by day 2 postinduction, PPAR γ 2, aFABP, GPDH, and Glut4 were expressed at higher levels in O/E-1-infected cultures, supporting the idea that O/E-1 stimulates adipogenesis. The time span until up-regulation suggests that O/E-1 does not directly control any of the examined genes but rather that the up-regulation of these genes at later stages is due to a more efficient differentiation of O/E-1-infected cells.

To further investigate the ability of O/E-1 to induce differentiation, we infected primary untransformed MEF with either the empty control virus or the O/E-1-encoding retrovirus. Oil Red O staining of these cell cultures (Fig. 5) indicated that

O/E-1-infected MEF differentiated more efficiently than the vector-infected MEF both in the absence and in the presence of darglitazone. These results suggest that O/E-1 has the ability to stimulate terminal adipocyte differentiation also in untransformed cells.

O/E-1 stimulates adipocyte differentiation in noncommitted fibroblastic cells. The results of retrovirus infection experiments with 3T3-L1 and MEF suggest that O/E-1 can enhance adipocyte differentiation in cells that are already committed to the adipocyte lineage or at least have a documented adipogenic potential. However, the most potent stimulators of adipogenesis also possess the ability to induce the differentiation of cells with a broader lineage potential, such as fibroblastic NIH 3T3 cells (33). To explore whether O/E-1 could initiate adipogenesis in cells with a low adipogenic potential, NIH 3T3 fibroblasts were infected with retrovirus vector alone or retroviruses harboring the gene encoding PPAR γ 2, known to be a powerful inducer of adipocyte differentiation (36, 37), or O/E-1. The expression levels of O/E-1 protein in the infected cultures before the addition of external stimuli were examined by EMSA. Results suggested that a low level of O/E-1-like binding activity could be detected in the vector-infected cells but that this binding activity was increased after transduction with O/E-1-encoding retrovirus to levels comparable to those observed in differentiated 3T3-L1 cells (Fig. 6A). Cells were allowed to differentiate in the presence of MDI with or without darglitazone, and lipid accumulation was measured by Oil Red O staining. Both O/E-1 and PPAR γ induced differentiation into a few lipid-containing cells that resembled cultured adipocytes (Fig. 6B) with MDI stimulation alone. Darglitazone dramatically increased lipid accumulation in O/E-1-infected as well as in PPAR γ 2-infected cultures, while very few lipid-containing cells developed in vector-infected cultures. To support the notion that O/E-1 induced differentiation in these

