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cAMP-induced Interleukin-10 Promoter Activation Depends on CCAAT/Enhancer-binding Protein Expression and Monocytic Differentiation*

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The molecular mechanisms underlying the regulation of interleukin (IL)-10 transcription in monocytic cells by various stimuli during inflammation and the stress reaction are not fully understood. Recently, we provided evidence that stress-induced IL-10 promoter activation in monocytic cells is mediated by catecholamines via a cAMP-dependent signaling pathway including CREB/ATF (cAMP-responsive element binding protein/activating transcription factor) binding to two CRE motifs. However, the mutation of these sites diminished cAMP responsiveness by only 50%, suggesting a role for additional transcription factors and elements in the cAMP-dependent regulation of the human IL-10 promoter. Here, we analyze the functional role of one such factor, C/EBP, in two cell lines of myelomonocytic origin, THP-1 and HL-60, which are known to differ in their differentiation status and C/EBP protein content. We show that the level of basal as well as cAMP-stimulated IL-10 transcription depends on the expression of C/EBPα and β and their binding to three motifs in the promoter/enhancer region. The C/EBP5 motif, which is located between the TATA-box and the translation start point, is essential for the C/EBP-mediated constitutive and most of the cAMP-stimulated expression as its mutation nearly abolished IL-10 promoter activity. Our results suggest a dominant role of C/EBP transcription factors relative to CREB/ATF in tissue-specific and differentiation-dependent IL-10 transcription.

An appropriate balance between pro- and anti-inflammatory cytokines during the immune response is critical in the resolution of many pathological conditions. In this context, interleukin (IL)[1]-10 is of special interest because of its anti-inflammatory and immunosuppressive properties. It is known that IL-10 synthesis in mononuclear cells can be induced or enhanced by various inflammatory stimuli and by sympathetic activation during the stress reaction or injury (1, 2), but the molecular mechanisms underlying the regulation of these processes are not fully understood. Recent reports demonstrate that lipopolysaccharide-induced IL-10 transcription may involve Sp1 activation by p38 mitogen-activated protein kinase (3) or the binding of Stat3 to the 5′-flanking promoter region of IL-10 (4). We showed previously that stress-induced IL-10 expression in monocytic cells is mediated by catecholamines via β_2-adrenoreceptors linked to a cAMP-dependent signaling pathway (5), which activates CREB/ATF by phosphorylation. These transcription factors act through two cAMP-responsive elements (CRE), CRE1 and CRE4, located within 1308 bp upstream from the translation start codon of the IL-10 gene (6). Yet, site-directed mutagenesis of these two CRE sites resulting in loss of protein binding was not sufficient to abolish cAMP responsiveness with 50% activity remaining. However, we previously described an additional putative CRE site (CRE3) that binds proteins that do not belong to the CREB/ATF or the activating protein-1 families of transcription factors. Although mutation of this CRE3 motif had no effect on protein binding or on promoter stimulation by cAMP, deletion of CRE3 by 5′-exonuclease digestion resulted in a significant reduction in activity (6). Therefore, we concluded that other cAMP-responsive transcription factors bind to CRE3, even if mutated, or to hitherto unknown regulatory elements in this region during stress/cAMP-dependent activation of the IL-10 promoter. At first, we examined the involvement of three putative activating protein-2 sites exhibiting protein binding, but we were not able to confirm their specificity in supershift assays. We next investigated CCAAT/enhancer-binding proteins (C/EBP), because these transcription factors are shown to be involved in cAMP-dependent gene expression, *e.g.* of IL-6 and IL-1, as well as of enzymes of catabolic metabolism in the liver (7–9). It is well established that C/EBP proteins mediate cAMP responsiveness by indirect mechanisms, which include their increased expression and trans-location into the nucleus in response to elevated cAMP levels. Moreover, they possess domains that contain cAMP-inducible activities that are independent of direct phosphorylation by protein kinase A (PKA) (10, 11). Similar to CREB/ATF, C/EBP proteins belong to the group of basic region/leucine zipper transcription factors that have the potential to form heterodimers with each other and bind to motifs of either specificity (10, 12). C/EBP proteins are known to regulate adipocytic and monocytic differentiation, and their expression patterns vary depending on the state of cellular differentiation (13–15). Recently, we found that promonocytic THP-1 cells

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[1] The abbreviations used are: IL, interleukin; CREB, cAMP-responsive element binding protein; ATF, activating transcription factor; AU, arbitrary units; CBP, CREB-binding protein; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP responsive element; dbcAMP, N^6,2′-*O*-butyryl-cAMP; EMSA, electrophoretic mobility shift assay; MSV, mouse sarcoma virus; PKA, protein kinase A; TFBS, transcription factor binding site; DBA, DNA block aligner; *hs*, *H. sapiens*; *mm*, *M. musculus*; *ma*, *M. monax*.

TABLE I

C/EBP sites, their positions in the 5′-untranslated region of the IL-10 gene and oligonucleotides used in EMSA and site-directed mutagenesis

Location of the elements is specified as the upstream position relative to translation start point. TFBS regions are underlined, and capital boldfaced letters indicate coincidence with the C/EBP consensus sequence in case of C/EBP1 with the CRE consensus sequence.

Site	Site position	Oligonucleotides
C/EBP1 (CRE3)	−865/−852	tgtac**agg**TGAt**GT**a***A***cat**c**tctg
mutC/EBP1		........gac.agtc........
C/EBP2	−584/−574	gggctgc**TTG**g**G**a**A**c**I**ttgagg
C/EBP3	−452/−439	caatta**TT**t**C**t**CAAT**cccatt
mutC/EBP3		......gactagtc.......
C/EBP4	−435/−418	ttgtattct**gga**a**TG**g**GCAAT**ttgtc
C/EBP5	−43/−30	cttgctc**TTGC**aa**AA**cca**a**acc
mutC/EBP5		.......gact.gtc.......
C/EBPcons		tgcaga**TTGCGCAAT**tctgca
CREcons		agagattgcc**TGACGTCA**gagagctga

harbor significantly more C/EBPα than the myelomonocytic progenitor cell line HL-60 (16) and that THP-1 cells, unlike HL-60 cells, produce high levels of IL-10 protein after cAMP treatment (data not shown). To gain insight into the components involved in cAMP/stress-induced IL-10 expression, we asked whether C/EBP transcription factors contribute to promoter stimulation through the CRE3 site and four putative C/EBP binding sites, which were predicted by computer analysis.

Using THP-1 and HL-60 cells, we demonstrate that C/EBPα and β are critical in both basal and cAMP/stress-dependent regulation of IL-10 expression during monocytic differentiation. As targets, we identified three previously unknown C/EBP motifs in the promoter/enhancer of the IL-10 gene, one of which corresponds to the recently described CRE3 site.

EXPERIMENTAL PROCEDURES

Electrophoretic Mobility Shift and Assay (EMSA) and Supershift Assay—Preparation of nuclear extracts and electrophoretic mobility shift assays were performed essentially as described elsewhere (16, 17). 10 μg of nuclear proteins extracted from THP-1 cells were incubated with 1 ng of radiolabeled oligonucleotides in a 20-μl reaction for 20 min at room temperature. The location of C/EBP binding motifs and the sequences of the used oligonucleotides are listed in Table I with the exception of CRE1 and CRE4, which we have described previously (6). For competition or supershifting, 100 ng of unlabeled oligonucleotide or 1.5 μl of the specific antiserum were added, respectively. The C/EBPα, β, and δ antisera and oligonucleotides corresponding to C/EBP and CRE consensus binding sites were obtained from Santa Cruz Biotechnology.

