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Structure and Function Analysis of the Human Myeloid Cell Nuclear Differentiation Antigen Promoter: Evidence for the Role of Sp1 and Not of c-Myb or PU.1 in Myelomonocytic Lineage-Specific Expression

Woei-Yau Kao, Judith A. Briggs, Marsha C. Kinney, Roy A. Jensen, and Robert C. Briggs*

Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tennessee

Abstract The human myeloid nuclear differentiation antigen (MNDA) is expressed specifically in maturing cells of the myelomonocytic lineage and in monocytes and granulocytes. Epitope enhancement was used to confirm the strict lineage- and stage-specific expression of MNDA in bone marrow as well as in other paraffin-embedded fixed tissues. A 1-kb region of the gene that includes 5′ flanking sequence was reported earlier to contain functional promoter activity and was specifically demethylated in expressing cells in contrast to null cells. Further analysis has revealed that this 1-kb fragment promotes higher reporter gene activity in MNDA-expressing cells than non-expressing cells, indicating cell-specific differences in transactivation. This sequence contains consensus elements consistent with myeloid-specific gene expression, including a PU.1 consensus site near the major transcription start site and a cluster of c-Myb sites located several hundred bases upstream of this region. However, analysis of deletion mutants localized nearly all of the promoter activity to a short region (−73 to −16) that did not include the cluster of c-Myb sites. A 4-bp mutation of the core Sp1 consensus element (GC box) (−20) reduced overall promoter activity of the 1-kb fragment. Mutation of the PU.1 site did not significantly affect promoter activity. Only a small region (−35 to +22) including the Sp1 element and transcription start site, but not the PU.1 site was footprinted. The 4-bp mutation of the core Sp1 consensus element abolished footprinting at the site and an antibody super-shift reaction showed that Sp1 is one of the factors binding the consensus site. The Sp1 site also co-localizes with a DNase I hypersensitive site. The results indicate that DNA methylation, chromatin structure, and transactivation at an Sp1 site contribute to the highly restricted expression of this myelomonocytic lineage specific gene. J. Cell. Biochem. 65:231–244.

Key words: monocytes; granulocytes; cell maturation; promoter; transcription

Hematopoiesis is a process in which stem cells give rise to morphologically and functionally distinct lineages. An essential feature of the process is the transcriptional activation of lineage-specific genes. A number of well characterized genes are expressed specifically in the myelomonocytic lineage [Lubbert et al., 1991] and the molecular basis for their restricted transcription has been examined in some cases [Feinman et al., 1994; Grove and Plumb, 1993; Pahl et al., 1993; Rosmarin et al., 1995; Shelley et al., 1993; Zhang et al., 1994a]. Both the c-Myb and PU.1 transcription factors have been implicated in regulating myeloid-specific transcription [Ahne and Stratling, 1994; Feinman et al., 1994; Grove and Plumb, 1993; Melotti et al., 1994; Ness et al., 1993; Nicolaides et al., 1991; Pahl et al., 1993; Rosmarin et al., 1995; Shelley et al., 1993; Zhang et al., 1994a]. Disruption of *c-myb* and PU.1 genes produced defects in the development of the myelomonocytic lineage, lending additional support for their importance in myelomonocytic lineage specific transcription [Mucenski et al., 1991; Scott et al., 1994]. However, expression of c-Myb or PU.1 is not restricted to the myeloid lineage [Alitalo et al., 1984; Griffin and Baylin, 1985; Thiele et al., 1988]. It appears that other regulatory factors/mechanisms act in conjunction with c-Myb and PU.1 to accomplish myelomonocytic-specific transcription.

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*Correspondence to: Dr. Robert C. Briggs, Department of Pathology, TVC 4918 Vanderbilt University School of Medicine, Nashville, TN 37232-5310.