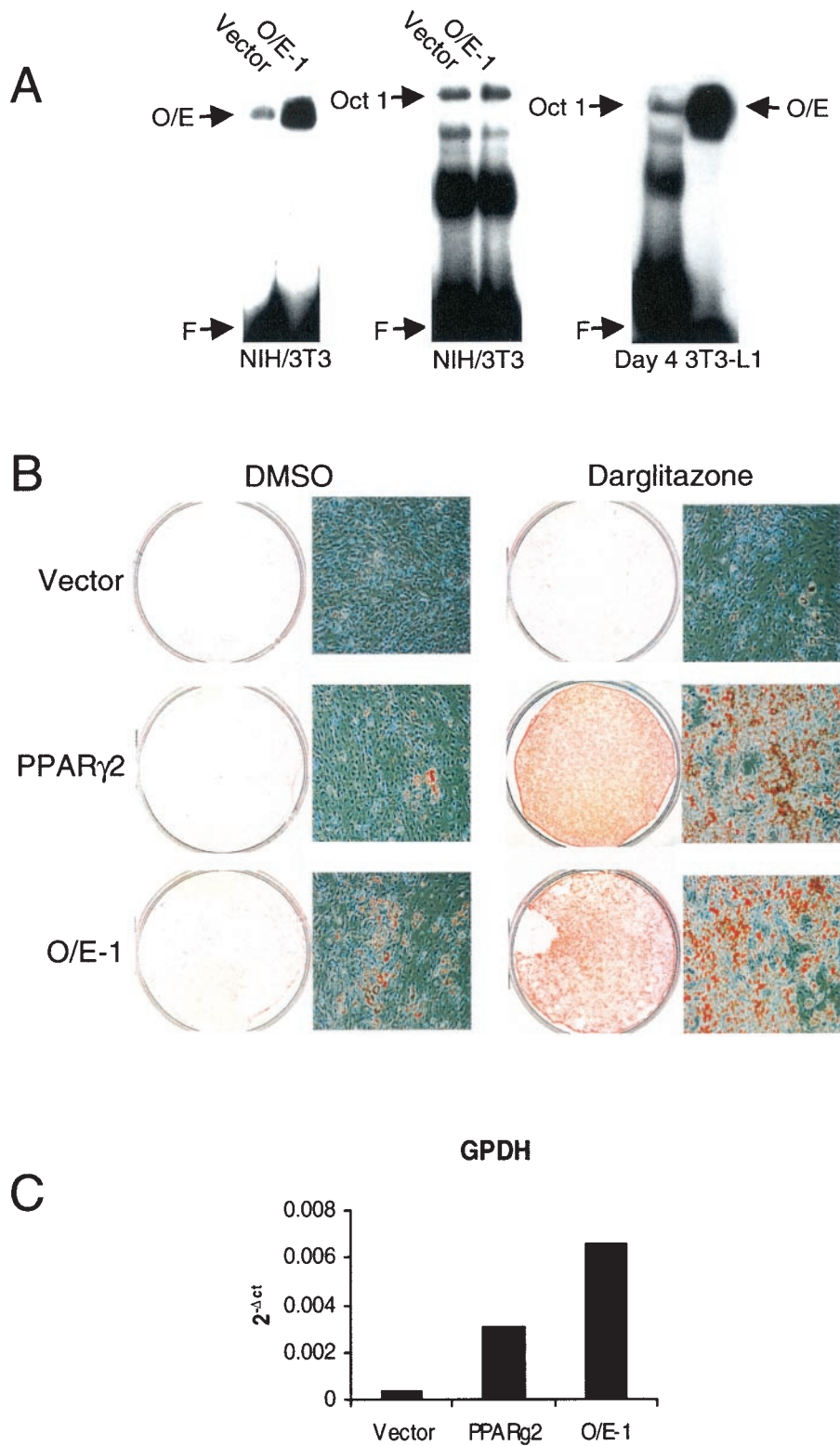


FIG. 6. O/E-1 promotes adipocyte differentiation of NIH 3T3 fibroblasts. (A) NIH 3T3 cells were infected with empty retroviruses (vector) or viruses containing O/E-1 or PPAR γ 2 cDNA. Nuclear extracts were prepared, and the presence of O/E protein was analyzed by EMSA with the *mb-1* O/E-1 probe. The Oct probe was included as a control for protein content, and nuclear extracts from 3T3-L1 cells, differentiated for 4 days by the addition of MDI, were included for a comparison of O/E levels. F indicates free probe. (B) Infected NIH 3T3 cells were treated with MDI and either darglitazone or the vehicle (DMSO) 2 days after confluence. The cultures were stained with Oil Red O after 10 days of differentiation. Petri dishes and micrographs from one representative experiment out of four are shown. (C) GPDH levels of infected cells after differentiation. Infected cells were induced to differentiate by the addition of MDI and darglitazone, and RNA samples were harvested after 4 days of differentiation. DNase-treated samples were used for cDNA synthesis and subsequent quantitative real-time-PCR analysis. The results of one representative experiment out of two are shown.

