CELL STRUCTURE AND FUNCTION 23: 109–118 (1998)

Transcriptional Regulation by C/EBPα and -β in the Expression of the Gene for the MRP14 Myeloid Calcium Binding Protein

Ryoko Kuruto-Niwa[1]*, Michi Nakamura[1], Keiichi Takeishi[2], and Ryushi Nozawa[1]

[1]Laboratory of Microbiology and Host Defenses and [2]Laboratory of Genetic Engineering, School of Food and Nutritional Sciences, University of Shizuoka, Yada, Shizuoka 422–8526, Japan

Key words: C/EBPα/C/EBPβ/MRP14/HL-60 cells/1,25-dihydroxyvitamin D_3/THP-1 cells

ABSTRACT. **Transcriptional regulation of the gene for the myeloid calcium binding protein, MRP14, was investigated in human monocytic leukemia cell lines. The MRP14 gene was not expressed in monoblastic ML-1 cells, promonocytic U-937 cells, or promyelocytic HL-60 cells. On the other hand, the gene was expressed in monocytic THP-1 cells and in the HL-60 cells treated with 1,25-dihydroxyvitamin D_3 (VD_3). The level of MRP14 in VD_3-treated HL-60 cells was two-fold higher than that in THP-1 cells. Among several known transcription factor binding motifs, nuclear protein(s) of VD_3-treated HL-60 cells and THP-1 cells bound to the CCAAT/enhancer binding protein (C/EBP)-binding motif that was located in the upstream region of the MRP14 gene (-81), as evidenced by the competitive gel mobility-shift assay. An antibody for C/EBPα supershifted the nucleoprotein complex in THP-1 cells but not in the VD_3-treated HL-60 cells, whereas an antibody for C/EBPβ blocked the formation of the complex with the nuclear factor of the HL-60 cells but not with that of THP-1 cells. An anti-C/EBPδ antibody had no effect on the complex in either cell. Thus, it was concluded that C/EBPα and -β were able to bind to the C/EBP motif, and that C/EBPα bound to the motif in THP-1 cells and C/EBPβ bound to that in the VD_3-treated HL-60 cells. Furthermore, to examine the transcriptional activity of the C/EBP motif, we transfected several constructed luciferase reporter DNAs into HL-60 cells and THP-1 cells. The luciferase activity of the C/EBP motif in HL-60 cells was increased by VD_3 treatment. The C/EBP motif in the MRP14 gene was confirmed to function as a regulatory region in VD_3-treated HL-60 cells and THP-1 cells by the assay. Since C/EBPβ was also detected in VD_3-untreated HL-60 cells by immunoblotting, VD_3 activated C/EBPβ to bind to the motif, probably through post-translational modification.**

Myeloid calcium binding proteins, MRP8 and MRP14 (25, 35), also designated cystic fibrosis antigen (5, 12), the leukocyte L1 molecule (4), the 60B8 antigen (34, 55), p8/p14 (17), calgranulin A/B (22), and calprotectin (51, 66), are members of the S100 protein family, whose genes are known to be localized in the cluster on human chromosome 1q21 (46). Therefore, it has been proposed that MRP8 and MRP14 be called S100A8 and S100A9, respectively (46). They are expressed in myeloid lineage cells (17, 34) and in certain epithelial cells (63, 68). MRP8 and MRP14 are not expressed in normal macrophages but are detected in macrophages that have infiltrated into inflammatory lesions (35, 40, 43, 68). Differential expression of MRP8 and MRP14 in such macrophages has been reported (11, 68), probably reflecting the clinical manifestation of inflammatory diseases. MRP proteins are also essential as a diagnostic marker being abundant in serum of patients with chronic inflammatory diseases such as cystic fibrosis (CF), rheumatoid arthritis (RA) and other connective tissue diseases (5, 14, 23) and with hematological disorders such as chronic myeloid leukemia (20). Furthermore, the protein was detected in feces of certain colon cancer patients (13, 42).

MRPs are induced in human promyelocytic leukemia HL-60 cells that have differentiated into macrophage-like cells by treatment with 1,25-dihydroxyvitamin D_3 (VD_3) (34, 55). The MRP8 and MRP14 mRNAs were co-transcribed starting one day after VD_3 treatment (23, 24). The mRNA level reached a peak on day two and then quickly declined. We have investigated how the expressions of MRP8 and MRP14 genes are co-expressed. Roth *et al.* suggested that a calcium-induced suppressor protein was responsible for suppression of MRP8 and MRP14 at the transcriptional level (44). In our previous study, differentia-

* To whom correspondence should be addressed.
Tel: +81–54–264–5554, Fax: +81–54–264–5553
E-mail: kuruto@fns1.u-shizuoka-ken.ac.jp

Abbreviations: VD_3, 1,25-dihydroxyvitamin D_3; C/EBP, CCAAT/enhancer binding protein; FBS, fetal bovine serum; GFP, green fluorescent protein; MAb, monoclonal antibody.

tion-specific binding of nuclear factors to the 5′-upstream regions of MRP8 and MRP14 genes were identified, together with consensus sequences for several known transcription factor binding motifs in these regions (24).

In this study we aimed to examine where the promoter elements in the upstream region of the MRP14 gene were located and what transcription factors bound to these elements. Two DNA fragments in the upstream region of the MRP14 gene were prepared, and factors that bound to these fragments were detected by the gel mobility-shift assay. A factor bound to the CCAAT/enhancer binding protein (C/EBP)-binding motif (-81) of a fragment, depending on the VD_3-induced differentiation. C/EBP family is a basic leucine zipper transcriptional factor and has several members (1, 6, 10, 26, 41). C/EBPα and -β are reported to recognize the same nucleotide sequence (1). We identified that the binding factor was C/EBPβ in VD_3-treated HL-60 cells, C/EBPα in human monocytic leukemia THP-1 cells, which constitutively expressed MRP proteins.

MATERIALS AND METHODS

Cells and culture

Human promyelocytic HL-60, monocytic THP-1, promonocytic U-937, and monoblastic ML-1 leukemia cells were cultured in F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Bioserum, Australia) and 100 μg/ml each of sodium ampicillin and kanamycin sulfate at 37°C in a 5% CO_2 incubator. The HL-60 cells (2×10^5 cells per ml) were treated with 10^{-7} M VD_3 (Chugai Pharmaceutical Co. Ltd., Tokyo) for up to 6 days with the medium renewed every 3 days. THP-1 and U-937 cells were obtained from the Japanese Cancer Research Resources Bank.

Human peripheral blood monocytes and neutrophils were purified as described previously (34).

Northern blot hybridization

Total cellular RNA was extracted from cells by the guanidinium/cesium chloride method (45). Northern blot hybridization was performed using ^{32}P-labeled MRP8, MRP14, and β-actin cDNA probes (23, 24).

Gel mobility-shift analysis

The 5′-upstream region of the MRP14 gene (sequences from −1,000 to +50) was amplified by PCR, using a sense primer; 5′-ATCACTGTGGAGTAGGGGAA-3′ and an antisense primer; 5′-GGGAAGCTGGCAGCTCACTT-3′ (24). The amplified MRP14 gene fragment was then cleaved with Sac I and Hae II (Takara Biomedicals, Kyoto) into 251 bp (−400 to −150) and 199 bp (−149 to +50) fragments, which are designated f251 and f199, respectively (Fig. 4A). The fragments were further purified by electrophoresis on DEAE cellulose membrane (S&S, Dassel, F.R.G.) (45) and used as probes for the gel mobility-shift analysis.

