Appearance of Nuclear Factors That Interact With Genes for Myeloid Calcium Binding Proteins (MRP-8 and MRP-14) in Differentiated HL-60 Cells

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Myeloid calcium binding proteins MRP-8 and MRP-14 were induced, and their genes were coordinately expressed, during differentiation of human leukemia HL-60 cells into macrophage-like cells after treatment with 1,25-dihydroxyvitamin D$_3$ (VD$_3$). Both MRP-8 and MRP-14 mRNAs appeared on the day after VD$_3$ treatment. Their level reached a peak on day 2, and then quickly declined. Nuclear factors that interact with the 5' upstream regions of MRP-8 and MRP-14 genes were studied with gel mobility-shift assays. Two factors (MP8FI and MP8FII) that interacted with 379 bp (426 ~ 48 bp upstream from the transcription-initiation site of MRP-8 gene) and 67 bp (−477 ~ +20) DNA fragments, respectively, were found in the cells treated with VD$_3$ for 1 day. MP8FI and MP8FII were present neither in the nuclei of untreated HL-60 cells, nor in the nuclei of the cells treated with VD$_3$ for 6 days. Human monocytic leukemia THP-1 cells, which constitutively expressed MRP genes, had MP8FI but not MP8FII. MP8FII was found to interact with the 19-mer sequence located just upstream of the TATA box. Also, two factors that bound to the different upstream regions (−400 ~ −150 and −149 ~ +50) of MRP-14 gene were detected in the differentiated HL-60 cells. One of these, MP14FII, appeared on day 1, but on day 6 its concentration greatly decreased. The other, MP14FII, was found in greater quantity on day 6 than on day 1. MP14FII, but not MP14FII, was found in THP-1 cells. These factors may be involved in the expression of MRP-8 and MRP-14 genes in VD$_3$-differentiated HL-60 cells.

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oligonucleotide primers, and was cleaved at Pvu II site into 379 bp (-426 ~ -48) and 67 bp (-47 ~ +20) fragments. Similarly, a DNA fragment that lies 1,050 bp (-1,000 ~ +50) upstream from the transcription start site of MRP-14 gene was synthesized by PCR, and digested by Sac I and Hae III into 251-bp (-400 ~ -150) and 199-bp (-149 ~ +50) fragments. These fragments were purified by electrophoresis on diethyl aminoethyl (DEAE)-cellulose membranes (Schleicher & Schuell, Tokyo, Japan). Each fragment was used as a probe in the gel mobility-shift assay.

Gel mobility-shift assay. Nuclear proteins were extracted from HL-60 cells and THP-1 cells as described. DNA fragments were end-labeled with 32P-adenosine triphosphate (ATP) using T4 polynucleotide kinase. The assay was performed as described with minor modifications. Briefly, the end-labeled DNA (1 ng) was incubated with 5 µg nuclear protein in a 10-µL reaction mixture (15 mmol/L HEPES [pH 7.9], 0.2 mmol/L MgCl2, 1 mmol/L dithiothreitol (DTT), 5% glycerol, 2.5 µg poly[d(dC)poly[d(dC)]] for 30 minutes at 20°C. Competition studies were performed by further adding 100 ng of nonlabeled probe DNA or herring-sperm (hs) DNA. The reaction products were then analyzed by 4% polyacrylamide gel electrophoresis and autoradiographed. The graphic image was analyzed by a bioimage analyser BAS 2000 (Fuji Film Co Ltd, Tokyo, Japan).

Fig 1. Northern blot hybridization of MRP-8 and β-actin mRNA in HL-60 cells differentiated with VD3. Total cellular RNA was extracted from VD3-treated cells for 0 to 10 days and analyzed with (A) MRP-8 cDNA probe and (B) β-actin probe.
man monocytic leukemia THP-1 cells, which constitutively expressed MRP-8 gene. Similarly, a nuclear factor of day 1 cells retarded the mobility of the 67-bp fragment (Fig 2B). The fragment was not retarded when incubated with the nuclear extract of undifferentiated HL-60 cells, indicating that this factor is correlated with the differentiation of HL-60 cells. We designate this factor MP8FII. No MP8FII was found in the nuclei of HL-60 cells treated with VD3 for 6 days. MP8FII was also found in the nuclei of THP-1 cells.

**Binding of MP8FII to DNA sequences.** To determine what DNA sequences interact with MP8FII, we synthesized three oligonucleotides A (−47 to −29), B (−22 to −1), and C (−34 to −17) in the 67 bp fragment (Fig 3). Corresponding antisense oligonucleotides were also synthesized and hybridized with each sense oligonucleotide. Competition by each nonlabeled hybrid for the interaction of 32P-labeled 67-bp fragment with MP8FII was then tested (Fig 4). Hybrid A but not B and C inhibited the interaction, indicating that MP8FII bound to the region included in the 19-mer oligonucleotide that is located just upstream from TATA box.

