Novel elements located at −504 to −399 bp of the promoter region regulated the expression of the human macrophage scavenger receptor gene in murine macrophages

Hai-Sun Liao,*,† Tatsuhiko Kodama,† Takefumi Doi,§ Mitsuru Emi, Hitoshi Asaoka,* Hiroshige Itakura,* and Akiyo Matsumoto[1,*]**

Division of Clinical Nutrition,* National Institute of Health and Nutrition, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162, Japan; Department of Molecular Biology and Medicine, †Research Center for Advanced Science and Technology, University of Tokyo, Japan; Faculty of Pharmaceutical Sciences, §Osaka University, Osaka, Japan; and Department of Molecular Biology, **Institute of Gerontology, Nippon Medical School, Kawasaki, Japan

Abstract. The expressions of type I and type II macrophage scavenger receptors (MSRs) are highly specific in macrophages and related cell types. Although some reports have described the regulation of MSR gene expression and proposed some *cis*-elements related to cell-specific expression, the regulation of MSR remains largely unclear. This is due, in part, to an unacceptably low efficiency of transfection into monocyte/macrophage cells. In the present study, we optimized the conditions of electroporation in murine macrophage (P388D1) cells. The efficiency of electroporation was increased 20-fold compared with previous methods. Using the optimized method, we focused on studying the regulation of the human MSR promoter in macrophages. We presently demonstrate that: *a*) the proximal −10 to +50 bp human MSR promoter region is necessary for the cell type-specific expression of human MSR; *b*) the 6.5 kbp upstream sequence suppresses the expression of human MSR; *c*) a promoter region extending from −504 to −399 bp produced the greatest increase in transcriptional activity; *d*) macrophage cell-specific transcription factors bind to the region as determined by electrophoretic mobility shift assay (EMSA) and a footprint assay; and *e*) mutations of the region reduced about 40–75% of the promoter activity in a transfecting assay. We concluded that novel elements located at the −504 to −399 bp region may play an important role in the regulation of the MSR gene expression in macrophages. We speculate that macrophage-specific factors binding to those elements may be responsible for the transcription regulation of the MSR gene in macrophages.—**Liao, H-s., T. Kodama, T. Doi, M. Emi, H. Asaoka, H. Itakura, and A. Matsumoto.** Novel elements located at −504 to −399 bp of the promoter region regulated the expression of the human macrophage scavenger receptor gene in murine macrophages. *J. Lipid Res.* 1997. **38:** 1433–1444.

Supplementary key words electroporation • monocyte/macrophage • scavenger receptor • transcriptional regulation

Type I and type II macrophage scavenger receptors (MSRs) are highly restricted in their patterns of expression in macrophages and related cell types (1–5). MSRs mediate the uptake of modified lipoproteins, including acetylated and oxidized low density lipoprotein (LDL), endotoxins, and other macromolecules with increased negative charges (6–9). MSR also plays a role in the divalent cation-independent macrophage adhesion to endothelial cells (10). Unlike the LDL receptor, MSR is not down-regulated by increments in the intracellular cholesterol level (7, 8). Therefore, macrophages will continue to take up and process modified LDL as long as it is present in the extracellular milieu. This results in an intracellular cholesterol accumulation and the transformation of macrophages into foam cells. This phenomenon may play a key role in the pathogenesis of early atherosclerotic lesions (2, 9). Previous studies have suggested that MSR expression is modulated by macrophage-colony stimulation factor, tumor necrosis factor, interferon-γ, and transforming growth factor-β1 (11–15). It has been observed that MSR expression is increased during the differentiation of monocytes into macrophages and when promonocytic cell lines such as THP-1 are stimulated by phorbol esters (16–18). Using

Abbreviations: MSR, macrophage scavenger receptor; LDL, low density lipoprotein; bp, base pair; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift assay.

[1]To whom correspondence should be addressed.

THP-1 cells as a model, a −179 to +112 bp region, including a Pu.1 site at position −133 and an AP-1/ets at position +1, has been shown to be sufficient for transcriptional regulation (19). Wu et al. (20) have reported that the upstream sequence from −6.5 to −4.8 kbp contained a silencing element and the sequence between −4.1 to −4.5 kbp contained a phorbol ester-responsive element. Little is known concerning the regulation of the MSR at the macrophage level. This is due, in part, to the low efficiency of the transfection into macrophages. Electroporation is a convenient method to introduce DNA into both plant and animal cells. A few reports about transfection into macrophage cell lines by electroporation have been published (21, 22); however, the electroporation conditions were not completely described and this led to difficulty in reproducing the findings.

In the present study, therefore, we first optimized the transfection conditions of electroporation in murine macrophage (P388D1) cells, which have been widely used for studying the gene expression and regulation in macrophages. Using wild and mutated types of human MSR promoter constructs, we presently demonstrate that maximal transcription activity requires the −504 to −399 bp promoter domain. Using an electrophoretic mobility shift assay (EMSA) and a footprint assay, we demonstrate that two protein binding sites are found in the −504 to −399 bp region of the MSR promoter. The putative transcription factor binding to the region was found by the EMSA to exist only in cell extract of P388D1 cells but not in Hela cells. This indicated that these factors might be macrophage-specific transcription factors. Mutations in these sites abolished about 40–75% of the promoter activity in a transfection assay. These results demonstrated the functional importance of the −504 to −399 bp region of the MSR promoter.

MATERIALS AND METHODS

Cell lines

P388D1, THP-1, CHO, and Hela cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The P388D1 and THP-1, cells were cultured in RPMI-1640 (IBL, Fujioka, Japan) medium supplemented with 10% fetal bovine serum (FBS, Multiser, Castle Hill, N.S.W.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37°C under 5% CO_2. The CHO cells were maintained in F12 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO_2. The Hela cells were grown in MEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO_2.

Construction of plasmids

The pGL2-Basic luciferase reporter vector and pCAT-Control vector were obtained from Promega (Madison, WI). A series of 5′ deletions was prepared by polymerase chain reaction (PCR) with a human MSR promoter clone as a template (23). Promoter fragments were cloned initially into pCR™ II (Invitrogen, San Diego, CA) and then were subcloned into the multi-cloning site of the promoterless luciferase reporter vector, pGL2-Basic, between the upstream Xho I and the downstream Hind III sites. Inserted DNA sequences were confirmed by dideoxy sequencing. A 6.5 kbp promoter (−6.5 k to +50 bp) was amplified by a long PCR kit (GeneAmp XL PCR kit, Perkin Elmer, Branchburg, NJ) with the forward primer, 5′-GTTTTCCCAGTCACGA-3′, and backward primer 5′-ATGTCGACTTATCTCATAG-TATTTCAG-3′ with a human MSR promoter clone as a template (23), and then subcloned into pGL2-Basic. The orientation of the inserted fragment was verified by agarose gel electrophoresis after digesting with asymmetric double-restriction enzymes. Site-directed mutageneses of the human MSR promoter fragments were performed by PCR using mutated primers and then the fragments were subcloned into pGL2-Basic using the same method of constructing the promoter deletion constructs. The mutation was confirmed by sequencing. Plasmid DNA was prepared with QIAGEN-tip (Qiagen, Chatsworth, CA). Plasmid DNA, the concentration of which was determined from the optical density at 260 nm, was dissolved in sterile Milli-Q water.

