PU.1 and USF Are Required for Macrophage-specific Mannose Receptor Promoter Activity*

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In the current study we report the isolation of 854 base pairs of the rat mannose receptor promoter. Analysis of the sequence revealed one Sp1 site, three PU.1 sites, and a potential TATA box (TTAAAA) 33 base pairs 5′ of the transcriptional start site. The tissue specificity of the promoter was determined using transient transfections. The promoter was most active in the mature macrophage cell line NR8383 although the promoter also showed activity in the monocytic cell line RAW. No activity was observed in pre-monocytic cell lines or epithelial cell lines. Mutation of the TTAAAA sequence to TTGGAA resulted in a 50% decrease in activity in transient transfection assays suggesting that the promoter contains a functional TATA box. Using electrophoretic mobility shift assays and mutagenesis we established that the transcription factors Sp1, PU.1, and USF bound to the mannose receptor promoter, but only PU.1 and USF contributed to activation. Transient transfections using a dominant negative construct of USF resulted in a 50% decrease in mannose receptor promoter activity, further establishing the role of USF in activating the rat mannose receptor promoter. Comparison of the rat, mouse, and human sequence demonstrated that some binding sites are not conserved. Gel shifts were performed to investigate differences in protein binding between species. USF bound to the rat and human promoter but not to the mouse promoter, suggesting that different mechanisms are involved in regulation of mannose receptor expression in these species. From these results we conclude that, similar to other myeloid promoters, transcription of the rat mannose receptor is regulated by binding of PU.1 and a ubiquitous factor at an adjacent site. However, unlike other myeloid promoters, we have identified USF as the ubiquitous factor, and demonstrated that the promoter contains a functional TATA box.

The maturation of phagocytes during the latter stages of myeloid cell differentiation is accompanied by the appearance of a number of myeloid-restricted proteins. One of these proteins, the mannose receptor, is absent from monocytes but is expressed on mature macrophages, appearing very late in the differentiation process (1, 2). The mannose receptor is a 175-kDa type I transmembrane protein and is a member of the C-type lectin family (3). The protein has been purified from a variety of species (4, 5), and the murine and human cDNA sequences have been reported (6, 7). Several in vivo functions have been proposed for the macrophage mannose receptor: endocytosis of extracellular peroxidases and hydrolases during the resolution phase of inflammation (8), phagocytosis of opsonized pathogens (9), and antigen capture for eventual presentation to T cells (10).

Studies in our laboratory for the past several years have focused on the mechanisms involved in regulation of expression of the mannose receptor in macrophages. Mannose receptor expression is a marker of the mature macrophage, although expression has also been reported on dendritic cells (11) and retinal pigmented epithelial cells (12). Mannose receptor protein is absent in freshly isolated monocytes and appears during in vitro differentiation in the presence of colony-stimulating factors (13). Recent studies from a number of laboratories have begun to shed some light on the mechanisms that regulate myeloid-restricted gene expression. Studies of the promoters of these genes have revealed a number of potential factors that modulate myeloid-specific gene expression, and a pattern of features required for activation of these genes during myeloid differentiation has emerged (14, 15). Control of tissue specificity typically resides in approximately 150 base pairs 5′ of the major transcriptional start site. All of the myeloid promoters contain consensus binding sites for PU.1, a member of theets family of transcription factors (16). A role for this factor in myeloid cell differentiation has been supported by the findings that macrophage development is restricted in PU.1 null mice, and that PU.1 expression is up-regulated during commitment of multipotential stem cells to the myeloid lineage (17). The activity of a number of myeloid promoters requires an intact PU.1 site (18–26), and the PU.1 sites are in close proximity to a site that binds a second transcription factor. Binding elements for four of these factors have been identified to date: AML1, a member of the Runt/PEBP2/CBF family; C/EBPα or C/EBPβ; Sp1; and AP-1 (14). Binding of both PU.1 and this second factor are involved in controlling maximal activity. In addition, the promoters for many of these myeloid-restricted proteins lack a TATA box and have multiple transcriptional start sites.

Some information on factors involved in mannose receptor regulation has come from the recent reports of the isolation and partial characterization of the promoters for the human and murine mannose receptor genes (27, 28). Potential regulatory mechanisms were identified in the murine sequence, and myeloid restricted activity was demonstrated for the human promoter. Both promoters contain PU.1 sites, and one of these

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF121966. ** To whom correspondence should be addressed: Dept. of Veterans Affairs Medical Center/Research Service; 1310 24th Ave. South, Nashville, TN 37212. Tel.: 615-327-4751 (ext: 5499); Fax: 615-321-6305; E-mail: virginia.l.shepherd@vanderbilt.edu.

