Incubation of 3T3-L1 preadipocytes with isobutylmethylxanthine (IBMX), dexamethasone, and insulin, alone or in combination, demonstrated that IBMX, which increased cAMP-response element-binding protein (CREB) phosphorylation, was the predominant regulator of Pde3b expression. Real-time PCR and immunoblotting indicated that in 3T3-L1 preadipocytes, IBMX-stimulated induction of Pde3b mRNA and protein was markedly inhibited by dominant-negative CREB proteins. By transfecting preadipocytes, differentiating preadipocytes, and HEK293A cells with luciferase reporter vectors containing different fragments of the 5'-flanking region of the Pde3b gene, we identified a distal promoter that contained canonical cis-acting cAMP-response elements (CRE) and a proximal, GC-rich promoter region, which contained atypical CRE. Mutation of the CRE sequences dramatically reduced distal promoter activity; H89 inhibited IBMX-stimulated CREB phosphorylation and proximal and distal promoter activities. Distal promoter activity was stimulated by IBMX and phorbol ester (PMA) in Raw264.7 monocytes, but only by IBMX in 3T3-L1 preadipocytes. Chromatin immunoprecipitation analyses with specific antibodies against CREB, phospho-CREB, and CBP/p300 (CREB-binding protein) showed that these proteins were associated with both distal and proximal promoters and that interaction of phospho-CREB, the active form of CREB, with both Pde3b promoter regions was increased in IBMX-treated preadipocytes. These results indicate that CRE in distal and proximal promoter regions and activation of CREB proteins play a crucial role in transcriptional regulation of Pde3b expression during preadipocyte differentiation.

Cyclic 3',5'-nucleotide phosphodiesterases (PDEs) catalyze hydrolysis of cAMP and cGMP, important intracellular second messengers that regulate many signal transduction pathways. The PDE superfamily includes at least 11 distinct, structurally related, and highly regulated mammalian PDE gene families (PDE1–11) (1). Multiple PDEs are found in most cells but in different amounts, proportions, and subcellular locations; little is known of the mechanisms that regulate their differential expression. PDE3 isozymes can be distinguished from other PDE families by their high affinities for cAMP and cGMP, and by their sensitivity to specific inhibitors such as milrinone and cilostamide (1). The PDE3 family includes two isozymes, PDE3A and PDE3B, that have very similar pharmacological and catalytic properties and exhibit distinct, but overlapping, patterns of expression (2, 3). PDE3A is relatively highly expressed in the cardiovascular system, platelets, various types of smooth muscle, and oocytes (4); PDE3B is relatively highly expressed in cells critical to the maintenance of energy homeostasis such as white and brown adipocytes (4–7), hepatocytes (4, 8), and pancreatic β-cells (9–11). PDE3B is also expressed in other tissues, including vascular smooth muscle (4, 12, 13).

cAMP seems to play a dual, if not seemingly paradoxical, role in adipocytes, and different PDEs may be involved in the regulation of these effects. cAMP is intimately involved in the differentiation program, presumably via activation of CREB, and also in promoting adipogenesis (14, 15). On the other hand, in mature or differentiated adipocytes, cAMP stimulates lipolysis via PKA-catalyzed phosphorylation of perilipin and phosphorylation/activation of hormone-sensitive triglyceride lipase (16). Expression of membrane-associated PDE3B is induced during differentiation of 3T3-L1 adipocytes (6, 17, 18). In differentiated adipocytes, insulin-induced phosphorylation and
Regulation of Murine Pde3b Gene Expression by CREB

EXPERIMENTAL PROCEDURES

Materials

Tissue culture reagents (Dulbecco’s modified Eagle’s medium, fetal bovine serum, and penicillin/streptomycin), GeneRacer™ kit, and TOPO TA Cloning® kit were from Invitrogen; [3H]cAMP was from PerkinElmer Life Sciences; rolipram, cilostamide, bisindolylmaleimide I, and anti-CREB antibodies were from Calbiochem; isobutylmethylxanthine, dexamethasone, and insulin (20). We and others (21, 22) have also reported that specific inhibitors of PDE3, not PDE4, block the antilipolytic action of insulin in differentiated or mature adipocytes. Taken together, these observations suggest that PDE4 isoforms may regulate cAMP pools involved in initiation of differentiation, whereas PDE3 isoforms may regulate cAMP pools that control lipolysis in differentiated adipocytes. Experiments with PDE3 inhibitors have also indicated an important role for PDE3 in regulation of insulin secretion in pancreatic islets (9–11, 23, 24) and, perhaps, oxygen consumption in human subjects (25).

Because the detailed molecular mechanisms that govern adipocyte Pde3b expression during adipogenesis are not known, and because terminal differentiation of 3T3-L1 adipocytes provides a well characterized model for the study of gene expression and regulation (26, 27), we initiated studies of mechanisms underlying transcriptional control of Pde3b expression in these cells. We isolated the 5‘-flanking region of the murine Pde3b gene, which included the putative first exon and an ~5-kb genomic fragment upstream of the translation start site, and which contained distal and proximal promoter regions and transcription initiation sites in the proximal promoter region (~0.5 kb upstream of the translation start site), close to those described by Niiya et al. (28). In addition, our results suggest that cis-acting CRE (cAMP-response) elements in both promoter regions and activation of CREB (CRE-binding proteins) are important in the regulation of induction of Pde3b by cAMP during differentiation of 3T3-L1 adipocytes.
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RNA Ligase-mediated Rapid Amplification of 5’ cDNA Ends (RLM-5’-RACE) for Identification of Distal Transcripts of the Pde3b Gene