Plasmids—Firefly luciferase reporter gene constructs containing 1308 bp (pGL2-1308) and 376 bp (pGL2-376) from the region upstream of the IL-10 translation start codon as well as the double CRE mutant of the 1308-bp fragment (pGL2-mutCRE1,4) have been described elsewhere (6). To obtain each single mutant pGL2-mutC/EBP1, pGL2-mutC/EBP3, and pGL2-mutC/EBP5, the plasmid pGL2-1308 was subjected to *in vitro* mutagenesis using the GeneEditor (Promega) and oligonucleotides as indicated in Table I. Successful mutagenesis was monitored by *Spe*I digestion, because the nucleotide exchange introduced an additional restriction site. The triple mutant pGL2-mutC/EBP1,3,5 was generated by cloning of a *Sac*I fragment from pGL2-mutC/EBP3 into pGL2-mutC/EBP5 followed by digestion with *Bsu*36I and ligation of the resulting fragment into pGL2-mutC/EBP1. pGL2-*Avr*IImutC/EBP5 was made from pGL2-mutC/EBP5 by digestion with *Avr*II and *Sma*I followed by blunt-ended ligation after refilling of the 3′ overhang and comprises 397 bp of DNA from upstream of the start codon. The sequences of triple mutated and *Avr*IImutC/EBP5 fragments were confirmed by sequence analysis (BigDye Terminator cycle sequencing kit, version 2.0, Applied Biosystems). The pMSV/EBPα and pMSV/EBPβ expression plasmids that contain the respective cDNAs from rat under the control of the mouse sarcoma virus (MSV) long terminal repeat were kindly provided by S. McKnight (University of Texas Southwestern Medical Center, Dallas, TX). To obtain the MSV promoter control plasmid pMSV, the pMSV/EBPα plasmid was digested with *Nco*I followed by religation. The expression plasmids for rat p30, pcDNA3/p30, and CREB133 (pCMV/CREB) were supplied by A. Leutz (Max-Delbrück-Centrum, Berlin, Germany) and Clontech (BD Biosciences), respectively. The mock plasmid pcDNA3 was from Invitrogen and pCMV was obtained by digestion with *Xho*I and subsequent religation.

Cell Culture, Transfection, and Luciferase Assay—THP-1 and HL-60 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and maintained in RPMI 1640 medium with 10% fetal calf serum certified for low endotoxin and free of mycoplasma (Biochrom, Berlin, Germany). Transfections were performed by electroporation using 10 μg of reporter gene plasmid and 5×10^6 cells/0.25 ml in 0.4-cm electroporation cuvettes with a gene pulser (Gene zapper 450/2500, IBI, Cambridge, United Kingdom) at 1200 microfarads, 100 ohm, and 300 V. Transfection efficiencies were found to be similar in THP-1 and HL-60 cells as determined by the expression of the green fluorescent protein (pEGFP-C1, Clontech). Dot blot analysis also showed comparable results for different reporter gene constructs. In co-transfection experiments, 5 μg of expression plasmid were used unless stated otherwise. HL-60 cells transiently transfected with 5 μg of pMSV/EBPα were designated as HL-60/EBPα. To account for possible promoter interference of reporter gene and expression plasmid and to equalize DNA concentrations, the reaction mixture in the transfection cuvette was topped up with mock plasmid. After electroporation, 1×10^6 cells/ml/well were cultured in the absence or presence of 500 μM dibutyryl-cAMP (dbcAMP, Sigma) unless indicated otherwise. Cells were harvested 24 h after transfection and assayed for luciferase expression using the Promega luciferase assay kit and the Autolumat LB 953 (Berthold, Bad Wildbad, Germany). The expression level of C/EBPα in THP-1 and HL-60 was verified by Western blotting. Specific luciferase expression or promoter activity was expressed in arbitrary units (AU) and assessed by normalizing relative light units (raw data) for the protein content of the lysate measured by the Bradford protein assay (Sigma). The luciferase stimulation factor was calculated from the ratio of luciferase activity in dbcAMP-treated or co-transfected cells *versus* basal activity without dbcAMP treatment or transfected with the respective mock plasmid obtained in the same experimental setting. Conversely, the inhibition factor was evaluated as the ratio between the higher basal activity (AU with mock plasmid) and the reduced activity, resulting from expression of p30 or CREB133. An example for the calculation of the experimental results is given in Table II. Significance was estimated by the Man-Whitney-*U* test.

Computational Analysis of the IL-10 Promoter Region—Human and murine genomic sequences were retrieved from the University of California, Santa Cruz Genome Browser (genome.ucsc.edu) datasets from April 2002 and February 2002, respectively. GenBank™ accession numbers for the other sequences are AF120030 (*Marmota monax* genomic IL-10 sequence), BG538741 (human EST), NM_000572 (human mRNA), Z30175 (human IL-10 promoter), NM_010548 (murine mRNA), and AF012909 (*M. monax* mRNA). Pairwise comparative analyses were performed with the program DNA Block Aligner (DBA) (18). The DBA input files were masked with Repeatmasker (ftp.genome-.washington.edu/RM/RepeatMasker.html). The MatInspector Professional program (www.genomatix.de) was used to predict transcription factor binding sites (TFBS). All of the positions in the promoter/enhancer refer to the translation start site of IL-10.

RESULTS

C/EBPα and β Bind to Three Motifs in the IL-10 Promoter/Enhancer Region—In this study, we analyzed five putative C/EBP sites named C/EBP1 to C/EBP5 according to their position in the enhancer region upstream of the human IL-10 promoter (Table I). Four of these motifs (C/EBP2–5) were newly predicted by the MatInspector Professional program with core similarity of 0.7 and optimized matrix similarity. One

