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Cloning and Expression of Two Human Genes Encoding Calcium-Binding Proteins That Are Regulated during Myeloid Differentiation

ERIC LAGASSE AND ROGER G. CLERC†*

Department of Biotechnology, CIBA-GEIGY, CH-4002 Basel, Switzerland

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The cellular mechanisms involved in chronic inflammatory processes are poorly understood. This is especially true for the role of macrophages, which figure prominently in the inflammatory response. Two proteins, MRP8 and MRP14, which are expressed in infiltrate macrophages during inflammatory reactions but not in normal tissue macrophages, have been characterized. Here we report that MRP8 and MRP14 mRNAs are specifically expressed in human cells of myeloid origin and that their expression is regulated during monocyte-macrophage and granulocyte differentiation. To initiate the analysis of *cis*-acting elements governing the tissue-specific expression of the MRP genes, we cloned the human genes encoding MRP8 and MRP14. Both genes contain three exons, are single copy, and have a strikingly similar organization. They belong to a novel subfamily of highly homologous calcium-binding proteins which includes S100α, S100β, intestinal calcium-binding protein, P11, and calcyclin (2A9). A transient expression assay was devised to investigate the tissue-specific regulatory elements responsible for MRP gene expression after differentiation in leukemia HL60 cells. The results of this investigation demonstrated that the *cis*-acting elements responsible for MRP expression are present on the cloned DNA fragment containing the MRP gene loci.

Cells of myeloid origin composing the monocyte-macrophage and granulocyte lineages exercise a wide range of functions in the physiology of the immune system. The regulation of the many accessory and effector roles of these cells is complex and subject to control by a variety of lymphokines and cytokines. Macrophage migration inhibitory factor is one such factor and is thought to play an important role in the differentiation of mononuclear phagocytes (34).

Using a monoclonal antibody directed against human macrophage migration inhibitory factor, two novel proteins were isolated and purified as part of a complex (4). These are referred to as MRP8 and MRP14 (migration inhibitory factor-related proteins) (41). The cloning and expression of human MRP8 and MRP14 cDNAs have allowed studies on the biological properties of these proteins to be done (41). Experiments employing immunohistochemical techniques have shown that these proteins are expressed exclusively in cells of myeloid origin. In peripheral blood they appear in monocytes and granulocytes (G. Zwadlo, J. Brueggen, G. Gerhards, R. Schlegel, and C. Sorg, Clin. Exp. Immunol., in press). In infiltrate of acute inflammatory tissues, MRP14 is expressed in certain subsets of macrophages, whereas MRP8 is absent. In contrast, both MRP8 and MRP14 are expressed in chronic inflammatory lesions, such as those observed in primary chronic polyarthritis. In resident macrophages of different tissues, neither of the proteins is detectable (Zwadlo et al., in press). Recently, it has been reported that MRP8 is present in elevated quantities in blood serum of patients suffering from cystic fibrosis (10).

The present study deals with the regulation of the genes encoding MRP8 and MRP14. Their expression is shown to be restricted to human blood monocytes and human cell lines of myeloid origin, the promyelocytic leukemia cell line HL60 (7), and the promonocytic leukemia cell line U937 (46). We further demonstrate that expression of both MRP8 and MRP14 mRNAs was enhanced or diminished during the induction of differentiation of HL60 cells depending on the inducing agent. The complete human genes for MRP8 and MRP14 were cloned and characterized, including the flanking transcriptional regulatory sequences. Furthermore, using a transient chloramphenicol acetyltransferase (CAT) assay in HL60 cells, we demonstrated that the regions responsible for the regulated expression of MRP8 and MRP14 are located in the cloned genes. The polypeptide sequences of MRP8 and MRP14 were compared with published data for other proteins, and it was found that the MRP proteins share extensive homology with small calcium-binding protein sequences such as S100α (25), S100β (23, 24, 27, 30), intestinal calcium-binding protein (ICaBP) (22, 32, 47), calcyclin (2A9) (5), and P11 (15, 17). We suggest that these closely related proteins belong to a new subfamily of calcium-binding proteins. Our studies provide a framework for the study of regulatory *cis*-acting elements activated during cell differentiation in the myeloid pathway.

MATERIALS AND METHODS

Cells. Human blood monocytes were isolated from healthy blood donors and successively purified on two Percoll (Pharmacia, Inc., Piscataway, N.J.) gradients according to the protocol of the manufacturer. One sample of monocytes was isolated and further purified in the presence of 5 μg of actinomycin D (Serva Heidelberg, Federal Republic of Germany) per ml to block transcription during the procedure. The following human cell lines were used in the study: HL60 (promyelocytic leukemia), U937 (promonocytic leukemia), Namalwa (B-cell lymphoma), and MOLT-4 (T-cell leukemia). These cells were grown in RPMI = 1640 medium supplemented with 10% fetal calf serum and antibiotics.

* Corresponding author.
† Present address: Center for Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139.

L132 (human embryonic lung epithelium-like) cells were grown in minimal essential medium supplemented with 10% fetal calf serum and antibiotics. Cultures were incubated at 37°C in tissue culture flasks initially equilibrated in an atmosphere of 95% air and 5% CO_2. For the induction of HL60 cell differentiation along the myelocytic lineage, either dimethyl sulfoxyde (DMSO) (8) or phorbol myristate acetate (PMA) (36) was added to a final concentration of 1.25% (vol/vol) or 20 ng/ml, respectively.