Poly(A)-mRNA was isolated from IBMX-treated 3T3-L1 preadipocytes using the Qiagen RNeasy mini kit and Oligotex mRNA mini kit. After first strand synthesis, cDNA was amplified by PCR using a GeneRacer 5’-primer and a gene-specific primer (5’-CAGCGGGCTACGCAGCTTCTCCACGTA-3’, reverse-complement 100–126 bp downstream of ATG start codon). Nested PCR was performed on purified PCR products (Qiagen PCR purification kit), using a GeneRacer 5’-nested primer and a nested, gene-specific primer (5’-AGCTTCTTACGTAGCCGGTCGCA-3’, reverse-complement 89–112 bp downstream of ATG start codon). After TOPO TA cloning of PCR products, positive clones were identified by PCR and sequenced.

Transient Transfection with Transfast™ Reagent (Promega) and the Dual Luciferase Assay

Cells were cotransfected with pRL-TK and pGL3 reporter plasmid vectors that expressed Renilla and firefly luciferase activities, respectively. For the different pGL3 reporter plasmids, firefly luciferase expression was driven either by Pde3b 5’-flanking region genomic fragments or constructs (Pde3b-pGL3), by the SV40 promoter (SV40 (pro)-pGL3) or the SV40 enhancer and promoter (SV40 (con)-pGL3) as positive controls, or by promoter-free pGL3 (Basic-pGL3) as a negative control.

Transfection was performed using TransFAST™ reagent according to the manufacturer’s instructions (Promega). On the day before transfection, 400 μl of nuclease-free water was added to the TransFAST™ reagent, and the lipid film was dispersed by vigorous vortexing for 10 s. After 24 h, a mixture of plasmid DNA, TransFAST™ reagent (3 μl), and serum-free growth medium (1 ml) was prepared, incubated (10–15 min, room temperature), and used to transfect cells, after removal of growth medium from the cells. Each DNA sample contained the pRL-TK plasmid vector (0.02 μg) and a pGL3 plasmid vector (1.0 μg). 3T3-L1 preadipocytes were cotransfected in 6-well culture dishes at 60–80% confluence, incubated for 1 h at 37 °C, and then overlaid with prewarmed DMEM containing 10% FBS (2 ml) and maintained for 48 h at 37 °C. For transfection of differentiating 3T3-L1 preadipocytes, 3T3-L1 preadipocytes were grown in DMEM containing 10% FBS to about 80% confluence (day 0), when differentiation was induced by addition of fresh DMEM containing 10% FBS and MDI (where MDI is IBMX, Dexam, and insulin in differentiating preadipocytes). Three days later (day 3), differentiating 3T3-L1 preadipocytes were cotransfected by the procedure described above but were then overlaid with 2 ml of prewarmed DMEM containing 10% FBS and 1 μg/ml insulin and maintained for 48 h at 37 °C. After 48 h at 37 °C, the cotransfected cells were then washed three times with PBS, disrupted in 250 μl of Passive Lysis Buffer (PLB, Promega), and centrifuged (1,000 × g, 5 min). Supernatant firefly and Renilla luciferase activities were measured with the dual luciferase reporter assay system (Promega) using a luminometer (Lumat LB 9501). Luciferase reagent substrate (100 μl) was...
mixed with cell lysates (20 μl), and firefly luciferase activity was measured (10 s, twice). To terminate this reaction and initiate the Renilla luciferase reaction, Stop & GloR Solution (100 μl) was added to the mixture for 20 s, and Renilla luciferase activity was measured (10 s, twice). The firefly luciferase activity in lysates from cells transfected with Pde3b-pGL3 reporter constructs, and positive and negative controls, was normalized to Renilla luciferase activity values, and the ratio between firefly and Renilla luciferase activities was referred to as relative luciferase activity (RLA). RLA (mean ± S.D., n = 3) was assayed (in duplicate) in lysates from triplicate transfections as described above. Unless otherwise noted, experiments presented in the various figures are from single experiments, which were replicated once or twice.

For comparison of transcriptional activities in different cell types, HEK293A, 3T3-L1, and RAW264.7 cells were transfected with pGL3 plasmid DNAs containing various lengths of 5’-flanking regions of the Pde3b gene. After transfection, the cells were treated with IBMX (300 μM) for 24 h and assayed for luciferase activities.

**Construction of Pde3b-luciferase Vectors and Reporter Plasmids**

In general, Pde3b reporter plasmids were generated by insertion of Pde3b genomic fragments and constructs into pGL3-Basic vectors.

**Distal Region (−5.1 to −3.4 kb Upstream of ATG)—**The 1.7-kb distal SalI/XbaI fragment from the 5’-flanking region of the Pde3b gene (−5.1 to −3.4 kb upstream of ATG) (Figs. 3A and 4) was ligated into SalI/XbaI sites of pBluescript, which were located between the KpnI and Nhel restriction sites of pBluescript. The SalI/XbaI fragment (−1.7 kb) was then excised from pBluescript by cleavage with KpnI/Nhel and ligated into pGL3-Basic digested with KpnI/Nhel, yielding SX-pGL3 (Figs. 4 and 5A). Direct ligation of the SalI/XbaI fragment into pGL3-Basic digested with Nhel/Xhol yielded SX*-pGL3, with the genomic fragment in the reverse orientation (Fig. 5A). The SalI/XbaI fragment in pBluescript was cleaved with KpnI/HindIII, KpnI/EcoRI, or KpnI/BamHI, and the fragments were ligated into pGL3-Basic vector at appropriate restriction sites to yield SH-pGL3 (350 bp), SE-pGL3 (−1.1 kb), and SB-pGL3 (∼1.2 kb), respectively (Figs. 4 and 5A). The size and orientation of all constructs were verified by digestion with KpnI/BglII or KpnI/HindIII.

