Journal of Cellular Biochemistry 73:49–55 (1999)

Identification of Epithelial and Myeloid-Specific DNA Elements Regulating MRP14 Gene Transcription

Martin Klempt,[1,2*] **Harutyun Melkonyan,**[1] **Heiko A. Hofmann,**[1] **and Clemens Sorg**[1]

[1]Institute of Experimental Dermatology, University of Münster, 48149 Münster, Germany
[2]Institute of Physiology and Biochemistry of Nutrition, Alliance Product and Nutrition Research, Federal Dairy Research Center, 24103 Kiel, Germany

Abstract Macrophage migration inhibition factor-related protein 14 (MRP14) is a Ca^{2+}-binding protein of the S-100 family highly abundant in myelomonocytic and epithelial cells. The expression pattern is restricted to myeloid and epithelial cells and therefore the MRP14 gene is ideally suited to study the regulation of gene expression in these cells. We characterized the human MRP14 promoter by using the chloramphenicol acetyltransferase reporter assay system. The analysis was performed in epithelial (TR146) and myeloid (HL-60) cells, and we were successful in mapping positive and negative regulatory elements. The region -114/-419 contains strong myeloid-specific regulatory elements, whereas the domain -600/-1000 enhances the MRP14 transcription in epithelial cells. The sequence -462/-600 accommodates a regulatory element that enhances the promoter activity in both myeloid and epithelial cells. Regions -114/-419 and -1000/-4500 reduce the expression of MRP14 only in epithelial cells, whereas the domains -419/-462 and -4500/-6500 contain down-regulating elements in both, epithelial and myeloid cells. The presented data demonstrate that transcription of the human MRP14 gene is regulated in a complex manner enabling the precise control of the MRP14 level in epithelial and myeloid cells. J. Cell. Biochem. 73:49–55, 1999.

Key words: myeloid promoter, endothelial promoter, MRP14

Macrophage migration inhibition factor-related proteins MRP8 and MRP14 are two Ca^{2+} binding proteins of the S-100 protein family expressed in myeloid cells [Odink et al., 1987; Lagasse and Clerc, 1988; Dorin et al., 1990] and in certain epithelial and endothelial cells [Gabrielson et al., 1986; Yen et al., 1997]. The expression of MRP14 is usually accompanied by the expression of MRP8. Both proteins form heterodimers that have been proposed to represent the biologically active form. The presence in monocytes and granulocytes coincides with the state of differentiation [Roth et al., 1994]. A high intracellular Ca^{2+} level diminishes MRP mRNA expression by a currently unknown suppressor mechanism and is responsible for biochemical alterations like its translocation to the cell membrane, or association to the cytoskeleton [Roth et al., 1994]. In the promyeloid cell lines HL-60 or U937 the expression of MRPs was highly upregulated by dimethylsulfoxide (DMSO) and vitamin D_3 [Koike et al., 1990; Roth et al., 1993], indicating a preferential expression in cells representing an advanced differentiated phenotype. Multiple functions of MRPs have been described: They participate in phosphorylation, cell cycle progression, cell differentiation, and cytoskeleton-membrane interaction, and in addition have antifungal, antibacterial, and chemotactic properties [for review, see Schäfer and Heizmann 1996; Zimmer et al., 1995; Murao et al. 1990; Hessian et al., 1993]. Besides the expression in monocytes and granulocytes, MRPs have also been found in endothelial cells [Yen et al., 1997; Hogg et al., 1989].

Epithelial cells express MRPs under pathophysiological circumstances such as systemic lupus erythematosus, psoriasis, or cutaneous malignancies [Gabrielson et al., 1986; Brandtzaeg et al., 1987; Kelly et al., 1991]. Since strati-

Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant number: SFB 293; Contract grant number: Kl 723/2–1, 2, 3.