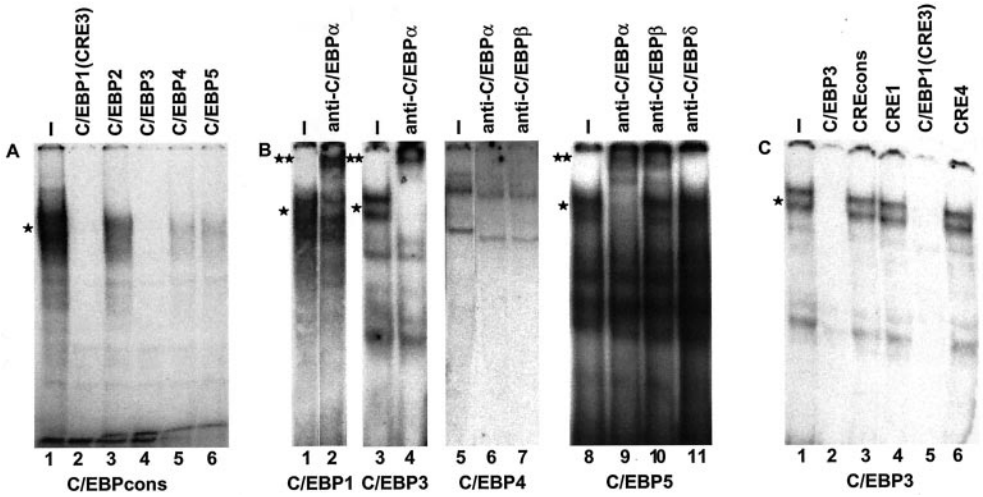


FIG. 1. **Protein binding to putative C/EBP motifs of the IL-10 promoter/enhancer.** Radiolabeled oligonucleotides as indicated at the *bottom* were incubated with nuclear extracts from THP-1 cells and subjected to competition with a 100-fold molar excess of unlabeled oligonucleotide (*panels A* and *C*) or supershifted with specific antiserum (*panel B*). Competitor oligonucleotides and antibodies are shown at the *top*. The shifted DNA·C/EBP protein complex is indicated by *one asterisk*, and the supershifted complex is indicated by *two asterisks*. The same binding patterns with C/EBP1, C/EBP3, and C/EBP5 oligonucleotides were distinguishable. Therefore, the reaction of C/EBPβ- and δ-specific antisera with C/EBP5 oligonucleotide is given as a representative example.

motif, C/EBP1, is identical to the previously analyzed CRE3 site, which was shown not to be involved in CREB/ATF-induced promoter activation (6). Oligonucleotides harboring presumptive C/EBP motifs (Table I) were initially examined for their ability to compete with the consensus sequence using THP-1 nuclear extracts that are known to contain considerable amounts of C/EBP proteins. Unlabeled oligonucleotides consisting of C/EBP1, 3, 4, and 5 competed efficiently with the C/EBP consensus oligonucleotide for C/EBP protein binding (Fig. 1*A*, *lanes 2, 4, 5*, and *6*), whereas C/EBP2 failed to compete effectively (*lane 3*). Supershift assays with labeled C/EBP1, 3, and 5 oligonucleotides revealed that DNA-protein complexes contained C/EBPα (Fig. 1*B*, *lane 2*, *4*, and *9*) and C/EBPβ but not C/EBPδ. Data obtained using C/EBP5 oligonucleotide are shown as a representative example for the reaction with antisera against C/EBPβ and δ (*lanes 10* and *11*). The proteins shifting the C/EBP4 oligonucleotide failed to react with C/EBPα or β antiserum (Fig. 1*B*, *lanes 6* and *7*). Since we found that the C/EBP1 motif, which is identical to the previously analyzed CRE3 site binds C/EBPα and β, we wanted to exclude this possibility for the other CRE motifs. Competition experiments revealed that protein binding to labeled C/EBP3 does not interfere with unlabeled CRE consensus, CRE1 or CRE4 oligonucleotides (Fig. 1*D*, *lanes 3*, *4*, and *6*). Supershift analysis confirmed that protein complexes bound to CRE1 and CRE4 oligonucleotides do not contain C/EBPα or β (data not shown). The location of C/EBP binding sites in the 1308-bp IL-10 promoter/enhancer fragment is shown with respect to CRE motifs and the TATA box in Fig. 2.

cAMP-stimulated IL-10 Promoter Activation in THP-1 Cells Involves C/EBPα—To investigate the role of C/EBPα in cAMP-induced IL-10 promoter activation in THP-1 cells, we co-transfected increasing amounts of an expression plasmid for the 30-kDa truncated isoform of C/EBPα (pcDNA3/p30) along with the reporter plasmid pGL2-1308. This isoform acts as a partial C/EBPα antagonist by retaining its binding capacity but with significantly reduced trans-activation ability (19–21). Overexpression of p30 in THP-1 cells significantly reduced both the cAMP stimulation of the IL-10 promoter and its basal activity. The cAMP stimulation rates adjusted from the p30 inhibition of the basal promoter activity are shown in Fig. 3 (see also Table II). These results indicate that the binding of endogenous C/EBPα to the enhancer region and its trans-activation play a yet unknown role in constitutive and cAMP-stimulated IL-10 expression.

cAMP-stimulated IL-10 Promoter Activation in THP-1 Cells Depends on the Integrity of C/EBP Binding Sites—To understand the functional role of each individual C/EBP motif, we assessed the impact of C/EBP site mutations on cAMP-mediated IL-10 promoter activation in THP-1 cells. The oligonucleotides chosen for *in vitro* mutagenesis (Table I) were tested for loss of their C/EBP protein binding activity by EMSA (data not shown). In Fig. 4, *A* and *B*, the deleted or mutated promoter fragments are shown together with their residual promoter activity following cAMP-stimulation in THP-1 cells relative to the native fragment representing 100% activity. The mutation of C/EBP5, which lies closest to the translation start point, decreased cAMP-responsiveness to 22%, whereas the mutation of C/EBP3 and C/EBP1 reduced promoter activity to 42 and 57%, respectively. Subsequent experiments with the triple mutant pGL2-mutC/EBP1,3,5 revealed no further reduction compared with pGL2-mutC/EBP5. The truncated promoter/enhancer fragment comprising 376 bp upstream from the translation start point and lacking CRE and C/EBP motifs with the exception of C/EBP5 still showed 48% residual activity. Abolition of C/EBP5 in a deletion mutant of similar length (pGL2-*Avr*IImutC/EBP5 comprising 397 bp) reduced cAMP responsiveness further to 17%. These results emphasize a major functional role for the C/EBP5 motif in the trans-activation of the IL-10 gene by C/EBPα and β. As outlined in *panel C*, the mutation of C/EBP motifs also reduced the basal promoter activity in THP-1 cells, suggesting that endogenous C/EBP proteins are involved in stimulated as well as in basal activity. Finally, as shown in Fig. 5, cAMP-induced promoter activity in THP-1 cells was almost completely abolished (residual activity 12%) when the binding of both C/EBPα and β as well as CREB/ATF was prevented by co-transfection of pGL2-mutCRE1,4 and pcDNA3/p30. Overexpression of p30 inhibited the cAMP effect on promoter activity of pGL2-1308 to the same degree as the destruction of all three C/EBP motifs (25 and 23% residual activity), but as expected, it had no effect on the activity of pGL2-mutC/EBP1,3,5 (Fig. 5).

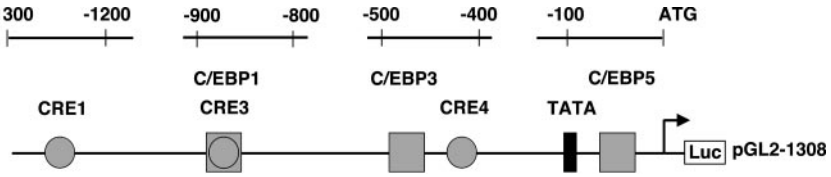


FIG. 2. **1308-bp fragment of the IL-10 promoter/enhancer region as cloned in pGL2-basic.** The C/EBP and CRE motifs within the IL-10 promoter/enhancer region are delineated in relation to the TATA box (*black box*) and the IL-10 translation start (*arrow*) point. *Gray circles* and *squares* represent CRE and C/EBP motifs, respectively.