**Proximal Region (−3.4 kb Upstream of ATG)—**To prepare constructs containing different portions of the 5’-flanking region of the Pde3b gene downstream of the distal SalI/XbaI fragment (Figs. 3A and 4), the 6-kb XbaI/XbaI fragment (Fig. 3A), which contained a BamHI restriction site just upstream of the initiation ATG codon (Fig. 3B), was subcloned, isolated, and digested with BfrII, Ndel, EcoRI, BstII, PshAI, NotI, BglII, PvuII, or Smal. The products with 5’- and 3’-overhang ends, together with the parent XbaI/XbaI fragment, were blunt-ended with Klenow enzyme and digested with BamHI to yield fragments with a 5’-blunt-end and a BamHI-restricted 3’-end (Figs. 3B and 4). These were subjected to electrophoresis (1% agarose gel), extracted (Qiagen, Quick Gel kit), and ligated to Smal/BgIII-digested pGL3-Basic (which contained a 5’-blunt end compatible with the 5’-blunt ends of the Pde3b genomic fragments and 3’-end compatible with BamHI) to form Xba-pGL3, Bfr-pGL3, Nde-pGL3, EcoR-pGL3, Bst-pGL3, Psh-pGL3, Not-pGL3, Bgl-pGL3, Pvu-pGL3, and Sma-pGL3 constructs (Figs. 3B and 4). A 0.8-kb Apal/BamHI fragment from the XbaI genomic clone was ligated into pBluescript II SK(−), which was then digested with KpnI/BamHI. The excised fragment was then ligated into pGL3-Basic vector that had been treated with KpnI/BglII, generating Apa-pGL3. The sizes and orientation of the constructs (Fig. 4) were confirmed by digestion with KpnI/HindIII.

The entire 5’-upstream genomic Sall-BamHI fragment (∼5 kb) was ligated into pGL3-Basic vector (SX-pGL3) via a three-fragment ligation, i.e. Sal-Xba (−1.7 kb) fragment excised from the MG171 genomic clone with KpnI-XbaI, the XbaI/BamHI fragment cut from the XbaI/XbaI genomic fragment, and pGL3-Basic digested with KpnI and BglIII (Figs. 3B and 4). The size and orientation of constructs were verified by digestion with KpnI/EcoRI, ApaI/EcoRI, and other restriction enzymes.

**Distal-Proximal Region Fusion Constructs—**The 1.1-kb Sall-EcoRI portion (SE) from the Sall/XbaI distal genomic fragment (Figs. 4 and 5A), containing AP-2, Sp-1, and CRE cis-acting elements, exhibited strong promoter activity when expressed as the SE-pGL3 reporter plasmid (Fig. 5A). SE was subcloned into Sall/EcoRI sites of pBluescript in between KpnI/Smal sites, and then excised with KpnI/Smal. Pde3b pGL3-Basic plasmids containing proximal promoter regions (Fig. 4) were digested with KpnI and MluI (restriction sites in the multicloning region of pGL3); the 5’-overhang resulting from MluI digestion was blunt-ended. The KpnI/Smal fragment that contained the distal SE promoter region was ligated into the plasmids previously digested with KpnI/MluI (blunted) (Fig. 7). The resulting distal-proximal region fusion constructs contained a 13-bp (CCCGTGCTAGCCC) linker between the SE fragment of the distal promoter and the proximal promoter constructs (Fig. 7). One construct, SEEB, from which this linker was removed, was generated by digesting SE-Bfr-pGL3 with EcoRI to remove the Bfr-EcoRI portion of Bfr-pGL3, as well as the linker between distal and proximal promoter regions of the Pde3b gene, and by self-ligating at EcoRI sites (Fig. 7).

**Construction of Expression Vectors Containing CRE, Sp-1, and AP-2 Sequences—**A series of luciferase expression vectors, containing either the AP-2 (activator protein 2), Sp-1, or CRE sequences found in the Sall/XbaI fragment (Figs. 4 and 5C), were generated by PCR (forward and reverse primers are given in Table 1) from the Sall/XbaI fragment and named A15-pGL3 (214 bp), AT4-pGL3 (125 bp), and AT1-pGL3 (224 bp), respec-
Regulation of Murine Pde3b Gene Expression by CREB