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The myeloid cell nuclear differentiation antigen (MNDA) is expressed exclusively in cells of the human myelomonocytic lineage. Expression of MNDA in cases of myeloid leukemia was reported earlier [Cousar and Briggs, 1990]. The appearance of MNDA within this lineage coincides with cessation of cell proliferation and the final stages of maturation. MNDA appears to function through interactions with a number of other nuclear proteins [Xie et al., 1995]. MNDA is encoded by a single-copy gene located on chromosome 1q21-22 belonging to a family of interferon-regulated genes [Briggs et al., 1994a]. To delineate the mechanisms responsible for the tight regulation of this gene, an extensive study of the 5′ flanking sequence isolated from a genomic clone [Briggs et al., 1994a] was initiated. This sequence contains functional promoter activity and several regulatory elements consistent with myelomonocytic lineage-specific gene transcription [Kao et al., 1996]. In addition, the DNA is demethylated in expressing cells [Kao et al., 1996]. The present investigation demonstrates that the level of promoter activity is higher in expressing cells than in non-expressing cells suggesting altered status or availability of key transactivating factors. Analysis of deletion mutations identified a short segment of sequence responsible for nearly all of the promoter activity associated with the 1-kb fragment. Site-directed mutagenesis and DNase I footprinting provided strong evidence for the importance of Sp1, but not PU.1, in regulating MNDA transcription. Furthermore, Sp1 was identified as one of the proteins binding to the Sp1 consensus site. The results do not support involvement of a cluster of c-Myb binding sites located approximately 600 bp upstream of the major start site in the regulation of constitutive MNDA transcription.

METHODS

Immunohistochemistry

Normal human bone marrow, tonsil (B5 fixed tissues) and a ruptured epidermal inclusion cyst (formalin fixed) were embedded in paraffin and sectioned at 5 μm. Sections were subjected to microwave epitope enhancement [Shi et al., 1991] in Citra Solution (BioGenex, San Ramon, CA) followed by methanol-H_2O_2 quenching of endogenous peroxidase activity. MNDA was detected using rabbit polyclonal antiserum against recombinant MNDA [Xie et al., 1995] diluted 1:1,000. Pre-immune serum (1:1000) was used as a negative control. A peroxidase based avidin-biotin detection system was used in an automated slide stainer (Ventana, Medical Systems, Inc., Tucson, AZ).

Cell Lines and Culture

The MNDA positive, U937 and HL-60, and negative, KG-la and K562, leukemic cell lines (American Type Culture Collection, Rockville, MD) were maintained in RPMI-1640 medium supplemented with 15% (U937, KG-la, and K562) or 20% (HL-60) fetal bovine serum (Gibco BRL, Grand Island, NY), and antibiotics (penicillin, 50 U/ml and streptomycin, 50 μg/ml). MNDA negative HeLa cells, obtained from Dr. Wallace LeStourgeon (Vanderbilt University, Nashville, TN), were grown in DMEM medium supplemented with 10% defined bovine calf serum (Hyclone Laboratories, Logan, UT) and 2 mM glutamine with antibiotics as described above.

MNDA Promoter/Luciferase Reporter Gene Construct and Deletion Mutations

pGL2-MNDA, a pGL2 Basic (Promega, Madison, WI) construct containing 856 bp of MNDA 5′ flanking sequence and 161 bp of untranslated exon 1 sequence inserted upstream of the luciferase reporter gene was described in detail previously [Kao et al., 1996]. Truncations with the prefix "tpGL2-" were constructed from pGL2-MNDA by removing a series of segments upstream of native restriction sites including *Spe* I (−527), *Age* I (−226), and *Pst* I (+141) or engineered sites (inserted as described in the next section), namely, *Eco*R V (−73) and *Xba* I (−16). An internal deletion mutation, prefixed by ΔpGL2-, was constructed by removing the segment between restriction sites *Xba* I (−16) and *Pst* I (+141).