Northern (RNA) blot hybridization. The cells were pelleted by centrifugation and dissolved in 4 M guanidinium thiocyanate. Total RNA was isolated by centrifugation through a 5.7 M CsCl cushion (18). RNA samples (10 to 20 μg each) were electrophoresed through 1.5% agarose–formaldehyde gels and blotted onto nitrocellulose membranes in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by standard procedures (6). Hybridizations were performed with α-^{32}P-labeled MRP8 and MRP14 cDNA probes (see Southern blot procedure) in 50% formamide–5× SSC–5× Denhart solution–50 mM sodium phosphate buffer (pH 6.8)–10 mM EDTA–0.1% sodium dodecyl sulfate–200 μg of carrier DNA per ml for 12 h at 42°C. Membranes were washed successively in 6× SSC–0.1% sodium dodecyl sulfate and 1× SSC–0.1% sodium dodecyl sulfate at room temperature. The amount of RNA loaded was scored by hybridizing the membranes to a control cDNA probe, EF4 (mouse protein synthesis initiation factor 4A) (39). The EF4 cDNA probe was a 1.4-kilobase (kb) *Eco*RI DNA fragment (39) and was labeled with [α-^{32}P]dATP (Amersham Corp., Arlington Heights, Ill.) (42).

Southern blot hybridization. Human placental genomic DNA was isolated by the method of Nathans and Hogness (37). Human placental DNA (10 to 15 μg) was digested to completion with *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst*I and blotted by the method of Southern (45). The [α-^{32}P]dATP-labeled MRP8 and MRP14 cDNA probes (42) were hybridized to the digested genomic DNA by standard methods. The MRP8 cDNA probe was a 369-base-pair (bp) *Pvu*II-*Pst*I fragment derived from pMRP8-3 containing a complete MRP8 cDNA (41). The MRP14 cDNA probe was a 364-bp *Dra*III-*Ava*I fragment derived from pMRP14-10 containing the MRP14 cDNA (41). After hybridization, membranes were washed successively at low stringency (6× SSC, 65°C) and at high stringency (0.1× SSC, 65°C).

Cloning and sequencing of MRP8 and MRP14 genes. A human fetal liver DNA library established in the λ Charon 4A bacteriophage vector (31) was screened by plaque hybridization by the method of Benton and Davis (2). With the MRP8 cDNA probe, six positively hybridizing clones were identified from 6×10^5 independent phage plaques. Southern blot analysis of the purified DNA from clones λMRP8-1 to -6 showed that all six recombinant phage contained the complete MRP8 gene. A 5-kb *Hpa*II DNA fragment encompassing the MRP8 gene was subcloned from phage λMRP8-3 into pBR322 linearized with *Cla*I (pBRMRP8). A *Hin*dIII-*Eco*RI fragment containing the MRP8 gene was similarly subcloned in *Hin*dIII-*Eco*RI-cut pUC8 (pUCMRP8). With the MRP14 cDNA probe, four positive phage plaques were identified and plaque purified. One clone, λMRP14-2, containing the complete MRP14 gene on a 6-kb *Pst*I DNA fragment was subcloned into the *Pst*I site of pUC9 (pUCMRP14). The strategy used for sequencing the MRP8 and MRP14 genes in a plasmid subclone is depicted in Fig. 3; arrows indicate the direction of DNA sequencing. DNA sequencing was performed on bacteriophage M13 single-stranded templates according to the protocol of the manufacturer (Amersham). Most of the DNA sequence of both MRP genes was confirmed by sequencing the opposite strand.

Plasmid construction. The expression vector pCMVCAT is described in reference 12 and was kindly provided by K. Foecking and H. Hofstetter. The clones pUCMRP8dCAT and pUCMRP14dCAT were constructed by first deleting the region in pUCMRP8 and pUCMRP14 which extends from the *Bgl*II sites (position 791 for MRP8 and positions 559 and 2375 for MRP14; see Fig. 4 and 5) to the untranslated region of the exon 2 with exonuclease III and mung bean nuclease (Stratagene). After the linearized vectors were ligated in the presence of *Bgl*II linkers (Amersham), the appropriate plasmids were selected by sequencing the endpoints of the deletion. The plasmids pUCMRP8d and pUCMRP14d containing the *Bgl*II linker inserted at positions 536 and 420 (see Fig. 4 and 5), respectively, were then used as expression plasmids. The 2.2-kb *Hin*dIII-to-*Eco*RI fragment from pCMVCAT containing the *cat* gene was inserted into the pUCMRP8d and pUCMRP14d vectors at the *Bgl*II site by blunt-end ligation. The resulting CAT expression plasmids pUCMRP8dCAT and pUCMRP14dCAT were verified by sequencing through the 5′ boundary of the *cat* gene.

DNA transfection. The promyelocytic cell line HL60 was transfected by the DEAE-dextran procedure (13) with minor changes. The cells (10^7) were washed once with Tris-buffered saline and suspended in 1 ml of transfection mixture (5 μg of supercoiled DNA per ml and 0.5 mg of DEAE-dextran per ml in Tris-buffered saline) at room temperature. After 30 min, 10 ml of culture medium containing 0.1 mM chloroquine was added followed by incubation for 1 h at 37°C in the presence of 5% CO_2. The medium was replaced with 10 ml of culture medium, and the cells were incubated for 72 h at 37°C in the presence of 5% CO_2. For HL60 induction, DMSO (1.25% [vol/vol]) was added after transfection. The lung embryonal cell line L132 was transfected by the DEAE-dextran method described previously (47).

CAT assay. The cells were washed twice with cold Tris-buffered saline and then lysed by three cycles of freeze-thawing in 150 μl of 250 mM Tris hydrochloride (pH 7.5). Cellular debris was pelleted by centrifugation. Portions of the cellular supernatants were assayed for CAT activity as described previously (19).