FIG. 3. **Influence of p30 overexpression on basal and cAMP-stimulated IL-10 promoter activity in THP-1 cells.** Cells were co-transfected with pGL2−1308 along with increasing amounts of pcDNA3/p30 and incubated in the absence or presence of dbcAMP. Positive values indicate fold stimulation of the basal promoter activity obtained with the mock plasmid or the respective concentration of pcDNA/p30 by cAMP. Negative values represent the ratio (inhibition factor) between the basal and the reduced activity obtained with the mock plasmid and pcDNA/p30 in the absence of cAMP. Mean values ± S.D. of three independent experiments are shown.

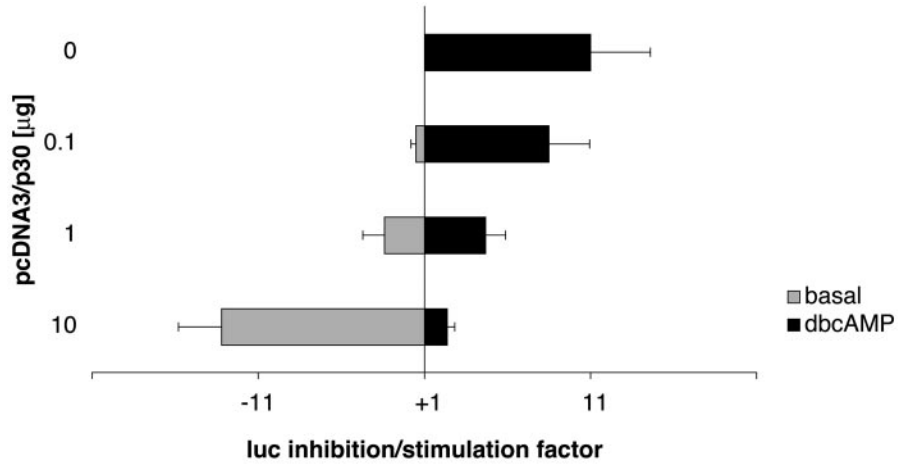


TABLE II
Inhibition of basal and cAMP-stimulated promoter activity in THP-1 cells by p30

Results and the calculation of one typical co-transfection experiment using 10 μg of pGL2–1308 and 1 μg of p30 expression plasmid or the respective mock plasmid and 500 μM cAMP are shown. SF-stimulation factor is the ratio between promoter activity achieved by addition of cAMP and the basal activity obtained with the respective plasmid. IF-inhibition factor obtained by p30 expression is shown. SF in percent is determined on the premise that a factor of one means no stimulation (0%). Therefore, percentage from the reference in a given experiment is calculated from SF reduced by one. Basal activity in percent is calculated directly from arbitrary units.

Plasmid	AU basal	AU cAMP	SF	SF	Basal
			fold	*%*	*%*
pcDNA3	122	1492	12,2	100	100
pcDNA3/p30	45	235	5,2	38	37
IF	2.7-fold	6.3-fold			

C/EBPα Influences Constitutive IL-10 Promoter Activity in HL-60 and THP-1 Cells—We recently showed that the promyelomonocytic cell line HL-60 contains significantly less endogenous C/EBPα compared with THP-1 cells (16). To study the influence of C/EBP on basal IL-10 promoter activity, we co-transfected pGL2-1308 along with increasing amounts of pMSV/EBPα in HL-60 cells. The data revealed that the expression of C/EBPα enhanced IL-10 promoter activity in a concentration-dependent manner up to 17-fold (Fig. 6). In contrast, C/EBPα expression over and above the high endogenous C/EBPα levels in THP-1 cells only increased IL-10 transcription approximately 3-fold (data not shown). Similar results were obtained by overexpression of C/EBPβ (data not shown). The respective mock plasmid had no significant effect, indicating that the observed promoter activation resulted from C/EBP expression and was not the result of promoter interference of co-transfected plasmids. To examine the role of constitutively phosphorylated endogenous CREB in the C/EBP-mediated promoter activation, we co-transfected the expression plasmids for C/EBPα and CREB133 in HL-60 cells. This dominant negative CREB comprises a serine-to-alanine mutation, is therefore unable to be phosphorylated, and thus known to almost completely inhibit CREB-dependent trans-activation (22, 23). As shown in Fig. 7, the C/EBP-mediated IL-10 promoter activation was significantly impaired but not completely prevented by CREB133. Considering the reduced basal activity in the presence of CREB133, the genuine C/EBPα stimulation rate decreased from 9.6 (994/103) to 5.6 (208/37). The inhibitory effect of CREB133 was dependent on the molar concentration ratio of both co-transfected expression plasmids (data not shown). These data suggest that the phosphorylation of CREB is involved in basal as well as in C/EBPα-mediated promoter activation in HL-60 cells. To verify the role of C/EBPα in constitutive IL-10 promoter activity in HL-60 and THP-1 cells, we again co-transfected the p30 expression plasmid. As expected, p30 had a relatively minor influence on the basal promoter activity in original HL-60 cells because of their low endogenous C/EBP expression level. On the other hand, p30 significantly reduced constitutive IL-10 transcription in THP-1 cells in a manner comparable to HL-60/EBPα cells transiently expressing exogenous C/EBPα (Fig. 8). These results underline the role of C/EBPα in constitutive IL-10 expression.

C/EBPα-dependent Constitutive IL-10 Promoter Activity Is Mediated Mainly through C/EBP5—Our aim was to evaluate the role of each individual C/EBP motif in C/EBPα-dependent constitutive IL-10 expression. For this purpose, we used HL-60/EBPα cells because of the direct correlation between C/EBP expression and promoter activity. C/EBP5 had the greatest impact on C/EBP-dependent promoter stimulation, since its mutation nearly abolished promoter activity (residual activity 7%) while the mutation of C/EBP3 and C/EBP1 reduced it only to 38 and 56%, respectively (Fig. 4, *panel D*). Similar to cAMP-stimulated THP-1 cells, no further reduction was seen with the triple mutant pGL2-mutC/EBP1,3,5 compared with pGL2-mutC/EBP5 in HL-60/EBPα cells. The residual activity of the truncated promoter/enhancer fragment (pGL2-376) in HL-60/EBPα cells can be attributed to the effect of C/EBP5, because its mutation almost eliminated promoter activity. This confirms the essential role of C/EBP5 in the trans-activation of the IL-10 gene.

