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Regulated Expression of the *MRP8* and *MRP14* Genes in Human

Promyelocytic Leukemic HL-60 Cells Treated with the Differentiation-

Inducing Agents Mycophenolic Acid and $1\alpha,25$ -Dihydroxyvitamin D_3

Audrey L. Warner-Bartnicki, Shinichi Murao, Frank R. Collart, and Eliezer Huberman*

The Biological and Medical Research Division, Argonne National Laboratory, 9700

South Cass Avenue, Argonne, Illinois, 60439

Abstract

The calcium-binding proteins *MRP8* and *MRP14* are present in mature monomyelocytic cells and are induced during differentiation. Previous studies have demonstrated that the proteins may mediate the growth arrest in differentiating HL-60 cells (S. Murao, F. Collart, and E. Huberman, *Cell Growth & Differ.*, 1: 447-454, 1990). We determined the levels of a protein complex (PC) containing *MRP8* and *MRP14* and investigated the mechanism by which the genes encoding these proteins are regulated in HL-60 cells treated with the differentiation-inducing agent mycophenolic acid (MPA). While the PC was barely detectable in untreated cells, MPA treatment resulted in elevated levels of the PC which were maximal at 3-4 d, and were found to directly parallel gains in the steady-state levels of *MRP8* and *MRP14* mRNA. Transcription studies with the use of nuclear run-on experiments revealed increased transcription initiation at the *MRP8* and *MRP14* promoters after MPA treatment. $1\alpha,25$ -Dihydroxyvitamin D_3 , which induces HL-60 cell differentiation by another mechanism, was also found to increase transcription initiation at the *MRP8* and *MRP14* promoters. Our results suggest that this initiation is the major control of maturation agent-mediated increases in *MRP8* and *MRP14* gene expression, and support a role for the PC in terminal differentiation of human monomyelocytic cells.

Running Title: Regulated Expression of the *MRP8* and *MRP14* Genes

*To whom correspondence should be directed.

Telephone: (708) 252-3819

Fax: (708) 252-3387

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The abbreviations used are: MPA, mycophenolic acid; 1,25-(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; PC, protein complex; SDS, sodium dodecyl sulfate; mAB, monoclonal antibody.

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Introduction

The molecular events that govern cellular differentiation are poorly understood but are surely complex and involve the regulated and progressive expression of a large number of genes. While recent work has suggested that genes expressed early in the response to chemical agents (e.g., *c-fos* and *c-jun*) may play a functional role in cell differentiation (1), a role could similarly be proposed for genes expressed later in the differentiation cascade. Two genes that may prove relevant to human monomyelocytic cell maturation are those encoding the calcium-binding proteins MRP8 and MRP14. These proteins are members of the S-100 protein family (which includes calcyclin and S-100 α and β) that has been postulated to be involved in cell differentiation, cell cycle progression, transmission of biological calcium signals, and cytoskeletal membrane interactions (2).

Intracellularly, MRP8 and MRP14 comprise noncovalently linked complexes, the major forms of which appear to be a heterodimer, a trimer, and a tetramer (2, 3, 4). Different names have been used for the proteins and protein complex: protein complex (PC) (5); p8,14 (4); calprotectin (3); cystic fibrosis antigen (6); macrophage migration inhibitory factor-related proteins (7); L1 light and heavy chain (8); and calgranulin A and B (9). While the PC constituent proteins are barely detectable in untreated cultured human monomyelocytic leukemic cells, they are induced during the terminal differentiation of such cells and, *in vivo*, are present in mature cells of monomyeloid origin (6, 7). Recently, we demonstrated that the PC is part of a regulated differentiation program since treatment of monomyelocytic leukemic cells

with an agent or condition that causes suppression of growth but does not induce differentiation does not result in an increase in the PC level (10). Moreover, the PC added exogenously functions as an effective inhibitor of cell growth (10), and inhibits casein kinase II (5), an enzyme implicated in the control of cell proliferation by its ability to phosphorylate substrates including RNA polymerase II, ribosomal proteins PO, P1, and P2, DNA topoisomerase II, Myc and Myb oncoproteins (11), and the tumor suppressor p53 (12). These findings suggest the PC may in fact mediate the growth inhibition that is intrinsic to terminal differentiation of monomyelocytic cells (10).