Oligonucleotides containing IRF-1 (half-site), Sp1, or C/EBP element (Table I) and each complementary oligomer were synthesized and annealed by heating both oligonucleotides at 90°C for 2 min and then at 70°C for 10 min followed by cooling to room temperature. These DNA fragments were used as competitors for the gel mobility-shift analysis.

Nuclear extracts were prepared from cultured HL-60 and THP-1 cells according to the method of Schreiber *et al.* (47). The DNA fragments were end-labeled with [γ-^{32}P] ATP (DuPont, NEN, F.R.G.) by T4 polynucleotide kinase (Takara Biomedicals, Kyoto) and purified by polyacrylamide gel electrophoresis. Gel mobility-shift analysis was performed as described previously (24). Competition experiments were performed by adding excess amounts of synthetic oligonucleotides or herring sperm DNA for 10 min prior to addition of the labeled probe. For supershift experiments, 1 μl of polyclonal rabbit antibody specific to C/EBPα, -β, or -δ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that specifically reacts with human C/EBPα, -β, or -δ, respectively, and that is non cross-reactive with other members of C/EBP family (supplier's manual), was added to the reaction mixture for 1 hour at 4°C before the addition of the probe. As a control, the heated (10 min) anti-C/EBPβ antibody or normal rabbit serum (1:100 dilution) was added. The reaction products were then analyzed by 4% polyacrylamide gel electrophoresis. The radiographic image was analyzed by a BAS2000 imaging analyzer (Fuji Film Co. Ltd., Tokyo, Japan) or autoradiographs.

Plasmids

For the construction of the green fluorescent protein (GFP) expression vector, the Pst I-Sal I sGFP(S65T) fragment (0.7 kb) was inserted between the Pst I site and Xho I site of the πH3M plasmid containing the chimeric CMV/HIV promoter

Table I. OLIGONUCLEOTIDES USED FOR THE COMPETITION ASSAY[a)]

oligonucleotide	motif	location in the MRP14 gene
5′-ATAGAAGGGAAATGAACTAA-3′	IRF-1 (half site)	from −235 to −216
5′-CTGTGAAGCAATCTTCCGGA-3′	C/EBP	from −88 to −69
5′-TTTCCTGCCCCGCCCCAGCC-3′	Sp1	from −139 to −120

[a)] The 20-mer oligonucleotides each containing a transcription factor-binding element (underlined) were synthesized. The sequence of the MRP14 gene refers to Lagasse and Clerc (25).

Table II. OLIGONUCLEOTIDES USED FOR THE C/EBP BINDING MOTIF

	Sequence[a]
Wild type	GATCTGTGAAGCAATCTTCCG ACACTTCGTTAGAAGGCCTAG
Mutant	GATCTGTGAAG**AA**CTCTTCCG ACACTTC**TT**GAGAAGGCCTAG

[a] The mutated substitutions are represented in boldface.

(3, 9, 16). This plasmid was designated πH3M-GFP (4.2 kb).

The πH3M-luc plasmid (5.4 kb) was constructed by insertion of luciferase cDNA (1.9 kb) between the blunt-ended Pst I site and the Xho I site of the πH3M plasmid and was used as a positive control. pIGCAT-MRP14 was constructed by fusing the PCR-amplified MRP14 gene (−1,000 to +430) and pIGCAT (18). The blunt-ended Sal I-Bal II fragment including CAT cDNA and the SV40 polyadenylation site of pIGCAT-MRP14 was replaced by the blunt-ended Hind III-BamH I fragment (2.8 kb) of πH3M-luc, including luciferase cDNA, pSV2 splice and polyadenylation signals. The resultant plasmid was named p1000-luc (7.8 kb). pIG-luc was promoterless and had a defect in the p1000-luc region of the MRP14 gene, and was used as a negative control (6.4 kb). p73-luc plasmid included the region of the MRP14 gene from −73 to +430, which is the downstream region than the C/EBP binding motif. The p1000(-C/EBP)-luc plasmid, which was defective in the C/EBP binding motif, was constructed by inserting an upstream region of the C/EBP binding motif in the MRP14 gene (−1,000 to −89), which was amplified in a Thermo Processor (TAITEC) using a sense primer; 5′-ATAAAGCTTCTAGATCACTGTGGAGTAGG GGAA-3′ and an antisense primer; 5′-AAAGGATCCGTGA GCAGTGTGGTAATGCT-3′, into the p73-luc plasmid. Monomers and multimers (three copies) of the wild type and mutated C/EBP oligonucleotides (Table II) were fused to the p73-luc construct (Fig. 5).

Transfections

Cells were resuspended at 1×10^7/200 μl in serum-free F12 medium (pH 7.2 with NaOH). In some experiments, FBS was added to 10%. Twenty-five micrograms of luc plasmid DNA, 10 μg of πH3M-β-gal as an internal control, and 15 μg of herring sperm DNA in 50 μl of 1/10 TE were added to the cell suspension in a 0.4-cm electroporation cuvette (BioRad). The cells and plasmids were then preincubated for 10 min at room temperature. Transfection of THP-1 cells was carried out by electroporation with a BioRad gene pulser at 300 V and 250 μF. Transfection of HL-60 cells was conducted at 300 V and 500 μF. Cuvettes were allowed to stand for 10 min at room temperature after transfection. The cells were then transferred to 10 ml of F12 containing 10% FBS and incubated at 37°C in a CO_2 incubator.

Luciferase and β-galactosidase assays

Twenty-four (for HL-60 cells) or forty-eight hours (for THP-1 cells) after transfection, cells were harvested and washed twice with phosphate-buffered saline (PBS). The cells were then lysed in lysis solution (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100, 1 mM dithiothreitol) and the luciferase activity was assayed using a PicaGene kit (Toyo ink). β-galactosidase activity in cell extracts, which had been preheated at 48°C for 50 minutes to inactivate endogenous β-galactosidase activity (65), was measured by a Galacto-Light chemiluminescent reporter assay kit (Tropix, Inc.) in order to normalize the transfection efficiency in each experiment (21).

Western blot analysis

Nuclear extracts were prepared from HL-60, THP-1, U-937, and ML-1 cells and after being subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions, were electrotransfered to a PVDF membrane, Trans-Blot (BioRad). The membrane was treated with the anti-C/EBPβ or -α antibody, or normal rabbit serum followed by alkaline phosphatase immunostaining with NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate).

RESULTS

Expression of MRP14 in human monocytic leukemia cells

We examined the mRNA expression in human monocytic leukemia cell lines and peripheral blood leukocytes by Northern blot analysis using MRP14, MRP8 and β-actin cDNA as probes (Fig. 1). MRP14 mRNA was not expressed in HL-60 cells but was induced in the cells after VD_3 treatment, as previously described (23, 24). No MRP14 mRNA was also detected either in ML-1 or U-937 cells, while THP-1 cells constitutively expressed the mRNA (Fig. 1A). Its level in THP-1 cells was 54% of that in VD_3-treated HL-60 cells according to the scanning by a densitometer. The pattern of MRP8 mRNA expression was identical with that of MRP14 (Fig. 1B).