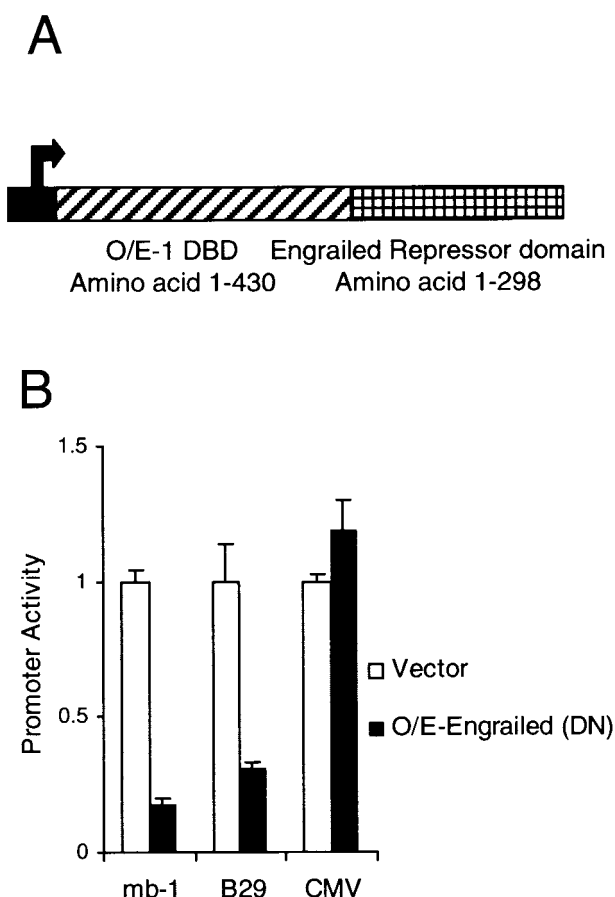


FIG. 7. A dominant negative (DN) O/E-1-Engrailed fusion protein represses O/E-1-dependent target promoters. (A) Schematic drawing of the O/E-1-Engrailed construct. Amino acids 1 to 430 of mouse O/E-1 were N-terminally fused to amino acids 1 to 298 of the *Drosophila* Engrailed protein. DBD, DNA binding domain. (B) The O/E-1-Engrailed hybrid cDNA was cloned into the pcDNA3 expression vector and used in cotransfection experiments. 70Z/3 pre-B cells were transfected with empty pcDNA3 (vector) or pcDNA3 containing O/E-1-Engrailed (O/E-Engrailed) in conjunction with reporter plasmids containing the luciferase gene driven by the *mb-1*, *B29*, or CMV promoter. The resulting luciferase activities from three transfections are indicated.

cells, we performed real-time-PCR analysis of the expression of the GPDH message 4 days after stimulation (Fig. 6C). The results suggested that both O/E-1- and PPAR γ 2-infected cells expressed higher levels of this gene than the control-infected cells, supporting the idea that they differentiate into adipocytes. These results suggest that ectopic expression of O/E-1 promotes adipogenesis and lipid accumulation also in cells that are not committed to the adipocyte lineage.

Expression of a dominant negative form of O/E-1 impairs the differentiation of 3T3-L1 cells. Overexpression of O/E-1 was able to stimulate adipogenesis in preadipocytes as well as in cells with low adipogenic potential, and we thus wanted to investigate whether the O/E proteins were required for terminal adipocyte development. To examine this issue, we designed a dominant negative form of O/E-1 by using a truncated O/E-1 cDNA that lacked the C-terminal transactivation domain while

retaining the DNA binding and dimerization domains (amino acids 1 to 430) (34) and that was fused to the repressor domain of the *Drosophila* protein Engrailed (amino acids 1 to 298) (Fig. 7A). To verify that this protein possessed the ability to suppress the activities of O/E target promoters, we transiently transfected reporter constructs carrying either the mouse *mb-1* or *B29* promoter, both of which are targets for O/E-1 (17, 1), or a control CMV promoter into an O/E-1-expressing pre-B cell line (70Z/3). The reporter constructs were cotransfected with either an empty vector or an O/E-1-Engrailed-encoding expression vector, and the relative functional activities of the promoters were determined (Fig. 7B). The *mb-1* promoter activity was reduced to 19% and the *B29* promoter activity was reduced to 31% by the inclusion of the dominant negative O/E protein, while no repression of the CMV promoter could be detected. The same basic strategy has earlier been used to construct a dominant negative E47 protein with the ability to impair muscle cell differentiation. (4). Dominant negative O/E-1 will likely affect the functions of all three O/E proteins by repressing the activities of target promoters, since they are reported to interact with the same consensus DNA binding site (40). Retroviruses carrying O/E-1-Engrailed were used to infect 3T3-L1 cells, and their differentiation capabilities were compared with that of vector-infected cells. Differentiation was induced with MDI, and cultures were stained with Oil Red O after 5 days. As shown in Fig. 8A, expression of the dominant negative form of O/E-1 in 3T3-L1 cells reduced the capacity of preadipocytes to develop into terminally differentiated adipocytes, without any apparent signs of the general toxicity of the fusion protein. Differentiation was not blocked completely in O/E-1-Engrailed-infected cultures since some cells still accumulated lipids, but the number of differentiating cells was reduced. To further verify that the dominant negative form of O/E-1 was able to impair the ability of 3T3-L1 cells to terminally differentiate, we performed real-time-PCR analysis of O/E-1-Engrailed- and vector-infected cells 2 and 5 days after stimulation with MDI as described above (Fig. 8B). The results indicated that the expression of late-stage markers, such as GPDH, Adipsin, and Glut4, was reduced in the cells expressing the dominant negative protein. We also observed reduced expression of aFABP, PPAR γ 1, PPAR γ 2, c/EBP α , and, to a lesser extent, SREBP1. These data indicate that O/E target genes are important and required for the differentiation of 3T3-L1 preadipocytes into lipid-containing adipocytes.