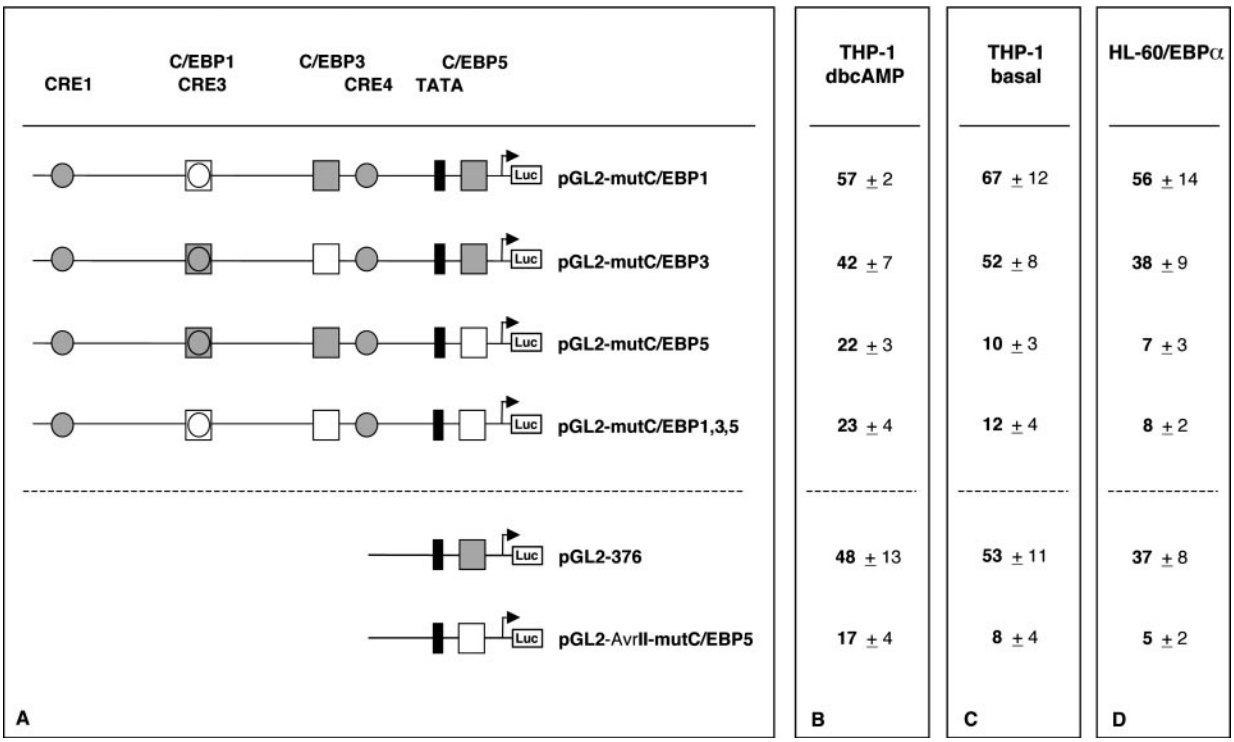
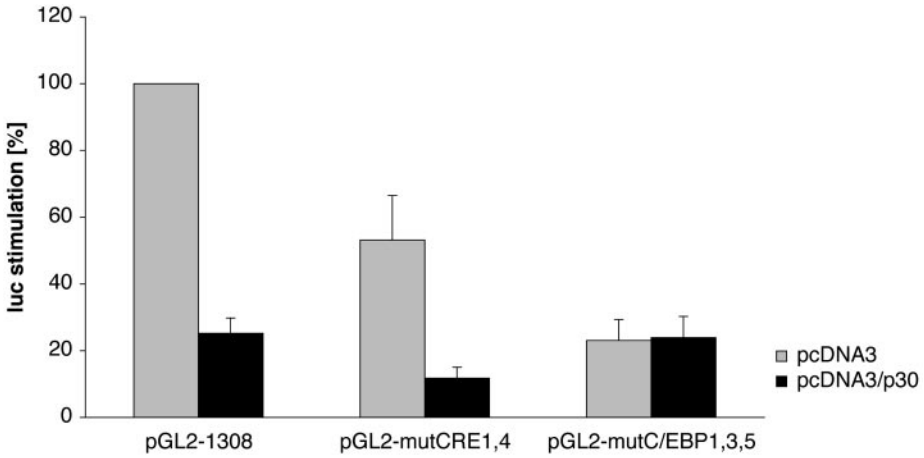


FIG. 4. **Involvement of C/EBP binding sites in basal and cAMP-stimulated IL-10 promoter activity in THP-1 cells and in C/EBPα-transfected HL-60 cells (HL-60/EBPα).** Luciferase reporter gene plasmids containing mutated or deleted promoter fragments (*A*) were transfected into THP-1 and into HL-60/EBPα cells. THP-1 cells were incubated in the presence or absence of dbcAMP. Promoter activity of the mutants is expressed as the percentage of cAMP-stimulated (*B*) and of basal (*C*) luciferase expression in THP-1 cells or as a fraction of C/EBPα-mediated luciferase expression obtained in HL-60/EBPα cells (*D*) with pGL2-1308 (representing 100%). Mean values ± S.D. of ten experiments are presented along. *Gray circles* and *squares* represent CRE and C/EBP motifs, respectively; *blank symbols* indicate mutated ones.

FIG. 5. **cAMP-induced IL-10 promoter activation is prevented by double mutation of CRE1 and CRE4 motifs and simultaneous overexpression of p30 in THP-1 cells.** Cells transfected with pGL2-1308, pGL2-mutCRE1,4, or pGL2-mutC/EBP1,3,5 were either co-transfected along with pcDNA3/p30 or pcDNA3 and incubated in the presence of dbcAMP. Promoter activity is expressed as a percentage of cAMP-stimulated luciferase expression obtained with pGL2−1308 and pcDNA3. Results (mean values ± S.D.) of at least five independent experiments are shown.



Efficient IL-10 Promoter Activation by cAMP Depends on C/EBPα—From p30 overexpression experiments and functional analysis of C/EBP site mutations, we know that C/EBPα has a significant effect on cAMP stimulation in THP-1 cells. We now wanted to investigate whether promoter responsiveness is impaired in HL-60 cells because of the low C/EBP protein content in this cell line. In fact, comparing the cAMP responsiveness of the IL-10 promoter in both cell lines, we found that the elevation of intracellular cAMP levels had only a small effect in HL-60 cells, whereas in THP-1 cells, elevated cAMP levels induced a steep rise in promoter activity (Fig. 9). As already shown in Fig. 6, promoter activity was substantially increased, supplying HL-60 cells with exogenous C/EBPα. Starting from a higher level of constitutive promoter activity, it could be further stimulated by cAMP in HL-60/EBPα cells up to the same level as that achieved in THP-1 cells expressing endogenous C/EBPα (Fig. 9). Even though the cAMP stimulation rate remains nearly unchanged in HL-60 and HL-60/EBPα cells, together, our data indicate that for maximal promoter activity, both the presence of C/EBP protein and the phosphorylation of CREB are necessary.

Comparative Analysis of the IL-10 Promoter/Enhancer Region—The transcription start site of the human IL-10 gene has not yet been identified experimentally, but a human EST and 5′ ends from mRNA of other species (*Sus scrofa* L20001, *Ovis aries* Z29362, and *Trichosurus vulpecula* AF026277) suggest that the transcription start site lies at position −59. Three IL-10 sequences were analyzed: human (*hs*), murine (*mm*), and the *M. monax* (*ma*) promoter/enhancer. All of the three sequences contain a TATA-box located at similar distances from the ATG: *hs* −89; *mm* −87; and *ma* −86. Pairwise comparison with DBA revealed two blocks of similarity that appeared in all of the three pairs: Block A at approximately −500 to −300 bp and Block B from −100 bp to the ATG start codon containing