We examined the expression and regulation of the *MRP8* and *MRP14* genes in the human leukemic cell line HL-60 (13) treated with the differentiation-inducing agents mycophenolic acid (MPA)¹ and the biologically active form of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃). The HL-60 cell line is a useful model for the study of monomyelocytic cell differentiation, as treatment with MPA induces features of both mature granulocytes and monocytes (14, 15), and 1,25-(OH)₂D₃ treatment induces expression of differentiation markers characteristic of monocytic-like cells (16).

Results

Time- and Dose-Dependent Increase in PC Levels during MPA Treatment of HL-60 Cells. PC levels in HL-60 cells were monitored by indirect immunofluorescence using the NM-6 mAb that detects the heteromeric PC complex.

While detection of the PC complex was limited in untreated cells (Fig. 1A), the complex was detected in the nucleus and cytoplasm of MPA-treated cells (Fig. 1B). During 4 d in culture, less than 7% of untreated HL-60 cells exhibited reactivity with the antibody (Fig. 2A). Treatment of HL-60 cells with MPA for up to 4 d resulted in a time- and dose-dependent increase in the percentage of reacting cells (Fig. 2A). After 4 d of treatment with 3 μ M MPA, more than 85% of the cells reacted with the NM-6 mAb (Fig. 2A). Under these conditions, HL-60 cells express antigenic and enzymatic markers of both monocytic and granulocytic differentiation (15). The MPA-mediated increase in reactivity with the NM-6 mAb was accompanied by an inhibition of HL-60 cell proliferation, which was also dose-dependent (Fig. 2B). As we previously demonstrated, this growth inhibition may be a consequence of the maturation agent-mediated increase in the PC level (10).

Time- and Dose-Dependent Increase in MRP8 and MRP14 Steady-State mRNA Levels during MPA Treatment of HL-60 Cells. To determine whether changes in the amount of PC complex were due to an increase in PC constituent mRNA steady-state levels, we carried out Northern blot analyses of RNA isolated from untreated and MPA-treated HL-60 cells with the use of radiolabeled *MRP8* and *MRP14* cDNA probes (Fig. 3). These probes detected the 0.55-kbp *MRP8* and 0.75-kbp *MRP14* transcripts (10). For comparison, we included a 1,25-(OH)₂D₃ treatment condition which in HL-60 cells can increase the levels of the PC and both *MRP8* and *MRP14* mRNA (10). Treatment of the HL-60 cells with MPA for up to 4 d resulted in a time- and dose-dependent increase in the steady-state levels of *MRP8* and

MRP14 mRNA (Fig. 3). While *MRP8* mRNA levels exhibited a 2-fold greater increase than *MRP14* mRNA levels after MPA treatment (i.e., 65- as compared to 35-fold), the genes were coordinately expressed. Similar to what was observed for the PC level, the highest induction of both species of mRNA was found to occur at 2-3 μ M MPA. Induction with MPA exceeded the 1,25-(OH)₂D₃-mediated increases in the steady-state levels of *MRP8* and *MRP14* mRNA and occurred with faster kinetics. Whereas MPA-generated increases were observed as early as 1 d after treatment, 1,25-(OH)₂D₃-generated increases took approximately twice as long to occur. For both maturation agents, transcript induction appeared maximal on either the third or fourth day after treatment.

Increased Transcription of *MRP8* and *MRP14* Genes in HL-60 Cells.

Two common ways in which mRNA accrual can occur are through an increase in mRNA half-life, or through an increase in transcription initiation events at relevant promoters. This former regulatory scheme was investigated for the *MRP8* and *MRP14* genes by monitoring mRNA half-lives 1 d and 4 d after MPA and 1,25-(OH)₂D₃ treatment. The half-lives in untreated HL-60 cells were not measured because of the low steady-state *MRP8* and *MRP14* mRNA levels. Both mRNAs exhibited half-lives of about 6–8 h, which did not vary markedly with length or type of treatment (data not shown).