DISCUSSION

We here report data suggesting that the O/E proteins are directly involved in terminal adipocyte differentiation and also participate in the initiation of adipocyte development. These findings place the O/E proteins in a group of adipogenic transcription factors, including PPAR γ s, C/EBPs, and SREBP1 (6, 33). The existence of a set of proteins sharing the ability to stimulate adipogenic differentiation makes it interesting to consider transcription factor hierarchies and networks. SREBP1 has been suggested to act directly upstream of PPAR γ by binding to its promoter (12) but also in parallel with the same protein to possibly enhance the activation of PPAR γ target genes (22). PPAR γ transcription is also enhanced after ectopic expression of C/EBPs (41), possibly via binding sites in

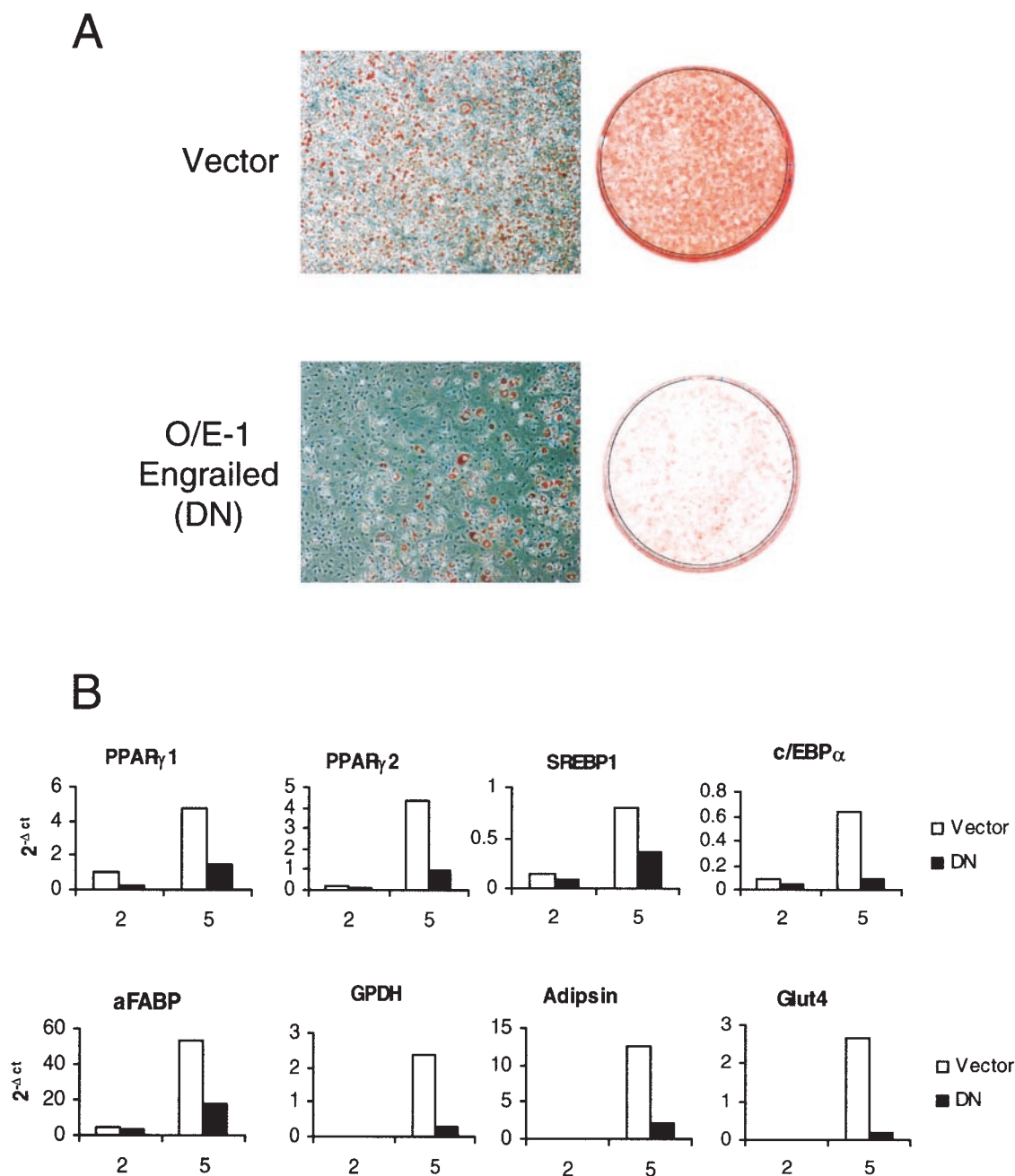


FIG. 8. Dominant negative O/E-1 reduces the level of adipogenesis of 3T3-L1 preadipocytes. (A) 3T3-L1 cells were infected with empty retrovirus (vector) or retroviruses harboring the O/E-1-Engrailed hybrid cDNA (O/E-1-Engrailed) and treated with MDI 2 days postconfluence. Adipocyte differentiation was assessed by Oil Red O staining 5 days after induction. Petri dishes and micrographs from one representative experiment out of four are shown. (B) Total RNA samples were extracted from cultures at 2 and 5 days postinduction (lanes 2 and 5, respectively). cDNAs were synthesized from DNase-treated RNA, and quantitative real-time PCR was performed with primers for the indicated adipocyte differentiation markers.