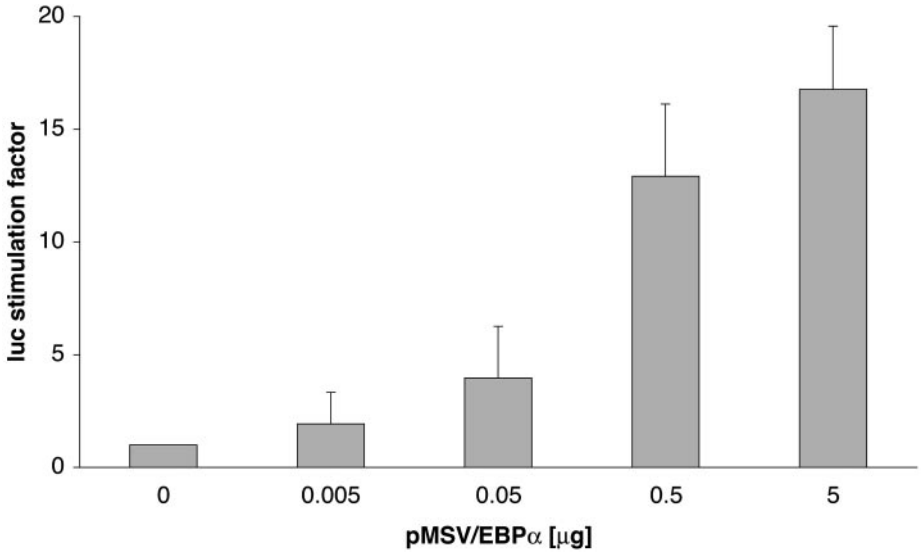


FIG. 6. **Effect of exogenous C/EBPα expression on basal IL-10 promoter activity in HL-60 cells.** HL-60 cells were co-transfected with pGL2–1308 along with increasing amounts of pMSV/EBPα. Promoter activity obtained by supplying exogenous C/EBPα is expressed as multiples of the basal luciferase expression level in the presence of the mock plasmid. Results of at least three independent experiments are displayed.

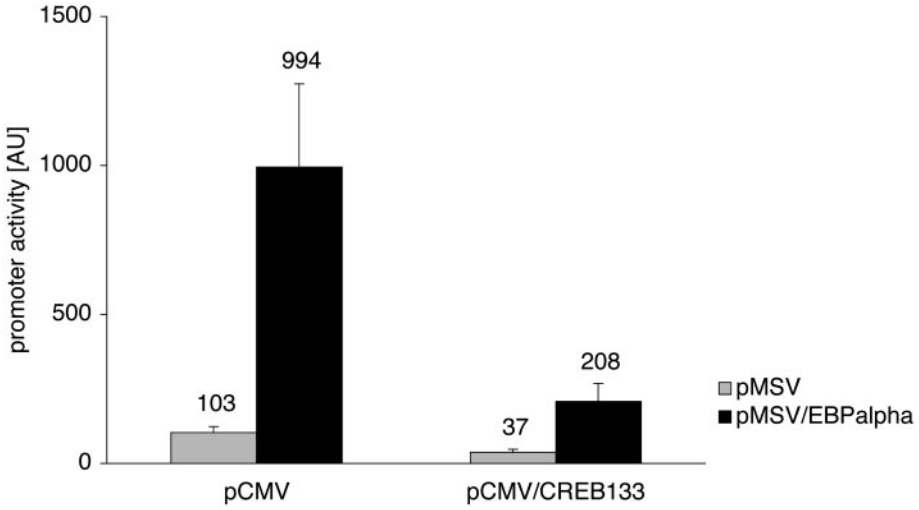


FIG. 7. **Effect of exogenous C/EBPα expression is inhibited by CREB133.** Promoter activity (AU) from HL-60 cells transfected with pGL2−1308 and 5 μg of pMSV/EBPα or pMSV and additionally co-transfected with 5 μg of pCMV/CREB133 or pCMV is shown (mean values ± S.D. of seven independent experiments).

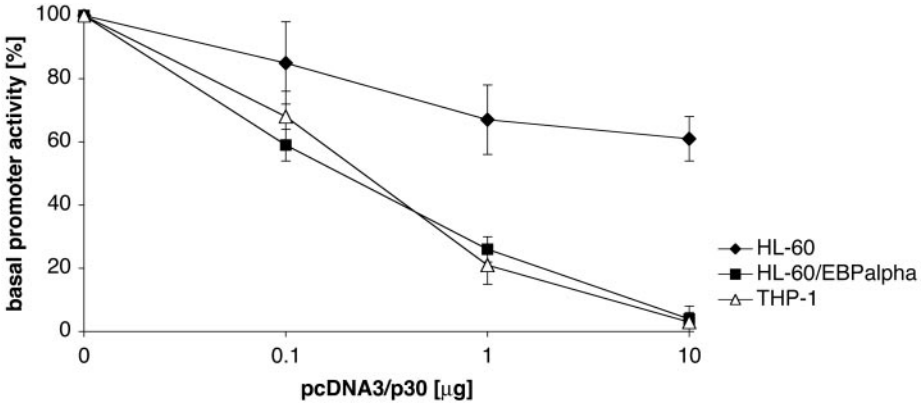


FIG. 8. **Influence of p30 overexpression on basal IL-10 promoter activity in THP-1, HL-60, and HL-60/EBPα cells.** pGL2−1308 was co-transfected into THP-1, HL-60, or HL-60/EBPα cells along with increasing amounts of pcDNA3/p30. Basal promoter activity is expressed as a percentage of the relative luciferase light units obtained with pGL2−1308 and pcDNA3 normalized for protein content. The mean values ± S.D. of at least three experiments are shown.

the TATA-box. These conserved sequences were analyzed by the MatInspector Professional program (core similarity 0.95, optimized matrix). Block A in human, mouse, and *M. marmota* contains one C/EBP binding site (binding site matrix CEBP.01 in *hs* at −439 to −452, in *mm* at −450 to −463, and in *ma* at −422 to −435) and one CREB binding site (binding site matrix ATF.01 in *hs* at −401 to −414, CREB.01 in *mm* at −415 to −422, and CREB.04 in *ma* at −385 to −396), corresponding to the C/EBP3 and CRE4 motifs analyzed in the human IL-10 promoter (Fig. 10). In Block B, another C/EPB site (C/EBP5 in the human sequence) was identified: in *hs* at −30 to −43 and in *ma* at −39 to −52 (both matched the C/EPB.01 matrix). Initially, in the murine sequence, this matrix was not detected, but after lowering the search parameters, the equivalent sequence was found at positions −39 to −52 (Fig. 10). The quality of local alignments (data not shown) and TFBS matrix similarity suggest a closer relationship of the human and the *M. monax* IL-10 gene *versus* human and mouse or mouse and *M. monax*.