To determine whether the observed increases in *MRP8* and *MRP14* mRNA steady-state levels were due to increased transcription initiation at their promoters, we used nuclear run-on transcription assays to monitor the transcription rates of the

MRP8 and *MRP14* genes in untreated cells, and in HL-60 cells treated for 1, 3, or 4 days with either MPA or 1,25-(OH)₂D₃ (Fig. 4). We standardized *MRP8* and *MRP14* transcription rates using two common positive controls: β -actin and 28S rRNA. While little or no increase in transcription initiation at *MRP8* and *MRP14* promoters could be observed at 1 d after treatment of HL-60 cells with MPA, an increase was observed 3–4 d after treatment (Fig. 4A, Table 1). In comparison, 1,25-(OH)₂D₃-mediated gains in transcription initiation frequency were detected as early as 1 d after treatment, and were maximal 3–4 d after treatment (Fig. 4B, Table 1). The magnitudes of increase in the *MRP8* and *MRP14* gene transcription rates were found to be comparable (about an order of magnitude) for both MPA and 1,25-(OH)₂D₃ (Table 1).

Examination of *MRP8* and *MRP14* mRNA in Granulocytes. *MRP8* and *MRP14* transcripts have been examined in normal blood monocytes (17, 18), but not in normal blood granulocytes. This is relevant as some studies have reported an additional larger *MRP8* transcript in monocytes (6, 17). We therefore examined *MRP8* and *MRP14* transcripts in normal blood granulocytes and compared them with mRNA obtained from untreated HL-60 cells, and HL-60 cells treated with MPA or 1,25-(OH)₂D₃ (Fig. 5). The granulocytes clearly expressed the same size transcripts as did HL-60 cells, and did not transcribe additional higher molecular weight *MRP8* and *MRP14* mRNAs.

Discussion

In humans, the *MRP8* and *MRP14* genes are transcribed in cells of monomyeloid origin (7) and may mediate cell growth inhibition during monomyeloid cell differentiation (10). While the two genes are primarily coordinately expressed, differential expression can be observed in certain cell lines (17) and in macrophages infiltrating acutely inflamed tissues (7). To investigate the level at which these genes are controlled, we examined *MRP8* and *MRP14* expression in the promyelocytic HL-60 cell line in response to treatment with the differentiation agents MPA and 1,25-(OH)₂D₃. These maturation agents coordinately induced *MRP8* and *MRP14* expression in a time- and dose-dependent fashion. The inductive response of both genes was comparable for the two agents. Additionally, the observed MPA-generated increases in PC levels were found to roughly parallel increases in the transcript levels of both genes.

The results of our nuclear run-on transcription assays attest to the transcriptional regulation of *MRP8* and *MRP14* genes by MPA and 1,25-(OH)₂D₃. While a small increase in the frequency of transcription initiation could be detected as early as 1 d after treatment, the most pronounced gains occurred 3 and 4 d after treatment. These increases in transcription initiation frequency were in agreement with the increments in *MRP8* and *MRP14* mRNA steady-state levels. Even though the increase in transcription initiation frequency (about an order of magnitude) is not large when compared with the increase in *MRP8* and *MRP14* transcript levels (as much as 65-fold after MPA treatment), the long half-lives (6–8 h) of both transcripts

suggest that relatively small increases in the frequency of transcription initiation can result in substantial accumulation of *MRP8* and *MRP14* mRNA.

While *MRP8* and *MRP14* are clearly transcriptionally regulated by MPA and 1,25-(OH)₂D₃, other mechanisms may contribute to the regulation of these genes. This is supported by our finding that *MRP8* and *MRP14* transcripts are barely detectable in untreated HL-60 cells, even though transcription initiation at both promoters occurs at a low constitutive level. One possible mechanism might involve stabilization of nascent messages, since a small change in *MRP8* or *MRP14* half-life as a consequence of treatment or rapid degradation of transcripts in untreated HL-60 cells was not ruled out in the present study. Some additional mechanisms that could modulate the *MRP8* and *MRP14* transcriptional response to differentiation agents include regulation of transcript elongation, differential transport of messages from the nucleus to the cytoplasm, varying efficiency of polyadenylation, and preferential splicing of nascent transcripts in the nucleus.