Transfection by electroporation

Cells were split to 10^6 cells/ml 16–24 h prior to transfection. At the time of transfection, the THP-1 cells were harvested by centrifugation at 500 *g* for 5 min at room temperature. The P388D1 cells were scraped and centrifuged at room temperature. The electroporation was carried out as follows. Cells were washed twice with RPMI-1640 medium without FBS and were resuspended in the same medium. Seven hundred microliters of suspension, containing 5×10^6 cells and 30 μg of indicated luciferase construct DNA and 5 μg of pCAT-Control plasmid DNA was placed in a 0.4 cm electroporation cuvette (Bio-Rad, Richmond, CA). The cuvette was incubated at room temperature for 10 min. The electroporation was performed at room temperature, 960 μF and 400 V. After electroporation, the cuvette was placed on ice for exactly 10 min. The cells were transferred into 5 ml of RPMI-1640 containing 10% FBS. The CHO

and Hela cells were transfected by the method of Chu, Hayakawa, and Berg (24).

Relative cell viability was measured by plating cells exposed and not exposed to the electric field on parallel plates, allowing them to grow for 72 h, and then measuring the relative cell number directly by counting after trypan blue solution staining.

Luciferase and CAT assays

The cells were harvested 24 h after electroporation. They were then washed twice with phosphate-buffered saline (PBS) free of Mg^{2+} and Ca^{2+} and resuspended in 100 μl of PBS. The cell extracts were prepared by four cycles of a freeze-thawing procedure. Luciferase activities were determined by a Lumat LB 9501 (Berthold, Pittsburgh, PA), using a PicaGene kit (Wako, Osaka, Japan). In brief, 10 μl of cell extracts was mixed with 100 μl of luciferin (0.5 mg/ml) at room temperature, and the light emission for 10 seconds was immediately determined. The relative luciferase activity was normalized for 20 μg of total protein of cell extracts. Chloramphenicol acetyltransferase (CAT) enzyme activity was determined by the method of Sleigh (25). All experiments were carried out in triplicate.

Preparation of cell extracts

Cell extracts were prepared by the method of Dignam et al. (26): 1×10^9 cells were collected by scraping and washed twice with PBS at 4°C. The cells were suspended in 5 volumes of buffer A containing 10 mM HEPES–NaOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, and 0.5 mM DTT. The cells were then transferred into a glass Dounce homogenizer and then homogenized with ten up-and-down strokes using a type B pestle. After centrifugation at 25,000 *g* at 4°C for 30 min, the nuclear pellets were resuspended in 1 volume of buffer C containing 20 mM HEPES–NaOH (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, and 0.5 mM DTT. The cells were homogenized again at 4°C. After centrifugation at 25,000 *g* at 4°C for 30 min, the supernatant was dialyzed at 4°C for 16 h against a 100-fold volume of buffer D containing 20 mM HEPES–NaOH (pH 7.9), 1.5 mM $MgCl_2$, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, and 0.5 mM DTT. Buffers A, C, and D were added to protease inhibitors (2 μg/ml leupeptin, 0.5 mM phenylmethysulfonyl fluoride, and 1 μg/ml pepstatin) before using. The cell extracts were collected by centrifugation and the protein concentration was determined with a Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL).

Probes for gel mobility shift and footprint assays

The backward primers used in PCR reactions were labeled into the 5′ end of the primer with [γ-^{32}P]ATP and T4 polynucleotide kinase. Each ^{32}P-labeled probe for the footprint assay was synthesized by PCR with GeneAmp Reagent kit (Perkin Elmer) using an MSR promoter clone as a template (23). The oligonucleotide probes (25–30 bp) used in the gel mobility shift assay were labeled into the 5′ end with [γ-^{23}P]ATP and then purified by electrophoresis with a 20% polyacrylamide gel.

Gel electrophoretic mobility shift assay and immunosupershift assay

The gel electrophoretic mobility shift assay (EMSA) was performed by the method of Ausubel et al. (27) with a slight modification. The binding reaction was carried out in a 12.5 μl reaction mixture containing 4 mM Tris-HCl (pH 7.9) 10 mM HEPES (pH 7.9), 1 mM DTT, 1 mM EDTA, 60 mM KCl, 0.2 mg/ml poly(dI-dC), 10% glycerol, 0–4 μg of cell extract protein, and 20,000 cpm of indicated ^{32}P-labeled probe. Each reaction mixture was incubated at room temperature for 30 min and then loaded directly onto a 6% polyacrylamide gel with 1 × TAE buffer containing 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA. The electrophoresis was carried out at a constant voltage of 150 V at room temperature for 1.5–2 h. The gel was dried and then exposed to X-ray film overnight at −80°C. The antisera against NF-kappa B subunits, including those against the C terminus of murine c-Rel, against the N terminus of human p65 (the murine sequence is slightly different, but the serum does recognize the murine protein), against the N-terminus of murine p50, against the N-terminus of murine IkB-α, and against the C terminus of murine IkB-β, were a generous gift from Dr. Nance Rice (NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, Frederick, MD). The immunosupershift assay was performed with the same procedure as that of EMSA except for an addition of indicated antibody against the corresponding transcription factor.

Footprint assay

The footprint assay was performed by the method of Ausuble et al. (27) with a slight modification. The binding reaction of DNA and protein was carried out at room temperature for 30 min in a volume of 25 μl containing 5–10 fmoles of DNA probe with 100,000 cpm, 10 μl of 10% polyvinyl alcohol, 0–20 μg of nuclear extract protein, 10 mM HEPES (pH 7.9), 1 mM DTT, 1 mM EDTA, 0.2 mg/ml poly(dI-dC), 10% glycerol, and 60 mM KCl. Limited DNase I digestion was performed at room temperature after adding 25 μl of a solution containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$, and then 1 μl (1 unit) of DNase I. After 2 min at room temperature, the reaction was stopped by adding 100 μl of a solution con-

taining 200 mM NaCl, 20 mM EDTA, 1% SDS, and 100 μg/ml yeast RNA. Samples were extracted by phenol-chloroform, precipitated, and loaded onto a 6% denaturing polyacrylamide gel. The gel was dried and then exposed to X-ray film overnight at −80°C.

RESULTS

Optimization of electroporation for transient transfection of P388D1 cells

In the past, only a low efficiency of transfection in monocyte/macrophage cells could be obtained due to large-scale cell death after electroporation (22). To increase the efficiency of transfection, we focused on increasing the cell viability after transfection. Using a firefly luciferase report gene as an indicator of transfection efficiency, we optimized the voltages, capacitance, DNA concentration, electroporation medium, and temperature in P388D1 cells. When the electroporation was carried out at room temperature, with an optimal DNA concentration (≤50 μg/ml), and full culture medium (RPMI-1640) at 960 μF, 400 V, the cell viability increased by 22% after electroporation, and the efficiency of transfection increased 20-fold compared with the previous method (**Fig. 1**). Thus, a useful system for transient expression of the macrophage cell lines has been optimized. The system is very sensitive and reproducible and suitable for studies of a weak promoter in macrophages. We also obtained very good results with the optimized conditions in THP-1 cells (data not shown). Although the optimal voltage should be determined for other monocyte/macrophage cells, the optimized conditions such as capacitance, temperature, full medium and DNA concentration are useful for further studies in other monocyte/macrophage cell lines.