*Correspondence to: Martin Klempt, Institute of Physiology and Biochemistry of Nutrition, Aliance Product and Nutrition Research, Federal Dairy Research Center, Hermann-Weigmann-Str. 1, D-24103 Kiel, Germany.
E-mail: klempt@bafm.de

Received 21 July 1998; Accepted 22 October 1998

fied squamous epithelia of tongue, esophagus, and buccal cells express MRP constitutively [Wilkinson et al., 1988; Hitomi et al., 1996], they represent an ideal model to study the regulation of MRP expression in epithelial cells. To date, the regulation of MRP expression in epithelial cells is not well characterized despite the high expression of these proteins during prenatal development in amniotic epithelial cells [Hitomi et al., 1996]. To analyze the transcriptional control of MRP14, the following two cell lines were transfected with different MRP14 promoter constructs containing chloramphenicol acetyltransferase as reporter gene: TR146, a squamous carcinoma cell line originating from bucal cells [Rupniak et al., 1985], and HL-60, a promyeloid cell line [Collins et al., 1979]. We demonstrate that the expression of MRP14 is regulated differently on the transcriptional level in myeloid and epithelial cells.

MATERIALS AND METHODS

Constructs

A 7-kb *Eco*RI fragment containing the MRP14 gene was identified by screening a human genomic library (Stratagene, Heidelberg, Germany) and subsequently cloned into pSP72 [Melkonyan et al., 1998]. This fragment was used to construct the plasmids, as indicated in Figure 1. To obtain promoter constructs containing >1 kb of the 5′ flanking region (pHM-6500, pHM-4500, pHM-1000), the restriction sites found within the gene were used, and the fragments were cloned into pCAT3-Basic-Vector (Promega, Mannheim, Germany). To obtain constructs containing promoter regions shorter than 1 kb, exonuclease digestion of pHM-1000 was performed. After *Kpn*I/*Nhe*I digestion 20 µg plasmid DNA was incubated at 37°C with 500 U exonuclease III (MBI Fermentas, St. Leon-Rot, Germany). Aliquots of DNA were removed from the reaction at different time points and cooled on ice to stop the reaction. The overhanging single strand DNA was digested using S1 nuclease (MBI Fermentas), the plasmids were religated and finally transformed into heatshock-competent *Escherichia coli*.

The constructs lacking the first intron and the first exon were cloned after generating suitable restriction sites with polymerase chain eaction (PCR). The correct insertion of all DNA fragments into the vectors was confirmed by extensive restriction analysis and DNA sequencing, which showed the correct size of the exonuclease generated molecules. The sizes of the used fragments are indicated in Figure 1; pHM-40 contains the basic promoter with the TATA box.

Cell Culture

HL-60 cells (ATTC CCL 240) and TR 146 cells [Rupniak et al., 1985] were cultured using RPMI