**Differentiation-dependent nuclear factors for the expression of MRP-14 gene.** Next, we explored nuclear factors that interact with DNA in the upstream region of exon 1 of MRP-14 gene. A DNA fragment with 1,050 bp (−1,000 to +50) from the transcription-initiation site of the gene) was cleaved with SacI and HaeII, and the resulting two fragments (251 bp, −499 to −150; and 199 bp, −149 to +50) were used for the study. When nuclear extract of HL-60 cells treated with VD3 for 1 day was incubated with the 251-bp fragment, the gel mobility of the fragment was retarded (Fig 5A). However, the fragment was not retarded after incubation with the extract from undifferentiated cells, indicating that a factor having affinity with the 251-bp fragment appeared in the nuclei of VD3-differentiated HL-60 cells. We designated this differentiation-dependent factor MP14FI. In the nuclei of cells treated with VD3 for 6 days, much smaller amounts of MP14FI were present, or the activity of MP14FI was diminished. MP14FI was also found in the nuclei of THP-1 cells. Similarly, the 199-bp DNA fragment was retarded when incubated with nuclear extracts of HL-60 cells treated for 1 and 6 days with VD3 (Fig 5B). Nuclear extracts of undifferentiated cells had no such effect. We designated the factor in the differentiated cells MP14RI. The level of MP14RI apparently increased more on day 6 than on day 1. Little, if any, MP14RI was detected in THP-1 cells. Therefore, MP14RI seems different from MP14FI.

**DISCUSSION**

We studied differentiation-dependent nuclear factors that interact with the 5'-upstream regions in MRP-8 and MRP-14 genes. Two factors (MP8FII and MP8FII) were found to
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Fig 4. Competition by oligonucleotides for the interaction of MP8FII with the 67-bp fragment of MRP-8 gene. MP8FII complex with the 67-bp fragment (lanes 1, 4, and 7) was coincubated with oligonucleotide A hybrid (lane 2), B hybrid (lane 5), C hybrid (lane 8), or hs DNA (lanes 3, 6, and 9). The pointer indicates MP8FII complex with the 67-bp fragment.

bind specifically to 379-bp and 67-bp fragments, respectively, of MRP-8 gene. These factors were not in the nuclei of undifferentiated HL-60 cells, but appeared 1 day after the treatment of HL-60 cells with VD₃ (Fig 2B). No factor was found in cells treated with the vitamin for 6 days. The time of appearance of those factors coincided with the level of MRP-8 mRNA during VD₃-induced differentiation of HL-60 cells (Fig 1). Further, MP8FII was detected in the nuclei of THP-1 cells in which the MRP-8 gene was constitutively expressed, whereas MP8FI was not detected in THP-1 cells. Thus, MP8FI seems to be required for VD₃-induced transcription of MRP-8 gene in HL-60 cells, but MP8FII is produced by any cell in which the MRP-8 gene is expressed. The MRP-8 gene may be under a positive control by MP8FII. The gene may be expressed when MP8FII is present in the nucleus and binds to a specific DNA sequence of the 67-bp fragment, which contains the partial sequence of exon 1 and the TATA box. Our attempts to pinpoint an MP8FII-binding sequence in the 67-bp fragment indicated that MP8FII interacts with the 19-mer sequence (oligonucleotide A) located upstream from TATA box (Figs 3 and 4). Because oligonucleotide C did not compete for the complex between MP8FII and the 67-bp fragment, MP8FII may bind 13-mer nucleotide sequence (CTGGCCAAGCCTA), 47 bp to 35 bp upstream from the transcription-initiation site of MRP-8 gene. Two additional factors bound to the 379-bp region of MRP-8 gene. These factors might also be required for MRP-8 gene expression, but they were not differentiation-dependent in HL-60 cells. Two factors (MP14FI and MP14FII) were found to bind to specific DNA sequences of 5'-upstream region of MRP14 gene, and were detected in the nuclei of VD₃-differentiated HL-60 cells. MP14FII levels increased on day 6, when MRP-14 mRNA disappeared, compared with levels on day 1, when the MRP-14 mRNA transcription started. Little, if any, MP14FII was present in THP-1 cells that constitutively express MRP-14 gene, probably indicating that

Fig 5. Nuclear factors that bind the 5'-upstream of MRP-14 gene. (A) Gel mobility-shift assay using 251-bp fragment (-400 ~ -150 from the transcription-initiation site of MRP-14 gene). Assay was performed as mentioned in Fig 2. The pointer indicates the complex with MP14FI. (B) Gel mobility-shift assay using 199-bp fragment (-149 ~ +50). Assay was performed as described above. The pointer indicates the complex with MP14FII.
MP14FII is correlated with the disappearance of MRP-14 mRNA. Similar factors were found in HL-60 cells on the thymidylate synthase gene, the expression of which was quickly blocked after VD3-differentiation. On the other hand, MP14FII appeared on day 1 and its level declined on day 6 in the differentiated HL-60 cells. MP14FII was detected in TPH-1 cells. Thus, this factor seems to control positively the MRP14 gene. It is uncertain how our nuclear factors appeared in the nuclei of differentiated HL-60 cells. These factors might be newly synthesized, modified, or translocated from the cytosol to the nucleus after VD3 treatment of HL-60 cells. These investigations are in progress.

Sequence-specific DNA binding proteins are known to regulate the initiation of transcription in various mammalian cells. Lagasse and Clerc identified the SP-1 transcription factor-binding site (GGGGCGGGGC) at position −133 in the MRP-14 gene after they determined the genomic sequences of MRP-8 and MRP-14. We reexamined other transcription factor-binding sites in these genes (Fig 6). There were seven sites to which transcription factors (IRF-1, CDP/NF-κB, CBP, GTI, and Pu motif) could bind in the 379-bp fragment of MRP-8 gene. On the other hand, no sequence to which known transcription factors could bind was found in the 67-bp fragment of MRP-8 gene. Therefore, MP8FII may be a new transcription factor. It is essential to further characterize MP8FII and to pinpoint the MP8FII-specific sequence. There was a repeated sequence for IRF-1 at −231 and a CBP sequence at −81 in addition to the SP-1 site in the MRP14 gene. We would like to determine whether these known transcription factors are identical with MP8FII, MP14FII, and MP14FIII in competition experiments with those transcription factor-specific sequences.

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