1 The abbreviations used are: bp, base pair(s); CMV, cytomegalovirus; MR, mannose receptor; USF, upstream stimulatory factor; EMSA, electrophoretic mobility shift assays; PCR, polymerase chain reaction.
sites appears to be involved in murine promoter activity. Using transient transfection of mannose receptor-negative myeloid cells, Eichbaum et al. (28) reported that activity of the murine promoter required binding of PU.1 and Sp1 at sites within the first 200 bp. What has hampered further studies on a complete promoter required binding of PU.1 and Sp1 at sites within the mature macrophages. We have focused on sites that are potentially involved in myeloid-restricted expression of the rat mannose receptor, and have extended the findings from both the human and murine systems to include regulation and function of the mannose receptor promoter in a mouse receptor-positivemacrophase cell line.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—NR8383 cells were obtained from Dr. R. Helmeye (University of Texas Health Science Center) and maintained in Ham’s F-12 medium with 15% fetal bovine serum (Life Technologies, Inc.). The murine macrophage cell line RAW264, the human monocytic line HL60, and the human epithelial line A549 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). RAW and HL60 cells were maintained in RPMI 1640 with 10% fetal bovine serum and antibiotics. A549 cells were maintained in F-12K supplemented with 15% fetal bovine serum and antibiotics. A549 epithelial cells were maintained in 2% FBS in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Life Technologies, Inc.). The murine macrophage cell line RAW264, the human monocytic line HL60, and the human epithelial line A549 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). RAW and HL60 cells were maintained in RPMI 1640 with 10% fetal bovine serum and antibiotics. A549 cells were maintained in F-12K supplemented with 15% fetal bovine serum and antibiotics. Rat bone marrow macrophages were prepared from Sprague-Dawley rats as described previously (34) by culture for 5–7 days in the presence of L-929 cells (kind gift from Dr. R. Helmeye (Institut Cochin de Genetique Moleculaire)).

**Promoter Isolation**—The strategy for isolation of the rat mannose receptor promoter was a modification of the procedure used for cloning the human mannose receptor promoter (27) as follows: a synthetic oligonucleotide of known sequence (Integrated DNA Technologies) was ligated to the ends of rat genomic DNA pieces generated by digestion with HindIII. This oligonucleotide formed an “anchor” sequence to which a complementary primer could be hybridized as primer 1 for generation of mannose receptor promoter fragments by PCR using a mouse receptor-specific oligonucleotide as primer 2. The 854-bp mannose receptor promoter was cloned into pGEM-T (Promega) and sequenced using the dideoxynucleotide chain-termination method.

**Plasmid Constructs**—The 854-bp mannose receptor promoter was cloned into the pGL2 basic vector (Promega) containing the luciferase cdNA after BglII/Sphi digestion (MR554). The proximal 656-bp promoter fragment (MR656) was generated by HindIII digestion of the 854-bp promoter with subsequent ligation into a HindIII-digested pGL2 vector. A construct containing the proximal 228 bp of the mannose receptor promoter (MR228) was generated by digestion of the 854-bp construct with Smal and PluI, followed by re-ligation. The 1–108 (MR108) and 1–458 (MR48) constructs were generated by PCR using the MR656 as a template, the appropriate sense primers, and a vector-based antisense primer. The products were digested with HindIII, and ligated into the pGL2 basic vector at the HindIII/Smal site. Mutations in the mannose receptor promoter were introduced using a PCR-based approach as described by Ho et al. (35). Complimentary sense and antisense primers containing the desired mutations were obtained from Integrated DNA Technologies (Corvalia, IA). The products of the PCR reactions containing the mutations were isolated, digested with the appropriate restriction enzymes, and ligated into MR554 following removal of the specific mutated region. The dominant negative USF1 expression vector, AbTDU1, was a kind gift from Dr. Raymondjean (Institut Cochin de Genetique Moleculaire).

**Transfection and Reporter Gene Assays**—Electroporation was used for transfection of the cells lines under the following conditions: man-
nose receptor promoter DNA (5 μg) was ligated into the pGL2 luciferase (pRL-CMV, Promega) (1 μg) were added to 250 μl of serum-free media. RAW cells were transfected at a concentration of 105 cells/l reaction at 280 V and 960 μfardas. NR8383 cells were transfected at a concentration of 5 × 105 cells/reaction at 250 V and 960 μfardas. A549 cells (5 × 105) were transfected at 250 V and 960 μfardas. NR8383 and HL60 cells were also transfected using DEAE-dextran as follows: cells were incubated with phosphatase, then electroporated in the presence of a plasmid at 300 μg/ml aprotinin, 5 μg/ml leupeptin, and 500 μg/ml pepstatin, and placed on ice for 10 min. After electroporation for 1 min at 14,000 rpm, the pellet containing the nuclei was washed once with buffer A, then suspended in 200 μl of buffer B (20 mM Hepes, pH 7.9, with 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiorthiole, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml apro tin, 5 μg/ml leupeptin, and 500 μg/ml pepstatin), and placed on ice for 10 min. After centrifugation for 1 min at 14,000 rpm, the pellet containing the nuclei was washed once with buffer A, then suspended in 200 μl of buffer B (20 mM Hepes, pH 7.9, with 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiorthiole, plus levels of protease inhibitors as in A) and vortexed for 15 min. Cell debris was removed by centrifugation and nuclear protein was quantified using the Bio-Rad Protein Assay (Bio-Rad).

Single-stranded oligonucleotides (Integrated DNA Technologies) were used as a negative control in transfection assays. The pGL2-CMV vector was used to correct for transfection efficiency. Data are expressed as the luciferase activity for each sample normalized to the Renilla luciferase activity.

**Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extract was isolated from cell preparations as follows: 105 cells were suspended in 300 μl of lysis buffer A (10 mM Hepes, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.4% Nonidet P-40, 1 mM dithiorthiole, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml apro tin, 5 μg/ml leupeptin, and 500 μg/ml pepstatin), and placed on ice for 10 min. After centrifugation for 1 min at 14,000 rpm, the pellet containing the nuclei was washed once with buffer A, then suspended in 200 μl of buffer B (20 mM Hepes, pH 7.9, with 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiorthiole, plus levels of protease inhibitors as in A) and vortexed for 15 min. Cell debris was removed by centrifugation and nuclear protein was quantified using the Bio-Rad Protein Assay (Bio-Rad).

Single-stranded oligonucleotides (Integrated DNA Technologies) were used as a negative control in transfection assays. The pGL2-CMV vector was used to correct for transfection efficiency. Data are expressed as the luciferase activity for each sample normalized to the Renilla luciferase activity.
were annealed and 5' nucleotides were added using Klenow (Promega). The resulting double-stranded oligonucleotides were end-labeled using T4 polynucleotide kinase (Promega) and [γ-32P]ATP (NEN Life Science Products Inc.). Labeled oligonucleotide (2 × 106 cpm), single-stranded nonspecific DNA (200 ng), nuclear extract (10 μg), and binding buffer (3 μl) (20 mM Tris-HCl, pH 7.5, with 20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 0.25 mg/ml poly(dI-dC)) were incubated at room temperature for 20 min in a total reaction volume of 15 μl. A 200-fold excess of unlabeled double-stranded oligonucleotide was used in competition assays. For supershift analysis, 2 nM were annealed and 5' nucleotides were added using Klenow (Promega).

**RESULTS**

**Identification of the Rat Mannose Receptor Transcriptional Start Site**—Primer extension analysis using rat bone marrow macrophage RNA was performed using a probe derived from the rat cDNA spanning the region from +23 to +46 bp (5'–CCGGGTTGC–CCCTTGAGTTACTGCC–3'). The DNA was extended using avian myeloblastosis virus reverse transcriptase, then analyzed on a 9% polyacrylamide nondenaturing gel. Lanes 3 and 4 show the products of the extension reaction. Lanes 1 and 2 show that no extension occurred using a primer with the sequence 5'-ATCGAGGCGGTCGCCAGACC-3' which was designed with the 3'-end at the predicted start site. A sequencing ladder is shown on the left for size identification of the products. The site labeled 46 is the major product, with minor products indicated at 52 and 58 bp.

**TABLE I**

| Probe | Sequence | Notes |
|-------|----------|-------|
| 108/71 | 108–AATTCCTGTTTTTCTAACGCCCACTGTGACAGGAT-71 | Base pairs in bold and underlined represent the specific mutations in each oligonucleotide. |
| P106m | 108–AATTCCTGTTTTTCTAACGCCCACTGTGACAGGAT-71 | Base pairs in bold and underlined represent the specific mutations in each oligonucleotide. |
| S91m | 108–AATTCCTGTTTTTCTAACGCCCACTGTGACAGGAT-71 | Base pairs in bold and underlined represent the specific mutations in each oligonucleotide. |
| E74m | 108–AATTCCTGTTTTTCTAACGCCCACTGTGACAGGAT-71 | Base pairs in bold and underlined represent the specific mutations in each oligonucleotide. |
| 108/82 | 108–AATTCCTGTTTTTCTAACGCCCACTGTGACAGGAT-71 | Base pairs in bold and underlined represent the specific mutations in each oligonucleotide. |
| 96/71 | 96–TTCTAACCGCCCCCATGTGACAGGAT-71 | Base pairs in bold and underlined represent the specific mutations in each oligonucleotide. |
| US3 | CCCCCCAGATGACCCCCC | Base pairs in bold and underlined represent the specific mutations in each oligonucleotide. |
| Sp1 | GAGGAAACAGCAAGGACGGGGGG | Base pairs in bold and underlined represent the specific mutations in each oligonucleotide. |
| PU.1 | GGGGGGGAACAGCAAGGACGGGGGG | Base pairs in bold and underlined represent the specific mutations in each oligonucleotide. |

**FIG. 1.** Identification of the transcriptional start site in the rat mannose receptor gene by primer extension. Total RNA was prepared from rat bone marrow macrophages, then incubated with an end-labeled oligonucleotide derived from the rat mannose receptor cDNA spanning the region from +23 to +46 bp (5'–CCGGGTTGC–CCCTTGAGTTACTGCC–3'). The DNA was extended using avian myeloblastosis virus reverse transcriptase, then analyzed on a 9% polyacrylamide nondenaturing gel. Lanes 3 and 4 show the products of the extension reaction. Lanes 1 and 2 show that no extension occurred using a primer with the sequence 5'-ATCGAGGCGGTCGCCAGACC-3' which was designed with the 3'-end at the predicted start site. A sequencing ladder is shown on the left for size identification of the products. The site labeled 46 is the major product, with minor products indicated at 52 and 58 bp.