We find it intriguing that the differentiation-inducing agents MPA and 1,25-(OH)₂D₃, which do not have the same mode of action in cells, are capable of precipitating similar substantial increases in the *MRP8* and *MRP14* proteins during monomyelocytic maturation of HL-60 cells. Whereas 1,25-(OH)₂D₃ binds to an intracellular receptor and directly interacts with responsive genes to modulate their transcription (19), MPA depletes guanine nucleotides necessary for cellular metabolism by inhibiting IMP dehydrogenase, the rate-limiting enzyme in *de novo* guanine nucleotide synthesis (20). Additionally, the observed elevated *MRP8* and

MRP14 mRNA and protein levels are a consequence of an increase in transcription initiation. This may indicate that the PC complex functions as a common mediator in the maturation response to both agents. Our findings, as well as the abundance of expression of these genes in normal monocytes and granulocytes, substantiate that the *MRP8* and *MRP14* genes play a role in the terminal cell differentiation of human promyelocytes. These findings are consistent with our previously suggested role for the PC (10), that of mediating the growth inhibitory response to differentiation agents.

Materials and Methods

Reagents. MPA (Sigma Chemical Co., St. Louis, MO) was dissolved in 150 mM NaHCO₃. Actinomycin D (Sigma) and 1,25-(OH)₂D₃ were solubilized in 70% ethanol. 1,25-(OH)₂D₃ was used at a final concentration of 0.4 μM in all experiments. Restriction enzymes were purchased from either the Promega Corporation (Madison, WI) or Boehringer-Mannheim (Indianapolis, IN). All other chemicals were obtained from either Sigma; Research Organics, Inc. (Cleveland, OH); or Fisher Scientific Co. (Fairlawn, NJ).

Cell Culture. The human leukemic HL-60 cell line (from R.C. Gallo, National Cancer Institute, Bethesda, MD) was cultured in RPMI 1640 medium (Whittaker Bioproducts, Walkersville, MD) supplemented with 15% heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin (Gibco Laboratories, Grand Island, NY), and 100 μg/ml streptomycin (Gibco). Cells were maintained in

a humidified incubator at 37 °C with 8% CO₂ in air and were used at passages 50 to 90. Exponentially growing cells were inoculated at 2×10^5 cells/ml into 10 ml medium in 100-mm Petri dishes for immunofluorescence experiments, and into 50 ml medium in 150-mm Petri dishes for all other experiments. Cells were incubated 3–6 h before treatment with chemical agents. Cell cultures were at a density of 2×10^5 cells/ml at the time of treatment.

Northern Blot Analysis. Total RNA isolated by ultracentrifugation through a cesium chloride cushion (21) was fractionated by formaldehyde-agarose gel electrophoresis (22), transferred to a Magnagraph nylon membrane (Micron Separations, Inc., Westboro, MA), and adhered to the membrane by ultraviolet crosslinking (UV Stratalinker 2400; Stratagene, La Jolla, CA). Filters were prehybridized at 42 °C in a buffer consisting of 5× Denhardt's solution, 50% (v/v) deionized formamide, 20% (v/v) sodium phosphate buffer (50 mM sodium phosphate [pH 6.5], 0.5% [w/v] tetrasodium phosphate, 5 M sodium chloride), 1% (w/v) sodium dodecyl sulfate (SDS), and 250 µg/ml sheared and denatured herring sperm DNA. *MRP8* and *MRP14* cDNA probes consisting of the approximate 375-base pair (bp) internal *PvuII*-*PstI* fragment of pUCmrp8-3 (17) (top band of doublet), and the approximate 200-bp internal *PvuII* fragment of pUCmrp14-10 (17) (lowermost band of triplet), respectively, were labeled for 3 h at room temperature by the random primer method (23). Hybridization with a terminal deoxynucleotidyl transferase 3' end-labeled 28S rRNA oligonucleotide probe (Clonetech, Palo Alto, CA) was used to standardize RNA amounts in each lane. Filters hybridized at 42 °C were washed in