A nuclear factor of C/EBP family binds to the 5′-upstream region of MRP14 gene

To examine the transcriptional regulation of MRP14 gene, we surveyed the DNA sequence of the known regulatory elements in the 5′-upstream region (−400 to +50) of the MRP14 gene (25), and the putative binding sites for three transcription factors (IRF-1 half-site, Sp1, and C/EBP) were found (Fig. 2A). In order to investigate which transcription factor is involved in the induction of MRP14 in VD_3-treated HL-60 cells, two DNA fragments [−400 to −150 (f251 fragment) and −149 to +50 (f199 fragment)] were prepared as probes and we performed competition gel shift experiments using an oligonucleotide containing the IRF-1 (half-site), Sp1, or C/EBP binding elements (Table I).

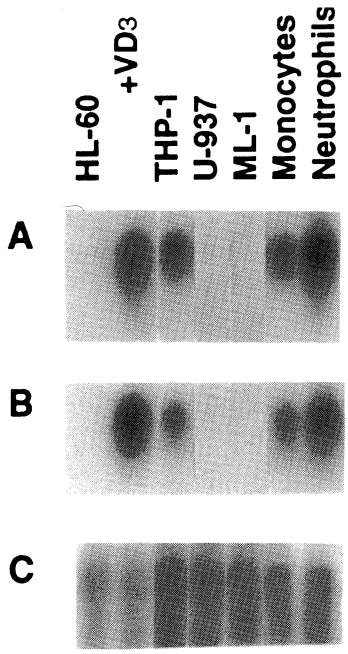

Fig. 1. Northern blot hybridization of MRP-14 and -8 mRNA in human leukemia and peripheral blood cells. Total cellular RNA extracted from untreated and VD_3-treated (day 2) HL-60 cells, and THP-1, U-937, ML-1 cells, monocytes, and neutrophils was hybridized with (A) MRP14 cDNA probe, (B) MRP8 cDNA probe, and (C) β-actin cDNA probe.

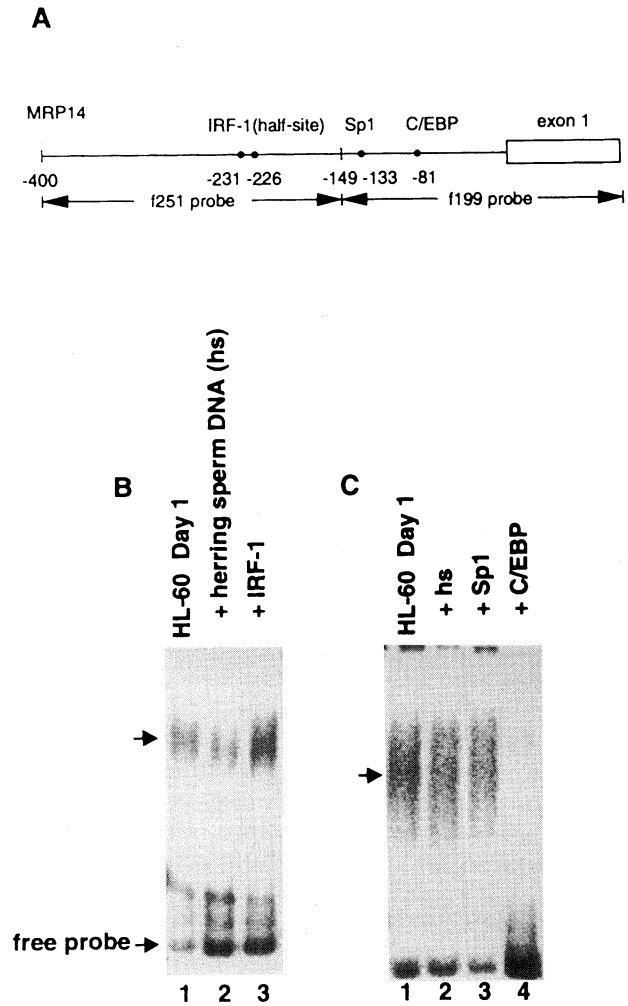

Fig. 2. Gel mobility-shift assays for the MRP14 gene. Nuclear extracts were prepared from HL-60 cells treated with VD_3 for 1 day. (A) Location of elements for some transcription factors (24) in the 5′-upstream region of the MRP14 gene. (B) Gel mobility-shift analysis using the 251 bp fragment (f251) as a probe. Herring sperm DNA and an oligonucleotide with the IRF-1 half-sites were used as competitors (100 ng, 1300-fold molar excess). (C) Gel mobility-shift analysis using the 199 bp fragment (f199) as a probe. Herring sperm DNA, and oligonucleotides with the Sp1- or C/EBP-binding motif were used as competitors (50 ng, 500-fold molar excess). An arrow indicates the position of shifted band.

An excess amount of a 20-mer oligonucleotide containing the IRF-1 half-sites could not compete with the binding of nuclear factors to the f251 probe (Fig. 2B), indicating that no factor bound to the region from −235 to −216 with the IRF-1 half-sites in the differentiated HL-60 cells. On the other hand, the binding to the f199 probe was competed for by the 20-mer oligonucleotide containing the C/EBP binding motif, but not by the oligonucleotide containing the Sp1 motif (Fig. 2C), suggesting that a factor of C/EBP family binds to f199.

Differential binding of C/EBPβ in HL-60 and C/EBPα in THP-1 cells to the C/EBP motif in the 5′-upstream region of the MRP14 gene

To further characterize the nuclear factors, the 20-mer oligonucleotide with the C/EBP element (C/EBP probe) instead of f199 was used as a probe in the gel shift assay. Nuclear extracts from HL-60 cells treated with VD_3 for indicated days were incubated with the C/EBP probe (Fig. 3A). The nuclear factor that bound to the C/EBP probe did not exist in the nuclei of untreated HL-60 cells, but was detected in the nuclei of HL-60 cells one day after the VD_3 treatment. The factor was most abundant in the nuclei on day 3 and had decreased greatly by day 6, indicating that this factor is differentiation-dependent. The binding of the factor to the C/EBP probe was competed for by the unlabeled C/EBP probe but not by the Sp1 oligonucleotide, confirming that the factor bound specifically to the C/EBP motif. The shifted band was also present in THP-1 cells (Fig. 3B).