RESULTS

Expression of MRP8 and MRP14 mRNA in human blood monocytes and leukemia cell lines. To identify cell lines that can be used to study MRP gene regulation, we analyzed a number of human cell lines for expression of MRP8 and MRP14 mRNA. We primarily tested cells of myeloid origin since it has been previously reported that the expression of MRP8 and MRP14 proteins is specific for cells of myeloid origin (i.e., granulocytes, monocytes, and macrophages), whereas lymphocytes and platelets do not show detectable levels of expression (41). Purified human blood monocytes, leukemia cells (HL60 and U937), human B lymphocytes (Namalwa) and T lymphocytes (MOLT-4), and human lung epithelial cells (L132) were tested. Hybridization of MRP cDNAs to these RNA samples revealed that MRP8 and MRP14 are detectable only in cell lines of myeloid origin (Fig. 1A and B). In the blood monocytes and in the promyelocytic cell line HL60, MRP8 and MRP14 mRNAs (550 and 750 bases long, respectively) were present. However, for the promonocytic cell line, U937, a clear signal for MRP14, but not MRP8, mRNA was observed. No hybridization was detected in Namalwa, MOLT-4, and L132 cell lines. Hybrid-

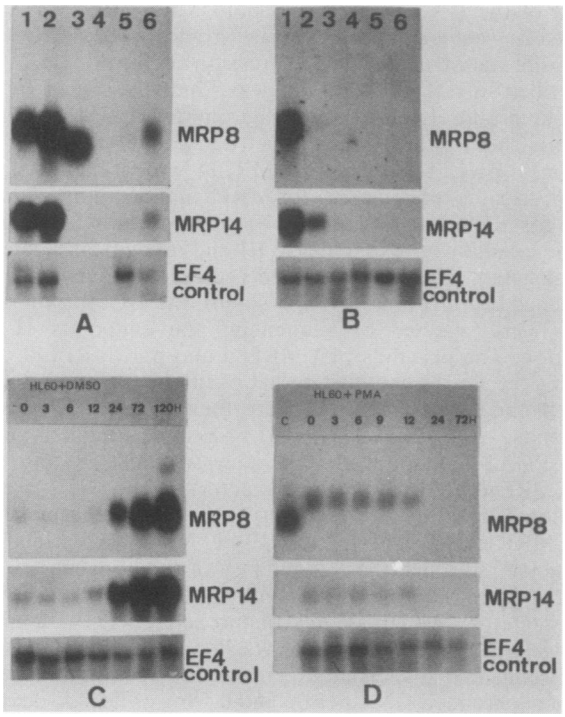

FIG. 1. Northern blot analysis of human cells. The blots were hybridized with MRP8, MRP14, or EF4 (control) cDNA probes. (A) Lanes: 1, total cellular RNA from purified human blood monocytes; 2, total RNA purified from human blood monocytes in the presence of actinomycin D; 3, control lane, SP6 synthetic MRP8 RNA (500 bases); 4, RNA molecular weight standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); 5, total cellular RNA from HL60 cells after 12 h of PMA treatment; 6, total cellular RNA from uninduced HL60 cells. (B) Total cellular RNA. Lanes: 1, uninduced HL60 cells; 2, U937 cells induced with PMA; 3, uninduced U937 cells; 4, MOLT-4 cells; 5, Namalwa cells; 6, L132 cells. (C) Total cellular RNA from HL60 cells after 0, 3, 6, 12, 24, 72, and 120 h of DMSO treatment. (D) Total RNA from HL60 cells after 0, 3, 6, 9, 12, 24, and 72 h of PMA treatment. Lane c, Control lane, same as for panel A, lane 3. Given differences in exposure time and specific activity of the probes, the absolute levels of hybridization signal shown in panels A through D cannot be compared (e.g., the level of MRP8 mRNAs in panels C and D [zero time points] is the same, whereas the signal is higher in panel D).

ization to the control cDNA probe EF4, the mouse protein synthesis initiation factor 4A, revealed a transcript of 2 kb in all cells (39).

Association of regulation of MRP8 and MRP14 expression with differentiation of HL60 leukemia cells. To follow the temporal pattern of expression of the MRP8 and MRP14 mRNA in a myeloid cell line during differentiation, we analyzed RNA from the HL60 cell line. This cell line is a model system for myeloid differentiation. Proliferating HL60 cells can be stimulated to terminally differentiate into non-proliferating monocytic (36) or granulocytic (8) lineages by treatment with PMA or DMSO, respectively. Within 24 h of induction of HL60 cell differentiation with PMA, the levels of MRP8 and MRP14 mRNA essentially disappear (Fig. 1D). In contrast, with DMSO treatment, a marked expression of both MRP mRNAs was observed after 24 h. For MRP8, this was concomitant with the progressive appearance of a larger mRNA transcript, approximately 1 kb in length (Fig. 1C). These results indicate that MRP8 and MRP14 expression is regulated in HL60 cells that are induced to differentiate into either the monocytic or granulocytic cell lineage.

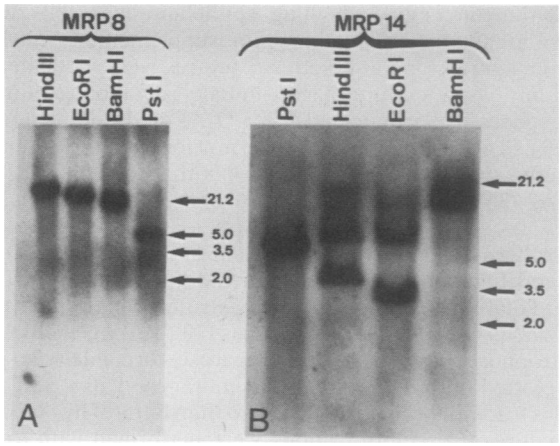

FIG. 2. Southern blot hybridization analysis of human chromosomal DNA. High-molecular-weight DNA prepared from human placenta was digested with various restriction enzymes as indicated. (A) The genomic DNA was probed with a human MRP8 cDNA probe. The *Bam*HI digest yields an 18-kb hybridizing DNA fragment, *Hin*dIII yields a 22-kb fragment, *Eco*RI gives a 21-kb fragment, and *Pst*I yields a 5-kb DNA fragment. (B) Same as for panel A but with a human MRP14 cDNA probe. The *Bam*HI digest yields an 11.6-kb fragment, and *Pst*I yields a 6-kb fragment. The *Hin*dIII and *Eco*RI digests both yield two fragments: for *Hin*dIII, 5.7 and 3.6 kb, and for *Eco*RI, 5.4 and 2.7 kb. The membranes were washed in 0.1× SSC at 65°C. Numbers to right of panels A and B are in kilobases.