Effect of Differentiation Agents on Pde3b Expression and Lipid Accumulation in 3T3-L1 Preadipocytes—As seen in Fig. 1A (lane 2), immunoreactive PDE3B protein was significantly increased during incubation of preadipocytes with IBMX alone (p < 0.01 compared with control). Dex and/or insulin, alone (Fig. 1A, lanes 3 and 4) or in combination (lane 7), only slightly increased Pde3b expression but significantly potentiated the effect of IBMX (lane 8) (p < 0.01). As shown in Fig. 1B, cilostamide-inhibited PDE activity, which in adipocytes represents mainly PDE3B activity (2-4, 6), was also increased in cells treated with IBMX, alone (lane 2) or in combination with Dex and/or insulin (lanes 5, 6, and 8). These results suggested that the PDE inhibitor IBMX, which increased (most likely via cAMP and PKA) phosphorylation of CREB in 3T3-L1 preadipocytes (cf. Fig. 8A), played a major role in the induction of Pde3b during differentiation and that insulin and dexamethasone (via unidentified signaling pathways) potentiated, or added to, the effects of cAMP. To assess effects of differentiation agents on neutral lipid accumulation, preadipocytes were incubated for 12 days in the presence of IBMX, Dex, and insulin, alone or in combination, and then stained with Oil Red O. As shown in Fig. 1C, in contrast to the effects on Pde3b expression, insulin, alone (lane 4) or in combination with IBMX and Dex (lanes 6–8), increased neutral lipid accumulation (quantitated by extraction of Oil Red O from the cells) to a much greater extent than IBMX (lane 2).

Inhibitory Effects of Dominant-negative CREB on Induction of Pde3b by IBMX—CREB is apparently a crucial transcriptional activator of mitotic clonal expansion and the differentiation program in adipocytes, because of its role in regulating transcription of C/EBPβ (14, 15, 30, 31). To examine further the role of CREB on Pde3b expression, without triggering differentiation, 3T3-L1 preadipocytes were infected with adenovirus constructs expressing β-galactosidase (adeno-β-galactosidase),
dominant-negative forms of CREB (MCREB and KCREB), or active forms of CREB (V16-CREB, D-CREB) and treated with 300 μM IBMX alone for 5 days. As seen in Fig. 2, IBMX increased expression of PDE3B immunoreactive protein (Fig. 2A) and PDE3 activity (Fig. 2B) in control preadipocytes (lane 3) and preadipocytes infected with adeno-β-galactosidase (lane 4), indicating that infection did not affect the induction process. IBMX-induced expression of Pde3b was enhanced by overexpression of active V-16 and D-CREB (Fig. 2B, lanes 5 and 8), and markedly decreased by dominant-negative forms of CREB, MCREB, or KCREB (lanes 6 and 7). As shown in Fig. 2C, IBMX (in preadipocytes) and MDI (IBMX, Dex, and insulin in differentiating preadipocytes) induced Pde3b gene transcription (quantified by real time PCR), which was inhibited by overexpression of KCREB. These data suggest that IBMX-induced expression of Pde3b in both preadipocytes and differentiating preadipocytes requires activation of CREB.

Characterization of the 5′-Flanking Region of the Murine Pde3b Gene—A genomic Sall restriction fragment (~14 kb), which contained 5′-flanking region (~5.1 kb upstream from the ATG initiation codon) and putative exon 1 of the Pde3b gene (Fig. 3A), was cloned from murine 129/Sv and Balb/c...
The 5'-flanking region of the murine Pde3b gene was sequenced (GenBank™ accession numbers AF547434 and AY159890). To determine putative transcription initiation sites, 5'-RACE analysis and RT-PCR were performed using mRNA isolated from mouse 3T3-L1 preadipocytes and differentiated adipocytes (data not shown (32)). As depicted in Fig. 3A and supplemental Fig. 1A, five different transcription initiation sites in the 5'-flanking region of the murine Pde3b gene were identified and located at positions −346, −321, −293, −290, and −227 bp from the translation start site (ATG, +1), respectively. Results from ribonuclease protection assays (RPA) were consistent with those from 5'-RACE (RT)-PCR. Several transcripts of −320, −300, −280, and −240 bp were markedly increased in differentiated adipocytes and protected by an antisense RNA probe corresponding to −360 bp upstream of the translation start codon, consistent with the marked increase in Pde3b expression during differentiation (data not shown). By using primer extension assays,
Niiya et al. (28) reported a transcription initiation site (TIS) ~195 bp upstream of the translation start site of Pde3b. In mouse EST databases, a unique EST was found in RIKEN (4931402H15) with a TIS at ~322, and which spans the first ATG codon site in known putative exon 1 of Pde3b. In addition, the lengths of 5'-untranslated regions of human, mouse, rat, and chicken phosphodiesterase 3B mRNAs are reported in GenBankTM as 291 bp (AY459346), 270 bp (AF547435), 64 bp (Z22867), and 153 bp (AJ851613), respectively.

Promoter Activities in the 5'-Flanking Region of the Pde3b Gene—A series of reporter vectors, containing different portions of the 5'-flanking region of the Pde3b gene fused to the firefly luciferase gene (Fig. 4), were constructed and transfected into 3T3-L1 preadipocytes and differentiating preadipocytes (Fig. 3B). Direct effects of IBMX alone were examined in transfected, nondifferentiating HEK293A cells (Fig. 3C). As seen in Fig. 3, B and C, two regions in the 5'-flanking region of the Pde3b gene apparently were responsible for promoter activities. One region (the “distal” promoter region), which contained a canonical CRE, cis-acting sequence (supplemental Fig. S1B), was apparently located ~4 kb upstream of the TIS, in the distal SalI-Xbal (−5.1 to −3.4 kb) fragment (Fig. 3, B and C). Another, the proximal promoter region, which contained several atypical CRE cis-acting elements (supplemental Fig. S1A), was located close to the identified putative TIS, <1 kb upstream from them. Promoter activity was enhanced during differentiation of 3T3-L1 preadipocytes (Fig. 3B) or in transfected HEK293A cells incubated with IBMX (Fig. 3C). The two regions seemed to be separated by a gene segment with reduced promoter activity or a negative regulatory region. Removal of the distal SalI-Xbal fragment (~1.7 kb) from SXB-pGL3 produced a fragment, Xba-pGL3, with very low luciferase activity, and subsequent deletion of the downstream XbaI-Apal fragment (~2.6 kb) was required to allow expression of proximal promoter activity in the Apal-PvuI region, i.e. with Apa-pGL3, Psh-pGL3, Not-pGL3, and Bgl-pGL3 reporter vectors (Fig. 3, B and C). Compared with Basic-pGL3, luciferase activity of all expression vectors was much greater in HEK293A cells (Fig. 3C) than 3T3-L1 preadipocytes (Fig. 3B), but the relative pattern and differences in the activities of the individual
reporter vectors were similar in the two cell lines (Fig. 3, B and C).