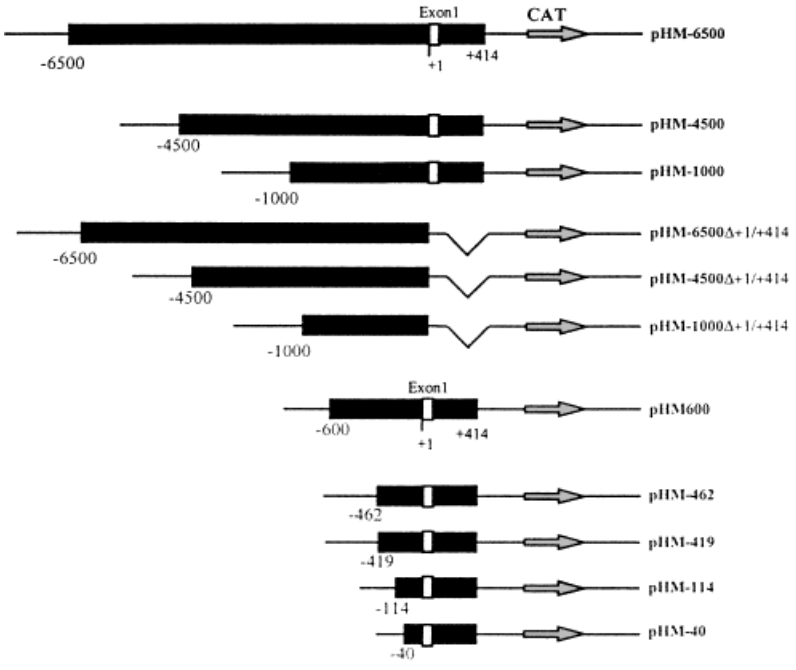

Fig. 1. Human MRP14 promoter-CAT reporter constructs. The full-length pHM-6500 construct was cloned as described under Materials and Methods. Constructs containing shorter fragments of the human MRP14 gene (solid black boxes) were derived of pHM-6500 using appropriate restriction enzymes (pHM-1000 and pHM-4500) or of pHM-1000 using exonuclease digestion. Gray arrows, coding region of chloramphenicol acetyltransferase (CAT).

1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% nonessential amino acids, and 1 mM Na-pyruvic acid. TR146 cells were treated with 1.25% DMSO(Boehringer Ingelheim Bioproducts, Heidelberg, Germany), 10^{-7} M vitamin D_3 (Sigma, Deisenhofen, Germany) or 20 ng/ml 12-O-tetradecanoylphorbol 13-acetate (TPA, Sigma) for 3 days.

Electroporation and CAT assay

Transfection was performed as described previously [Melkonyan et al., 1996]. Briefly, 7×10^6 cells were electroporated with 30 µg DNA, 960 F at 250 V for HL-60 and 960 F at 260V for TR146 using the Gene pulser (BioRad, München, Germany). After electroporation, the cells were incubated in the appropriate medium for 24 h at 37°C and 5% CO_2. To analyze the transcriptional activity of the constructs, the activity of chloramphenicol acetyltransferase was determined by using ^{3}H-coenzyme A (Hartmann Analytic, Braunschweig, Germany) as substrate in 10 mM Tris pH 7.8 [Sambrook et al., 1989]. All transfections were performed at least 12 times in three completely independent experiments.

Northern Blotting

Cells were extracted by a modification of the method of Chomzynski and Sacchi [1987], using guanidinium thiocyanate-phenol-chloroform extraction to provide total RNA samples. Aliquots of the RNA (20 µg) were separated on a denaturing 1.5% agarose gel, blotted onto nylon membrane, and probed with ^{32}P human MRP14 cDNA according to standard methods [Sambrook et al., 1989]. Finally, the membrane was washed twice with 0.1% SSC at 65°C and exposed to an X-ray film for 2 days.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) for comparison between the data obtained from different construct, followed by t-test [Zar, 1984]. All values are presented as mean ±SD.

RESULTS

MRP14 is highly abundant in myeloid and epithelial cell, and the myeloid expression is very well characterized [Roth et al., 1993]. Therefore, we studied the expression of MRP14 mRNA in epithelial cells. To further analyze the regulation of MRP14 expression, TR146 cells were incubated for 3 days with various agents and Northern blotting was performed. As demonstrated in Figure 2, TR146 cells do express MRP14 mRNA at moderate levels. The expression is not altered by the addition of TPA over 3 days, but the addition of vitamin D_3 and DMSO decreases the expression of MRP14 mRNA dramatically. The MRP14 mRNA in HL-60 cells is regulated inversely: MRP14 mRNA expression is upregulated by using DMSO or vitamin D_3 and downregulated by using TPA [Roth et al., 1993]. Therefore, we performed promoter analysis to identify cell-specific elements within the MRP14 promoter.