A comparison of the first 250 bp of the rat promoter sequence to both the human and murine mannose receptor promoter sequences is shown in Fig. 3. There is almost complete identity of murine and rat sequences over the first 150 bp, with a homology over the entire 250 bp of 87%. There is lower homology between the rodent sequences and the human, with very little agreement upstream of position −150. The homology of rat to human over the 250-bp sequence is 56%. The highest degree of homology among the three sequences occurs between −30 and −150, suggesting that important regulatory sites may be located within this region. Careful analysis of the first 250 bp of the rat sequence revealed a potential TATA box at −33, a core Sp1-binding site (CGGCCG) at position −91, and three potential PU.1-binding sites in the non-coding strand at −18 (GAGGAA), −106 (CAGGAG), and −163 (GAGGAA) which are conserved in the mouse and human sequences.

**Analysis of Rat Mannose Receptor Promoter Activity in Mannose Receptor-positive and Mannose Receptor-negative Macrophages and Non-macrophage Cells**—To determine if the 854-bp 5'-region of the rat mannose receptor gene directs cell-specific expression, promoter activity was determined in mannose receptor-positive macrophages, mannose receptor-negative macrophages, and mannose receptor-negative non-macrophage cell lines using a reporter gene assay. In Fig. 4A, NR8383 macrophages (open bars) and A549 epithelial cells (solid bars) were transfected with the pGL2-MR554, -MR656, -MR228, -MR108, and -MR48, and promoterless pGL2 basic vectors. PU.1 sites are represented by the closed ovals, and the Sp1 sites by the open ovals. Cells were co-transfected with pEL-CMV as an internal control for transfection efficiency. The 228-bp fragment was almost as active as the 854-bp promoter in NR8383 cells, suggesting that this region contains the necessary myeloid-specific sites as suggested by Clarke and Gordon (14) for myeloid-restricted gene expression. Interestingly, there was a slight decrease in activity with the MR656 construct. This supports the observation by Rouleux et al. (27) that the region from −250 to −750 may contain a repressor site that is overcome by
the additional 5' bases in MR854. In addition, Eichbaum et al. (28) reported that sequences of -200 bp in the murine promoter reduced activity. The MR108 construct showed approximately 50% of the activity of MR228. MR108 still contains two PU.1 sites and the Sp1 site, but maximal activity may require the upstream PU.1 site at -206.

![Figure 2](image1.png) Sequence of the 854-bp promoter region of the rat mannose receptor gene. The mannose receptor promoter region was isolated using PCR amplification of rat genomic DNA. The DNA was subcloned in pGEM-T and sequenced using vector-based primers. Consensus sites for PU.1 and Sp1, and a consensus E box motif are highlighted by the boxed areas. A potential TATA box is underlined.

![Figure 3](image2.png) Comparison of the proximal 250 bp of the rat, murine, and human mannose receptor promoters. Sequences of the mannose receptors promoters from rat, mouse, and human are shown with identical residues marked by the dots. PU.1, Sp1, and E box motifs in each sequence are in bold. The human sequence is from Rouleux et al. (27), and the murine sequence is from Eichbaum et al. (28).
The Mannose Receptor Promoter Activity Is Regulated by PU.1 and USF

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Fig. 4. Transient transfection assays of mannose receptor promoter-luciferase truncated constructs. The 854-bp mannose receptor promoter construct (MR854) was cloned into the pGL2 basic vector. Truncated fragments were produced from this construct by restriction digest and each was subcloned into the pGL2 vector. Cells were transfected with 5 μg of mannose receptor promoter-pGL2, and 1 μg of CMV-Renilla-luciferase to control for transfection efficiency. DNA was introduced by electroporation (NR8383, RAW, and A549 cells) or DEAE-dextran (HL60 cells) using the conditions described under “Experimental Procedures.” Cells were then plated in complete medium in 100-mm tissue culture dishes for 24 h prior to assaying luciferase activity. Panel A shows a comparison of the relative light units for firefly luciferase normalized to Renilla luciferase for each mannose receptor construct in both NR8383 macrophages and A549 epithelial cells. Data are the average of duplicate determinations and are representative of four separate experiments (MR854, MR656, and MR228) or two separate experiments (MR108 and MR48). Panel B shows the relative activity for the MR854 construct in NR8383 macrophages, RAW264 monocytes, and HL60 myeloid cells. Data are the average of triplicate determinations ± S.D. and are representative of three separate experiments.

Fig. 5. Transient transfection assays of mannose receptor promoter-luciferase truncated constructs containing the wild type, consensus, and mutated TATA box sequences. The 854-bp mannose receptor promoter construct (MR854) was cloned into the pGL2 basic vector. Mutations were introduced into this construct using a PCR-based method. Cells were transfected with 5 μg of mannose receptor promoter-pGL2 and 1 μg of CMV-Renilla-luciferase to control for transfection efficiency. DNA was introduced by electroporation using the conditions described under “Experimental Procedures.” Cells were then plated in complete medium in 100-mm tissue culture dishes for 24 h prior to assay of luciferase activities. Results are expressed as the relative light units for firefly luciferase normalized to Renilla luciferase. Data are the average ± S.D. of triplicate determinations, and are representative of two separate experiments. *, p < 0.05 for TTGGAA compared with TTTAAA.