Structure of human MRP genes. To initiate the analysis of *cis*-acting elements governing the tissue-specific expression of the MRP genes, we cloned the human genes encoding MRP8 and MRP14. Restriction analysis of human placental DNA revealed that both genes are represented as a single copy per haploid genome. For example, restriction analysis with *Hin*dIII, *Eco*RI, *Bam*HI, and *Pst*I revealed a single DNA fragment hybridizing with an MRP8 cDNA probe (Fig. 2A). Similarly, placental DNA restricted with *Bam*HI and *Pst*I also revealed a single DNA fragment hybridizing with the MRP14 cDNA probe, whereas two DNA fragments were detected after cleavage with *Hin*dIII and *Eco*RI (Fig. 2B). No additional bands of hybridization were detected even under low-stringency conditions (data not shown). To isolate the two MRP genes, we screened a human liver DNA library with MRP8 and MRP14 cDNA probes. Several overlapping clones, as determined by restriction analysis (data not shown), were isolated. For sequence analysis, a 5-kb *Hpa*II DNA fragment containing the MRP8 gene and a 4.2-kb *Pst*I DNA fragment encompassing the MRP14 gene were subcloned (Fig. 3). The entire sequences of the MRP8 and MRP14 genes including the introns and proximal 5′- and 3′-flanking regions are shown in Fig. 4 and 5. The MRP genes consist of three exons which have different lengths (33, 164, and 211 bp for MRP8 and 28, 165, and 380 bp for MRP14). In each gene, exon 1 encodes part of the 5′ untranslated region of the mRNA, whereas exon 2 contains the translation initiation site. Exon 3 includes the termination codon and the polyadenylation signal AATAAA. The exons are separated by two introns of different lengths (484 and 150 bp for MRP8 and 292 bp and ~2 kb for MRP14). Intron 2 of both the MRP8 and the MRP14 genes is of class 0 (44) since it interrupts the coding region between codons. All exon-intron junctions are in agreement with the consensus splice donor and acceptor sequences (35). Furthermore, the 3′ half of each intron contains a polypyrimidine stretch and a

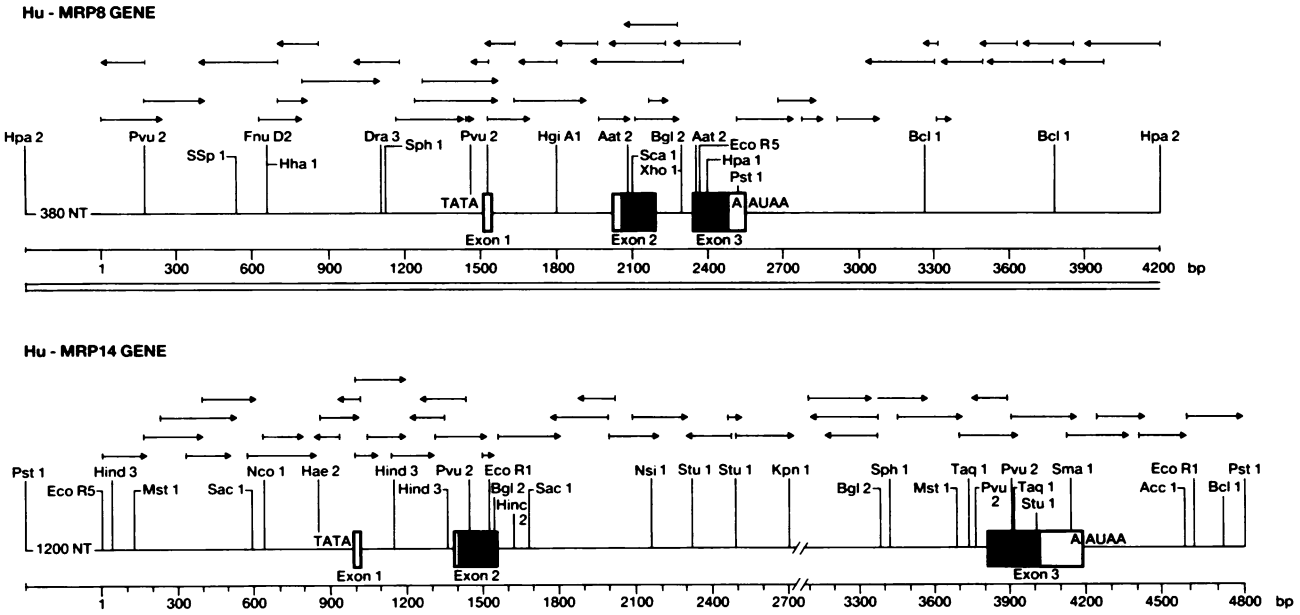

FIG. 3. Genomic organization and DNA-sequencing strategy of the human MRP8 and MRP14 genes. The black areas stand for the coding regions, and the boxed-in regions show the untranslated parts of the genes. The arrows point to the sequencing direction and end when the sequence is not clearly readable.

consensus DNA motif involved in lariat structure formation (5'-CTPuAPy-3') for splicing (43). A stretch of GTs and Ts is found 15 to 20 bp downstream of the poly(A) addition site (Fig. 4 and 5). Such DNA sequences were recently shown to be essential for the formation of genuine 3' mRNA termini (16; for a review, see reference 3). The DNA sequences of the exons were identical to the cDNA sequences described before (41).