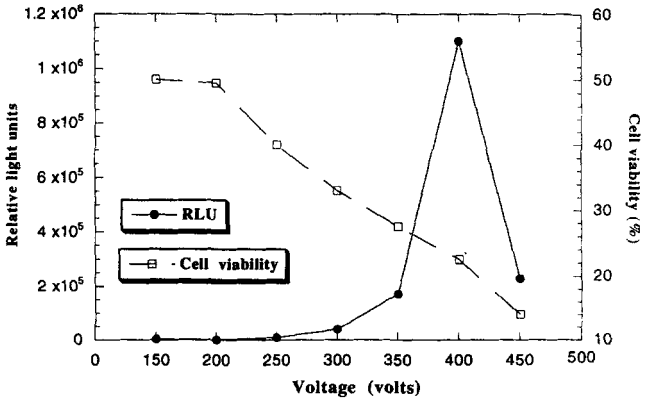

Fig. 1. Effect of voltage on the transfection of P388D1 cells. Twenty micrograms of pGL2 luciferase control DNA was transfected into P388D1 cells at the indicated voltage and 960 μF. Luminescence was determined 24 h after electroporation. The relative light units (RLU) of 20 μg of total protein of cell extracts are shown. The RLU data are the average of three independent experiments. Numbers on the right indicate the percent cell viability.

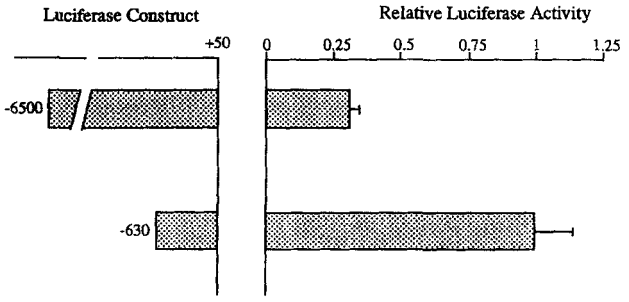

Fig. 2. Distal 5′-flanking sequence of human MSR inhibits MSR transcription in P388D1 cells. P388D1 cells were cotransfected by electroporation at 960 μF, 400 V with 30 μg of each construct and 5 μg of pCAT-Control as an internal control. The data are the mean ± SD of three independent experiments on the rates of luciferase activity/CAT activity.

Cell type-specific expression and effect of the distal *5′-flanking region*

To determine the cell type-specific expression of the human MSR gene, a construct extending from −630 to +112 bp and a promoterless pGL2-Basic vector were transfected into P388D1, CHO, and Hela cells. This promoter domain activated luciferase activity about 200-, 5-, and 1.2-fold over the promoterless vector in the P388D1, Hela, and CHO cells, respectively. This result indicated that the −630 to +112 bp promoter region was sufficient to induce the cell type-specific expression. Another construct (−10 to +50 bp) was also found to be restricted to express in P388D1 cells (data not shown). Consistent with the findings of Aftring and Freeman (28), we observed that the cell type-specific expression of MSR gene was regulated by a very short promoter in P388D1 cells. We next investigated the promoter activity of the 5′-distal promoter region. Minus 6.5 k to +50 bp and −630 to +50 bp fragments were fused to a promoterless luciferase gene. The plasmids were transiently transfected into P388D1 cells together with CAT-Control to correct variations in transfection efficiency. We found that the −6.5 kbp construct stimulated about 30% of the luciferase activity of the −630 bp promoter construct in P388D1 cells (**Fig. 2**). This result suggests that the 5′-distal flanking sequence suppressed MSR transcription in P388D1 cells.

Deletion analysis of the human MSR promoter

To further define the relationship of the −630 to +50 bp fragment with stimulated transcription in P388D1 cells, a series of 5′ deletion constructs was made

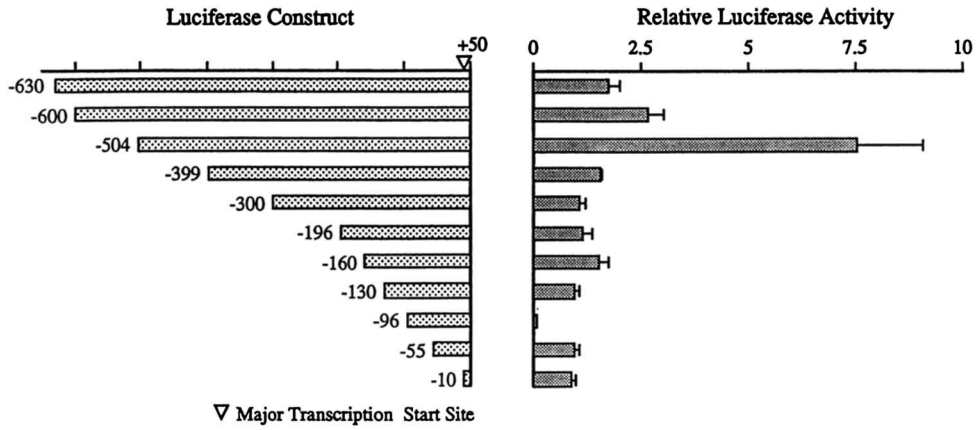

Fig. 3. Analysis of deletion constructs of human MSR promoter in P388D1 cells. P388D1 cells were cotransfected by electroporation at 960 μF, 400 V with 30 μg of each construct and 5 μg of pCAT-Control as an internal control. The data are the mean ± SD of three independent experiments on the rates of luciferase activity/CAT activity.

by a PCR method and transfected into P388D1 cells. **Figure 3** shows that the region included positively acting sequences at −504 to −399 bp and −130 to −96 bp, and negatively acting domains at −630 to −504 bp and −96 to −55 bp. A finding from these truncations was that a very short promoter fragment, −10 to +50 bp, stimulated luciferase expression nearly equivalent to the fragment from −130 to +50 bp. Moulton et al. (19) suggested that this region (−179 to +112 bp), including Pu.1 and AP-1/ets, is sufficient for human MSR expression in THP-1 cells. Our present result shows that the −10 to +50 bp promoter sequence may stimulate the basic transcription of MSR gene in P388D1 cells. In agreement with the findings of Moulton et al. (19) and Aftring and Freeman (28), the mutation of AP-1/ets reduced luciferase activity in P388D1 cells (data not shown). In contrast, although a −130 to +96 bp element including a Pu.1 site appeared a positive element in P388D1 cells, and Moulton et al. (19) have demonstrated that Pu.1 site involved cell-type specific expression in THP-1 cells, this element is not as important in P388D1 cells as it is in THP-1 cells. In P388D1 cells, the distal promoter domain (−504 to −399 bp) stimulated 8.6-fold higher transcription relative to the −10 to +50 bp construct in the present study. This indicates that in P388D1 cells, the maximal transcription activity requires the distal −504 to −399 bp promoter domain.