PCR-Based Site-Directed Mutagenesis

Four base pair mutations of the PU.1 and Sp1 binding sites and a site midway (−9 to −4) between the Sp1 binding element and the major transcription start site were generated by the oligo-directed two-step PCR mutagenesis [Zhao et al., 1993]. In the first-step PCR reaction, products carrying the mutation were produced from a linearized pGL2-MNDA DNA template primed by a forward oligo based on upstream non-mutated sequence, 5′-AAGATCCTTATTCAGCGGCTACCGG-3′ (−247 to −222) combined with reverse mutated primers in which a 4-bp segment was replaced with sequence resulting in an engineered restriction site. Specifically, these primers included a mu-

tated PU.1 oligo generating an *Eco* RV site (PU.1-EcoR V mut), 5′-AGAAAGTGGTCAGGATATCTTTGTAGTAAGTAATC-3′; a mutated Sp1 oligo generating an *Xba* I site (Sp1-XbaI mut), 5′-GAAACAATTTTGAAGTCTAGACTATCAGCCTTG-3′, and a third non-specific mutation generating an *Xba* I site a short distance downstream of the Sp1 site (NS-XbaI mut), 5′-GTTATGTAATCATGAATCTAGATTGAAGCCACGC-3′. Prior to the addition of 3 U Taq enzyme, 60 µl PCR reactions containing 4 fmol of linearized (*Bam*H I) pGL2-MNDA, 1 µM each forward and reverse primer, 200 µM each dNTP in Taq buffer containing 1.5 mM $MgCl_2$ (Perkin Elmer, Foster City, CA) were denatured at 95°C for 5 min. The mixture was subjected to 25 cycles of 95°C for 1 min, 65°C for 2.5 min, and 72°C for 1 min followed by extension at 72°C for 10 min. The first-step PCR reaction mixture was then diluted 1:40 with additional dNTPs and enzyme and recycled 7 times which allowed the first product strands to act as primers for extension. To generate sufficient amounts of fragment that would permit cleavage downstream of the mutated site, 1 µM each of the original upstream primer and a second reverse primer complementary to 5′ luciferase gene sequence [(luc-rev), 5′-CGCCGGGCCTTTCTTTATGTTTTTGGCGTCTTC-3′] was added and the mixture subjected to 15 additional rounds of cycling with reduced annealing temperature (50°C). Products of expected size were purified and restricted with *Age* I (−226) and *Hind* III (+160). These fragments were then isolated, purified, and cloned into the *Age* I/*Hind* III sites of pGL2-MNDA. Cloned plasmids carrying the mutation were selected by successful cleavage at the engineered site and further documented by sequencing. Site-mutated clones are designated by the prefix, mpGL2-. The mpGL2-PU.1/Sp2 double mutation was generated from the mutated mpGL2-PU.1 template and the Sp1-XbaI mut primer in the initial PCR step.

Transient Transfection Assays

All plasmid constructs were purified by anion exchange chromatography (Plasmid DNA Kit, Qiagen Inc., Chatsworth, CA). To assess the activity of MNDA promoter in MNDA-expressing and non-expressing cells, 40 µg of pGL2-MNDA or control pGL2-Basic plasmid was co-transfected with 20 µg of pSV-β-Gal plasmid into U937 or HeLa cells by electroporation using a Gene Pulser™ (Bio Rad, Hercules, CA) at 300 V for U937 and K562 and 150 V for HeLa at 960 µF as recommended by Pahl et al. [1991]. During electroporation, the cells were suspended in serum-free growth medium at a concentration of 2×10^7/0.5 ml for U937 and K562 and 1×10^6/0.5 ml for HeLa. After electroporation, the cells were incubated in 10 ml complete medium for 7 h prior to luciferase and β-galactosidase assay. Results were normalized for transfection efficiency based on β-galactosidase activity and the relative luciferase activity was reported as fold increase over the promoterless pGL2-Basic activity. The data represent the mean ± SD for three experiments each containing triplicate samples. The level of promoter activity of specific regions of the MNDA gene was determined in the same manner using the mutated plasmids transfected into U937 cells.