0.2× SSC/1% SDS at room temperature for 15 min and four times at 65 °C for 30 min. 1× SSC contained 150 mM NaCl, 15 mM sodium citrate. This wash protocol was abbreviated for hybridizations involving the oligonucleotide probe. Blots were exposed to Fuji Medical X-ray film (Fuji Film Co., Ltd., Japan) with intensifying screens for various amounts of time to allow comparison of autoradiograms in the linear range of the film. Several different exposures of film were scanned with a Shimadzu CS-910 chromatogram scanner (Shimadzu Co., Kyoto, Japan). Filters were stripped by boiling 20 min in 0.1× SSPE/0.1% SDS, and were then reused. 1× SSPE contained 150 mM NaCl, 10 mM sodium phosphate (pH 7.5), 1 mM ethylenediaminetetraacetic acid)

mRNA Decay Determination. Actinomycin D was added at a concentration of 5 µg/ml to HL-60 cell treated for 1 or 4 days with MPA- or 1,25-(OH)₂D₃. Aliquots were harvested at 0, 1, 2, 4, 6, and 8 h, and total RNA was isolated and examined by Northern analysis. Data obtained by densitometric scanning of autoradiograms was plotted semilogarithmically versus time, and the half-life was extrapolated as the value at which the transcript level decreased 50%.

Nuclear Run-on Transcription Assays. Nuclei from $(0.5-1) \times 10^8$ HL-60 cells were isolated by sedimentation through 0.8 M sucrose containing 1 mM phenylmethyl-sulfonyl fluoride as described previously (5). Run-on transcription assays were performed as indicated by Datta *et al.* (24), with the following modifications. The RNase inhibitor RNasin (Promega) was added at 200 U/ml to the incorporation reaction, which contained 250 µCi [α -³²P] UTP (3000 Ci/mmol;

Amersham Corp., Arlington Heights, IL). The reaction was terminated by the addition of 50 U of RQ1 DNase (Promega) and 50 μ l DNase buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl, 20 mM $MgCl_2$). DNase digestion was allowed to proceed 30 min at 37 °C and was followed by proteinase K digestion for 45 min at 37 °C. For this second digestion, 10 μ l of 10 mg/ml proteinase K, 7 μ l of 10 mg/ml yeast tRNA, and 37 μ l of 10 $\times$ SET were added. 1 $\times$ SET contained 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.5% SDS. After phenol:chloroform extraction and ethanol precipitation, pellets were resuspended in 200 μ l of 40% formamide.

Total label incorporated into nuclear RNA was determined by scintillation counting of washed and unwashed Whatman DE81 filters onto which 1 μ l of RNA solution had been absorbed. Roughly equivalent amounts of nuclear RNA per condition were hybridized to 5 μ g of each of the following DNAs: pUCmrp8-3; pUCmrp14-10; pUC9 or pBluescript; pHF β A-3'ut, which contains the 3' untranslated region of the human β -actin gene (25); and the 1.5-kbp *Bam*HI fragment of pRR228, an internal fragment of the rat 28S rRNA gene (26). DNA samples were denatured and adhered to Magnagraph membrane with the use of a Minifold II slot blotter (Schleicher & Schuell, Inc., Keene, NH) according to the recommendations of the manufacturer. Hybridizations were performed with at least 3×10^7 total counts of $\alpha^{32}P$ -labeled RNA in the prehybridization buffer described above, in which formamide was decreased to 40% and herring sperm DNA was increased to 600 μ g/ml. After at least a 65 h incubation at 42 °C, filters were washed in (a) 2 $\times$ SSC/0.1% SDS at 42 °C for 30 min; (b) 2 $\times$ SSC containing 2 μ g/ml DNase-free RNase (Boehringer

Mannheim Biochemicals) at room temperature for 10 min; and (c) 0.1× SSC/0.1% SDS at 42 °C for 30 min. These washes were followed by additional RNase washes as necessary to reduce background. Relative signal was determined by densitometric scanning of autoradiograms as described above.

Other Methods. The detection of the PC complex by indirect immunofluorescence with the use of the NM-6 mAb (10) was conducted as described previously (27). Granulocytes were isolated from fresh blood by the standard Ficoll-Hypaque method accompanied by dextran sedimentation (28). Total RNA was obtained from granulocytes as indicated above.

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Table 1 Relative transcription rates of the *MRP8* and *MRP14* genes

HL-60 cells were incubated in the presence or absence of the differentiation agents for 4 d, and the transcription rate of the *MRP8* and *MRP14* genes was quantified at 0, 1, 3, and 4 d by densitometric scanning of autoradiograms. Values reported are the maximum fold induction observed at 3-4 d after treatment in two experiments.