Nuclear proteins of P388D1 cells interact with the MSR promoter

The results of the above-described deletion analysis of the MSR promoter showed that the region extending from −630 to −400 bp is very important in transcription regulation in the MSR gene in P388D1 cells. The activation and repression of MSR gene transcription are probably mediated by transcription factors and repressors binding to the regions. To identify these factors, we performed a gel EMSA. We first prepared the cell extracts from P388D1 cells. Nuclear proteins of Hela cells were purchased from Stratagene (La Jolla, CA). We then performed the gel EMSA with a ^{32}P-labeled −630 to −350 bp fragment of the MSR promoter as a probe. As shown in **Fig. 4**, two slowly migrating complexes were found in the P388D1 cell extract but not in the extract of Hela cells, which are not monocyte/macrophage-like cells. The density of the complex signals increased with the amount of nuclear proteins. The formation of the complexes was completely inhibited by a 100-fold molar excess of unlabeled −630/−350 fragments, but not by poly (dI-dC). These results indicated that the formation of the complexes was sequence-specific and was found only in monocyte/macrophage-like cells.

DNase I footprint

According to the MSR promoter activity findings, the −504 to −399 bp region contains strong positive elements. The EMSA data showed the existence of transcription factors in the region. We speculated that the protein binding sites were in the −504 to −399 bp region. To test this hypothesis, we performed a DNase I footprint assay. Nuclear extract was prepared from P388D1 cells. Nuclear proteins of Hela cells were purchased from Stratagene (La Jolla, CA). The ^{32}P-labeled −630 to −350 bp fragment of MSR promoter was prepared by PCR and used as a probe. **Figure 5** illustrates the results. Two footprints were found. The first one was located at −504 to −485 bp of the MSR promoter and the other was observed at the −475 to −450 bp. The results indicated that the strong positive *cis*-ele-

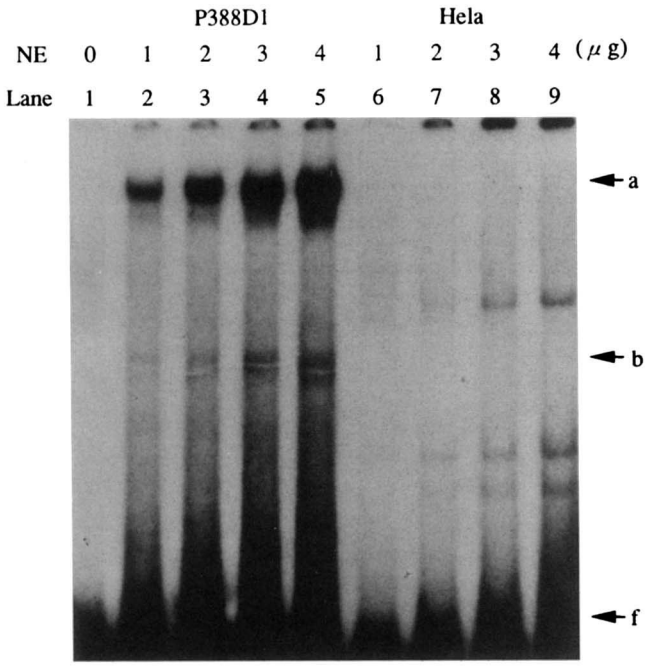

Fig. 4. Electrophoretic mobility shift assay of nuclear proteins from macrophage cell line P388D1 and no-macrophage Hela cells. A ^{32}P-labeled −504 to −350 bp fragment of human MSR promoter was used as a probe. Lane 1 shows the probe alone (no protein control). Lanes 2–5 represent 1–4 μg of nuclear protein (NE) of P388D1 cells. Lanes 6–9 represent 1–4 μg of nuclear protein of Hela cells. EMSA assays were performed as described in Materials and Methods. The "a" and "b" indicate shift bands. The "f" indicates free probe.

ment located at the −504 to −399 bp region resulted from the binding of nuclear proteins (or transcription factors). To determine the function of these footprints in the expression of the MSR gene in P388D1 cells, we transfected the −510 to +50, −475 to +50, and −399 to +50 bp luciferase constructs into P388D1 cells. **Figure 6** shows that the −510 to +50 and −475 to +50 constructs stimulated 5- and 2.9-fold higher luciferase activity compared compare with the −399 to +50 bp construct in P388D1 cells, respectively. These results indicated that both −510 to −485 bp and −475 to −450 bp regions were enhancer elements in the expression of the MSR gene in P388D1 cells.

Gel electrophoretic mobility shift competitive assay and supershift assay

To identify the protein binding to the −504 to −485 promoter region, we performed a gel electrophoretic mobility shift competitive assay in the presence of a 100-fold molar excess of unlabeled popular oligonucleotides with the ^{32}P-labeled −510 to −485 bp oligonucleotide as a probe. **Figure 7** illustrates that the AP-3 and NF-κB consensus sequence partially abolished the protein complex formation with nuclear extracts. Because

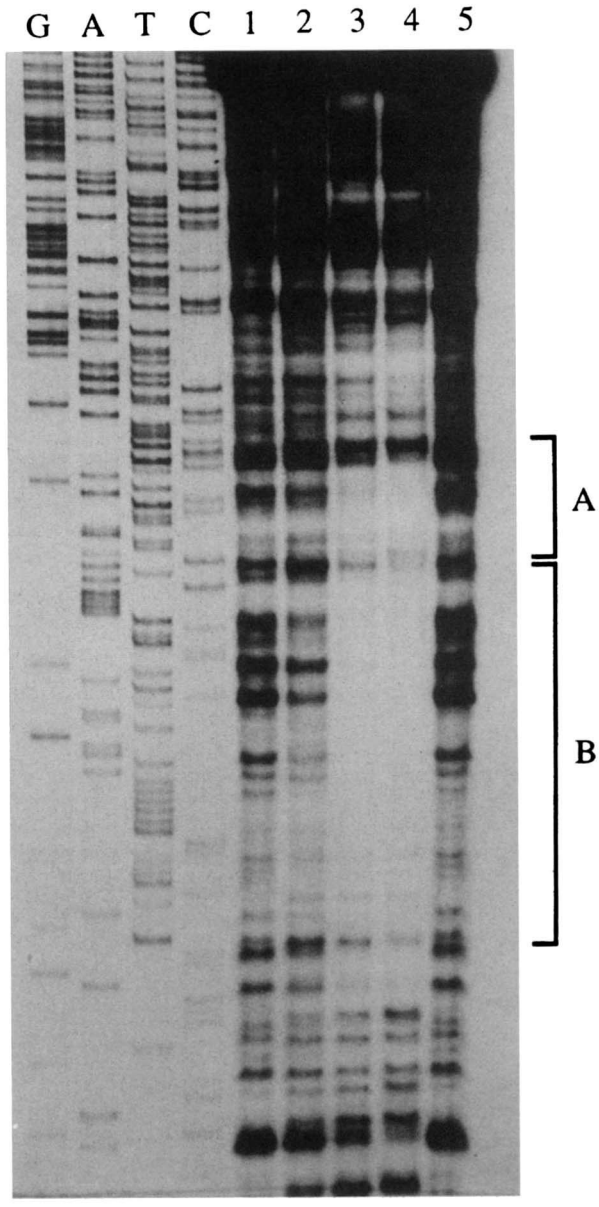

Fig. 5. DNase I footprinting analysis of the human MSR promoter demonstrates the presence of two DNA binding activities in the −630 to −350 bp promoter region. The ^{32}P-labeled −630 to −350 bp fragment of human MSR promoter was used as a probe. Nuclear extracts derived from P388D1 cells were incubated at increasing concentrations (0, 5, 10, and 20 μg). The DNA-protein complexes were subsequently subjected to DNase digestion and analyzed by electrophoresis through denaturing polyacrylamide gels. G, A, T, and C represent the sequence of the promoter region. A and B indicate the footprint regions.