The proposed locations of the distal and proximal promoter regions (upstream of the ATG translation start codon) are presented in Fig. 3A; their nucleotide sequences, which are homologous (∼99% identity) to the corresponding mouse genomic sequences deposited in GenBank™, are presented in Fig. S1, A and B. Alignment of GenBank™ sequences indicated limited homology among the 5′-flanking regions of the mouse, rat, chicken, and human PDE3B gene; only the mouse contained the canonical CRE sequences in the distal promoter region. Atypical CREB-binding sites, however, were predicted in putative distal and proximal promoter regions in the 5′-flanking regions of the four species. Consistent with our experimental results, two promoter regions (beginning at position −4061 and another at −364) were predicted when 5105 bp of the 5′-flanking sequence of the murine Pde3b gene was queried online. On the other hand, analysis at another on-line site predicted one promoter (at −532).

Distal Promoter Activity—To characterize the distal promoter region, 3T3-L1 preadipocytes or HEK293A cells were transfected with Pde3b-pGL3 constructs generated from the −1.7 kb SalI/XbaI fragment in the upstream or distal promoter region (−5.1 kb to −3.4 kb) of the 5′-flanking region of the Pde3b gene (Figs. 3A, 4, and 5, A and B). As depicted in Fig. 5, A and B, SX-pGL3, SB-pGL3, and SE-pGL3, but not SH-pGL3, exhibited strong promoter activity, indicating that active cis-elements were located between HindIII and BamHI sites in the SalI/XbaI fragment. The promoter activity of this region, which contains a canonical CRE site (TGACGTCA) and Sp-1 and AP-2 cis-acting elements, as well as a putative TATA box (Fig. 3A, Fig. 5A, and Fig. S1B), was much higher than that of the SV40 promoter-pGL3 construct (Fig. 5, A and B) and was increased in differentiating preadipocytes (Fig. 5A), as well as in

FIGURE 3—continued
3T3-L1 preadipocytes and HEK293A cells (Fig. 5B) incubated with IBMX. As also seen in Fig. 5A, in preadipocytes, the activities of the distal promoter fragments (SX-pGL3, SB-pGL3, and SE-pGL3) were orders of magnitude greater than Basic-pGL3. This suggested that activities of these distal promoter fragments were also much greater than the promoter activities (Fig. 3B) of the entire full-length 5’-flanking promoter region (SXβ-pGL3) or proximal promoter fragments (Apa-, PshA-, Not- and Bgl-pGL3), because, in preadipocytes, the luciferase activities of the latter constructs were only severalfold greater than Basic-pGL3 (note the differences in scale in Figs. 3B and 5A). Distal promoter activity was orientation-dependent because the SX*-pGL3 construct, with the same SalI-XbaI fragment as in SX-pGL3, but in reverse orientation, did not exhibit promoter activity (Fig. 5, A and B).

As seen in Fig. 5B, mutations of the CRE sites in SX-pGL3 caused a drastic reduction in basal as well as IBMX-stimulated promoter activities of the SX mutCRE-pGL3 reporter vector, demonstrating that the cis-acting CRE are critical for distal promoter function. Mutation of the putative TATA site exerted a much smaller effect on promoter activity (Fig. 5B). A series of luciferase-expression vectors containing the AP-2, Sp-1, and CRE sequences (alone and in combinations) were also generated by PCR. As seen in Fig. 5C, in transfected HEK293A cells incubated with IBMX, the relative luciferase activity of AT1-pGL3 reporter constructs containing CRE alone (AT1-pGL3), or in combination with Sp-1 and AP-2 elements (AT3-, 6-, 7-pGL3), was much higher than that of the SV40 promoter-pGL3. AT2-, -4-, and -5-pGL3 vectors, luciferase constructs containing AP-2 and Sp-1 binding sequence(s) (alone or in combination), demonstrated little or no promoter activity (Fig. 5C). Promoter activity related to CRE was reduced by the presence of AP-2 and/or Sp-1 elements (AT3-, -6-, and -7-pGL3 constructs) (Fig. 5C). In addition, mAT1-pGL3, an expression luciferase vector containing mutated CRE sequences, exhibited no promoter activity. Similar results were demonstrated with these expression vectors in differentiating preadipocytes (data not shown).