TATA Box—Based on the position of the start site in the rat gene, a potential TATA box (TTTTAAA) was identified at −33 bp. Rouleux et al. (27) suggested that the sequence at −27 in the human sequence (−48 in the rat sequence) of TAAATT might be a potential TATA box, while Eichbaum et al. (28) reported that the murine promoter was TATA-less. To determine if the sequence at −33 bp in the rat promoter is a functional TATA box, we prepared mannose receptor promoter-luciferase constructs with the TT7AAA sequence mutated to TTGGAA or changed to a consensus TATA sequence (TATAAA). NR8383 cells were transiently transfected with the wild type MR854 or mutated constructs. As shown in Fig. 5, insertion of the TATAAA sequence increased activity by 25% compared with the activity of the wild type sequence (TTTTAAA). However, activity of the TTGGAA mutant was reduced by approximately 50%, suggesting that the proposed TATA box (TTTTAAA) in the rat mannose receptor promoter is functional but not essential. It has been suggested that a characteristic of myeloid-restricted promoters is the absence of a TATA box (14). However, neutrophil elastase (36) and proteinase-3 (26) promoters contain the sequence TATAA, and the CD14 promoter has a potential TATA box (CATATAA) −30 bp upstream of the start site (37). A recent study by Hoopes et al. (38) examined the binding of TATA-binding protein to a variety of potential TATA-box sequences. They classified the sequences CATATAA (CD14) and TT7AAA (mannose receptor) as intermediate TATA boxes compared with the strong TATAAA sequence. The observation that mutation of the TT7AAA sequence in the rat mannose receptor promoter reduced promoter activity by 50% (Fig. 5) suggests that this sequence may bind TATA-binding protein with an intermediate affinity to enhance transcription.

In addition, the demonstration that this site plays a role in promoter activity supports the major transcriptional start site identified by primer extension analyses. Since this TT7AAA sequence is conserved across rat, mouse, and human, the major start site in the mouse and human promoters may be the same as in the rat promoter, and the TT7AAA sequence may function as a TATA box in these promoters.

Characterization of Binding of Transcription Factors to the Rat Mannose Receptor Promoter by EMSA—Based on the above observations, we chose to examine more closely the region within the 228-bp fragment that contains conserved sequences...
known to bind transcription factors involved in myeloid-restricted expression of other genes (14, 15), and that contains sites conserved in the rat, human, and murine mannose receptor promoters. A double-stranded oligonucleotide was prepared spanning the region from −108 to −71 (108/71). This region contains a consensus PU.1 site (CAGGAA) on the non-coding strand (PU106) and a core Sp1 site (CCGCCC) on the coding strand (Sp91). To assess which sites in the rat mannose receptor promoter are potentially functional, we performed EMSAs using nuclear extracts from mannose receptor-positive NR8383 cells. In the experiments shown in Fig. 6, nuclear extract from NR8383 cells was incubated with labeled oligonucleotides and competitors as indicated. Labeled 108/71 oligonucleotide was bound by three major proteins from nuclear extracts as shown in lane 1. The top band was identified as Sp1 by competition with a consensus Sp1 oligonucleotide (lane 2), and by partial supershift using an Sp1 antibody (lane 3). The bottom band was identified as PU.1 by competition with a consensus PU.1 oligonucleotide (lane 4) and a supershift of the complex with anti-PU.1 antibody (lane 5). Competition with an oligonucleotide mutated at the Sp1 site (CCGCCG → CCGAGC) (S91M) reduced PU.1 but not Sp1 binding (lane 6), and competition with an oligonucleotide mutated at the PU.1 site (CAGGAA → CACCAA) (P106M) inhibited the binding of Sp1 but not PU.1 (lane 7). A labeled oligonucleotide mutated at the PU.1 site (P106M) did not bind PU.1 (lane 8) and a labeled Sp1 mutant (S91M) did not bind Sp1 (lane 9). These results demonstrate that both PU.1 and Sp1 bind to the rat mannose receptor promoter, and suggest that these two factors may play a role in the regulation of mannose receptor promoter activity.

A prominent band that migrated slightly faster than the Sp1 complex was seen in all gels (Fig. 6, lane 1). Several oligonucleotides were used as competitors in order to determine the sequence to which the unidentified factor was binding. First, two truncated oligonucleotides were made spanning the regions from −108 to −82 (108/82) and −96 to −71 (96/71). As shown in Fig. 7, competition with the 96/71 oligonucleotide completely inhibited binding of this factor to labeled 108/71