DNase I Digestions

To detect DNase I hypersensitive sites in intact chromatin, freshly prepared nuclei were digested with the enzyme for various times. Nuclei were prepared from rapidly growing cells. After washing with PBS containing 3 mM $MgCl_2$, the cells were lysed (10^7 cells/ml) in 10 mM NaCl, 10 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$, and 0.5% Nonidet-P40 at 4°C. Nuclei were pelleted and resuspended in the same buffer without detergent at a concentration of 10^7 nuclei/ml. RNase-free DNase I, at levels given in the figures, was added and the suspension mixed gently at 25°C. One-milliter samples were withdrawn at various time points and immediately injected into 5 ml of lysis buffer [800 mM guanidine HCl, 30 mM EDTA, 30 mM Tris (pH 8.0), 5% Tween-20, and 0.5% Triton X-100]. Genomic DNA was isolated by anion exchange chromatography (Genomic DNA Purification Kit, Qiagen) resulting in an average of 60 µg DNA/10^7 nuclei. Preparation of control non-digested genomic DNA, conditions for restriction enzyme digestion, sample preparation, and Southern blot analysis were essentially as described previously [Briggs et al., 1994a]. Two non-overlapping DNA fragments, as illustrated in Figure 2, were used to prepare ^{32}P-labeled probes by random hexamer priming. A specific activity of 1.5×10^6 cpm/ml was used in hybridizations.

Isolation of Nuclear Extracts

Nuclear protein extracts were prepared using the Dignam method [Dignam et al., 1983]

with the exception that hematopoietic cells were lysed using the method of Hallick and Namba [1974].

DNase I Footprinting Assay

The DNA fragment flanked by *Age* I/*Hind* III (−226 to +160) (both normal and mutant) were used for DNase I footprinting analysis. The constructs tpGL2-Age I and mpGL2-Sp1 were linearized with *Hind* III and end-labeled by Klenow fill-in using both [α-^{32}P]-dATP and -dCTP [Ausubel et al., 1994]. After labeling, the DNA was digested at a 5′ site (vector site, *Kpn* I in the case of tpGL2-Age I and *Age* I for mpGL2-Sp1) and the resulting labeled fragments were gel-purified. DNA (2×10^4 cpm) was mixed with 80 µg U937 nuclear extract in a total volume of 50 µl containing 10 mM Hepes (pH 7.8), 30 mM KCl, 12% glycerol, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 0.2 mM PMSF, and 4 µg poly (dI.dC) and then incubated on ice for 30 min prior to digestion with 0.15 units of DNase I for 1 min at room temperature. The reaction was terminated by adding 50 µl of stop solution [0.2 M Tris (pH 7.5), 25 mM EDTA, 0.3 M NaCl, 2% SDS] and then digested with 20 µg of proteinase K at 50°C for 45 min. DNA was purified by phenol/chloroform extraction and ethanol precipitated. The products were loaded on a 6% denaturing polyacrylamide gel next to an internal control in which both nuclear extract and DNase I were omitted. G and A sequence generated from the same DNA by the Maxam-Gilbert chemical method [Maxam and Gilbert, 1980] was used to identify protected sites.

EMSA

A 26-bp sequence (−34 to −9) containing the consensus Sp1 element with internal GC box, 5′-CAAAGGCTGATAGGCGTGGCTTCAAA-3′ was synthesized (complementary oligonucleotides) and used as the probe for electrophoretic mobility shift assay (EMSA). Sense and antisense oligonucleotides were annealed as described [Hornstra and Yang, 1993; Mueller and Wold, 1989], polished with T4 DNA polymerase, and gel-purified. The annealed oligo, termed M-Sp1, was ^{32}P-end-labeled by T4 DNA kinase and purified using Sephadex G-25. The 20-µl binding reaction included 4 µg nuclear extract, 2 µg poly (dI.dC), 10 mM Hepes (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 4% Ficoll (w/v), and 1 µl of labeled probe (1.0 to 1.5×10^5 cpm). The reactions were incubated for 30 min on ice. In competition assays, cold M-Sp1 or non-related MNDA sequence (−92 to −58) was added at 50–100-fold molar excess and incubated for 10 min before adding the labeled probe. Super-shift assays included 3 µl of specific antiserum against Sp1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or antiserum against MNDA added just prior to addition of the probe. Reactions were electrophoresed at 170 V on a 6% acrylamide, 0.5XTBE gel for 2.5 h at 4°C. Gels were dried and exposed to Kodak XAR film.