Effects of PMA and IBMX on Promoter Activities and CREB Protein Phosphorylation in Raw264.7 Monocytes and 3T3-L1 Preadipocytes—As seen in Fig. 6, promoter activity of several distal region luciferase reporter vectors was stimulated by IBMX, but not phorbol ester (PMA) (50 nM), in transfected 3T3-L1 preadipocytes (Fig. 6A), whereas distal promoter activity was, under these conditions, stimulated by both IBMX and PMA in Raw264.7 monocytes (Fig. 6B). Mutation of CRE in the...
distal promoter SX mutCRE-pGL3 reporter vector significantly reduced the effect of PMA on luciferase reporter gene activities (Fig. 6C), suggesting that the CRE are important for the effects of PMA on Pde3b gene expression (Fig. 6B). In HEK293A cells transfected with SX-pGL3 (Fig. 6C) or with the NotI-pGL3 reporter vector (Fig. 6D) from the proximal promoter region (which contained an atypical CRE site), the effects of PMA on luciferase reporter gene activities were inhibited by bisindolylmaleimide I (a specific PKC inhibitor) and H89. The effects of IBMX were also blocked by H89 (Fig. 6D). PMA-induced CREB phosphorylation in monocytes (Fig. 6E) was more effectively inhibited by BisI than H89, suggesting that effects of PMA on CREB phosphorylation were mainly mediated by activation of PKC. ([R]p)-cAMPS and H89 blocked the effects of IBMX. In HL-60 cells, PMA-induced expression of ganglioside GM3 synthase is thought to be mediated via PKC/mitogen-activated protein kinase-dependent CREB phosphorylation (33).

**Promoter Activity of Distal/Proximal Fusion Constructs—** The results in Fig. 3, B and C, indicated that the negative regulatory region affected proximal promoter activities. To observe the effects of this gene segment (i.e. the XbaI-PshAI portion of the 5′-flanking region in Fig. 3, B and C) on distal promoter activity (cf. Fig. 5A), 3T3-L1 preadipocytes were transfected with reporter vectors in which the active SE fragment from the distal promoter region (1.7-kb SalI/XbaI fragment) was coupled with various downstream portions of the 5′-flanking region (XbaI-BamHI (122 bp)) of the Pde3b gene (Fig. 7). As seen in Fig. 7, distal promoter activity of the SE

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**Fig. 5—continued**

**FIGURE 5.** Promoter activities of constructs from the distal portion of the 5′-flanking region of the Pde3b gene. Luciferase reporter expression vectors were constructed with indicated portions of the distal 1.7-kb Sal/XbaI fragment, which contained the indicated restriction sites and AP-2, SP-1, and CRE cis-acting elements. RLA (mean ± S.D., n = 3 experiments) was measured (in duplicate) in lysates from triplicate transfections of 3T3-L1 preadipocytes and differentiating preadipocytes (A) and preadipocytes and HEK293A cells incubated without or with IBMX (B). SX*-pGL3 is orientation-reversed SX-pGL3 construct; mutCRE, mutTATA, and mutCRE/TATA are SX-pGL3 reporter vectors with mutations in CRE, TATA, and CRE and TATA sequences, respectively. C, HEK293A cells were transfected with luciferase reporter expression vectors that contained CRE, Sp-1, and AP-2 elements, alone and in combination, and that were generated by PCR from the 1.7-kb Sal/XbaI distal fragment, using primers listed in Table 1. AT1-pGL3 and mAT1-pGL3 reporter vectors contained wild-type and mutant CRE sequences, respectively. Transfected cells were incubated with IBMX. RLA (mean ± S.D., n = at least three separate experiments) was measured (duplicate assays) in lysates from triplicate transfections. A–C, SV40 (Pro)-pGL3 refers to pGL3 expression vector driven by the SV40 promoter; SV40(Con)-pGL3, pGL3 expression vector driven by both the SV40 promoter and SV40 enhancer; Basic-pGL3, the pGL3-promoter- and enhancer-free vector. **, p < 0.01, statistically different from control.
Regulation of Murine Pde3b Gene Expression by CREB

A. Preadipocytes

B. RAW 264.7 Monocytes

C. HEK293A Cells

D. HEK293A Cells

E. RAW 264.7 Monocytes
Regulation of Murine Pde3b Gene Expression by CREB

PDE3B appears or is markedly increased during differentiation of 3T3-L1 adipocytes in the presence of MDI (6, 17, 18). In adipocytes, PDE3B regulates β3 AMP pools important for lipolysis and the antilipolytic action of insulin but not for initiation of differentiation (20–22). In addition, the presence of fat depots in PDE3B-deficient mice, recently generated in our laboratory (data not shown), also indicates that PDE3B is not required for differentiation. These data, however, do not rule out an important role(s) for Pde3b expression during differentiation or its impact on adipogenesis.

During differentiation in the presence of MDI, IBMX, which increased CREB phosphorylation, was the predominant regulator of Pde3b expression (Figs. 1 and 2). ChIP analysis indicated that phosphorylated CREB interacts with CRE cis-acting elements in the proximal and distal promoter regions of the 5′-flanking region of the Pde3b gene and that phosphorylated CREB interacts with and thereby recruits the multifunctional coactivator, CBP/p300 histone acetyltransferase, to Pde3b promoter regions. In keeping with the current hypotheses concerning coactivators and histone acetyltransferases (35, 36), CBP/p300, by acetylating nucleosomal histone tails, could make the Pde3b promoter regions more accessible to other regulatory factors. CBP/p300 might also function as a scaffold for other transcription factors/coactivators or a bridge between Pde3b-specific factors and the general transcription factors and RNA polymerase II, and thus initiate transcription of Pde3b. In addition, CBP/p300 can catalyze covalent acetylation of proteins, including transcription factors and coactivators. Although CBP/p300 most likely is a critical coactivator for CREB-mediated regulation of Pde3b transcription, the relative importance of histone acetyltransferase activities, scaffold functions, and protein acetyltransferase activities in regulation of assembly of the transcriptional complex and Pde3b gene expression is not known.