Promoter analysis was performed using a 7-kb fragment of the human MRP14 gene containing 6.5 kb of the 5′ flanking region and exon/intron I. This fragment was cloned in 5′ position of the CAT gene to evaluate the promoter activity (Fig. 1). Additional reporter constructs containing progressively shorter segments of the 5′ flanking region were also used for transcription experiments. After electroporation, we found that the construct containing 40 base pairs upstream and exon/intron I was able to stimulate the expression in TR146 and HL-60 cells (Fig. 3). The activity in HL-60 cells is

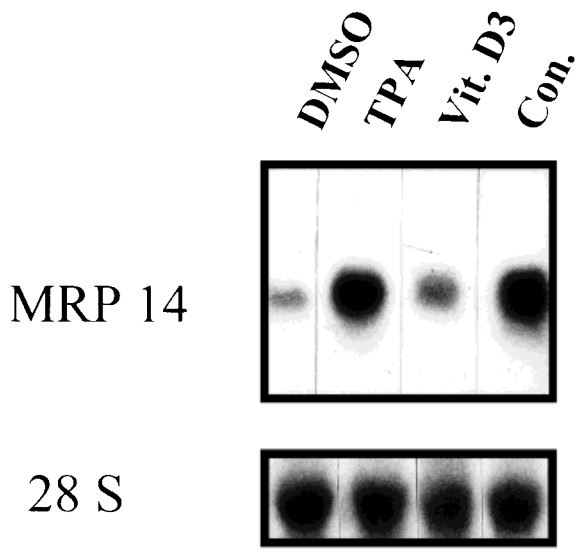

Fig. 2. Northern blot hybridization of MRP14 mRNA from TR146 cells treated with various agents. TR146 cells were treated with 1.25% dimethylsulfoxide (DMSO), 20 ng/ml TPA, or 10^{-7} M vitamin D_3 for 3 days and total RNA was probed with MRP14 cDNA. The control cells did not receive any treatment. The lower lane shows the 28S ribosomal RNA to demonstrate equal loading.

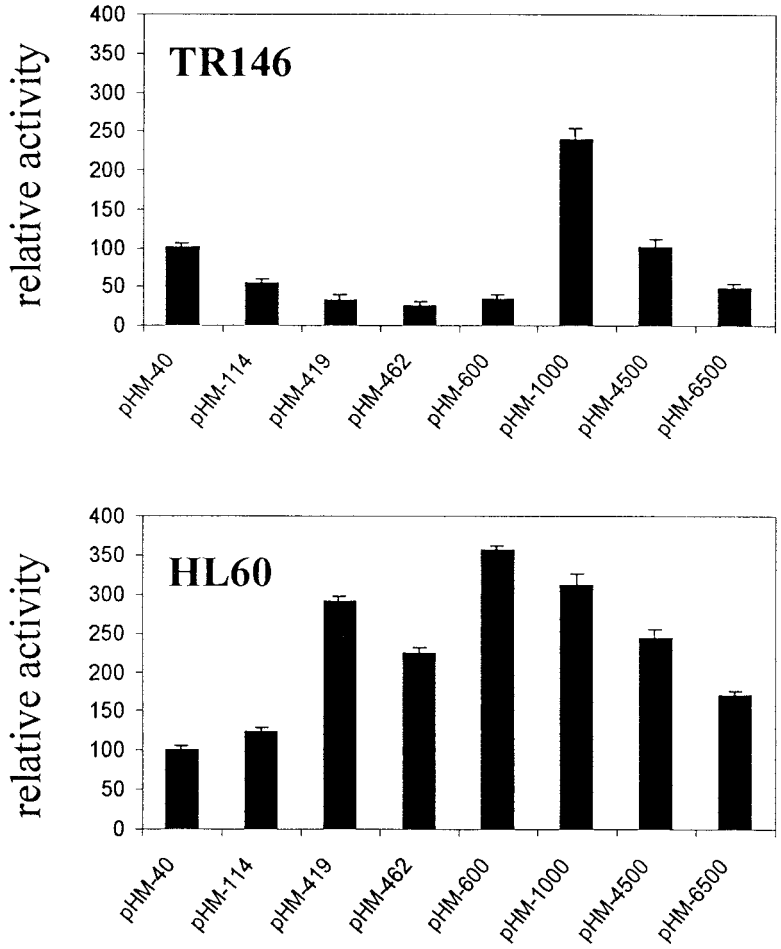

Fig. 3. Functional characterization of the human MRP14 promoter in epithelial (TR146) and myeloid (HL-60) cells. The human MRP14 promoter-CAT reporter constructs were transfected into TR146 (top) or HL-60 (bottom) cells. Data are normalized to the basic promoter (pHM-40). The data for each construct were found to be significantly different from the data obtained from the next longer and shorter construct ($P < 0.05$, t-test).

increased, whereas in TR146 the activity is decreased with increasing length of the promoter (Fig. 3). This is observable up to a length of 600 base pairs (bp)of the 5′ flanking region of the MRP14 gene.