DISCUSSION

With this study, we add new results to our previous findings concerning the important role of cAMP and cAMP-inducing

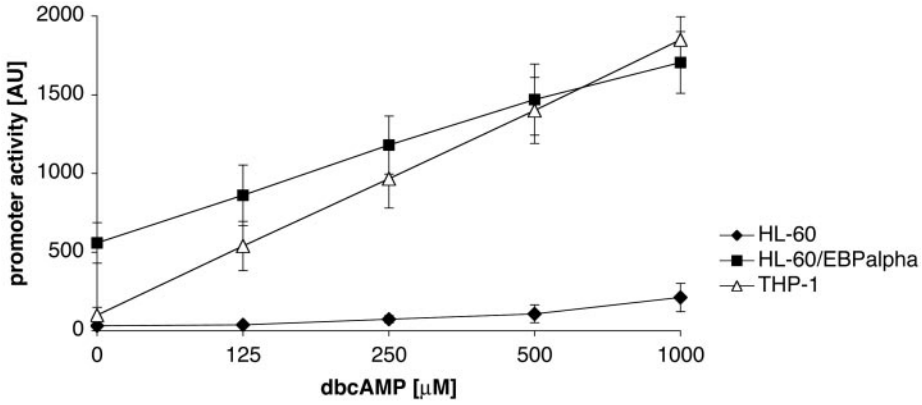


FIG. 9. **The level of cAMP-stimulated IL-10 promoter activity depends on the expression of C/EBPα.** pGL2−1308 was transfected into THP-1, HL-60, or HL-60/EBPα cells and incubated with increasing concentrations of dbcAMP. Promoter activity is expressed in AU assessed by normalizing relative luciferase light units for protein content. The *graph* represents results of ten independent experiments.

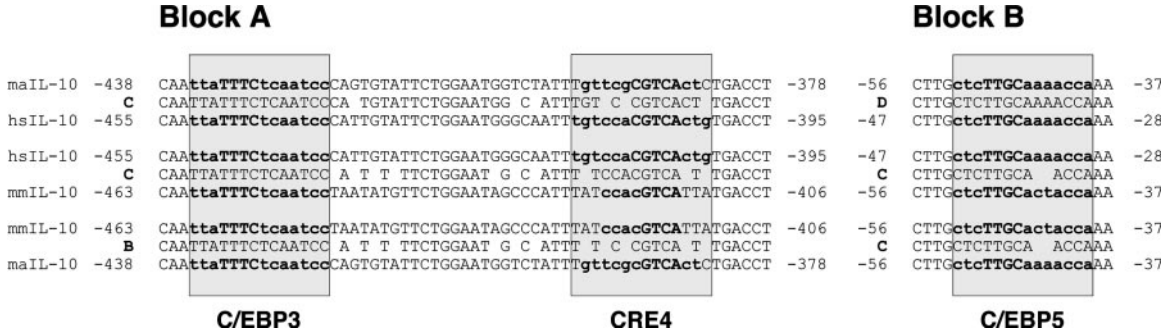


FIG. 10. **Localization of DBA blocks and the predicted TFBS in the IL-10 promoter region.** 1.5 kb upstream of the translation start codon of *Homo sapiens* (hsIL-10), *Mus musculus* (mmIL-10) and *M. monax* (maIL-10) were compared with the program DBA. The categories *B*, *C*, and *D* represent whole DBA block sequence identity (*B*, 70–80%; *C*, 80–90%; *D*, 90–100%). *Boldface letters*, nucleotides belonging to the whole TFBS region (*gray boxes*) identified by MatInspector; *boldface capitalized letters*, nucleotides of the TFBS core sequence defined by Genomatix. Only the parts of the blocks with the candidate TFBS are shown.

agents in the trans-activation of the human IL-10 gene in monocytic cells. Here, we show that the binding of the transcription factors C/EBPα and β to three motifs in the IL-10 promoter/enhancer region contributes to basal activity of the IL-10 promoter and is essential for maximal cAMP stimulation in differentiated monocytic cells. Each of the individual C/EBP motifs appeared to have a different impact on the trans-activation of the IL-10 gene. The most important effector site seems to be C/EBP5. Because of the proximity of C/EBP5 to the TATA box, an interaction of C/EBPα bound to this motif with the basal transcription-initiation complex can be envisaged (24, 25). Conversely, this model explains the comparatively high trans-activating capacity of the 376-bp fragment lacking CRE1, CRE4, C/EBP1, and C/EBP3 sites but retaining C/EBP5. The C/EBP3 motif, close to the main CRE4 site, had a moderate influence, whereas C/EBP1 seems to be less important. C/EBP1 coincides with the CRE3 site that failed to bind CREB/ATF (6) despite its greater sequence similarity to the CRE rather than to the C/EBP consensus motif. The binding of C/EBP to nonconsensus CRE sites and the functional substitution of CREB by C/EBP family members have been described previously (7, 26). It is not surprising that the functionally relevant motifs C/EBP3, CRE4, and C/EBP5 fall into regions that are evolutionary conserved between human, mouse, and *M. monax*, while the less important C/EBP1 and CRE1 sites are not conserved. These findings together with the fact that plasmids with single site mutation of C/EBP3 and CRE4 (6) retained similar cAMP-responsiveness as pGL2-376, bearing only the intact C/EBP5 motif hint at C/EBP-CREB interaction at multiple sites in a hierarchical and cooperative manner. We assume that the function of C/EBP3 requires prior occupation of the downstream C/EBP5 motif and the proximal CRE4 site, which implicates potential DNA looping and/or co-activator protein binding (19, 25, 27).

To study the role of C/EBPα in basal and cAMP-stimulated promoter activation, we used the truncated 30-kDa C/EBPα isoform (p30), which bears a conserved binding domain but lacks N-terminal regions required for its trans-activation and differentiation potential (19–21). Overexpressed p30 reduced the cAMP stimulation rate beyond the inhibition of basal activity and abolished the remaining promoter responsiveness of the double CRE mutant by competition with endogenous p42 C/EBPα in THP-1 cells. Data obtained from structure/function analysis of C/EBPα provide evidence that the mechanisms of constitutive and cAMP-inducible trans-activation are different and involve distinct albeit overlapping domains in the N-terminal portion of the protein (amino acids 55–86 and 52–124, respectively), which are both deleted in p30 (9, 19, 28, 29). The cAMP-mediated promoter stimulation was consistently stronger in THP-1 cells than in HL-60 cells, in agreement with their higher differentiation status and endogenous C/EBPα/β expression levels. When supplementing HL-60 cells with exogenous C/EBPα, we found that basal/constitutive promoter activity rose in a concentration-dependent manner and the cAMP response in HL-60/EBPα cells reached similar levels as in THP-1 cells. However, given the substantially higher basal level, the cAMP stimulation rate seems to be independent of C/EBPα overexpression. The reason for this remains unclear. We suggest that the increasing stimulation of the basal activity in THP-1 cells in response to rising cAMP concentrations involves enhanced expression, trans-location, and the binding of C/EBPα/β as well as phosphorylation of CREB, C/EBP and cofactors, and their interaction (11, 30–32). The model of transient overexpression of C/EBPα in HL-60 cells can only partially reproduce these processes. Co-transfection experiments with the dominant negative inhibitor of CREB in HL-60/EBPα cells revealed that trans-activation by C/EBPα to some extent depends on phosphorylated CREB being constitutively present