**FIGURE 6. Effects of PMA or IBMX on distal and proximal promoter activities.** 3T3-L1 preadipocytes (A) and Raw264.7 monocytes/macrophages (B) were transfected for 6 h with luciferase reporter expression vectors containing portions of the distal promoter (−1.7 kb Sal/XbaI fragment or orientation-reversed Sal/XbaI (SX) fragment). Cells were incubated with either IBMX (300 μM) for 3 days or PMA (50 nM) for 24 h and then lysed, and RLA was assayed. C and D, HEK293A cells were transfected for 6 h with luciferase reporter expression vectors containing either the −1.7 kb Sal/XbaI reporter vector (SX-pGL3) with or without mutations at the CRE site (C) or the NotI/BamHI fragment which contains the proximal promoter region (D). C and D, cells were incubated for 24 h with either PMA (50 nM) alone, PMA + Bis (2.5 μM), PMA + H89 (10 μM) (C) or PMA (50 nM) alone, PMA + Bis (2.5 μM), PMA + H89 (10 μM), IBMX (300 μM), IBMX + H89 (10 μM) (D) and then lysed, and RLA was assayed (triplicate assays). Data are presented as mean ± S.D. (n = 3 experiments). *p < 0.05; **p < 0.01, comparison of treated to control or comparison between treated with or without inhibitors. E, effects of inhibitors on PMA- and IBMX-induced CREB phosphorylation in Raw264.7 monocytes. Raw264.7 monocytes/macrophages were incubated with DMEM for 16 h and then with H89 (10 μM), (R)-cAMPS (10 μM), or BisI (2.5 μM) for 20 min, and then for 2 h with IBMX (300 μM) or PMA (50 nM), alone or in combination. Cells were lysed in protein sample buffer containing 2% SDS, 10 mM β-glycerol phosphate, 5 mM benzamidine, 50 mM sodium fluoride, and 1 mM sodium orthovanadate. Samples (30 μg of protein) were subjected to SDS-PAGE and immunoblotted with anti-phospho-CREB or -CREB1 antibodies; relative immunoreactive band intensities were quantitated by densitometry scanning. Data, mean ± S.D. (n = 3 experiments), were statistically analyzed with ANOVA, and Bonferroni-Dunn post hoc tests were performed with StatView (version 5.0.1). *p < 0.05; **p < 0.01. A representative blot from three separate experiments is shown. Ctrl, control.
Although the precise role of cAMP signals in initiating adipocyte differentiation and adipogenesis in the intact rodent has not been defined, transgenic and knock-out models have identified components of cAMP-signaling pathways that are critical regulators of adipogenesis (37, 38). For example, targeted disruption of the RII \( \alpha \) (H9252) gene of PKA produced mice with markedly reduced white adipose tissue depots that were resistant to diet-induced obesity and that exhibited increased basal lipolysis but reduced \( \beta \)-receptor stimulated lipolysis (37). cAMP has also been implicated in the expansion of brown adipose tissue in rodents, following activation of sympathetic innervation (response to cold temperature) or administration of \( \beta \)-receptor agonists (39). Much of our current knowledge of mechanisms of adipogenesis has relied on use of model systems, including 3T3-L1 cells (26). Treatment of quiescent 3T3-L1 preadipocytes with dexamethasone, insulin, and an agent such as IBMX, which can increase cAMP, initiates the adipocyte differentiation program. Phosphorylation of CREB, mediated by cAMP and insulin (14, 34, 40, 41), is crucial for initiation of differentiation, and phosphorylated CREB (presumably via recruitment of CBP) transcriptionally activates C/EBP \( \beta \) via cis-acting CRE in its promoter. The activated preadipocytes re-enter the cell cycle and undergo mitotic clonal expansion, during which time C/EBP\( \beta \) is phosphorylated, acquires DNA binding activity, and, along with C/EBP\( \delta \), transcriptionally activates the critical adipocyte transcription factors, C/EBP\( \alpha \) and PPAR\( \gamma \) (26).

**FIGURE 7. Promoter activities of proximal/distal promoter fusion reporter vectors expressed in preadipocytes and differentiating preadipocytes.** The SE fragment from the distal SalI/XbaI region (Figs. 4 and 5A) was coupled to the indicated fragments from the proximal promoter region (Figs. 3B and 4). Restriction sites used to generate the constructs are indicated. Promoter activity of the transfected proximal/distal promoter fusion constructs was measured as RLA (mean ± S.D., n = 3 experiments) as described under “Experimental Procedures.” *, the 13-bp linker present between the SE fragment and the proximal promoter fragments. *, p < 0.05.
critical because AP-2 and Sp-1 can inhibit expression of C/EBPα (26, 31). The cAMP-induced decrease in AP-2 and Sp-1 may promote access of C/EBP to the proximal promoter elements and derepression of the C/EBPα regulatory elements that are required for 3T3-L1 differentiation. C/EBPα and PPARγ, along with SREBP-1c and C/EBPβ, coordinately regulate genes responsible for acquiring and maintaining the adipocyte phenotype, perhaps including PDE3B (26, 27).