RESULTS

Immunohistochemical Localization of MNDA Positive Cells

Immunoperoxidase staining of MNDA positive cells in human bone marrow, tonsil, and skin showed nuclear marking consistent with expression in cells of the myelomonocytic lineage including macrophages and granulocytes found outside of the bone marrow (Fig. 1). No signal was observed when these fixed tissues were processed without microwave epitope enhancement (data not shown) or when reacted with pre-immune serum (Fig. 1b).

The 1-kb 5′ Flanking Sequence Functions as a Stronger Promoter in MNDA-Expressing Cells

The sequence of the 5′ end of the MNDA genomic clone was previously reported [Kao et al., 1996] and a schematic representation of the 1.0-kb promoter region is provided in Figure 2. To assess whether the 1.0-kb promoter region contains sequences that direct the lineage-specific expression of the MNDA gene, the promoter activity was compared in MNDA-expressing and non-expressing cells using a reporter gene assay. The pGL2-MNDA and pGL2-Basic plasmids were electroporated into MNDA expressing U937 cells and non-expressing K562 and HeLa cells. The promoter is more active in MNDA-expressing cells (Fig. 3) indicating that regulatory elements present contribute to the lineage-specific expression of the MNDA gene.

Functional Assessment of the 5′ Flanking MNDA Regulatory (Promoter) Region

To determine the contribution of specific regions of sequence to the functional promoter activity observed, three 5′ truncated sequences and one internal deletion mutation were generated. A comparison of reporter gene activity