In order to identify which member of C/EBP transcriptional factor binds to the motif, the nucleoprotein complex was incubated with anti-C/EBPα, -β, or -δ antibody. The formation of this specific complex in VD_3-

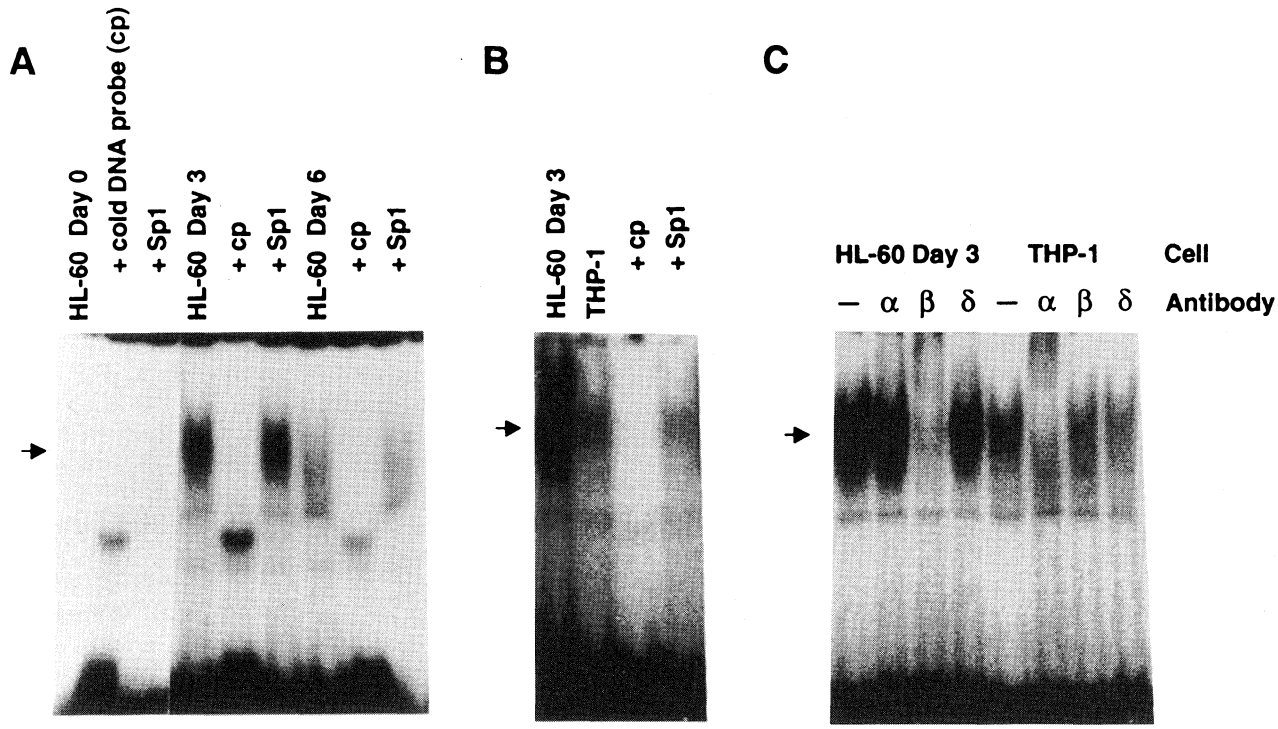

Fig. 3. Gel mobility-shift assays for the C/EBP motif of the MRP14 gene. The 20-mer synthetic oligonucleotide containing the C/EBP binding motif was used as a probe in the assay. (A and B) Competition analysis with the cold (unlabeled) DNA probe and Sp1 oligonucleotide (50 ng). Nuclear extracts were prepared from HL-60 cells treated with VD_3 for 0, 3, and 6 days (A) and THP-1 (B) cells. (C) Gel mobility-shift assays with the anti-C/EBPα (α), anti-C/EBPβ (β), or anti-C/EBPδ (δ) antibody. An arrow indicates the position of the shifted band.

treated HL-60 cells was inhibited by an anti-C/EBPβ antibody, but not affected by either anti-C/EBPα or -δ antibody (Fig. 3C), heated (100°C, 10 min) anti-C/EBPβ antibody, or normal rabbit serum (data not shown). However, the nucleoprotein complex in THP-1 cells was supershifted by an anti-C/EBPα antibody instead of an anti-C/EBPβ antibody. These results indicated that the complex contains C/EBPβ in VD_3-treated HL-60 cells and C/EBPα in THP-1 cells. In U-937 cells, which did not express MRP14 (Fig. 1A), weak shifted band was detected and the formation was inhibited by an anti-C/EBPα antibody (data not shown). No nuclear complex was found in ML-1 cells (data not shown).

Transcriptional activity of the C/EBP binding motif in the MRP14 gene

To characterize the transcriptional activity of the C/EBP binding motif, luc plasmid DNA was transfected into HL-60 cells treated with or without VD_3 and THP-1 cells (Fig. 5). We preliminarily tested the transfection efficiencies of πH3M-GFP into HL-60 and THP-1 cells, since HL-60 cells are suspected to have a low transfection efficiency (38). πH3M-GFP, which includes a visual reporter, GFP (7), was poorly transfected by electroporation into HL-60 cells compared with THP-1 cells (Fig. 4). The poor transfection of HL-60 cells was also shown by the β-galactosidase assay using the πH3M-β-gal construct (data not shown). However, the addition of serum (10%) to the electroporation medium improved the transfection efficiency of HL-60 cells up to 10% of that of THP-1 cells (data not shown). Therefore, the following luciferase assays of THP-1 cells were performed in serum-free electroporation medium, whereas that of HL-60 cells was done in the presence of 10% serum.

The results of the luciferase activity in HL-60 cells were indicated in Fig. 5A. We constructed the p1000 (-C/EBP)-luc that was defective in the C/EBP binding motif, in order to examine the function of the motif by comparison with p1000-luc. The luc activity of p1000 (-C/EBP)-luc was decreased compared with that of p1000-luc [Fig. 5A(a)], suggesting that the C/EBP motif was a positive functional regulatory region in the expression of the MRP14 gene. In VD_3-treated HL-60 cells, the luc activity of p1000-luc was approximately 1.5-fold greater than in the untreated cells. To further characterize the involvement of the C/EBP motif, single and multimerized, wild type or mutated C/EBP oligonucleotides were fused in the ordinary (sense) or

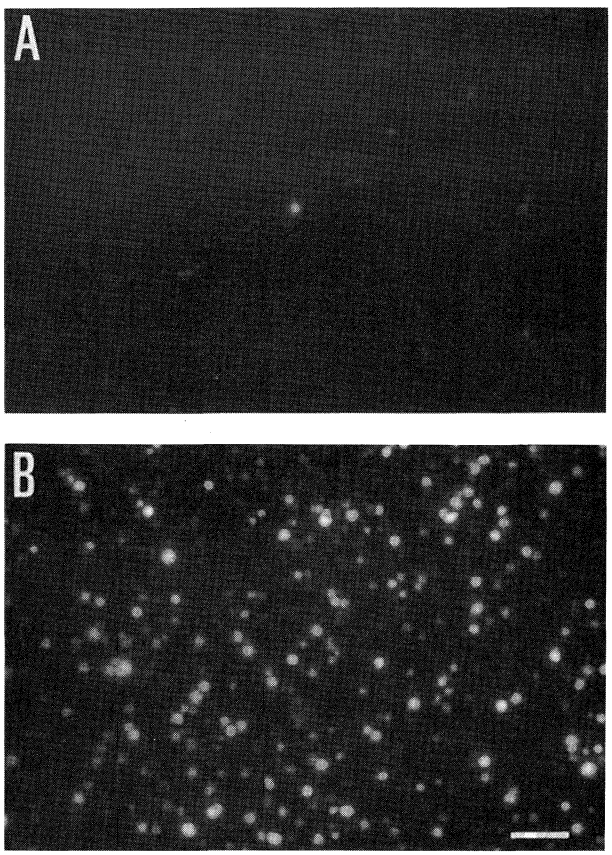

Fig. 4. Expression of GFP in HL-60 (A) and THP-1 (B) cells. The cells were electroporated and incubated with a plasmid DNA construct carrying sGFP(S65T). The GFP in transfected cells was visualized after 48 h incubation using a fluorescence microscope under blue light (490 nm). Bar = 100 μm.