**Fig. 6. Electrophoretic mobility shift assays.** Nuclear extracts were prepared from NR8383 cells as described under “Experimental Procedures,” and incubated with end-labeled double-stranded probes as indicated in Panel B. Probe 108/71 spans the −108 to −71 region of the rat mannose receptor promoter; probe 106M has a mutated PU.1 site (CC residues at −103 and −102 mutated to GG); probe 91M has a mutated Sp1 site (CC residues at −87 and −86 changed to AG). Single-stranded oligonucleotide probes were annealed and end-labeled. The labeled probes (2 × 10^5 cpm) were then incubated with single-stranded nonspecific DNA (200 ng), and nuclear extract (10 μg) in a total volume of 15 μl in Tris buffer with EDTA, dithiothreitol, and poly(dI-dC) for 20 min at room temperature. Samples were electrophoresed in a 5% polyacrylamide nondenaturing gel. The gels were dried and bands were visualized by autoradiography. Sequences of competitor oligonucleotides are listed in Table I. Panel A, positions of Sp1 and PU.1 are indicated. The arrow marks the position of the supershifted antibody-protein-DNA complex. The asterisk marks the position of a prominent band seen in all gel shift using the labeled 108/71 probe. Lanes 1–7 are reactions containing labeled 108/71 probe plus nuclear extract with the following additions: lane 1, buffer; lane 2, excess unlabeled Sp1 consensus oligonucleotide (Table I); lane 3, anti-Sp1 antibody; lane 4, excess unlabeled PU.1 consensus oligonucleotide (Table I); lane 5, anti-PU.1 antibody; lane 6, excess unlabeled S91M oligonucleotide; lane 7, excess unlabeled P106M oligonucleotide. Lanes 8 and 9 are nuclear extract plus labeled P106M oligonucleotide or S91M oligonucleotide, respectively.

**Fig. 7. Electrophoretic mobility shift analysis for identification of USF binding.** Gel shift assays were run as described in the legend to Fig. 6. The labeled probe used for all lanes was 108/71. The positions of the Sp1, PU.1, and USF bands are noted. The arrows mark the position of the anti-USF-USF-DNA complexes. Oligonucleotides used as competitors in lanes 2–5 are shown in Table I and Panel B. Each reaction contained the labeled 108/71 oligonucleotide plus nuclear extract with the following additions: lane 1, buffer; lane 2, excess unlabeled probe spanning −96 to 71; lane 3, excess unlabeled probe spanning −108 to −82; lane 4, excess unlabeled E74M oligonucleotide; lane 5, excess unlabeled E box consensus oligonucleotide (U83; Table 1). Lanes 6 and 7 contain antibodies against USF1 (lane 6) and USF2 (lane 7).
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Fig. 8. Transient transfection assays to determine the contribution of the PU.1, Sp1, and USF sites to mannose receptor promoter activity. NR8383 cells were transfected with full-length 854 mannose receptor-luciferase constructs in pGL2, or with constructs containing site mutations in the PU.1-106 site (second bar), the Sp1-91 site (third bar), the PU.1-106 and Sp1-91 sites (fourth bar), the PU.1 site at −163 (fifth bar), or the USF site at −83 (sixth bar). Relative light units were normalized to the activity of the co-transfected CMV-Renilla-luciferase construct. DNA was introduced as described in the legend to Fig. 4 and under “Experimental Procedures.” Results are expressed as the percent of activity of the MR854 construct as 100% and are representative of four separate experiments. Error bars are the S.D. of triplicate or quadruplicate determinations. * p < 0.05 for the second, fourth, fifth, and sixth bars compared with control (first bar).

(lane 2), while competition using the 108/82 fragment did not compete with the unknown factor (lane 3), narrowing the region for binding to −82 to −71. A potential ets site exists in this sequence between −74 and −71 (GGAT) (39). The addition of a cold 108/71 oligonucleotide with 75GG74 mutated to 75CC74 was still capable of competing for the unknown factor (lane 4), thus ruling out the possibility that this protein was binding the potential ets site. Based on the above competitions we determined that the third factor was binding somewhere in the sequence CATGTGACA (−83 to −74). This stretch contains a sequence (CATGTG) that fits the core ets hexamer sequence (50). This protein retains the helix loop helix and leucine zipper domains.

Species-specific Differences between the Rat, Mouse, and Human Mannose Receptor Promoters—Comparison of the results of the gel shift assays and transfection experiments presented in this study to the results from the murine mannose receptor promoter study reported by Eichbaum et al. (28) reveals some significant differences. First, activity of the murine mannose receptor promoter was found to involve two PU.1 sites at −164 and −177 (−150 and −163 in the rat sequence). The murine PU164 site bound PU.1 in gel shifts, and mutation of the core sequence reduced activity in transfection assays by 50%. This binding site is not conserved in either the rat or human sequences, suggesting that this site may not be critical for mannose receptor promoter activity. No binding of PU.1 to the P177 site (−163 in the rat) was reported in the murine study, but mutation of the core sequence reduced promoter activity to basal levels. Mutation of this site in the rat promoter reduced activity by only 25%. This site is also conserved in the human mannose receptor sequence, but the role of this site in the

Fig. 9. Effect of a dominant negative USF on MR854 promoter-reporter expression. NR8383 cells were transfected by electroporation as described in the legend to Fig. 4 and under “Experimental Procedures” with 5 μg of mannose receptor promoter-pGL2, 1 μg of CMV-Renilla-luciferase, and 4 μg of pGL2 basic vector (open bar), or 5 μg of mannose receptor promoter-pGL2, 1 μg of CMV-Renilla-luciferase, and 4 μg of the ΔDTDU vector (hatched bar). Activity is expressed in relative light units (RLU) for firefly luciferase normalized to Renilla luciferase. Data are the average of triplicate determinations ± S.D., and are representative of two separate experiments. p = 0.0002 for comparison of MR854 (open bar) to MR854+ΔDTDU1 (hatched bar).