Alignment of the MRP DNA sequences 5' of the initiation site of transcription revealed several sequence motifs conserved in MRP8 and MRP14. The promoter region of each of the MRP genes contains a sequence at −29 that fulfills the criteria for a TATAAA box as found in many polymerase II promoters as well as a CAAT box at position −92 in MRP8 and −76 in MRP14. The transcription initiation site, numbered +1 in Fig. 4 and 5, was determined by primer extension (data not shown).

Furthermore, the flanking sequences include an 11-bp stretch of homology which could be involved in the tissue-specific regulation of these genes. The 11-bp sequence reads 5'-AGGAGCTGCCT-3' and is placed immediately adjacent to, but on opposite sides of, the TATA box when both genes are compared. Other more inconspicuous stretches of DNA might be involved in the regulation of some of the clearly different patterns of expression of the two genes. First, the MRP8 gene is flanked by a pair of 325-bp inverted repeats (Fig. 4). These share 78% sequence homology, but the copy at the 3' end of the gene is interrupted by a 300-bp element that belongs to the *Alu*I family (26). The inverted repeat bears no significant homology with other known sequences in the EMBL DNA libraries. A second, 16-bp inverted repeat, 5'-TGAGTGCATGCACTCA-3', was identified at position −400 in the MRP8 gene (Fig. 4). This repeat contains an octamer, $(TGCA)_2$, which is also found as four tandemly arranged copies in the XU1b small nuclear RNA gene of *Xenopus laevis* (29). Its sequence is reminiscent of a transcriptional enhancer since it is composed of multiple alternate Pu and Py, an arrangement which is expected to fold into a Z-DNA conformation (38, 40). Finally, another potential regulatory DNA sequence is located 380 bp downstream of the 3' end of the MRP8 mRNA (Fig. 4). This 32-bp sequence contains mostly $(GT)_n$, yielding a potential Z-DNA structure.

As shown overlined in the MRP14 gene promoter (Fig. 5), there is a putative SP1 transcription factor-binding site (5'-CCGCCC-3') at position −130. In the mRNA 3' untranslated region, a stretch of DNA contains several direct repeats, 5'-GGCCAC-3' (overlined in Fig. 5). This motif is also found upstream of the TATA box in the interleukin-1α gene promoter (14) and was described as a potential transcriptional regulatory signal because of its homology with the adenovirus type 2 major late promoter sequence. This tandem array of direct repeats may explain the failure to isolate full-length cDNAs clones: five independent cDNA isolates end in the region containing these direct repeats (41). As a consequence, the 3' end of the MRP14 mRNA was placed 20 bp downstream of the poly(A) addition site, as for the MRP8 gene.

MRP8 and MRP14 are both members of a new family of small calcium-binding proteins. A computer-assisted protein sequence comparison of MRP8 and MRP14 peptides with the protein data base of the National Biomedical Research Foundation (United States) revealed strong homologies between MRP8 and MRP14 peptides and small calcium-binding proteins including S100α, S100β, ICaBP, calcyclin (2A9), and P11. The homologies are concentrated in the calcium-binding domains of these proteins (Fig. 6). These domains are known as EF hands or calcium-binding fingers and consist of two very characteristic α-helices interrupted by a turn (28), the calcium ion being bound to the turn by oxygen-containing residues. This motif is repeated two to four times in these calcium-binding proteins, and it is believed that the proteins have evolved from a common ancestor (1, 9). S100α, S100β, and ICaBP, like MRP8 and MRP14, contain two such EF hand structures; one in the N-terminal part of the proteins is 30 amino acids long (a