in low amounts. Beyond this, C/EBPα overexpression seems to up-regulate the IL-10 promoter activity by itself. Taken together, our data from the mutation and co-expression experiments with p30 and CREB133 indicate a co-operative interaction of CREB and C/EBP transcription factors both in basal and in cAMP-stimulated promoter activation. In contrast to CREB, the cAMP-inducible domain of C/EBPα lacks a PKA phosphorylation site; thus, the mechanism of its activation by cAMP is yet unclear (33). It has been suggested that a co-activator could interact with the cAMP-inducible domain of C/EBP transcription factors only after it itself has been phosphorylated by PKA. This model is similar to that of CREB activation recruiting CREB-binding protein (CBP)/p300 after PKA phosphorylation with the exception that phosphorylation of the co-activator rather than the DNA-binding protein would regulate the interaction (9, 28, 31). In addition, CBP/p300 is a possible mediator between CREB and C/EBP. Indeed, CBP/p300 comprises a binding domain for C/EBPα/β (amino acids 1752–1859), which is distinct from its CREB binding domain (amino acids 552–660) (34). On the basis of our experimental data, we conclude that CREB/ATF mediates a relatively low activating potential in the absence of C/EBPα/β proteins, which are necessary for maximal IL-10 promoter activation. In our view, multiple enhancer binding proteins need to be available simultaneously in differentiated monocytes to achieve stable binding site occupancy required for maximally activated IL-10 transcription. This suggestion is in agreement with the observation that C/EBPα expression is tissue-specific and varies during differentiation, while CREB/ATF is ubiquitously expressed (35, 36). Therefore, C/EBP-mediated transcription of a gene, in particular IL-10, is regulated in a tissue-specific manner and depends on the state of differentiation of a given cell. Experimental data from a mouse model, which revealed immediate IL-10 production in response to lipopolysaccharide and cAMP-elevating agents by hepatocytes known to contain high amounts of C/EBPα, support this hypothesis (37, 38).

In contrast to pro-inflammatory cytokines, which are mainly regulated by transcription factors such as NFkB and activating protein-1, the transcription of the prototypic anti-inflammatory cytokine IL-10 in monocytic cells, as shown here, is activated by cAMP or cAMP-inducing agents, *e.g.* catecholamines during sympathetic activation through transcription factors C/EBP and CREB. The mechanism of their interaction remains to be investigated.

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REFERENCES

1. Woiciechowsky, C., Asadullah, K., Nestler, D., Eberhardt, B., Platzer, C., Schoning, B., Glockner, F., Lanksch, W. R., Volk, H. D., and Döcke, W. D. (1998) *Nat. Med.* **4,** 808–813
2. Ziegler-Heitbrock, H. W. (1995) *J. Inflamm.* **45,** 13–26
3. Ma, W., Lim, W., Gee, K., Aucoin, S., Nandan, D., Kozlowski, M., Diaz-Mitoma, F., and Kumar, A. (2001) *J. Biol. Chem.* **276,** 13664–13674
4. Benkhart, E. M., Siedlar, M., Wedel, A., Werner, T., and Ziegler-Heitbrock, H. W. (2000) *J. Immunol.* **165,** 1612–1617
5. Platzer, C., Döcke, W., Volk, H., and Prösch, S. (2000) *J. Neuroimmunol.* **105,** 31–38
6. Platzer, C., Fritsch, E., Elsner, T., Lehmann, M. H., Volk, H. D., and Prösch, S. (1999) *Eur. J. Immunol.* **29,** 3098–3104
7. Tsukada, J., Saito, K., Waterman, W. R., Webb, A. C., and Auron, P. E. (1994) *Mol. Cell. Biol.* **14,** 7285–7297
8. Grassl, C., Luckow, B., Schlondorff, D., and Dendorfer, U. (1999) *J. Am. Soc. Nephrol.* **10,** 1466–1477
9. Wilson, H. L., and Roesler, W. J. (2002) *Mol. Cell. Endocrinol.* **188,** 15–20
10. Akira, S., and Kishimoto, T. (1997) *Adv. Immunol.* **65,** 1–46
11. Berrier, A., Siu, G., and Calame, K. (1998) *J. Immunol.* **161,** 2267–2275
12. Vinson, C. R., Sigler, P. B., and McKnight, S. L. (1989) *Science* **246,** 911–916
13. Umek, R. M., Friedman, A. D., and McKnight, S. L. (1991) *Science* **251,** 288–292
14. Scott, L. M., Civin, C. I., Rorth, P., and Friedman, A. D. (1992) *Blood* **80,** 1725–1735
15. Tenen, D. G., Hromas, R., Licht, J. D., and Zhang, D. E. (1997) *Blood* **90,** 489–519
16. Prösch, S., Heine, A. K., Volk, H. D., and Krüger, D. H. (2001) *J. Biol. Chem.* **276,** 40712–40720
17. Prösch, S., Staak, K., Stein, J., Liebenthal, C., Stamminger, T., Volk, H. D., and Krüger, D. H. (1995) *Virology.* **208,** 197–206
18. Jareborg, N., Birney, E., and Durbin, R. (1999) *Genome Res.* **9,** 815–824
19. Erickson, R. L., Hemati, N., Ross, S. E., and MacDougald, O. A. (2001) *J. Biol. Chem.* **276,** 16348–16355
20. Lekstrom-Himes, J., and Xanthopoulos, K. G. (1998) *J. Biol. Chem.* **273,** 28545–28548
21. Lin, F. T., MacDougald, O. A., Diehl, A. M., and Lane, M. D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9606–9610
22. Alberts, A. S., Arias, J., Hagiwara, M., Montminy, M. R., and Feramisco, J. R. (1994) *J. Biol. Chem.* **269,** 7623–7630
23. Roesler, W. J., Graham J. G., Kolen, R., Klemm, D. J., and McFie, P. J. (1995) *J. Biol. Chem.* **270,** 8225–8232
24. Pedersen, T. A., Kowenz-Leutz, E., Leutz, A., and Nerlov, C. (2001) *Genes Dev.* **15,** 3208–3216
25. Nerlov, C., and Ziff, E. B. (1995) *EMBO J.* **14,** 4318–4328
26. Bakker, O., and Parker, M. G. (1991) *Nucleic Acids Res.* **19,** 1213–1217
27. Hernandez-Munain, C., Roberts, J. L., and Krangel, M. S. (1998) *Mol. Cell. Biol.* **18,** 3223–3233
28. Wilson, H. L., McFie, P. J., and Roesler, W. J. (2001) *Mol. Cell. Endocrinol.* **181,** 27–34
29. Roesler, W. J., Park, E. A., and McFie, P. J. (1998) *J. Biol. Chem.* **273,** 14950–14957
30. Metz, R., and Ziff, E. (1991) *Genes Dev.* **5,** 1754–1766
31. Tae, H. J., Zhang, S., and Kim, K. H. (1995) *J. Biol. Chem.* **270,** 21487–21494
32. Roesler, W. J. (2001) *Annu. Rev. Nutr.* **21,** 141–165
33. Mahoney, C. W., Shuman, J., McKnight, S. L., Chen, H. C., and Huang, K. P. (1992) *J. Biol. Chem.* **267,** 19396–19403
34. Mink, S., Haenig, B., and Klempnauer, K. H. (1997) *Mol. Cell. Biol.* **17,** 6609–6617
35. Roesler, W. J. (2000) *Mol. Cell. Endocrinol.* **162,** 1–7
36. Sieweke, M. H., and Graf, T. (1998) *Curr. Opin. Genet. Dev.* **8,** 545–551
37. Jilg, S., Barsig, J., Leist, M., Kusters, S., Volk, H. D., and Wendel, A. (1996) *J. Pharmacol. Exp. Ther.* **278,** 421–431
38. Schrem, H., Klempnauer, J., and Borlak, J. (2002) *Pharmacol. Rev.* **54,** 129–158