opposite direction (antisense) to p73-luc construct, and the luciferase activity was assayed [Fig. 5A(b)]. The p73-luc plasmid that was fused with a single copy of the sense C/EBP motif (C/EBP-p73-luc) in VD_3-treated HL-60 cells had 3- to 4-fold higher activity than that in the untreated cells, and 5-fold higher activity than p73-luc (without the C/EBP motif) in the VD_3-treated cells. Similar results were obtained by the insertion of an antisense copy. Introduction of a two-base pair mutation in either C/EBP motif lowered the luc activity in both cells. Three copies of the wild type sense C/EBP oligonucleotide increased the activity to approximately 3-fold by VD_3 treatment, and 8-fold that of the p73-luc in the VD_3-treated cells. However, three copies of the antisense C/EBP motif as well as the mutation had no so stimulatory effect. In untreated HL-60 cells, the C/EBP motif raised the luciferase activity to a certain extent (Fig. 5A). It might be due to the artificial event that C/EBPβ with low affinity showed the activity by the transfection of multicopy DNA, because C/EBPβ was detected in untreated HL-60 cells by Western blot analysis as described below (Fig. 6). The luc activity of p1000-luc was a half of that of C/EBP-p73-luc in VD_3-treated HL-60 cells, suggesting that there might be silencer elements in the other region except the C/EBP motif in the MRP14 gene.

Fig. 5B showed the results in THP-1 cells. The luc activity of p1000(-C/EBP)-luc was decreased compared with that of p1000-luc [Fig. 5B(a)]. The activity of C/EBP-p73-luc was 3- to 4-fold greater than that of p73-luc [Fig. 5B(b)]. Moreover, three copies of the wild type sense C/EBP motif increased the activity to approximately 10-fold that of the p73-luc in THP-1 cells. These data demonstrated that the C/EBP binding motif was the functional regulatory region of the MRP14 gene in VD_3-treated HL-60 cells and THP-1 cells.

Expression of C/EBPβ in human monocytic leukemia cells

We investigated the nuclear expression of C/EBPβ in human monocytic leukemia cell lines by Western blot analysis with the anti-C/EBPβ antibody (Fig. 6). The antibody stained discrete two protein bands with molecular masses of 46 and 44 kDa; the 44 kDa band was stained much stronger by the antibody. These two proteins are considered to be derived from the first two AUG codons of C/EBPβ mRNA (1, 60). The intensity of the two signals did not significantly change in HL-60 cells after VD_3 treatment (Fig. 6). C/EBPβ was strongly expressed in all cell lines except for ML-1 cells. However, C/EBPβ in the nuclei of untreated HL-60 cells and THP-1 cells could not bind to the C/EBP motif in the MRP14 gene (Fig. 3, A, C).

With the anti-C/EBPα antibody, no specific band appeared in either cell by the Western blot analysis (data not shown). No band was detected with normal rabbit serum (data not shown).

DISCUSSION

We examined MRP expression in human monocytic leukemia cells. The MRP genes were not expressed in monoblastic ML-1 cells and promonocytic U-937 cells as well as promyelocytic HL-60 cells (Fig. 1). Other investigators have reported that MRPs are expressed in U-937 cells (25, 49). The discrepancy is probably due to variations of U-937 phenotype under different culture conditions. On the other hand, monocytic THP-1 (58) and VD_3-treated HL-60 cells expressed the MRP genes, but with differential levels (Fig. 1). Thus, it is apparent that MRP genes are expressed at the post promonocytic stage in monocytic differentiation and the VD_3 treatment induced the differentiation of HL-60 cells beyond the promonocytic stage.

It is known that binding of nuclear transcriptional

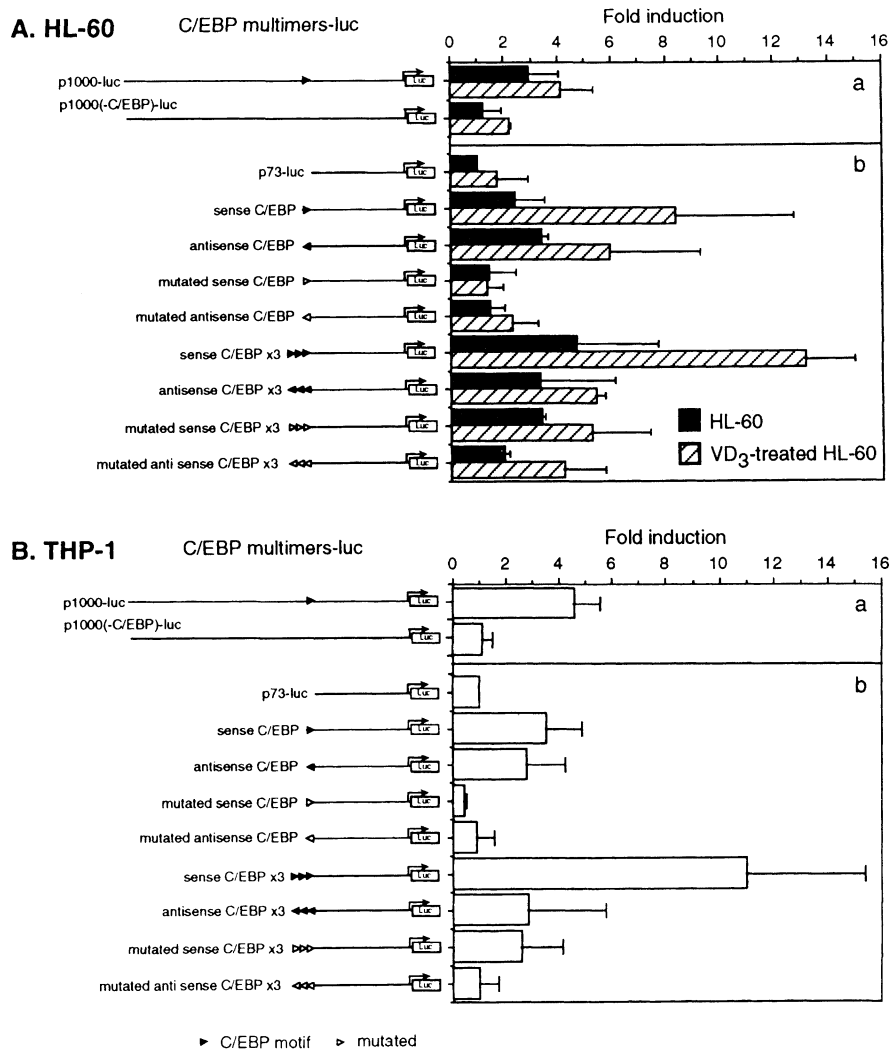

Fig. 5. Functional analysis of the C/EBP binding motif in the MRP14 gene by luciferase assay. (A) HL-60 cells were treated with or without VD_3 for 2 days and then transfected. The results are expressed relative to the activity obtained with the p73-luc construct in untreated HL-60 cells. (B) THP-1 cells were transfected and harvested 48 h later. The results are expressed relative to the luciferase activity obtained with the p73-luc construct. The values are means of three independent experiments; error bars represent standard deviations of the means. A schematic representation of each luc reporter construct is shown on the left. (a) p1000-luc and p1000(-C/EBP)-luc construct. (b) p73-luc and p73-luc fused with single and three copies, wild type or mutated C/EBP oligonucleotides.