a large number of proteins, some unrelated to NF-κB, can bind to the KB motif (29–31) the above data do not prove that an NF-κB actually binds to the motif. We therefore performed an immunosupershift assay with cell extracts of murine P388D1 cells. We found that the addition of the antibody to NF-κB: p50, P65, c-Rel and

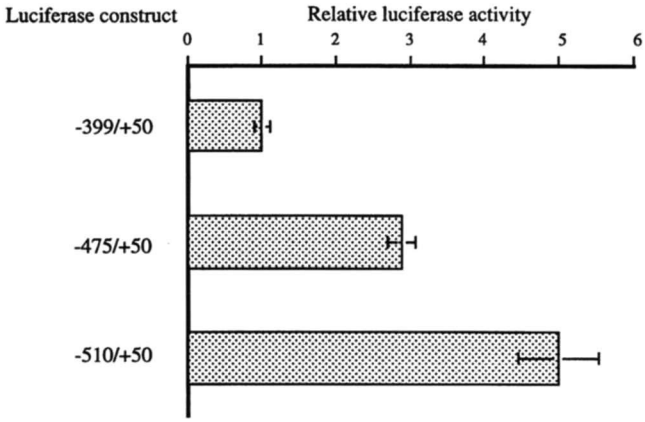

Fig. 6. Effects of footprint A and B of the human MSR promoter on the expression of the MSR gene in P388D1 cells. The human MSR promoter fragments from −510 to +50, −475 to +50 and −399 to +50 bp were cloned upstream of the luciferase gene of pGL2-Basic vector, respectively. P388D1 cells were transfected by electroporation at 960 μF, 400 V with 30 μg of each construct and 5 μg of pCAT-Control as an internal control. The results are the mean ± SD of three independent experiments on the rates of luciferase activity/CAT activity.

IκB α and β, respectively, to the EMSA mixture did not result in a specific retardation band. This result suggested that the binding protein to the −504 to −485 bp domain was not an NF-κB protein. It may be a novel transcriptional factor which also binds to AP-3 and NF-kappa B consensus. To precisely map the binding motif of the −504 to −485 bp region, we tested the ability of a series of mutated the −510 to −485 bp oligomers to inhibit the DNA/protein complex formation, using the EMSA with a ^{32}P-labeled −510 to −485 bp oligomer as a probe. The result clearly showed that a nuclear protein of macrophages bound in vitro to a 9 bp motif, 5′-ATCATGAGA-3′, located at −504 to −495 bp of the MSR gene (**Fig. 8**). Similar experiments to determine the protein binding to the −475 to −450 promoter region were carried out with unlabeled consensus oligonucleotides to popular elements and a ^{32}P-labeled −475 to −450 bp domain of the MSR promoter as a probe. No competition was found (data not shown). TATA box and ets motifs were identified by comparison to the database in the −475 to −450 bp region of the MSR promoter. We next studied whether the region binds to a

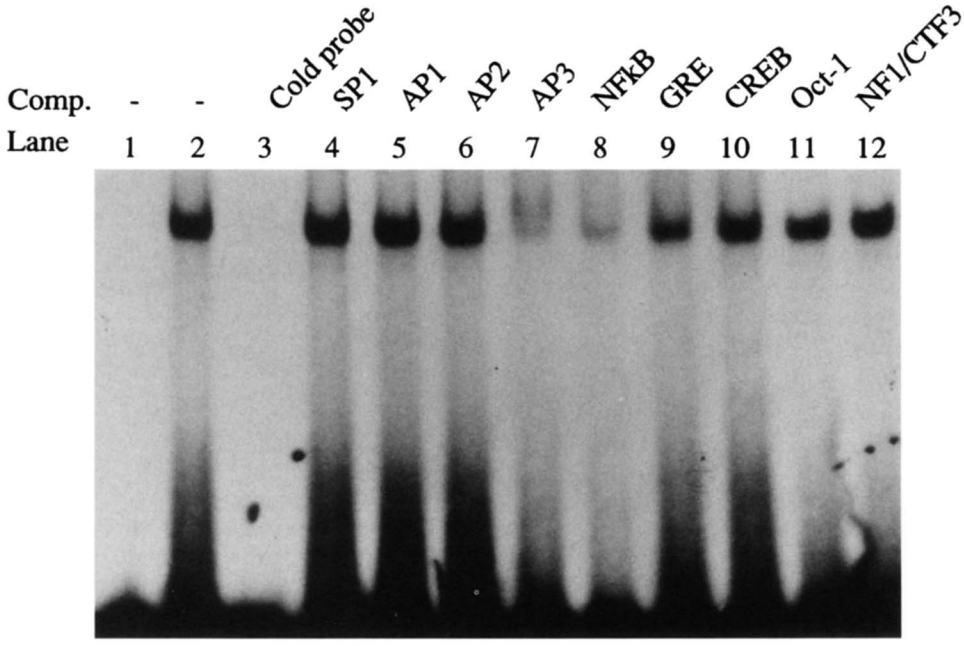

Fig. 7. Inhibition of protein complex formation at region A of the footprint by NF-kappa B and AP-3 consensus sequences. A ^{32}P-labeled −510 to −485 bp oligonucleotide corresponding to the human MSR promoter sequence was incubated with a nuclear extract derived from P388D1 cells, and the complexes were analyzed by EMSA. The first two lanes show the controls without and with the nuclear protein. Lanes 3–12 are the same as lane 2 except that they include a 100-fold molar excess of unlabeled competitor oligonucleotides containing the binding sites of several known transcription factors as indicated (lanes 4–12).

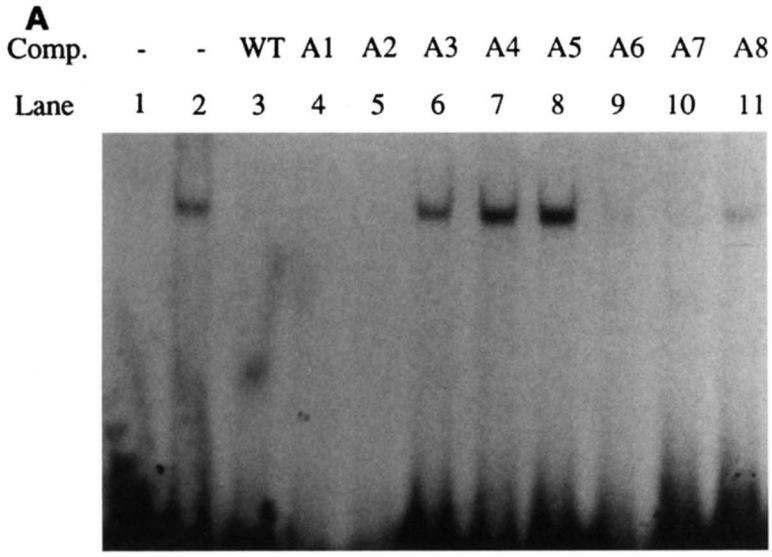

Fig. 8. Mapping of the protein binding motif in footprinting region A. A ^{32}P-labeled −510 to −485 bp domain of human MSR promoter was incubated with a nuclear extract derived from P388D1 cells, and the complexes were analyzed by EMSA. The first two lanes show the controls without and with the nuclear protein. Lanes 3–12 are the same as lane 2 except that they include various competitors. The sequences of the wild type A and its various deletion mutants (A1–A8, underlined deleted nucleotides) are listed in panel B, which also summarizes the abilities of the various competitors to inhibit protein complex formation at region A. Note the clear definition of a 9-bP binding motif (5′-ATCATGAGA-3′).