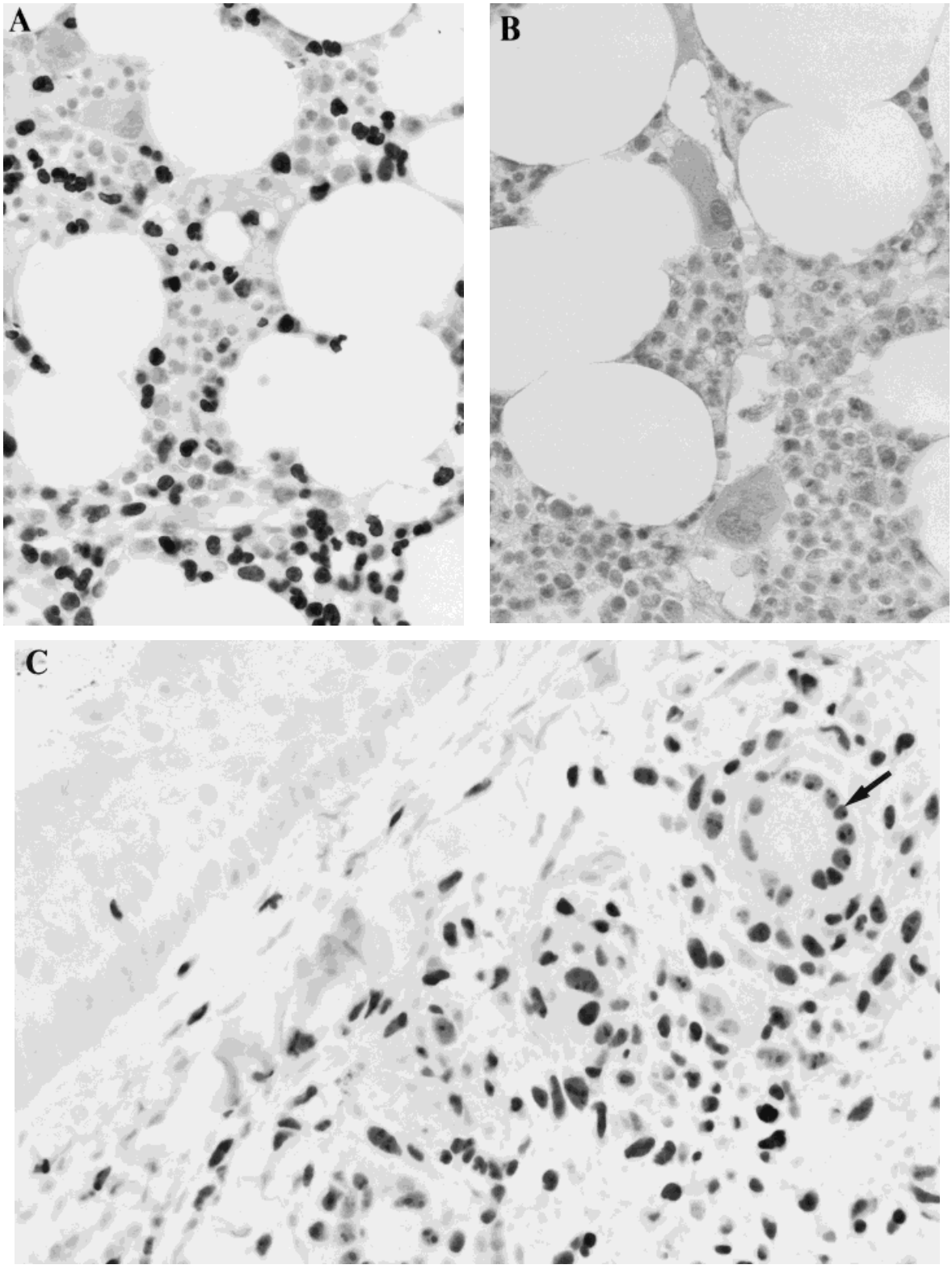


Fig. 1. Immunohistochemical detection of MNDA in fixed tissue sections. **A:** Normal human bone marrow reacted with antiserum against rMNDA. Strong nuclear staining in mature granulocytes and less intense reactions in mononuclear cells. The number of positive cells and the morphology of the reactive nuclei and cells are consistent with MNDA expression in cells of the myelomonocytic lineage. Magnification, ×400. **B:** Normal human bone marrow reacted with pre-immune serum. Magnification, ×400. **C:** A region of ruptured epidermal inclusion cyst containing a large number of MNDA positive macrophages including giant cells (*arrow*). However, most cell nuclei in the section are non-reactive (*top left*). ×400. Reactive tonsil stained with antiserum against rMNDA showed scattered mononuclear cells, probably histiocytic, in the parafollicular region with light staining nuclei (data not shown).

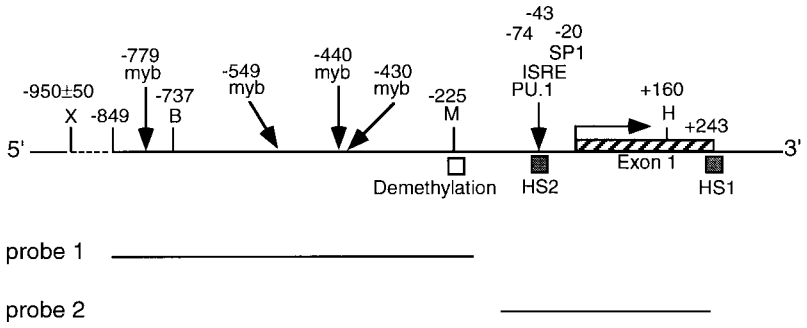


Fig. 2. Schematic representation of the promoter region of the MNDA gene. The positions of cis-elements, c-myb, PU.1, ISRE and Sp1, and unique restriction sites (*A, Age* I; *B,* B *Bsp* 1286 I; *M, Msp* I; *S, Spe* I; *P, Pst* I; *H, Hind* III) are designated. *Shaded rectangles:* The locations of DNase I hypersensitive sites HS1 and HS2.