		Induction ^a (fold increase)	
	Concentration (μ M)	<i>MRP8</i>	<i>MRP14</i>
1,25-(OH) ₂ D ₃ ^b	0.4	7.8 $\pm$ 2.4	11.5 $\pm$ 1.1
MPA ^c	3.0	12.0 $\pm$ 7.0	16.6 $\pm$ 5.4

^a Values are mean $\pm$ range.

^b Transcription rates were normalized to the β -actin transcription rate. Similar to Soloman *et al.* (29), we observed less than 2-fold differences between the β -actin and 28S rRNA transcription rate after 1,25-(OH)₂D₃ treatment.

^c Transcription rates were normalized to the 28S rRNA transcription rate. Upon normalization to the β -actin transcription rate, *MRP8* and *MRP14* fold induction values are respectively: 1.8 $\pm$ 0.6 and 4.1 $\pm$ 0.2.

Figure Legends

Fig. 1. Detection of the PC complex by indirect immunofluorescence. *A*, untreated HL-60 cells. *B*, HL-60 cells treated with 2 μ M MPA. The PC-specific mAb NM-6 was used as described in Experimental Procedures to examine cells after 3 d in culture. Quantitative results are presented in Figure 2.

Fig. 2. *A*, PC levels and *B*, cell growth in untreated and MPA-treated HL-60 cells. PC levels are presented as the percentage of cells reacting with the NM-6 mAb. O, untreated; Δ , 0.5 μ M MPA; Δ , 1.0 μ M MPA; Π , 2.0 μ M MPA; Π , 3.0 μ M MPA. Results are the mean of two experiments in which the variation of the data was within 20%.

Fig. 3. *A*, *MRP8* and *B*, *MRP14* steady-state mRNA levels in untreated and MPA- or 1,25-(OH)₂D₃-treated HL-60 cells. Graphic representation of quantitation by densitometric scanning of autoradiograms. O, untreated; O, 0.4 μ M 1,25-(OH)₂D₃; Δ , 0.5 μ M MPA; Δ , 1.0 μ M MPA; Π , 2.0 μ M MPA; Π , 3.0 μ M MPA. Results are expressed as the fold increase in the steady-state mRNA levels of *MRP8* and *MRP14* genes relative to the levels present in the untreated cells at time zero. Amounts of RNA in each lane were standardized with the use of the 28S rRNA probe. Results depicted are from a representative experiment. In two additional experiments, the fold increase in *MRP8* transcript levels was equivalent to or exceeded the fold increase in *MRP14* transcript levels.

Fig. 4. Nuclear run-on assays of *MRP8* and *MRP14* gene transcription rates in HL-60 cells. *A*, nuclei were isolated from cells treated with MPA for 1, 3, and 4 d, and from control cells after 4 d in culture. *B*, nuclei were isolated from cells treated with 1,25-(OH)₂D₃ for 1, 3, and 4 d, and from control cells after 4 d in culture. Radiolabelled nascent RNA was hybridized to 5 µg of the following plasmids immobilized on nylon membrane: pUCmrp8-3 (*MRP8*), pUCmrp14-10 (*MRP14*), pHFβA-3'ut (β-actin), pRR228 (28S rRNA), and the negative controls pUC9 or pBluescript (pBS⁺). In panel *A*, approximately twice as many incorporated counts were applied to the control and 1 d filters to allow visualization of *MRP8* and *MRP14* hybridizing bands. Filters were exposed to film for 31 h. In panel *B*, filters were exposed to film for 4 days.

Fig. 5. *MRP8* and *MRP14* steady-state mRNA levels in normal blood granulocytes and in HL-60 cells. The HL-60 cells were untreated or treated for 3 d with either 0.4 µM 1,25-(OH)₂D₃ or 2 µM MPA. Total RNA was isolated and analyzed as indicated in Experimental Procedures. *G*, granulocytes; *U*, untreated HL-60 cells; *D*, 1,25-(OH)₂D₃-treated HL-60 cells; *M*, MPA-treated HL-60 cells.

A

B

A **B**

G U D M

MRP8 –

MRP14 –

rRNA –

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