B

	Competitor
WT	5'-GAGGAGATCATGAGAATTAATGTAT-3'
A1	5'-GAGGAGATCATGAGAATTAATGTAT-3'
A2	5'-GAGGAGATCATGAGAATTAATGTAT-3'
A3	5'-GAGGAGATCATGAGAATTAATGTAT-3'
A4	5'-GAGGAGATCATGAGAATTAATGTAT-3'
A5	5'-GAGGAGATCATGAGAATTAATGTAT-3'
A6	5'-GAGGAGATCATGAGAATTAATGTAT-3'
A7	5'-GAGGAGATCATGAGAATTAATGTAT-3'
A8	5'-GAGGAGATCATGAGAATTAATGTAT-3'

TATA binding protein or/and an ets protein. We performed an EMSA in the presence of a 100-fold molar excess of unlabeled −475 to −455 bp mutant without ets motifs with a ^{32}P-labeled −475 to −450 bp oligonucleotide as a probe. **Figure 9** illustrates that the −475 to −455 bp oligonucleotide completely inhibited the formation of complexes in the −475 to −450 region, indicating that the binding protein may not be ets protein. We also performed an EMSA using TATA-to-CATA and TATA-to-GATA mutants of the −475 to −450 bp as competitors. The two mutated oligonucleotides also inhibited the formation of complexes in the −475 to −450 bp region. These results indicated that the −475 to −450 bp region may not bind to a TATA binding protein. Therefore, we speculate that a novel transcription factor may bind to this region.

Mutations of the −510 to −485 bp region abolished 45–75% of the MSR promoter activity

To determine the functional significance of the −510 to −485 bp region, we constructed a set of −504 to +50 bp luciferase constructs containing the 3 bp-deletion mutations in a 5′-ATCATGAGA-3′ oligomer which abolished protein binding to the sites in gel retardation experiments (Fig. 8). These constructs were transfected into P388D1 cells by electroporation. The results indicated that the mutations of 3 bp reduced 45–75% of the MSR promoter activity (**Fig. 10**). These results strongly suggest that the −504 to −485 bp region is necessary for the MSR gene to function as a promoter in transfected P388D1 cells.

DISCUSSION

Studies of the control of gene expression in mammalian cells rely heavily on the ability to induce DNA into cultured mammalian cells. The methods of gene transfer that work well with some cell lines, i.e., DEAE dextran, calcium phosphate co-precipitation, and liposomes, have been found to be refractory when attempted with monocyte/macrophage cells (32–35). Electroporation can be a highly efficient method for in-

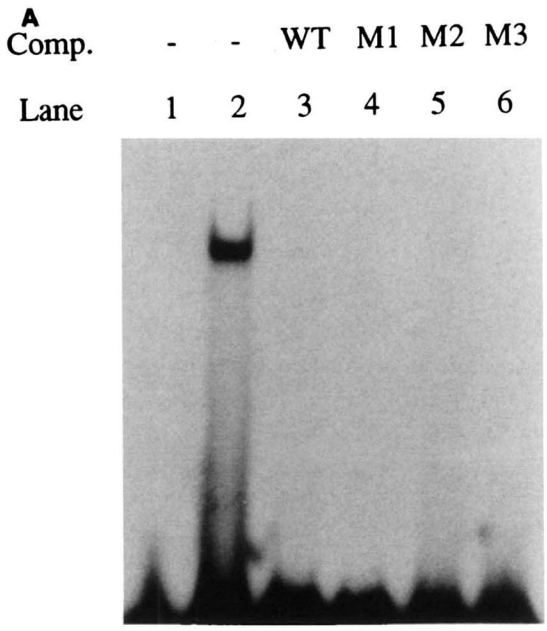

Fig. 9. A protein complex at region B in footprinting is neither TATA-binding protein nor ets-binding protein. A ^{32}P-labeled −475 to −450 bp oligonucleotide probe corresponding to human MSR promoter was incubated with a nuclear extract derived from P388D1 cells in the presence of a 100-fold excess of unlabeled competitors. Lane 1 represents the probe alone. Lane 2 shows the positive control without competitor. Lanes 3–6 show the competitors of unlabeled probe (WT), TATA-mutant (M1 and M2) and ets-mutant (M3) oligonucleotides, respectively.

troducing DNA molecules into cultured cells for the transient expression of genes (36). However, effective transfection by electroporation requires careful optimization of all parameters. Using a number of published methods, we have tried to introduce DNA molecules into THP-1 and P388D1 cells. However, a very low efficiency of gene expression was obtained. In the present study, therefore, we optimized all parameters for P388D1 cells. The efficiency of transfection was increased by 20-fold compared with previous methods. Although the electroporation voltage should be optimized, the results of temperature, medium, DNA concentration, and capacitance obtained here are likely to apply to all monocyte/macrophage cells.

Using murine P388D1 cells as a macrophage model, we studied the regulation of the human MSR gene. Moulton et al. (19) reported that nearly the same activities were found in their transfection study of human MSR into P388D1 and Hela cells, but in the present study, the −630 to +112 bp fragment of human MSR stimulated very high luciferase activity in P388D1 cells but only a background level in Hela cells. The difference in results may be due to the efficiency of transfection. Hela cells are easily transfected but P388D1 cells are very difficult to transfect using previously published methods. Studies of cell-specific expression have indicated that the −179 to +112 bp region contained sufficient information to give rise to cell-type specific expression in THP-1 cells (19). In the present study, we also found that the −179 to +112 promoter fragment stimulated significant luciferase activity in P388D1 cells. Further, we found that the essential information for macrophage-specific expression of the MSR gene may reside in a short fragment (−10 to +50 bp) in macrophage cells. Our results, consistent with the findings of Aftring et al. (28), show that the information of cell-specific expression may exist in a short promoter fragment. In this present study, the −6.5 kb upstream of the MSR pro-

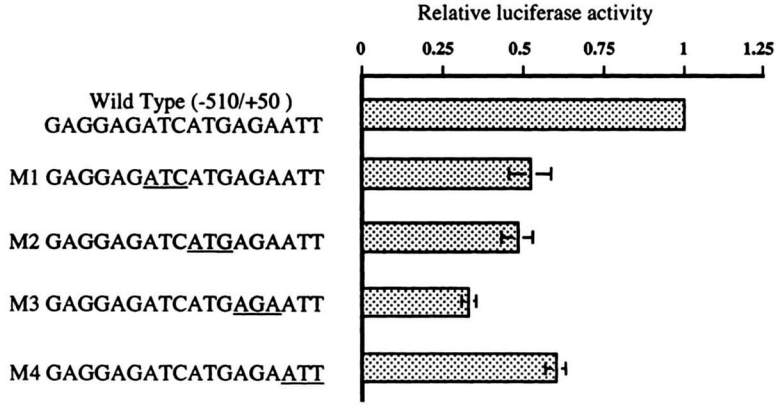

Fig. 10. Effect of mutations of the region A of human MSR on luciferase activity in P388D1 cells. The −510 to +50 bp promoter construct containing the region A was analyzed. The −510 to −493 sequence of human scavenger receptor promoter is shown. The native sequence is shown at the top left. Mutated sequences are shown adjacent to their represent luciferase activity, with the deleted nucleotides underlined. P388D1 cells were cotransfected with −510 to +50 bp promoter construct by electroporation at 960 μF, 400 V with 30μg of each construct and 5 μg of pCAT-Control as an internal control. The results are the mean ± SD of three independent experiments on the rates of luciferase activity/CAT activity.