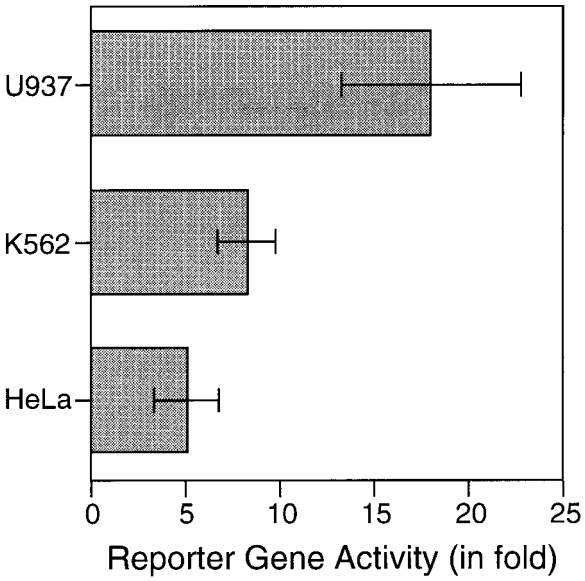


Fig. 3. The cloned 1.0-kb promoter is more active in MNDA-expressing cells. The pGL2-MNDA and pGL2-Basic were transfected separately into U937 (MNDA-expressing), K562 (non-expressing), and HeLa (non-expressing) cells as described in Methods. Forty micrograms of pGL2-MNDA or control pGL2-Basic supercoiled plasmid were cotransfected with 20 µg of pSV-b-Gal plasmid into U937 cells. Relative luciferase activity is presented as folds increased over pGL2-Basic for each cell line after correction for transfection efficiency. The means ± SD of three independent experiments (triplicate samples in each) are plotted.

relative to the intial 1-kb fragment in MNDA-expressing cells, U937, is shown in Figure 4. Truncation of the 5′ flanking sequence through the Sp1 site (tpGL2-Xba I) dramatically decreased promoter activity. The results locate the region between −73 through −16 as important for MNDA promoter activity. The internal deletion mutant (ΔpGL2-XbaI/Pst I) further confirms that a region between −16 nt (*Xba* I) and +141 nt (*Pst* I) is responsible for most of the observed 1-kb promoter activity.

Identification of DNase I Hypersensitive Sites

Further evidence that the region of the MNDA promoter containing the Sp1 consensus element is involved in the expression of *MNDA* gene in vivo was obtained through DNase I digestion of the promoter in nuclei from MNDA-expressing and non-expressing cells. As shown in Figure 5, DNase I digestion of nuclei from K562 cells released a 1.2-kb and a 0.9-kb subfragment detected with probe 1 (Fig. 2). The same sized fragments were released from nuclei isolated from MNDA expressing U937 cells (Fig. 5) and HL-60 cells (data not shown). Further analysis of the subfragments provided relative locations of the cutting sites, HS1 and HS2 (Fig. 2), giving rise to the fragments. Probe 2 (Fig. 2) did not hybridize to the 0.9 kb fragment (data not shown). Analysis of the cutting sites within a second set of restriction fragments generated by *Bsp* 1286/*Xba* I or *Bsp* 1286/*Hind* III digestion of U937 cell nuclei further confirmed the locations of the two sites (data not shown). Neither site is specific for MNDA expressing cells as both were detected in MNDA negative cells (K562) and MNDA positive cells (HL-60 and U937) (Fig. 5). Neither HS1 or HS2 were detected in MNDA non-expressing KG-1a cell nuclei (data not shown). HS1 is located near the exon 1/intron 1 junction, while HS2 co-localized with the region containing the PU.1, ISRE, and Sp1 consensus elements (Fig. 2).