factors to the cis-element of a gene can promote the initiation of transcription of the gene (15, 54). To investigate the transcriptional factors regulating the MRP14 gene expression, we performed gel-shift competition assay using two probes, f251 (−400 to −150) and f199 (−149 to +50) in the 5′-upstream region of the MRP14 gene. A retarded complex shown in Fig. 2C, lane 1, which had been faintly detected and neglected in the previous study (24), was clearly detected in the current study and was competed with a C/EBP-specific oligonucleotide (Fig. 2C, lane 4). Therefore, we re-evaluated the factor that bound to the C/EBP motif and further studied in the following experiments. A retarded band close to the free f199 probe has been designated MP14FII in the previous report (24). In this study, however, the band was not consistently detected, and we concluded that MP14FII was a false-negative. In the previous gel shift assay, we designated a factor in the nuclei of HL-60 cells that retarded the f251 probe (Fig. 2B) as MP14FI (24), which was also present in THP-1 cells (data not shown). MP14FI did not bind to the IRF-1 half-sites, since the retardation of f251 by MP14FI was not affected by the addition of the competitor with the IRF-1 half-sites (Fig. 2B). Therefore, it is essential to determine where MP14FI binds in the f251 fragment in future experiments.

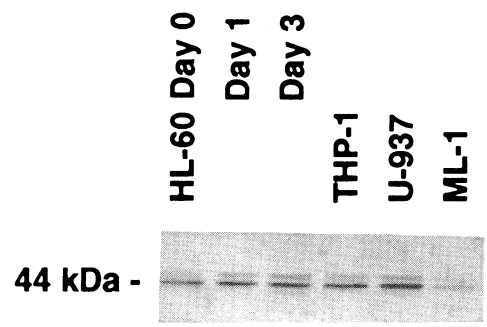

Fig. 6. Western blot analysis of C/EBPβ in human leukemia cells. Nuclear extracts were prepared for the detection of C/EBPβ from HL-60 cells treated with VD_3 for 0, 1, and 3 days, and from THP-1, U-937, and ML-1 cells. Blotted membrane was immunostained with an anti-C/EBPβ antibody.

The C/EBP-specific binding was further studied by the use of a C/EBP-specific oligonucleotide as a probe in the gel-shift assay (Fig. 3). The binding factor which is VD_3 differentiation-dependent in HL-60 cells (Fig. 3A) was immunologically identified as C/EBPβ, whereas C/EBPα bound to the same C/EBP motif in THP-1 cells (Fig. 3C).

C/EBPα, previously termed C/EBP, was identified originally as a heat-stable, sequence-specific DNA-binding activity present in soluble extracts of rat liver nuclei (26) and is characterized as a factor involved in cell differentiation and energy metabolism (28, 50, 53, 61). Several isoforms, such as C/EBPβ, -γ, -δ, -ε and CHOP 10, have been identified, and they are known as the C/EBP family (1, 2, 10, 26, 41, 54). These members can cross-dimerize and bind to DNA with similar specificity (1, 6, 37, 41, 64). C/EBPβ is known to regulate a number of cytokine genes and may contribute to normal myeloid development as well as to activation of mature myeloid cells in response to an inflammatory stimulus (1, 31, 39, 52, 53, 59). Furthermore, the expression of C/EBPβ increases during differentiation towards granulocytes as well as macrophages (31, 48).

The differential binding of C/EBPα and -β in our study may be important in the regulation of MRP14 expression since the level of MRP14 mRNA in THP-1 cells was half that in VD_3-treated HL-60 cells (Fig. 1A). Pope *et al.* reported that C/EBPβ was a stronger activator of the TNFα promoter than C/EBPα (39). It was also reported that C/EBPα attenuated the transcriptional activation by C/EBPβ (36). The transcriptional activities of the C/EBP binding motif were functional and showed similar patterns in VD_3-treated HL-60 and THP-1 cells (Fig. 5). It is possible that C/EBP synergistically interact with other transcription factors, which may bind to other promoter regions in the MRP14 gene, for example, an unknown factor that bound to the f251 fragment. It is known that C/EBPβ interacts with NF-κB, glucocorticoid receptor, and retinoblastoma protein (8, 27, 33), and that C/EBPα associates with TFIIB, TATA-binding protein, and AML (acute myelogenous leukemia)-1 (32, 67). The differential activity of C/EBP isoforms may be caused by distinct interactions with other proteins (37). We would like to examine the other negative or positive factors involved in MRP14 expression.

It was reported that no C/EBPβ mRNA was detected in untreated HL-60 and U-937 cells (31). In this study, however, C/EBPβ was detected in these cells by Western blot analysis (Fig. 6). This discrepancy may also due to different cell culture conditions as discussed above. We found that the DNA binding activity of C/EBPβ appeared in HL-60 cells after VD_3 treatment with little change in C/EBPβ level. Other investigators similarly reported that DNA binding activity of C/EBPβ increased after stimulation, whereas the amount of C/EBPβ remained unchanged, suggesting that C/EBPβ was activated through post-translational modification (1, 60). Similar post-translational modification of C/EBPβ may occur in VD_3-treated HL-60 cells. Several investigators have reported that C/EBPβ is activated through phosphorylation by calcium/calmodulin-dependent protein kinase (62), mitogen-activated kinase (30), protein kinase C (56, 57), or protein kinase A (57). It was also reported that the association with c-Fos or with c-Jun altered the DNA binding specificity of C/EBPβ and reduced its binding (19). Further studies will be needed to identify any modification in the HL-60 cells.

Acknowledgments. We are very grateful to Dr. Ken Takeda, Showa University, Tokyo for ML-1 cells, Dr. Hiroyasu Uchiyama, Hiroshima University, Hiroshima for πH3M-β-gal, and Dr. Yasuo Niwa, University of Shizuoka for providing sGFP(S65T) DNA with the permission of Dr. Brian Seed, Massachusetts General Hospital, Boston, and helpful suggestions of the plasmid construction. We thank Dr. Tetsuo Hirano of Hiroshima University for the advice on the transfection experiment and Dr. Kazunari Yokoyama, Tsukuba Life Science Center, RIKEN, for helpful discussions on the transfection.

REFERENCES

1. Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.*, **9**: 1897–1906.
2. Akira, S. and Kishimoto, T. 1992. IL-6 and NF-IL6 in acute-phase response and viral infection. *Immunol. Rev.*, **127**: 25–50.
3. Aruffo, A. and Seed, B. 1987. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA*, **84**: 8573–8577.
4. Berntzen, H.B. and Fagerhol, M.K. 1990. L1, a major granulocyte protein; isolation of high quantities of its subunits. *Scand. J. Clin. Lab. Invest.*, **50**: 769–774.
5. Brüggen, J., Tarcsay, L., Cerletii, N., Odink, K.,

RUTISHAUSER, M., HOLLÄNDER, G., and SORG, C. 1988. The molecular nature of the cystic fibrosis antigen. *Nature*, **331**: 570–573.