The transcriptional regulation of constructs containing >600 bp of the 5′ flanking region are also different in both myeloid and epithelial cells: in HL-60 cells, the CAT activity measured with 5′ flanking region of the human MRP14 promoter is constant over nearly 4,000 bp; the plasmid pHM-600 is 3.5-fold more active than the basal promoter (pHM-40), and pHM-4500 is 3.4-fold more active than the basal promoter (Fig. 3). In TR146 cells, the same region demonstrates a more complex regulation: a massive (6.8-fold) increase of transcriptional activity is found between position -1000 and -600 in TR146 cells (pHM-1000 vs pHM-600), whereas constructs containing 4,500 bp and 6,500 bp of the 5′ flanking region are less active than pHM-1000 (Fig. 3). In both cell lines, TR146 and HL-60, very long constructs (pHM-6500) initiate less CAT activity than the constructs with a 4,500 bp 5′ flanking region (pHM-4500).

The transcriptional activities of the constructs containing exon/intron I are superior to those without (Fig. 4). Interestingly, the relative enhancement, calculated as relation between the construct containing the first exon/intron and those of exactly the same size but without exon/intron I, is different in both cell lines. In TR146 cells, the first exon/intron is more potent with a weak promoter (activity of pHM-6500Δ+1/+414 is 6.8-fold higher than pHM-6500, but pHM-1000Δ+1/+414 is only 2.4-fold more active than pHM-1000Δ+1/+414). In HL-60 cells, the effect of exon/intron I appears to be different from the effect observed in epithelial cells: The consequence of exon/intron I are in the same range with all tested plasmids (2.0-fold increase with pHM-6500Δ+1/+414 compared with pHM-6500 vs 2.9-fold increase with pHM-1000Δ+1/+414 in relation to pHM-1000).