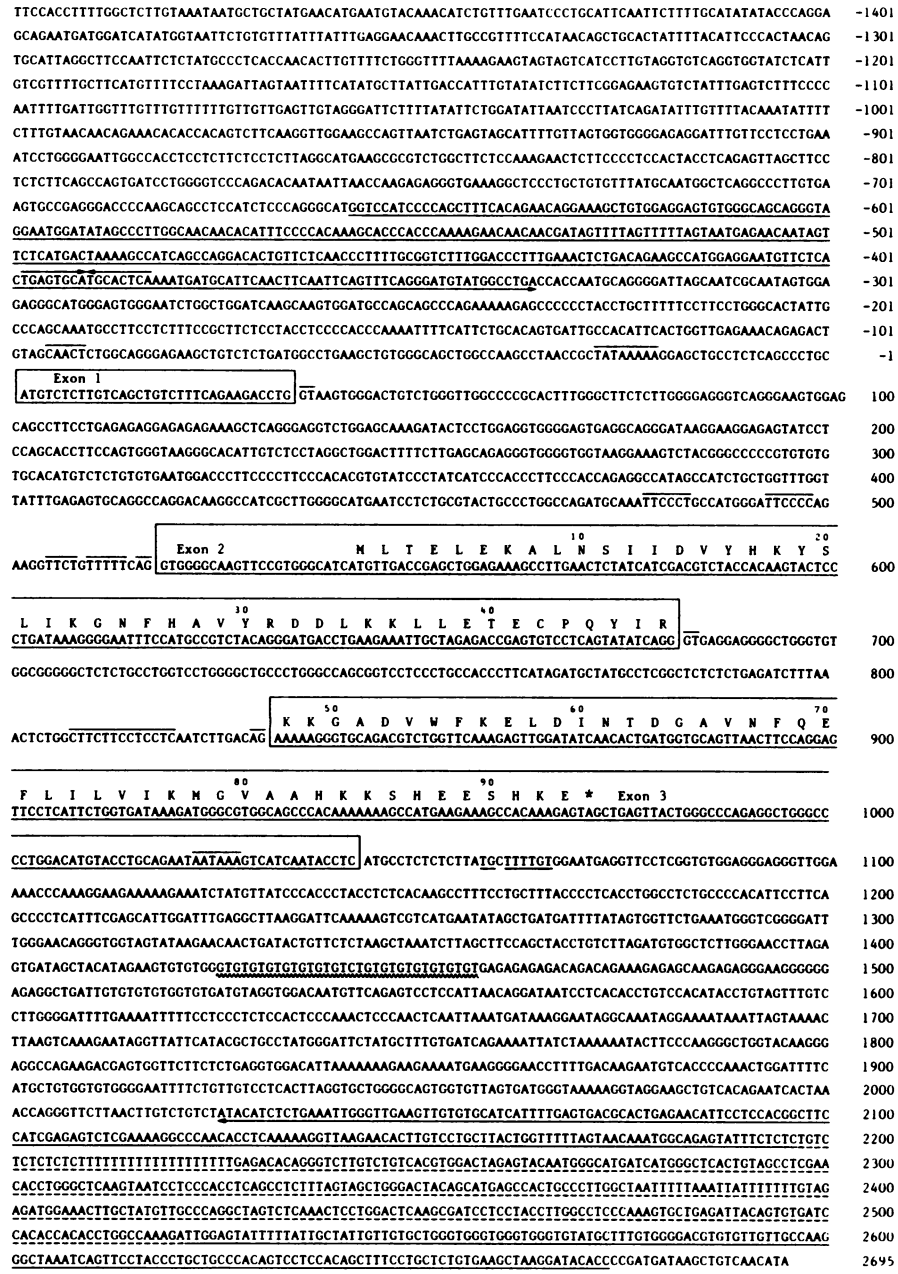

FIG. 4. Nucleotide sequence of the human gene encoding MRP8. The exon sequences are boxed, and the protein sequence is listed according to the one-letter amino acid code. Nucleotides are numbered according to the cap site (position +1). Conserved DNA motifs are overlined, and direct-indirect repeats are marked by arrows. The wavy line indicates a long Py-Pu stretch, and the dashed line splitting the 3′ long inverted repeat indicates the *Alu*I family repeated sequence.

variant EF hand), and the other in the C terminal part of the proteins is 28 amino acids long (a normal EF hand) (48). MRP8 protein is closely related to S100α. There are 32 residues identically aligned with respect to the sequence of the two proteins in Fig. 6, and the two additional residues of the variant EF hand in MRP8, serine and histidine, are aligned with respect to the sequence of the variant calcium-binding site of S100α. On the other hand, MRP14 is closely related to S100β. There are 34 amino acids aligned with respect to the sequences of the two proteins in Fig. 6. The two additional amino acids of the variant EF hand of MRP14 are serine and aspartic acid.

Transient expression of pUCMRP8dCAT and pUCMRP14 dCAT in the promyelocytic cell line HL60 but not in the epithelial cell line L132. A transient expression assay was devised to investigate the tissue-specific regulatory elements responsible for the induction or inhibition of MRP gene expression after differentiation in the leukemia HL60 cell. For both genes, we constructed two similar clones containing exon 1 and a large part of the untranslated region of exon 2 (Fig. 3; Materials and Methods) fused to the *cat* gene. The resultant clones, designated pUCMRP8dCAT and pUCMRP14dCAT, were transfected into HL60 cells (with or without DMSO induction) as well as nonmyeloid cells such as the epithelial cell line L132. Figure 7 shows the representative level of CAT activity detected in nine independent