moter was mainly inhibitory elements to human MSR expression in P388D1 cells. Aftring et al. (28) reported a 10-fold increase in luciferase activity for the murine promoter fragment of 5 kbp. Wu et al. (20) demonstrated that a silencer at −6.5 to −4.8 kbp and an enhancer at −4.1 and −4.5 kbp existed in human MSR and the upstream sequences resulted in 18- to 20-fold induction by phorbol esters. The difference between our data and those of Wu et al. (20) may be due to the different cell types; Wu et al. (20) used THP-1 cells as a model and they focused on the induction of the phorbol ester, tetradecanol phorbol acetate (TPA). We studied the transcription regulation of the MSR gene in macrophages. The difference between our data and those of Aftring et al. (28) may be due the length of the promoter fragments used. According to the finding of Wu et al. (20), −6.5 to −4.8 kbp contained a silencing element whereas Aftring et al. used a 5.0 kbp upstream sequence of mouse MSR gene, lacking a silencing element. Our deletion analysis of human MSR promoter demonstrated that the −10 to +50 bp fragment, which Moulton et al. (19) and Aftring et al. (28) suggested to be involved in AP-1/ets and GATA sites, was also sufficient to direct the significant expression of the luciferase gene in P388D1 cells. Moulton et al. (19) reported that the Pu.1 site in the proximal promoter is very important in the expression of the MSR gene in THP-1 cells and Horvai et al. (37) demonstrated that mutation of the Pu.1 binding site severely impairs MSR expression in peritoneal macrophages in transgenic mice. Aftring et al. (28) found that the cell-specific expression of the MSR gene in P388D1 cells does not appear to require the Pu.1 site. We found that Pu.1 at position −133 was a positive element but less important for luciferase activity in P388D1 cells. In accord with the finding of Wu et al. (20), we observed that the −130 to −55 bp region is a negative element. We found that the −504 to +50 bp promoter fragment stimulated luciferase activity about 8-fold over the −10 to +50 bp promoter, whereas the −600 to +50 bp promoter resulted in a loss of about two-thirds of the promoter activity of the −504 to +50 promoter fragment in P388D1 cells. These results indicated that the −504 to −399 bp element is a positive element and the −600 to −505 bp region is a negative element. Because of the abilities of MSR to bind and internalize ox-LDL (38), they have been proposed to provide an important pathway for the progressive accumulation of cholesterol by arterial wall macrophages and the formation of macrophage foam cells that characterize early atherosclerotic lesions. If the expression of the MSR gene in arterial wall macrophages is suppressed by transcriptional events mediated by the −600 to −505 bp negative element, the accumulation of cholesterol may be controlled. Therefore, the −600 to −505 bp element is also important. The −600 to −399 region of the human MSR promoter, which contained a 23-bp inverted repeat sequence (23), may play an key role in transcription regulation in macrophages. The deletion analysis of mouse MSR promoter by Aftring et al. (28) did not show the importance of the region in P388D1 cells because they destroyed this region while making the reporter gene constructs. The importance of the promoter regions should be examined in vivo.

We focused on examining which region binds to nuclear proteins from P388D1 cells and what proteins bind to the −504 to −399 bp fragment of MSR promoter. Our analysis of the DNA binding proteins by the EMSA and the footprint assay showed that the −504 to −399 bp region binds to transcription factors in P388D1 cells but not in Hela cells. These results indicated that a macrophage-specific transcription factor may bind to the region. According to our footprint data, two footprints (−504 to −485 bp and −475 to −450 bp) were found. Using the two elements as probes, we performed an EMSA. The retardation complexes were formed in P388D1 cell extract but not in Hela cell extract (data not shown). These results suggested that the binding protein may be a cell-specific protein. By a comparison with the database of the motif, we found that the −475 to −450 bp element contained a TATA box and an ets consensus sequence. However, oligonucleotides with a mutation of the TATA box and a deletion of the ets binding site completely inhibited the formation of retardation complexes in the −475 to −450 bp element. The proteins binding to the region may be other transcription factors. Further studies to determine the protein are currently underway. Although NF-kappa B consensus sequence and AP-3 consensus inhibited the formation of retardation complexes in the −504 to −485 bp promoter domain, our immunosupershift assay did not confirm that the proteins binding to the region are NF-kappa subunits. The analysis of the binding sites in the region showed that a 5′-ATCATGAGA-3′ domain, which is not consistent with AP-3 (TGTGGAAA/TTT) (39) and NF-κB consensus (GGGRTYYCC), plays an important role in the binding of the transcription factor. These results demonstrated that the proteins binding to −504 to −485 element may be NF-κB/AP-3-like transcription factor or proteins that can bind to AP-3 and NF-kappa B consensus sequences. We are now attempting to clone the transcription factor. Although we do not know yet which proteins bind to the region, mutations of the region reduced about 40–75% of the transcription activity in P388D1 macrophages and this finding indicated that the −504 to −399 bp region is important in the transcription regulation of the MSR gene in P388D1 macrophages. Thus, we conclude that novel elements regulate the expression of the MSR gene in P388D1 macrophages.

This study was supported by a grant from the Human Health Sciences Foundation and by a Grant for Research on Aging and Health from the Ministry of Health and Welfare, Japan.

Manuscript received 4 February 1997 and in revised form 1 April 1997.