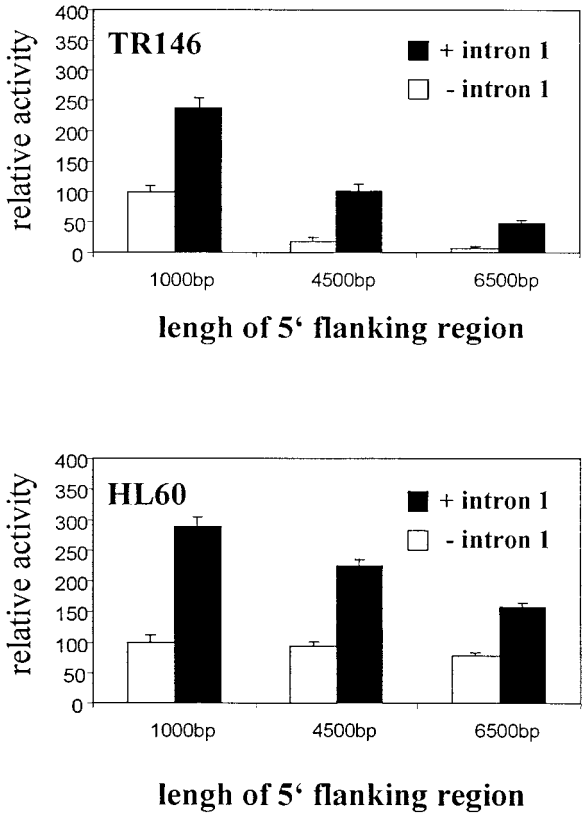

Fig. 4. Enhancing effect of human MRP14 exon I and intron I in epithelial and myeloid cells. The enhancing effect of exon/intron I was measured using constructs with 1.0, 4.5, and 6.5 kb of the 5′ flanking region of the human MRP14 gene as indicated. TR146 (left) and HL-60 (right) cells were transfected with the construct containing exon/intron I of the human MRP14 gene (solid boxes) and those lacking this region (open boxes). The data are presented as relation to the construct containing 1.0 kb of the 5′ flanking region of the MRP14 gene without exon/intron I. The data from the constructs with intron are always significantly different from the corresponding construct without intron ($P < 0.05$; t-test).

DISCUSSION

Applying Northern blot analysis and transient transcriptional analysis of the human MRP14 promoter in a CAT reporter system, we were able to characterize the basal transcriptional regulation of the human MRP14 gene in both epithelial cells (TR146) and myeloid cells (HL-60). Northern blot analysis shows that agents, well known to enhance the MRP14 mRNA in HL-60 cells like DMSO or vitamin D_3 [Roth et al., 1993], decrease the amount of MRP14 mRNA in TR146 cells, whereas TPA, an agent that decreases the MRP14 mRNA expression in HL-60 cells [Roth et al., 1993], has no effect on the MRP14 mRNA in TR146 cells. This clearly shows a distinct regulation of MRP14 expression in the two cell lines TR146 and HL60. By analyzing the promoter region of the MRP14 gene with the TRANSFAC database [Wingender et al., 1996], no vitamin D-responsive element could be found, demonstrating that the effect of vitamin D is probably due to a general cellular effect. This finding is in good agreement with the initial observation of Miyaura et al. [1981], which used vitamin D as an initiator for HL-60 cells to differentiate in the monocytic direction.

It was demonstrated earlier that the 1,000-bp 5′ region of human MRP14 gene is active only in myeloid/monocytic cells [Lagasse and Clerc, 1988; Melkonyan et al., 1998]. We were able to demonstrate that the basal promoter is active independent of any cell or tissue specificity and that the human MRP14 gene contains both negative and positive cell-specific regulatory elements. In agreement with the previous observations [Lagasse and Clerc, 1988; Melkonyan et al., 1998], regulatory elements are found upstream of the basal promoter. In summary, we found five non-cell-specific (Fig. 5A,B,E, positive; G,H, negative), two myeloid-specific (Fig. 5C,D), and one epithelial-specific (Fig. 5F) regulatory domains (Fig. 5).

A construct containing the proximal 40 nucleotides of the 5′ flanking region, exon I containing the 5′-untranslated region of the MRP14 mRNA and intron I (Fig. 5, elements A,B; −40/+414) was able to control the CAT expression in epithelial and HL-60 cells (pHM-40; Fig. 3). This construct contains the basal promoter accommodating the TATA box with initiator sequence. This expression can be altered by using constructs containing more of the 5′ flanking region. The increase of activity resulting from the fragment -462/-600 (Fig 5, element D) is 1.4-fold in TR146 cells (25% vs 35% of the basal promoter) and 1.6-fold in HL-60 cells (220% vs 358% of the basal promoter). A putative GATA1/2 binding site (5′ GGGGATGGGG beginning with position −301 of the MRP14 gene) and a putative MZF1 (myeloid zinc finger, 5′AAAGGG beginning with position −298 of the MRP14 gene) binding site are present within region D, as established by using the TRANSFAC database [Wingender et al., 1996]. The sequence derived probability that the mentioned GATA1/2 and MZF1 is binding to this segment is about 94% and 90%, respectively [Merika and Orkin, 1993; Morris et al., 1994]. Therefore, it is well conceivable that these proteins