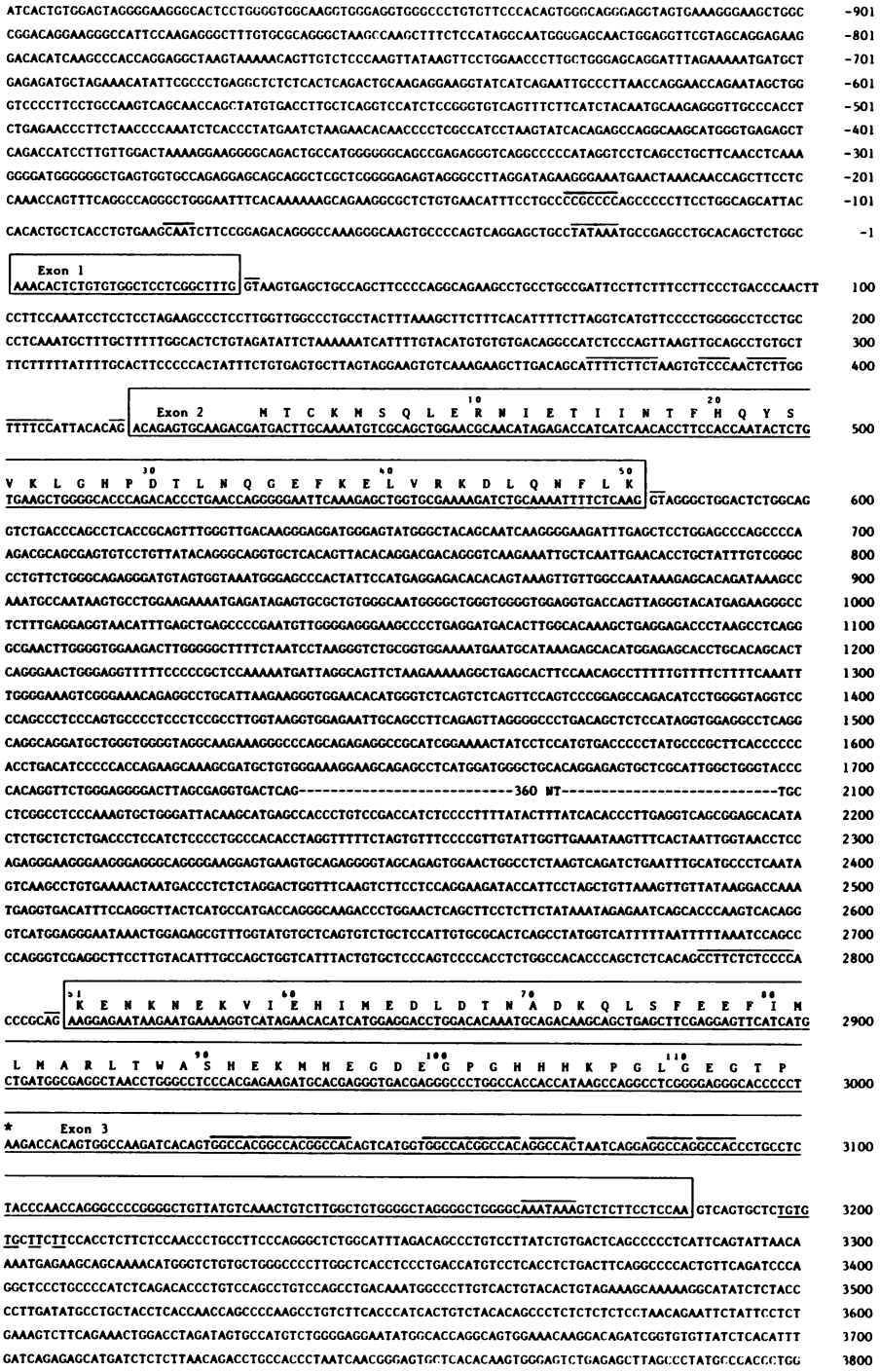

FIG. 5. Nucleotide sequence of the human gene encoding MRP14. The codes are as described in the legend to Fig. 4.

transfections in both HL60 and L132 cells. In each case, CAT expression was assayed relative to that of cells transfected with a pCMVCAT control plasmid. The cytomegalovirus promoter-enhancer routinely expressed high levels of CAT activity in both cell types. Both pUCMRP8dCAT and pUCMRP14dCAT expressed CAT activity in HL60 cells, and these levels increased three- to fourfold after induction by DMSO treatment (Fig. 7, lanes 3 to 6). This higher level of CAT activity was not observed for the pCMVCAT construct (lanes 1 and 2). In L132 cells, on the other hand, only the pCMVCAT vector generated a high level of CAT activity, while the pUCMRPdCAT constructs failed to yield detectable activity. From these results, we conclude that the regulatory *cis*-acting elements responsible for the regulated expression of the MRP genes in HL60 cells are present in the cloned MRP genes.

DISCUSSION

To study the tissue-specific regulation of the MRP8 and MRP14 genes, we analyzed mRNA derived from different

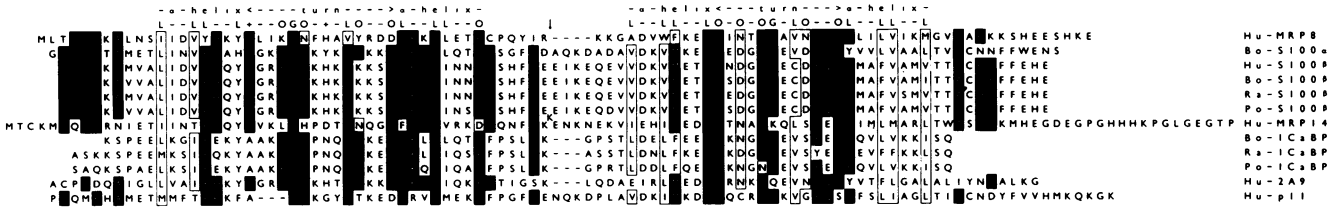

FIG. 6. BESTFIT alignment of calcium-binding proteins with two homologous calcium-binding sites (EF hands); one of them, in the N-terminal part of the proteins, is 30 amino acids long (variant EF hand), and the other, in the C-terminal part of the proteins, is 28 amino acids long (normal EF hand). The α-helices and turns of the EF hands are presented as predicted (28). The vertical arrow indicates the position where introns interrupt the coding sequences, except for the calcyclin (2A9) gene (11), in which it must be shifted one amino acid to the right. O stands for residues EDQNST (oxygen-containing residues), L stands for residues LVIFM (hydrophobic amino acids), and + stands for inserted amino acids. Identical amino acids are shown on black areas, and functionally homologous amino acids are boxed in. Hu, Human; Bo, bovine; Ra, rat; and Po, porcine.

human cell lines. We report here that MRP8 and MRP14 mRNA expression is only detected in leukemia cell lines. Promyelocytic HL60 cells expressed both MRP8 and MRP14 mRNAs. On the other hand, promonocytic U937 cells showed no detectable MRP8 mRNA expression and a weak level of MRP14 mRNA expression. The U937 cell is believed to belong to a monocytic lineage and shares properties with certain macrophage subsets (20). To investigate whether the different levels of MRP8 and MRP14 expression observed between HL60 and U937 leukemia cells correspond to their state of differentiation, we chose to follow the expression of MRP genes during the differentiation of the promyelocytic HL60 cell along the monocytic and granulocytic lineages. After 24 h of PMA-induced monocytic differentiation, HL60 leukemia cells no longer expressed MRP8 and MRP14 mRNA, whereas after granulocyte differentiation induced by DMSO, both MRP8 and MRP14 mRNAs were highly produced. Interestingly, MRP gene expression is only regulated as the promyelocytic cells begin to terminally differentiate into macrophages or granulocytes and therefore is not a direct effect of PMA or DMSO treatment. Granulocytic differentiation activates MRP mRNA expression strongly and in a strikingly parallel manner for both genes.

The low level of MRP8 and MRP14 mRNAs in monocytic U937 cells and in PMA-induced monocytic HL60 cells prompted us to analyze the mRNA levels in normal human blood cells because MRP8 and MRP14 mRNA expression observed in these leukemia cell lines is in contradiction with the high level of expression of the MRP8 and MRP14 proteins observed in normal blood monocytes and granulocytes (41). To determine whether this apparent contradiction was a result of using the leukemia cell lines, we initiated the analysis of the mRNA levels in freshly isolated human blood monocytes. RNA samples from those cells revealed high levels of MRP8 and MRP14 mRNAs. This result suggests that the regulation of the mRNA levels in U937 and in HL60 induced by PMA is different from that observed in normal blood monocytes.