6. CAO, Z., UMEK, R.M., and MCKNIGHT, S.L. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes & Dev.*, **5**: 1538–1552.
7. CHALFIE, M., TU, Y., EUSKIRCHEN, G., WARD, W.W., and PRASHER, D.C. 1994. Green fluorescent protein as a marker for gene expression. *Science*, **263**: 802–805.
8. CHEN, P.-L., RILEY, D.J., CHEN, Y., and LEE, W.-H. 1996. Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. *Genes & Dev.*, **10**: 2794–2804.
9. CHIU, W., NIWA, Y., ZENG, W., HIRANO, T., KOBAYASHI, H., and SHEEN, J. 1996. Engineered GFP as a vital reporter in plants. *Curr. Biol.*, **6**: 325–330.
10. CHUMAKOV, A.M., GRILLER, I., CHUMAKOVA, E., CHIH, D., SLATER, J., and KOEFFLER, H.P. 1997. Cloning of the novel human myeloid-cell-specific C/EBP-ε transcription factor. *Mol. Cell. Biol.*, **17**: 1375–1386.
11. DELABIE, J., DE WOLF-PEETERS, C., VAN DEN OORD, J.J., and DESMET, V.J. 1990. Differential expression of the calcium-binding proteins MRP8 and MRP14 in granulomatous conditions. *Clin. Exp. Immunol.*, **81**: 123–126.
12. DORIN, J.R., NOVAK, M., HILL, R.E., BROCK, D.J.H., SECHER, D.S., and VAN HEYNINGEN, V. 1987. A clue to the basic defect in cystic fibrosis from cloning the CF-antigen gene. *Nature*, **326**: 614–617.
13. GILBERT, J.A., AHLQUIST, D.A., MAHONEY, D.W., ZINSMEISTER, A.R., RUBIN, J., and ELLEFSON, R.D. 1996. Fecal marker variability in colorectal cancer: calprotectin versus hemoglobin. *Scand. J. Gastroenterol.*, **31**: 1001–1005.
14. GOLDEN, B.E., CLOHESSY, P.A., RUSSELL, G., and FAGERHOL, M.K. 1996. Calprotectin as a marker of inflammation in cystic fibrosis. *Archives of Disease in Childhood*, **74**: 136–139.
15. GRUNSTEIN, M. 1992. Histones as regulators of genes. *Scientific American* October, 40–47.
16. HASS, J., PARK, E., and SEED, B. 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr. Biol.*, **6**: 315–324.
17. HOGG, N., ALLEN, C., and EDGEWORTH, J. 1989. Monoclonal antibody 5.5 reacts with p8, 14, a myeloid molecule associated with some vascular endothelium. *Eur. J. Immunol.*, **19**: 1053–1061.
18. HORIE, N., CHIMOTO, M., NOZAWA, R., and TAKEISHI, K. 1993. Characterization of regulatory sequences and nuclear factors that function in cooperation with the promoter of the human thymidylate synthase gene. *Biochim. Biophys. Acta*, **1216**: 409–416.
19. HSU, W., KERPPOLA, T.K., CHEN, P.-L., CURRAN, T., and CHEN-KIANG, S. 1994. Fos and Jun repress transcription activation by NF-IL6 through association at the basic zipper region. *Mol. Cell. Biol.*, **14**: 268–276.
20. IVANOV, G.E., MISUNO, N.I., and IVANOV, V.E. 1996. Enzyme-linked immunosorbent assay for human MRP-8/MRP-14 proteins and their quantitation in plasma of hematological patients. *Immunol. Lett.*, **49**: 7–13.
21. JAIN, V.K. and MAGRATH, I.T. 1991. A chemiluminescent assay for quantitation of β-galactosidase in the femtogram range: Application to quantitation of β-galactosidase in lacZ-transfected cells. *Anal. Biochem.*, **199**: 119–124.
22. KELLY, S.E., JONES, D.B., and FLEMING, S. 1989. Calgranulin expression in inflammatory dermatoses. *J. Pathol.*, **159**: 17–21.
23. KURUTO, R., NOZAWA, R., TAKEISHI, K., ARAI, K., YOKOTA, T., and TAKASAKI, Y. 1990. Myeloid calcium binding proteins: Expression in the differentiated HL-60 cells and detection in sera of patients with connective tissue diseases. *J. Biochem.*, **108**: 650–653.
24. KUWAYAMA, A., KURUTO, R., HORIE, N., TAKEISHI, K., and NOZAWA, R. 1993. Appearance of nuclear factors that interact with genes for myeloid calcium binding proteins (MRP8 and MRP14) in differentiated HL-60 cells. *Blood*, **81**: 3116–3121.
25. LAGASSE, E. and CLERC, R.G. 1988. Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. *Mol. Cell. Biol.*, **8**: 2402–2410.
26. LANDSCHULZ, W.H., JOHNSON, P.F., ADASHI, E.Y., GRAVES, B.J., and MCKNIGHT, S.L. 1988. Isolation of a recombinant copy of the gene encoding C/EBP. *Genes & Dev.*, **2**: 786–800.
27. LECLAIR, K.P., BLANAR, M.A., and SHARP, P.A. 1992. The p50 subunit of NF-κB associates with the NF-IL-6 transcription factor. *Proc. Natl. Acad. Sci. USA*, **89**: 8145–8149.
28. MCKNIGHT, S.L., LANE, M.D., and GLUECKSOHN-WAELSCH, S. 1989. Is CCAAT/enhancer-binding protein a central regulator of energy metabolism? *Genes & Dev.*, **3**: 2021–2024.
29. MITCHELL, P.J. and TJIAN, R. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science*, **245**: 371–378.
30. NAKAJIMA, T., KINOSHITA, S., SASAGAWA, T., SASAKI, K., NARUTO, M., KISHIMOTO, T., and AKIRA, S. 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc. Natl. Acad. Sci. USA*, **90**: 2207–2211.
31. NATSUKA, S., AKIRA, S., NISHIO, Y., HASHIMOTO, S., SUGITA, T., ISSHIKI, H., and KISHIMOTO, T. 1992. Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. *Blood*, **79**: 460–466.
32. NERLOV, C. and ZIFF, E.B. 1995. CCAAT/enhancer binding protein-α amino acid motifs with dual TBP and TFIIB binding ability co-operate to activate transcription in both yeast and mammalian cell. *EMBO J.*, **14**: 4318–4328.
33. NISHIO, Y., ISSHIKI, H., KISHIMOTO, T., and AKIRA, S. 1993. A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat α1-acid glycoprotein gene via direct protein-protein interaction. *Mol. Cell. Biol.*, **13**: 1854–1862.
34. NOZAWA, R., KATO, H., ITO, T., and YOKOTA, T. 1988. Identification and characterization of a differentiation antigen human neutrophils and monocytes. *Blood*, **71**: 1288–1294.
35. ODINK, K., CERLETTI, N., BRÜGGEN, J., CLERC, R.G., TARCSAY, L., ZWADLO, G., GERHARDS, G., SCHLEGEL, R., and SORG, C. 1987. Two calcium-binding proteins infiltrate macrophages of rheumatoid arthritis. *Nature*, **330**: 80–82.
36. OSADA, S., TAKANO, K., NISHIHARA, T., SUZUKI, T., MURAMATSU, M., and IMAGAWA, M. 1995. CCAAT/enhancer-binding proteins α and β interact with the silencer element in the promoter of glutathione S-transferase P gene during hepatocarcinogenesis. *J. Biol. Chem.*, **270**: 31288–31293.
37. OSADA, S., YAMAMOTO, H., NISHIHARA, T., and IMAGAWA, M. 1996. DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. *J. Biol. Chem.*, **271**: 3891–3896.
38. PAHL, H.L., BURN, T.C., and TENEN, D.G. 1991. Optimization of transient transfection into human myeloid cell lines using a luciferase reporter gene. *Exp. Hematol.*, **19**: 1038–1041.
39. POPE, R.M., LEUTZ, A., and NESS, S.A. 1994. C/EBP beta

regulation of the tumor necrosis factor alpha gene. *J. Clin. Invest.*, **94**: 1449–1455.