REFERENCES

1. Kodama, T., P. Reddy, C. Kishimoto, and M. Krieger. 1988. Purification and characterization of a bovine acetyl low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA.* **85:** 9238–9242.
2. Matsumoto, A., M. Naito, H. Itakura, S. Ikemoto, H. Asaoka, I. Hayakawa, H. Kanamori, H. Aburatani, F. Takaku, H. Suzuki, Y. Kobari, T. Miyai, K. Takahashi, E. H. Cohen, R. Wydro, D. E. Housman, and T. Kodama. 1990. Human macrophage scavenger receptors: primary structure, expression, localization in atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **87:** 9133–9137.
3. Naito, M., H. Suzuki, T. Mori, A. Matsumoto, T. Kodama, and K. Takahashi. 1992. Coexpression of type I and type II human macrophage scavenger receptors in macrophages of various organs and foam cells in atherosclerotic lesions. *Am. J. Pathol.* **141:** 591–599.
4. Kodama, T., M. Freeman, L. Rohrer, J. Zabrecky, P. Matsudaira, and M. Krieger. 1990. Type I macrophage scavenger receptor contains α-helical and collagen-like coiled-coils. *Nature.* **343:** 531–535.
5. Rohrer, L., M. Freeman, T. Kodama, M. Penman, and M. Krieger. 1990. Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II. *Nature.* **343:** 570–572.
6. Hampton, R. Y., D. T., Golenbock, M., Penman, M., Krieger, and C. R. H., Raetz. 1991. Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature.* **353:** 342–344.
7. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–337.
8. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophages: implication for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223–261.
9. Ylä-Herttuala, S., M. E. Rosenfeld, S. Parthasarathy, E. Sigal, T. Särkioja, J. L. Witztum, and D. Steinberg. 1991. Gene expression in macrophage-rich human atherosclerotic lesions. *J. Clin. Invest.* **87:** 1146–1152.
10. Fraser, I., D. Hughes, and S. Gordon. 1993. Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. *Nature.* **364:** 343–346.
11. Fong, L. G., T. A. T. Fong, and A. D. Cooper. 1990. Inhibition of mouse macrophage degradation of acetyl-low density lipoprotein by interferon-γ. *J. Biol. Chem.* **265:** 11751–11760.
12. Geng, Y. J., and G. K. Hansson 1992. Interferon-γ inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. *J. Clin Invest.* **89:** 1322–1330.
13. Van Lenten, B. J., and A. M. Fogelman. 1992. Lipopolysaccharide-induced inhibition of scavenger receptor expression in human monocyte-macrophages is mediated through tumor necrosis factor-α. *J. Immunol.* **148:** 112–116.
14. Ishibashi, S., T. Inaba, H. Shimano, K. Harada, I. Inoue, H. Mokuno, N. Mori, T. Gotoda, F. Takaku, and Y. Yamada. 1990. Colony-stimulating factor enhances uptake and degradation of acetylated low density lipoproteins and cholesterol esterification in human-derived macrophages. *J. Biol. Chem.* **265:** 14109–14117.
15. Bottalico, L. A., R. E. Wager, L. B. Agellon, R. K. Assoian, and I. Tabas. 1991. Transforming growth factor-β1 inhibits its scavenger receptor activity in THP-1 human macrophages. *J. Biol. Chem.* **266:** 22866–22871.
16. Moulton, K. S., H. Wu, J. Barnett, S. Parthasarathy, and C. K. Glass. 1992. Regulated expression of the human acetylated low density lipoprotein receptor gene and isolation of promoter sequences. *Proc. Natl. Acad. Sci. USA.* **89:** 8102–8106.
17. Fogelman, A. M., M. E. Haberland, J. Seager, M. Hokom, and P. A. Edwards. 1981. Factors regulating the activities of the low density lipoprotein receptor and the scavenger receptor on human monocyte-macrophages. *J. Lipid Res.* **22:** 1131–1141.
18. Hara, H., H. Tanishita, S. Yokoyama, S. Tajima, and A. Yamamoto. 1987. Induction of acetylated low density lipoprotein receptor and suppression of low density lipoprotein receptor on the cells of human monocytic leukemia cell line (THP-1 cell). *Biochem. Biophys. Res. Commun.* **146:** 802–808.
19. Moulton, K. S., K. Semple, H. Wu, and C. K. Glass. 1994. Cell specific expression of the macrophage scavenger receptor gene is dependent on PU.1 and a composite AP-1/ets motif. *Mol. Cell. Biol.* **14:** 4408–4418.
20. Wu, H., K. Moulton, A. Horvai, S. Parik, and C. K. Glass. 1994. Combinatorial interactions between AP-1 and ets domain proteins contribute to the developmental regulation of the macrophage scavenger receptor gene. *Mol. Cell. Biol.* **14:** 2129–2139.
21. Pahl, H. L., T. C. Burn, and D. G. Tenen. 1991. Optimization of transient transfection into human myeloid cell lines using a luciferase reporter gene. *Exp. Hematol.* **19:** 1038–1041.
22. Stacey, K. J., I. L. Ross, and D. A. Hume. 1993. Electroporation and DNA-dependent cell death in murine macrophages. *Immunol. Cell Biol.* **71:** 75–85.
23. Emi, M., H. Asaoka, A. Matsumoto, H. Itakura, Y. Kurihara, Y. Wada, H. Kanamori, Y. Yazaki, E. Takahashi, M. Lepert, J-M. Lalouel, T. Kodama, and T. Mukai. 1993. Structure, organization, and chromosomal mapping of the human scavenger receptor gene. *J. Biol. Chem.* **268:** 2120–2125.
24. Chu, G., H. Hayakawa, and P. Berg. 1987. Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res.* **15:** 1311–1326.
25. Sleigh, M. J. 1986. A nonchromatographic assay for expression of the chloramphenicol acetyltransferase gene in eucaryotic cells. *Anal. Biochem.* **156:** 251–256.
26. Dignam, J. D., P. L. Martin, B. S. Shastry, and R. G. Roeder. 1983. Eukaryotic gene transcription with purified components. *Methods Enzymol.* **101:** 582–598.
27. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. Current Protocols in Molecular Biology. Chapter 12. 4. Greene Publishing Associates and Wiley-Interscience, New York.
28. Aftring, R. P., and M. W. Freeman. 1995. Structure of the

murine macrophage scavenger receptor gene and evaluation of the sequences that regulate expression in the macrophage cell line, P388D1. *J. Lipid Res.* **36:** 1305–1314.
29. Adams, B. S., K. Y. Leung, E. W. Haney, and G. J. Nabel. 1991. Cloning of R kappa B, a novel DNA-binding protein that recognizes the interleukin-2 receptor alpha chain kappa B site. *New Biologist.* **3:** 1063–1073.
30. Baldwin, A. G. J., and P. A. Sharp. 1988. Two transcription factors, NF-kB and H2TF1, interact with a single regulatory sequence in class I major histocompatibility complex promoter. *Proc. Natl. Acad. Sci. USA.* **85:** 723–727.
31. Lattion, A. L., E. Espel, P. Reichenbach, C. Fromental, P. Bucher, A. Israel, P. Baeuerle, N. R. Rice, and M. Nabholz. 1992. Characterization of a new tissue-specific transcription factor binding to the simian virus 40 enhancer TC-II (NF-kB) element. *Mol. Cell. Biol.* **12:** 5217–5227.
32. Chu, G., and P. A. Sharp. 1981. SV40 DNA transfection of cells in suspension: analysis of the efficiency of transcription and translation of T-antigen. *Gene.* **13:** 197–202.
33. Chen, C., and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7:** 2745–2752.
34. Fraley, R., S. Subramani, P. Berg, and D. Papahadjopoulos. 1980. Introduction of liposome-encapsulated SV 40 DNA into cells. *J. Biol. Chem.* **255:** 10431–10435.
35. Schaefer-Ridder, M., Y. Wang, and P. H. Hofschneider. 1982. Liposomes as gene carriers: efficient transformation of mouse L cells by thymidine kinase gene. *Science.* **215:** 166–168.
36. Andreason, G. L., and G. A. Evans. 1989. Optimization of electroporation of mammalian cell lines. *Anal. Biochem.* **180:** 269–275.
37. Horvai, A., W. Palinski, H. Wu, K. S. Moulton, K. Kalla, and C. K. Glass. 1995. Scavenger receptor A gene regulatory elements target gene expression to macrophages and to foam cells of atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **92:** 5391–5395.
38. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **78:** 6499–6504.
39. Mercurio, F., and M. Karin. 1989. Transcription factors AP-3 and AP-2 interact with the SV40 enhancer in a mutually exclusive manner. *EMBO J.* **8:** 1455–1460