Thus, IBMX-induced phosphorylation and activation of CREB, via cAMP and PKA (40, 41), is important in regulation of transcription of some early genes that trigger the adipocyte differentiation program. Similarly, treatment of murine wild-type and Kin−/− (cells that lack PKA) S49 lymphoma cells with the PKA-selective analog 8-CPT-cAMP, indicated that cAMP, via activation of PKA, regulated transcription of a large number of genes and gene “networks” in these cells. Primary transcriptional networks that were regulated within 2 h of exposure to 8-CPT-cAMP were enriched in CREB-binding sites in the proximal promoter region (C). In all cases, only the expected single 244 bp (B) and single 406 bp (C) PCR products were observed. Experiments in A–C were repeated, with similar results.

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reporter vectors (Figs. 5–8), demonstrated the presence of at least two distinct promoter regions in the 5′-flanking region of the Pde3b gene, separated by a negative regulatory gene segment. The proximal promoter region is located just upstream (within −0.5–1 kb) of the TIS, determined by 5′-RACE and RPA to be 300 bp upstream of the ATG translation initiation codon. It contains neither a typical TATA box nor a CAAT box but does contain atypical CRE cis-acting elements and GC-rich sequences with multiple housekeeper promoter elements, including Sp-1, GC box, TATA-like box, and an initiator motif (49–52). The distal promoter region, which contained a canonical CRE sequence, was mapped ~4 kb upstream of the ATG translation initiation codon. However, 5′-RACE (RT)-PCR, RLM-5′-RACE-PCR, and RPA analyses failed to detect transcripts in the distal promoter region or its immediate downstream region. After transfection of 3T3-L1 preadipocytes with a distal promoter reporter expression vector (SX-pGL3), however, a fusion transcript between the distal promoter region and the firefly luciferase gene was detected. This raised the possibility of the distal promoter generating Pde3b transcripts, still not identified, that might contain a small noncoding or alternative exon 1.

Canonical CRE cis-acting elements were clearly responsible for the strong activities of the distal promoter luciferase reporter vectors in transfected 3T3-L1 preadipocytes and differentiating adipocytes, as well as HEK293A cells. CREB-binding to target genes may be regulated in a cell-specific manner, and the ability of CREB to interact with specific CRE may be important in cell-specific regulation of gene expression (53). CRE may thus play a role in tissue-specific regulation of Pde3b gene expression, because PMA stimulated expression of several selected distal region reporter vectors (SE-pGL3, SX-pGL3, and SB-pGL3) in RAW monocytes, but not 3T3-L1 preadipocytes, whereas IBMX stimulated distal promoter activity in both cells. Mutation of the distal CRE sequences was associated with a marked reduction in distal promoter activities and loss of activation by IBMX in preadipocytes and HEK293A cells, and by MDI in differentiating adipocytes, as well as by PMA in HEK293A cells. IBMX- and PMA-stimulated expression of proximal promoter activity (Not1-pGL3 reporter vector) was inhibited by H-89 and BisI, respectively, further suggesting a role for CRE in regulation of Pde3b expression by both IBMX and PMA.

Because of the presence of the negative regulatory region and ambiguity regarding generation of transcripts from the distal promoter, the relative contributions of the distal and proximal regions in initiating transcription of the Pde3b gene have not been determined. Although the proximal promoter might drive transcription of the Pde3b gene, the canonical CRE in the distal promoter could function to generate alternate transcripts and/or to enhance regulation of Pde3b transcription through binding of transcription factors and coordination with the proximal promoter (35, 36, 41, 54). Interaction of CREB with CBP/p300 could increase the interaction/association of CBP with multiple transcription factors and regulators and RNA polymerase II, and their recruitment to the proximal promoter region of the Pde3b gene (55, 56), where they could collectively induce formation of a higher order complex, such as an enhanceosome (57). A loop formed between the distal and the proximal promoters could allow the distal promoter to interact-coordinate with the proximal promoter (54, 57). How-
ever, the identity of the transcription factors and coactivators, or protein complexes, which mediate CREB transactivation and Pde3b transcription, is not known.

Although insulin and dexamethasone alone were not as effective as IBMX in induction of Pde3b, both agents enhanced the effects of IBMX on Pde3b expression in differentiating preadipocytes. These agents might activate CREB via signaling pathways other than cAMP (40, 41, 56, 58–60) or act through transcription factors other than CREB. There are several consensus motifs of insulin-response elements and peroxisomal proliferator response elements in the 5′-flanking region of the Pde3b gene. By activating transcription factors that bind to insulin-response elements and peroxisomal proliferator response elements, insulin and dexamethasone can regulate the expression of other adipocyte genes, including, perhaps, PPARγ and C/EBPα (61, 62). Thus, C/EBP, insulin-response elements, and peroxisomal proliferator response element sites in the Pde3b gene might regulate Pde3b expression by acting coordinately with CRE. Taken together, our data suggest that cAMP-mediated CREB phosphorylation/activation plays a pivotal role in mediating Pde3b induction, whereas dexamethasone and insulin act as potentiators. Understanding the mechanisms of transcriptional control of Pde3b in adipose tissue, as well as pancreatic β-cells and liver tissues, could have important pathophysiological and therapeutic implications for obesity and non-insulin-dependent diabetes mellitus (63, 64), and pharmacological implications for potential therapeutic actions of specific PDE3 inhibitors (64).

Acknowledgments—We thank Dr. Martha Vaughan (NHHLI, National Institutes of Health) for critical review of the manuscript and Drs. A. Kristoff (NHHLI, National Institutes of Health) and Guang Gao (CBER, Food and Drug Administration) for helpful discussions. We also thank Carol Kosh for excellent secretarial assistance.

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