the PPAR γ promoter (12, 44). The initial effect on PPAR γ transcription is likely to be mediated by C/EBP β and $-\delta$, since expression of these factors precedes that of both PPAR γ and C/EBP α in differentiating adipocytes (5, 8, 43). PPAR γ also appears to act in functional synergy with C/EBP α , since coexpression of these proteins enhances the expression of adipocyte differentiation-linked genes (11). Even though the differentiation process induced by O/E-1 is greatly enhanced by

inclusion of a PPAR γ agonist (Fig. 6), O/E-1 does not appear to increase the early expression of PPAR γ s (Fig. 4). The levels of PPAR γ 2 transcripts are, however, increased in O/E-1-infected 3T3-L1 cells, but this effect is not obvious before day 2, a finding that therefore rather reflects a more efficient differentiation process than a direct effect of O/E-1 on PPAR γ transcription (Fig. 4). The overall mRNA levels of C/EBP α , PPAR γ 1, and SREBP1 were not dramatically affected by ec-

topic expression of O/E-1, arguing against a direct effect on the expression of either of these factors (Fig. 4). A possible interpretation is that O/E-1 acts in parallel with PPAR γ and/or other adipogenic transcription factors. This assumption is also supported by the results of a gene expression analysis of O/E-1- and PPAR γ 2-induced NIH 3T3 differentiation where large differences in expression profiles were found (P. Åkerblad, unpublished observations). We have not investigated how co-expression of O/E-1 and other proadipogenic proteins influences O/E-induced differentiation. Such experiments may, however, help to place O/E-1 in the hierarchy of transcription factors regulating adipogenesis. Of special interest to us would be an investigation of whether O/E-1 acts in concert with SREBP since collaboration between O/E-1 and basic helix-loop-helix proteins is suggested to be of key importance for B-cell development and the regulation of B-cell-restricted genes (30, 35).