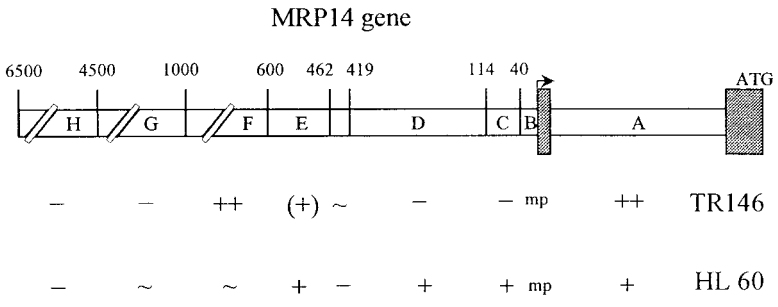

Fig. 5. Model of the regulatory domains of the human MRP14 gene. The locations of the minimal promoter (mp), and the upstream and downstream positive and negative regulatory elements are shown for TR146 and HL-60 cells. The numbers above the map represent the position relative to the start of transcription (+1); shaded boxes, exons I and II; letters, regulatory domains.

are responsible for the measured upregulation. This assumption appears reasonable, since both proteins are expressed in leukemia and other transformed cells [Zon et al., 1993; Lee et al., 1991]. The negative effect of transcription factors interacting with the DNA between positions -4500 and -6500 (Fig. 5, element H) is also present in both cell lines. In TR146 cells, the decrease is 2.1-fold (101% vs 47.8% of the basal promoter) and in HL-60 cells 2.0-fold (343% vs 171% of the basal promoter). Since both regions regulate the expression in different cells not only in the same direction, but in the same magnitude as well, we conclude that these regions are not cell specific.

Region A (Fig. 5; +1/+414; exon I/intron I) has positive effects on the transcription in both, the TR146 and HL-60 cells (Figs. 4, 5), which has been described earlier in other cells, even in cells not expressing MRP14. The enhancer could be confined to a 39-bp region within intron I to which a complex of four, currently unknown, proteins bind [Melkonyan et al., 1998].

Besides the elements effective in both cells, we found some regions that induce increased or decreased transcription rates in TR146 or HL-60 cells. Element F, which is obviously active only in TR146, but not in HL-60 cells, is located between positions -600 and -1000 of the human MRP14 gene. The construct pHM-1000 is 6.8-fold more active in TR146 but even slightly less active (0.9-fold) in HL-60 cells, compared to pHM-600. Therefore, region F contains an epithelial-specific DNA element, which obviously regulates the MRP14 expression. Myeloid-specific enhancing elements are located closer to the transcription initiation site (Fig. 5, element C, D). We found that pHM-419 increases the transcription rate 2.9-fold relative to the basal promoter, whereas in epithelial TR146 cells the effect is contrary (3.3-fold reduction; Fig. 3). It is tempting to speculate that the same stimulation might increase the human MRP14 mRNA expression in myeloid cells, and decrease the epithelial expression.

Our data demonstrate that the human MRP14 gene is under complex transcriptional control, with both negative and positive regulatory regions. Some of these regions are functional in a cell-specific manner, while others appear to be rather unspecific. These results have several implications to understand the regulation of MRP14 gene expression. First, the discovery of negative regulator elements G and H, which are operative in the tested cell lines, implicates the expression of protein binding to these elements in all cells and that cells expressing MRP14 require an appropriate agent to antagonize the action of that element. Second, the strong positive element A (+1/+414), which is functional in various different cells, may account for the strong expression of MRP14. Third, the presence of the cell-specific regions F in epithelial and C and D in myeloid cells indicate that interaction with multiple factors in individual cells types are required to obtain an optimal expression of MRP14. The precise mechanisms underlying the cell specificity requires further investigation to define the regulatory elements in more detail. Furthermore, the proteins, interacting with the MRP14 promoter and regulating the transcription of MRP14 specifically in myeloid or epithelial cells, need to be determined.

ACKNOWLEDGMENTS

The authors thank Mrs. B. Scheibel for excellent secretarial help, Dr. F. Schönlau and Dr. C. Kerkhoff for critical reading of the manuscript, and D. Wiesmann for superior technical assistance. This work is part of an HM Ph.D. thesis of the Westfälische Wilhems Universität Münster.

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