Approximately 80% reduction in mannose receptor promoter activity. Mutation of the Sp1 site had only a slight effect on promoter activity, while mutation in the PU163 site reduced mannose receptor activity by approximately 25%. Since Sp1 had been found to function in activation of the mouse promoter, we examined the possibility that the Sp1 and PU.1 sites in the rat promoter acted cooperatively. However, mutation at both the Sp1 site and the PU106 site showed no additional decrease in promoter activity compared with the single PU106 mutation. These data suggest that the PU.1 site at −106 and the E box at −83 are critical for rat mannose receptor promoter activity.

To further establish the role of USF in regulating mannose receptor promoter activity we used a dominant negative USF1 expression construct (ΔDTDU1) in transient transfections of NR8383 cells. The ΔDTDU1 is an expression vector encoding a USF1 protein lacking the basic region which is essential for DNA binding (50). This protein retains the helix loop helix and leucine zipper domains and can still dimerize with both USF1 and USF2 thus serving as a dominant negative of both factors. As shown in Fig. 9, MR854 promoter activity is reduced by 50% when co-transfected with the ΔDTDU1 construct. This does not seem to be a general effect since the activity of the internal control (pRL-CMV) was nearly equal in the presence or absence of the dominant negative (data not shown).
Mannose Receptor Promoter Activity Is Regulated by PU.1 and USF

The process of maturation of macrophages is controlled by a complex array of factors that are turned on sequentially as cells move through the maturation process to fully differentiated macrophages. The mannose receptor is expressed only on the end product of this differentiation cascade, the mature tissue macrophage, and thus is an excellent model gene to study the factors that control the transition from monocyte to mature macrophage. In the present study we report the isolation and characterization of the promoter region of the rat mannose receptor gene, and describe factors that are involved in regulating expression of this gene in mannose receptor-positive macrophages.

The rat mannose receptor 5′-flanking region contains multiple consensus sequences for the myeloid-specific PU.1 transcription factor. In addition, a potential binding site for the ubiquitous factor Sp1 is located just 3′ of PU.1–106. The presence of a second site bound by a ubiquitous factor is characteristic of myeloid-restricted expression. We examined the role of these sites in mannose receptor promoter activity by transient transfection assays using mannose receptor promoter-luciferase constructs and by gel shift assays. We focused our studies on the region from −108 to −71 that contains binding sites for PU.1 and Sp1. The PU.1 site is conserved in the rat, murine, and human mannose receptor promoters, while the Sp1 site is completely conserved in both rat and mouse sequences (27, 28). In addition, these sites are located within the first 108 bp of the mannose receptor promoter and are in close proximity to each other. All of these features have been suggested to be important for control of myeloid-restricted expression (14).

In gel shift assays using the labeled 108/71 oligonucleotide, we observed both Sp1 and PU.1 binding (Fig. 6, lane 1). These factors were competed by excess unlabeled consensus oligonucleotides and the protein-DNA complexes were supershifted with the appropriate antibodies. PU.1 binding was not seen in extracts from non-myeloid cells such as A549, Rat II, or HeLa cells (data not shown), while Sp1 binding was observed in all cells examined, supporting the reported expression patterns of these factors (51, 52).

PU.1 is a member of the ets family of transcription factors (16). PU.1 binding has been reported to be required for activity of a number of other myeloid promoters including the promoters for growth factor receptors (18, 19), integrins (20, 21), Fc receptors (22, 23), the scavenger receptor (24), myeloperoxidase (25), and human proteinase-3 (26). The rat mannose receptor promoter contains three PU.1 sites within the first 163 bases 5′ of the transcriptional start site. We examined the function of two of these sites by transfection assays using site-directed and truncated mutants of the MR854 construct. Mutation of the PU.1–106 site reduced promoter activity by approximately 80% of MR854, while mutating the PU.1 site at −163 resulted in a 25% decrease. Deletion of the 5′ 622 bp resulting in MR228 had little effect on promoter activity compared with MR854. This region contains the three PU.1 sites, and appears to contain all of the required sequences for maximal activity of the mannose receptor promoter. Removal of an additional 124 5′ bp (MR108) which includes one PU.1 site reduced activity by approximately 50%. Removal of the region containing the PU.1–106 site (MR48) further reduced activity to 25% of MR854. The MR48 construct retained myeloid activity and showed no activity in non-myeloid cells. These results suggest that all three PU.1 sites contribute to the control of myeloid-specific transcription driven by the mannose receptor promoter.

One characteristic feature of most myeloid promoters studied to date is the cooperation between the PU.1 site and another factor bound to a nearby site (14). Sp1 has been found to function as this second factor in a number of myeloid-specific promoters including the tyrosine kinase tec (53), CD11b (54), and c-fes (55). Sp1 was shown to be important in the mouse mannose receptor promoter, although the distance between the identified functional PU.1 site (at −163 in the rat sequence numbering) and the Sp1 site is significantly greater than is
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typical for myeloid promoters. It is possible that in the murine promoter the –106 PU.1 site is active in regulating promoter activity and cooperates with Sp1 binding at –91 to produce maximal activity, although this has not been tested. We examined the role of Sp1 binding in the regulation of rat mannose receptor promoter activity by transfection of MR854 with a mutation in the Sp1 binding sequence. Surprisingly, mutations within this site showed only a slight decrease (10%) in mannose receptor promoter activity and a mutation in the Sp1 site did not further decrease the reduced activity of the PU.1–106 mutant, suggesting that Sp1 does not play a role in the regulation of rat mannose receptor promoter activity. It is possible that this difference between the rat and murine studies is due to the longer promoter used for our studies or the use of mannose receptor-positive macrophages. However, another possible explanation is that an additional factor is functional in the rat mannose receptor promoter as discussed below.