O/E transcript levels are, when compared to those of the other known adipogenic transcription factors, rather modestly modulated during 3T3-L1 differentiation (Fig. 2A). A small change in O/E-1 mRNA expression levels may, however, have dramatic effects, since mono-allelic expression of the O/E-1 gene affects target gene activation in B-lymphoid cells (27). We have also analyzed by EMSA the expression of O/E-1 proteins in developing adipocytes, and even though the binding activity largely correlates with the level of mRNA, we cannot at this stage exclude posttranscriptional regulation of the DNA binding capacity. O/E mRNAs are present in undifferentiated 3T3-L1 cells. Connections between O/E proteins and external signaling pathways have been reported for *Drosophila*, where the O/E homologue Collier was shown to be involved in hedgehog signaling in the fly embryo (38). In addition, the O/E-interacting protein ROAZ is thought to be involved in BMP and SMAD signaling pathways (21), which suggests the interesting possibility that O/E-1 proteins integrate genetic predisposition and external signals in fibroblasts or preadipocytes to enhance entry into the adipogenic differentiation pathway. A role for O/E proteins in the response to external stimuli has also been suggested from experiments studying the insulin-dependent repression of the *Glut4* gene in adipocytes (9). A crucial repressor element flanking this gene was shown to interact with O/E proteins, suggesting that O/E proteins are involved in the repression of *Glut4* expression (9). We do not see any direct repressive effect on the endogenous gene after ectopic expression of O/E-1; instead, O/E-1-infected 3T3-L1 cells expressed higher levels of *Glut4*. This increase of *Glut4* expression is likely due to the augmented differentiation rate observed in O/E-1-infected cells. O/E-1 may, however, function as a negative regulator of the *Glut4* gene, but to study this effect, one needs to separate the adipogenic effect of O/E-1, which should lead to *Glut4* up-regulation, from its repressing effect on *Glut4* gene expression. This might be accomplished by overexpression of O/E-1 in later stages of adipogenesis.

The olfactory system is involved in the regulation of food intake, which influences the overall metabolism. The olfactory neuroepithelium of the brain has the capacity to detect and discriminate many different small organic and inorganic compounds. This ability is mediated through a large number of G protein-coupled receptors in olfactory sensory neurons that project into higher brain regions (13). The O/E proteins are

believed to be important for the regulation of genes in the olfactory system. Several promoter regions of odorant transduction pathway components have been shown to contain binding sites for the O/E transcription factors (23, 24, 28, 39). It is interesting that organs responsible for the sensing of food and the storage of energy excess have common transcription factors. The intake of food and signals of satiety are processes regulated by several stimuli, and olfactory neurons may play an important part in this process. It is possible that the use of some common transcription factors is part of a feedback mechanism that signals between adipose tissue and olfactory and possibly other parts of the brain.

The use of a dominant negative O/E-1 protein indicates that O/E-1 directly controls target genes crucial for terminal adipocyte differentiation. O/E-1 is known to control the expression of several genes in pre-B cells and olfactory neurons by direct interactions with the promoter elements (1, 2, 17, 23, 24, 28, 35, 39). We do not know the exact identities of the crucial genes that are under the control of O/E-1 in adipocytes, but a set of potential target genes has been defined by DNA microarray analysis and we are currently investigating their relevance in the adipocyte differentiation pathway.

ACKNOWLEDGMENTS

We thank K. Kristiansen, University of Southern Denmark, Odense, Denmark, for reagents and help with setting up the retrovirus infection system, R. Grosschedl and S. Johnson for the gift of cDNA fragments, and Simonetta Westerlund and Anki Lidberg for excellent technical assistance.

This work was supported by AstraZeneca and by the Medical Faculty at Lund University.