40. Postler, E., Lehr, A., Schluesener, H., and Meyermann, R. 1997. Expression of the S-100 proteins MRP-8 and -14 in ischemic brain lesions. *Glia*, **19**: 27–34.
41. Ron, D. and Habener, J.F. 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes & Dev.*, **6**: 439–453.
42. Roseth, A.G., Kristinsson, J., Fagerhol, M.K., Schjonsby, H., Aadland, E., Nygaard, K., and Roald, B. 1993. Faecal calprotectin: A novel test for the diagnosis of colorectal cancer?. *Scand. J. Gastroenterol.*, **28**: 1073–1076.
43. Roth, J., Sunderkotter, C., Goebeler, M., Gutwald, J., and Sorg, C. 1992. Expression of the calcium-binding proteins MRP8 and MRP14 by early infiltrating cells in experimental contact dermatitis. *Int. Arch. Allergy Immunol.*, **98**: 140–145.
44. Roth, J., Goebeler, M., Wrocklage, V., van den Bos, C., and Sorg, C. 1994. Expression of the calcium-binding proteins MRP8 and MRP14 in monocytes is regulated by a calcium-induced suppressor mechanism. *Biochem. J.*, **301**: 655–660.
45. Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 6.26–6.27.
46. Schäfer, B.W., Wicki, R., Engelkamp, D., Mattei, M.G., and Heizmann, C.W. 1995. Isolation of a YAC clone covering a cluster of nine S100 genes on human chromosome 1q21: rationale for a new nomenclature of the S100 calcium-binding protein family. *Genomics*, **25**: 638–643.
47. Schreiber, E., Matthias, P., Müller, M.M., and Schaffner, W. 1989. Rapid detection of octamer binding proteins with miniextracts, prepared from a small number of cells. *Nucleic Acids Res.*, **17**: 6419.
48. Scott, L.M., Civin, C.I., Rorth, P., and Friedman, A.D. 1992. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. *Blood*, **80**: 1725–1735.
49. Sellmayer, A., Krane, S.M., Ouellette, A.J., and Bonventre, J.V. 1992. 1α,25-$(OH)_2$ vitamin D_3 enhances expression of the genes encoding Ca^{2+}-binding proteins MRP-8 and MRP-14. *Am. J. Physiol.*, **262**: C235–C242.
50. Smith, L.T., Hohaus, S., Gonzalez, D.A., Dziennis, S.E., and Tenen, D.G. 1996. PU.1 (Spi-1) and C/EBPα regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood*, **88**: 1234–1247.
51. Steinbakk, M., Naess-Andresen, C.F., Lingaas, E., Dale, I., Brandtzaeg, P., and Fagerhol, M.K. 1990. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. *Lancet*, **336**: 763–765.
52. Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N., and Kishimoto, T. 1995. Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell*, **80**: 353–361.
53. Tenen, D.G., Hromas, R., Licht, J.D., and Zhang, D.-E. 1997. Transcription factors normal myeloid development, and leukemia. *Blood*, **90**: 489–519.
54. Thangue, N.B.L. and Rigby, P.W.J. 1988. Trans-acting protein factors and the regulation of eukaryotic transcription. In *Transcription and Splicing* (B.H. Hames and D.M. Glover, eds), IRL Press, Oxford, England 1–22.
55. Tobe, T., Murakami, K., Tomita, M., and Nozawa, R. 1989. Amino acid sequences of 60B8 antigens induced in HL-60 cells by 1,25-dihydroxyvitamin D_3. The antigens are identical with macrophage-related protein-14 and -8. *Chem. Pharm. Bul.*, **37**: 1576–1580.
56. Trautwein, C., Caelles, C., van der Geer, P., Hunter, T., Karin, M., and Chojkier, M. 1993. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature*, **364**: 544–547.
57. Trautwein, C., van der Geer, P., Karin, M., Hunter, T., Karin, M., and Chojkier, M. 1994. Protein kinase A and C site-specific phosphorylations of LAP (NF-IL6) modulate its binding affinity to DNA recognition elements. *J. Clin. Invest.*, **93**: 2554–2561.
58. Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T., and Tada, K. 1982. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res.*, **42**: 1530–1536.
59. Tsukada, J., Saito, K., Waterman, W.R., Webb, A.C., and Auron, P.E. 1994. Transcription factors NF-IL6 and CREB recognize a common essential site in the human prointerleukin 1β gene. *Mol. Cell. Biol.*, **14**: 7285–7297.
60. Wada, N., Matsumura, M., Ohba, Y., Kobayashi, N., Takizawa, T., and Nakanishi, Y. 1995. Transcription stimulation of the Fas-encoding gene by nuclear factor for interleukin-6 expression upon influenza virus infection. *J. Biol. Chem.*, **270**: 18007–18012.
61. Wang, N.D., Finegold, M.J., Bradley, A., Ou, C.N., Abdelsayed, S.V., Wilde, M.D., Taylor, L.R., Wilson, D.R., and Darlington, G.J. 1995. Impaired energy homeostasis in C/EBPα knockout mice. *Science*, **269**: 1108–1112.
62. Wegner, M., Cao, Z., and Rosenfeld, M.G. 1992. Calcium-regulated phosphorylation within the leucine zipper of C/EBPβ. *Science*, **256**: 370–373.
63. Wilkinson, M.M., Busuttil, A., Hayward, C., Brock, D.J.H., Dorin, J.R., and van Heyningen, V. 1988. Expression pattern of two related cystic fibrosis-associated tissues. *J. Cell Sci.*, **91**: 221–230.
64. Williams, S.C., Cantwell, C.A., and Johnson, P.F. 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes & Dev.*, **5**: 1553–1567.
65. Young, D.C., Kingsley, S.D., Ryan, K.A., and Dutko, F.J. 1993. Selective inactivation of eukaryotic β-galactosidase in assays for inhibitors of HIV-1 TAT using bacterial β-galactosidase as a reporter enzymes. *Anal. Biochem.*, **215**: 24–30.
66. Yui, S., Mikami, M., and Yamazaki, M. 1995. Purification and characterization of the cytotoxic factor in rat peritoneal exudate cells: its identification as the calcium binding protein complex, calprotectin. *J. Leukoc. Biol.*, **58**: 307–316.
67. Zhang, D.-E., Hetherington, C.J., Meyers, S., Rhoades, K.L., Larson, C.J., Chen, H.-M., Hiebert, S.W., and Tenen, D.G. 1996. CCAAT enhancer-binding protein (C/EBP) and AML1 (CBFα2) synergistically activate the macrophage colony-stimulating factor receptor promoter. *Mol. Cell. Biol.*, **16**: 1232–1240.
68. Zwadlo, G., Brüggen, J., Gerhards, G., Schlegel, R., and Sorg, C. 1988. Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Clin. Exp. Immunol.*, **72**: 510–515.

(Received for publication, January 14, 1998
and in revised form, April 2, 1998)