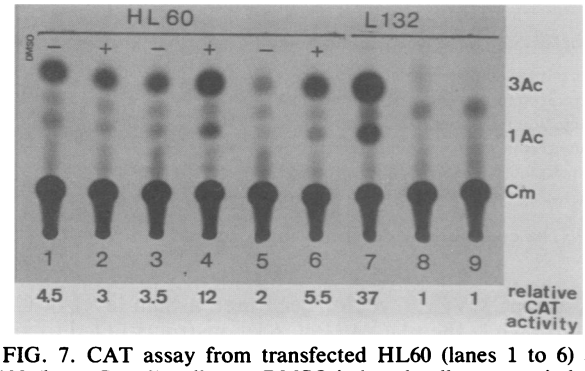

FIG. 7. CAT assay from transfected HL60 (lanes 1 to 6) and L132 (lanes 7 to 9) cells. +, DMSO-induced cells; −, noninduced cells. CAT activity is expressed relative to the activity measured with plasmids pUCMRP8dCAT and pUCMRP14dCAT (lanes 8 and 9) in L132 cells, for which it is arbitrarily set as 1. Lanes 1, 2, and 7, Cells transfected with pCMVCAT vector; lanes 3, 4, and 8, cells transfected with pUCMRP8dCAT; lanes 5, 6, and 9, cells transfected with pUCMRP14dCAT. Cm, Chloramphenicol; 1Ac and 3Ac, acetylated forms of chloramphenicol.

To further study this complex mechanism of MRP regulation during myeloid cell differentiation, we cloned the genes encoding human MRP8 and MRP14. MRP8 and MRP14 have a similar genomic organization: both are small genes containing three exons of approximately equivalent length and coding capacities (Fig. 3). While this manuscript was in preparation, the genomic structure of calcyclin (2A9) was described (11) and shown to be very similar to that of the MRP genes. In all cases, exon 1 is untranslated and exon 2 and 3 each delimit an entire calcium-binding domain. As presented in Fig. 6, MRP8 and MRP14 are calcium-binding proteins, closely related in structure to S100α, S100β, ICaBP, calcyclin (2A9), and P11. The MRP8 and MRP14 peptides contain two calcium-binding sites or EF hands; the normal EF hand with 28 amino acids resides in the C-terminal part of the protein and the variant EF hand with 30 amino acids is located in the N-terminal part of the protein (48). Experiments have shown that MRP8 and MRP14 proteins immobilized on Western blots (immunoblots) indeed bind $^{45}Ca^{2+}$ (J. Lucas et al., manuscript in preparation; data provided to the reviewer).

The linker sequences joining the two EF hands in all these proteins differ markedly. Since these linker regions are seemingly free to evolve, compared with the very conserved nature of calcium-binding domains, it was proposed that sequence divergence in the linkers results from the unique functions of these proteins and may be related to their specific interactions in different tissues (48). The C-terminal segment of MRP14 may similarly have some special function unique to this molecule since it is unusually long in comparison with other members of the family (Fig. 6).

At the gene level, we identified an intron in both the MRP8 and MRP14 genes located in the middle of the linker region. It is possible that this intron has had a role in the evolution of this protein family. The alignment of the EF hands in most of the protein sequences shown in Fig. 6 requires a gap or a deletion of amino acids in the region of this intron. Further,

exons 2 and 3 of MRP8 and MRP14 each encode a complete calcium-binding domain. The same is true for calcyclin (2A9) (11). In contrast, the large calcium-binding proteins such as calmodulin or parvalbumin (21) have introns which interrupt their calcium-binding domains. It is tempting to speculate that the presently uncharacterized genes encoding S100α, S100β, P11, and ICaBP will have genomic structures similar to those of the MRP8, MRP14, and calcyclin genes (see vertical arrow, Fig. 6) and therefore that all these closely related proteins belong to a new subfamily of calcium-binding proteins.

The promyelocytic cell line HL60 and the epithelial cell line L132 were transfected with the MRP genes fused with the *cat* gene. The two chimeric constructs pUCMRP8dCAT and pUCMRP14dCAT expressed CAT activity only in the HL60 cells. Furthermore, upon DMSO induction this activity was enhanced, whereas upon PMA induction no CAT activity was detected (data not shown). The mechanism of regulation seems to be specific for these two genes because the expression of CAT activity from the cytomegalovirus promoter-*cat* construct (pCMVCAT) was not affected. These results suggest that we cloned the tissue-specific regions which permit the MRP8 and MRP14 genes to be expressed in the promyelocytic HL60 cell. We also conclude that the *cis*-acting elements which enhance or inhibit MRP8 and MRP14 gene expression during HL60 differentiation into a granulocytic or monocytic lineage, respectively, are present. Interestingly, comparing MRP8 and MRP14 promoter regions, we found several conserved motifs. These homologies suggest that the two promoters could be recognized by common factors that bind these sequence motifs. We now have a framework for future investigation involving the characterization of the regulatory mechanisms responsible for these effects.

Data indicate that MRP8 and MRP14 are involved in inflammatory reactions and that MRP8 is found in elevated levels in serum from cystic fibrosis patients. Since MRP8 and MRP14 are calcium-binding proteins and since calcium metabolism is likely to be deregulated in the salivary glands of cystic fibrosis patients (33), it would be of interest to see whether the deregulation of these genes contributes to the etiology of this genetic defect.

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ADDENDUM

Brueggen et al. (J. Brueggen, L. Tarcsay, N. Cerletti, K. Odink, M. Rutishauser, G. Hollaender, and C. Sorg, Nature [London] **331**:570, 1988) recently reported that, instead of the MRP8 protein as reported here, MRP14 protein is present in elevated quantities in blood serum from cystic fibrosis patients as well as in blood serum from cystic fibrosis obligate heterozygotes. In the same publication, the human genes encoding MRP8 and MRP14 were reported to be localized on chromosome 1.

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