REFERENCES

1. Åkerblad, P., M. Rosberg, T. Leanderson, and M. Sigvardsson. 1999. The B29 (immunoglobulin beta-chain) gene is a genetic target for early B-cell factor. *Mol. Cell. Biol.* **19**:392–401.
2. Åkerblad, P., and M. Sigvardsson. 1999. Early B cell factor is an activator of the B lymphoid kinase promoter in early B cell development. *J. Immunol.* **163**:5453–5461.
3. Bally-Cuif, L., L. Dubois, and A. Vincent. 1998. Molecular cloning of Xco2, the zebrafish homolog of Xenopus Xco2 and mouse EBF-2, and its expression during primary neurogenesis. *Mech. Dev.* **77**:85–90.
4. Becker, J., C. Dorman, T. McClatterly, and S. Johnson. 2001. Characterisation of a dominant inhibitory E47 protein that suppresses C2C12 myogenesis. *Exp. Cell Res.* **267**:135–143.
5. Cao, Z., R. M. Umek, and S. L. McKnight. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* **5**:1538–1552.
6. Cowherd, R. M., R. E. Lyle, and R. E. McGehee, Jr. 1999. Molecular regulation of adipocyte differentiation. *Semin. Cell Dev. Biol.* **10**:3–10.
7. Crozatier, M., D. Valle, L. Dubois, S. Ibensouda, and A. Vincent. 1996. Collier, a novel regulator of *Drosophila* head development, is expressed in a single mitotic domain. *Curr. Biol.* **6**:707–718.
8. Darlington, G. J., S. E. Ross, and O. A. MacDougald. 1998. The role of C/EBP genes in adipocyte differentiation. *J. Biol. Chem.* **273**:30057–30060.
9. Dowell, P., and D. W. Cooke. 2002. Olf-1/early B cell factor is a regulator of *glut4* gene expression in 3T3-L1 adipocytes. *J. Biol. Chem.* **277**:1712–1718.
10. Dubois, L., L. Bally-Cuif, M. Crozatier, J. Moreau, L. Paquereau, and A. Vincent. 1998. Xco2, a transcription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in *Xenopus*. *Curr. Biol.* **8**:199–209.
11. El-Jack, A., J. Hamm, P. Pilch, and S. Farmer. 1999. Reconstitution of insulin sensitive glucose transport in fibroblasts requires expression of both PPAR gamma and C/EBP alpha. *J. Biol. Chem.* **274**:7946–7951.
12. Fajas, L., D. Auboeuf, E. Raspe, K. Schoonjans, A. Lefebvre, R. Saladin, J. Najib, M. Laville, J. Fruchart, S. Deeb, A. Vidal-Puig, J. Flier, M. Briggs, B. Staels, H. Vidal, and J. Auwerx. 1997. The organisation, promoter analysis, and expression of the human PPAR γ gene. *J. Biol. Chem.* **272**:18779–18789.
13. Firestein, S. 2001. How the olfactory system makes sense of scents. *Nature* **413**:211–218.
14. Garel, S., F. Marin, R. Grosschedl, and P. Charnay. 1999. Ebf1 controls