Binding of a third protein to the 108/71 oligonucleotide was detected in our gel shift analyses. Through competition using portions of the 108/71 oligonucleotide, protein binding was localized to the E box (CATGTTG) between positions –84 to –79. Mutation of the 5′-TG–79 to CC resulted in an oligonucleotide that did not bind this factor (data not shown), suggesting that these two bases are required for binding. The identity of this factor as the upstream stimulatory factor (USF) was confirmed by use of specific anti-USF1 and anti-USF2 antibodies to supershift the USF-DNA complex. In transient transfection assays, mutation of the TG residues resulted in loss of approximately 80% of promoter activity, and expression of a dominant negative USF decreased mannose receptor promoter activity by 50%, suggesting that USF binding is required for a functional rat mannose receptor promoter.

USF belongs to the family of basic helix loop helix transcription factors (40). Although these factors are ubiquitously expressed, an increasing number of studies have appeared in the past several years demonstrating a role for USF in the regulation of tissue-specific genes. USF binding has been shown to be required for human immunodeficiency virus type-1 enhancer activity in T cells (56) and for regulation of expression of the surfactant protein-A gene (57). Involvement of USF in myeloid cell differentiation was suggested by Kreider et al. (58) and Suzow and Friedman (25) reported that deletion of the E box in the myeloperoxidase gene reduced promoter activity by 5-fold. It is not yet known how USF might contribute to myeloid-specific control. In a recent study Feinman et al. (23) reported that USF bound to an E box region in the FcγRIII promoter and that binding of both USF and PU.1 were required for promoter activity. The PU.1 and USF proteins interacted to form a high affinity DNA-binding complex that appeared to be responsible for the myeloid specific activity. These authors further speculated that myeloid specificity resulted from post-translational modifications of either or both factors. This is supported by the finding that phosphorylation of Sp1 and PU.1 are increased as myeloid cells differentiate (52, 59). A second mechanism suggested by Sieweke et al. (56) suggests that eis-1 and USF-1 interact to form a complex, resulting in exposure of the respective DNA-binding domains of each factor. According to both of these models a ternary complex would exist that should be detectable by EMSA. We did not detect such a complex in our assays, although the possibility remains that under the specific conditions of our assay this complex was not detectable. A third model recently proposed by Rigaud et al. (60) might explain the mutually exclusive binding that we observed in gel shift assays with the rat mannose receptor promoter probe. In this model the tissue-specific factor PU.1 binds transiently to the promoter and induces a conformational change in the chromatin which in turn exposes the binding element of a ubiquitous factor, in this case USF.

Studies of myeloid promoters, including the mannose receptor promoter, have shown that a variety of ubiquitous factors such as Sp1 and USF interact with the myeloid-specific factor PU.1 to control transcription, suggesting that these ubiquitous factors share a common function. In support of this hypothesis, Pugh and Tjian (61) have proposed that Sp1 is involved in the assembly of the basal initiation factors, particularly through interactions with TFIIID. The role of Sp1 in pre-initiation complex formation has been demonstrated on both TATA-containing and TATA-less promoters (62). Similar experiments have shown that USF can function in the same manner, through interactions with TFIIID (63). This interaction appears to mediate the binding of TFIIID to the promoter, even in the absence of a functional TATA box. A comparison of binding elements in the rat, human, and murine mannose receptor promoter sequences shows complete conservation of the core Sp1 site in mouse and rat while the USF site is present only in the rat and human sequence. Furthermore, the Sp1 and USF sites are nearly overlapping suggesting that the function of these factors is position-dependent. The fact that the ubiquitous factor Sp1 is critical for mouse mannose receptor promoter activity while USF is required for rat promoter activity suggests that these two factors may be performing the same function in the two different species: namely, recruitment and stabilization of the pre-initiation complex.

In the present study we have demonstrated that maximal tissue specific activity is contained within the proximal 228 base pairs of the rat mannose receptor promoter. This region contains three PU.1 sites and a functional USF site. We propose that all three PU.1 sites are required for maximal expression. Mutation of PU.1–106 has the most marked effect, decreasing activity by approximately 80%. Mutation of the E box, which binds the ubiquitous factor USF, also decreases activity by approximately 80% while the expression of a dominant negative USF decreases activity by 50%. This finding is surprising in light of a recent study on the murine mannose receptor promoter which demonstrated a requirement for the ubiquitous factor Sp1. We demonstrate by EMSA that protein binding to the promoters of the rat, mouse, and human mannose receptor promoters are indeed different, with the most obvious difference being the absence of USF binding to the mouse promoter. We speculate that USF and Sp1 provide an essential and overlapping function that is required for activation from these promoters. Further studies will be required to determine the role that these transcription factors play in the different species.

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