- early cell differentiation in the embryonic striatum. *Development* **126**:5285–5294.
15. Garel, S., F. Marin, M. G. Mattei, C. Vesque, A. Vincent, and P. Charnay. 1997. Family of Ebf/Olf-1-related genes potentially involved in neuronal differentiation and regional specification in the central nervous system. *Dev. Dyn.* **210**:191–205.
 16. Gisler, R., S.-E. Jacobsen, and M. Sigvardsson. 2000. Cloning of human early B cell factor and identification of target genes suggest a conserved role for EBF in B cell development. *Blood* **96**:1457–1464.
 17. Hagman, J., C. Belanger, A. Travis, C. W. Turck, and R. Grosschedl. 1993. Cloning and functional characterization of early B-cell factor, a regulator of lymphocyte-specific gene expression. *Genes Dev.* **7**:760–773.
 18. Hagman, J., M. J. Gutch, H. Lin, and R. Grosschedl. 1995. EBF contains a novel zinc coordination motif and multiple dimerization and transcriptional activation domains. *EMBO J* **14**:2907–2916.
 19. Hagman, J., A. Travis, and R. Grosschedl. 1991. A novel lineage-specific nuclear factor regulates mb-1 gene transcription at the early stages of B cell differentiation. *EMBO J* **10**:3409–3417.
 20. Hansen, J. B., R. Peterson, B. Larsen, J. Bartkova, J. Alsner, and K. Kristiansen. 1999. Activation of peroxisome proliferator-activated receptor gamma bypasses the function of the retinoblastoma protein in adipocyte differentiation. *J. Biol. Chem.* **274**:2386–2393.
 21. Hata, A., J. Seoane, G. Lagna, E. Montalvo, A. Hemmati-Brivanlou, and J. Massague. 2000. OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell* **100**:229–240.
 22. Kim, J., and B. Spiegelman. 1996. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev.* **10**:1096–1107.
 23. Kudrycki, K., C. Stein-Izsak, C. Behn, M. Grillo, R. Akeson, and F. L. Margolis. 1993. Olf-1-binding site: characterization of an olfactory neuron-specific promoter motif. *Mol. Cell. Biol.* **13**:3002–3014.
 24. Kudrycki, K. E., O. Buiakova, G. Tarozzo, M. Grillo, E. Walters, and F. L. Margolis. 1998. Effects of mutation of the Olf-1 motif on transgene expression in olfactory receptor neurons. *J. Neurosci. Res.* **52**: 159–172.
 25. Lin, F., and M. Lane. 1994. CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3L1 adipocyte differentiation program. *Proc. Natl. Acad. Sci. USA* **91**:8757–8761.
 26. Lin, H., and R. Grosschedl. 1995. Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature* **376**:263–267.
 27. Lundgren, M., C. M. Chow, P. Sabbattini, A. Georgiou, S. Minaee, and N. Dillon. 2000. Transcription factor dosage affects changes in higher order chromatin structure associated with activation of a heterochromatic gene. *Cell* **103**:733–743.
 28. Margolis, F. L., K. Kudrycki, C. Stein-Izsak, M. Grillo, and R. Akeson. 1993. From genotype to olfactory neuron phenotype: the role of the Olf-1-binding site. *Ciba Found. Symp.* **179**:3–20.
 29. Nieminen, P., J. Liippo, and O. Lassila. 2000. Pax-5 and EBF are expressed in committed B-cell progenitors prior to the colonization of the embryonic bursa of fabricius. *Scand. J. Immunol.* **52**:465–469.
 30. O'Riordan, M., and R. Grosschedl. 1999. Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Immunity* **11**:21–31.
 31. Prasad, B. C., B. Ye, R. Zackhary, K. Schrader, G. Seydoux, and R. R. Reed. 1998. unc-3, a gene required for axonal guidance in *Caenorhabditis elegans*, encodes a member of the O/E family of transcription factors. *Development* **125**:1561–1568.
 32. Rosen, E. D., and B. M. Spiegelman. 2001. PPARgamma: a nuclear regulator of metabolism, differentiation, and cell growth. *J. Biol. Chem.* **276**:37731–37734.
 33. Rosen, E. D., C. J. Walkey, P. Puigserver, and B. M. Spiegelman. 2000. Transcriptional regulation of adipogenesis. *Genes Dev.* **14**:1293–1307.
 34. Sigvardsson, M. 2000. Overlapping expression of early B-cell factor and basic helix-loop-helix proteins as a mechanism to dictate B-lineage-specific activity of the $\lambda 5$ promoter. *Mol. Cell. Biol.* **20**:3640–3654.
 35. Sigvardsson, M., M. O'Riordan, and R. Grosschedl. 1997. EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrogate light chain genes. *Immunity* **7**:25–36.
 36. Tontonoz, P., E. Hu, and B. M. Spiegelman. 1995. Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor gamma. *Curr. Opin. Genet. Dev.* **5**:571–576.
 37. Tontonoz, P., E. Hu, and B. M. Spiegelman. 1994. Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated transcription factor. *Cell* **79**:1147–1156.
 38. Vervoort, M., M. Crozatier, D. Valle, and A. Vincent. 1999. The COE transcription factor Collier is a mediator of short-range Hedgehog-induced patterning of the *Drosophila* wing. *Curr. Biol.* **9**:632–639.
 39. Wang, M. M., and R. R. Reed. 1993. Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. *Nature* **364**:121–126.
 40. Wang, S. S., R. Y. L. Tsai, and R. R. Reed. 1997. The characterization of the Olf-1/EBF-like HLH transcription factor family: implications in olfactory gene regulation and neuronal development. *J. Neurosci.* **17**:4149–4158.
 41. Wu, Z., N. L. Bucher, and S. R. Farmer. 1996. Induction of peroxisome proliferator-activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBP β , C/EBP δ , and glucocorticoids. *Mol. Cell. Biol.* **16**:4128–4136.
 42. Wu, Z., Y. Xie, N. Bucher, and S. Farmer. 1995. Conditional ectopic expression of CEBP β in NIH-3T3 cells induces PPAR γ and stimulates adipogenesis. *Genes Dev.* **9**:2350–2363.
 43. Yeh, W., Z. Cao, M. Classon, and S. McNight. 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev.* **15**:168–181.
 44. Zhu, Y., C. Qi, J. R. Korenberg, X. N. Chen, D. Noya, M. S. Rao, and J. K. Reddy. 1995. Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR gamma) gene: alternative promoter use and different splicing yield two mPPAR gamma isoforms. *Proc. Natl. Acad. Sci. USA* **92**:7921–7925.