DNase I Footprinting Assay

To assess the protein-DNA interactions within HS2, a DNase I footprinting assay was per-

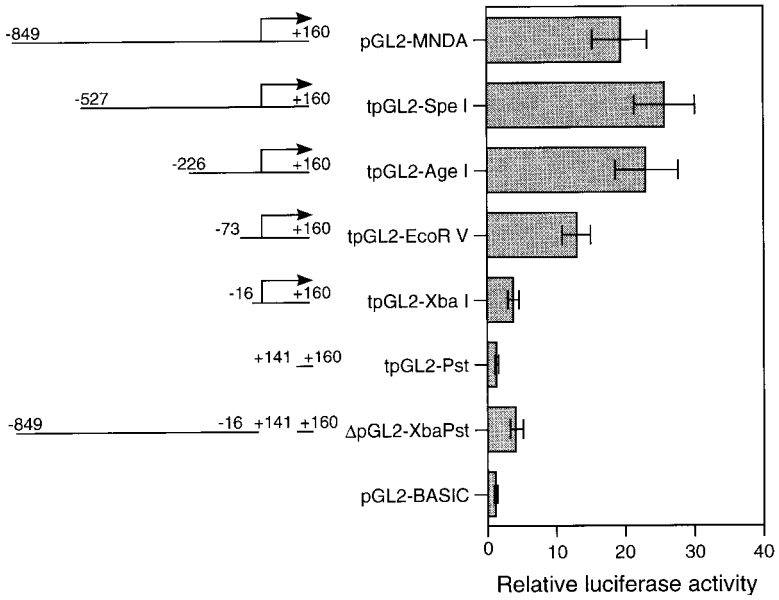


Fig. 4. Functional analysis of the MNDA promoter in MNDA-expressing cells. In addition to the wild type 1.0-kb construct (pGL2-MNDA) a series of mutated constructs were transfected into U937 cells by electroporating 40 μg of the luciferase reporter constructs and 20 μg of pSV-β-Gal as an internal control for transfection efficiency. The relative luciferase activity corrected for transfection efficiency was reported as fold increase over that of the promoterless construct, pGL2-Basic. The results are plotted as means ± SD for three experiments (triplicate samples).

formed over nt −226 to +160. A distinct region between −33 to +20, which includes the Sp1 DNA consensus binding site and major transcription start site, is protected in the presence of nuclear proteins isolated from U937 cells (Fig. 6). The Sp1 site binding proteins were shown to be only partly responsible for this large protected region, when the assay was carried out with DNA containing a mutated Sp1 element (mpGL2-Sp1). The results show that mutation of the Sp1 site abolished protection around the Sp1 site but a protected region was still evident just downstream of the Sp1 site (Fig. 6). This is consistent with the possibility that two proteins bind this region. The results also provide evidence that the protection at the Sp1 site is sequence-specific. Since the residual footprinted region includes the transcription start site, it most likely represents binding of the basal transcription factor complex. Sp1 has been reported to interact with the basal transcription complex [Gunther et al., 1995] and the close proximity of the Sp1 element to the start site in the MNDA promoter suggests a critical role for Sp1 in regulating the TATA-less MNDA promoter through binding to the GC box and interacting with the basal transcription machinery. The same pattern of footprinting was obtained using nuclear proteins from HeLa cells (data not shown) indicating that the proteins that bind to this region are not specific for MNDA-expressing cells.

Mutation of the Sp1 Site but Not the PU.1 Site Decreases MNDA Promoter Activity

As discussed above, PU.1 has been shown in other reports to be a myeloid/lymphoid-specific gene regulator of transcription. Although no footprinting was observed in the PU.1 region of the gene, mutation of this site was carried out to test the possibility of an effect on promoter activity. A 4-bp mutation in the consensus DNA binding sequence, GAGGAA [Karim et al., 1990; Shin and Koshland, 1993], in the MNDA promoter was analyzed by reporter gene assay. The results showed that the mutation of PU.1 did not significantly alter the 1.0-kb MNDA promoter activity. However, when the G/C rich sequence (GC box) within the Sp1 element was replaced with A/T bases, promoter activity was significantly decreased (Fig. 7). To further confirm the specificity of the mutation of the Sp1 element, the same mutated sequence was inserted at a position 5 bp downstream of the Sp1 site. This mutation also did not significantly alter promoter activity (Fig. 7).

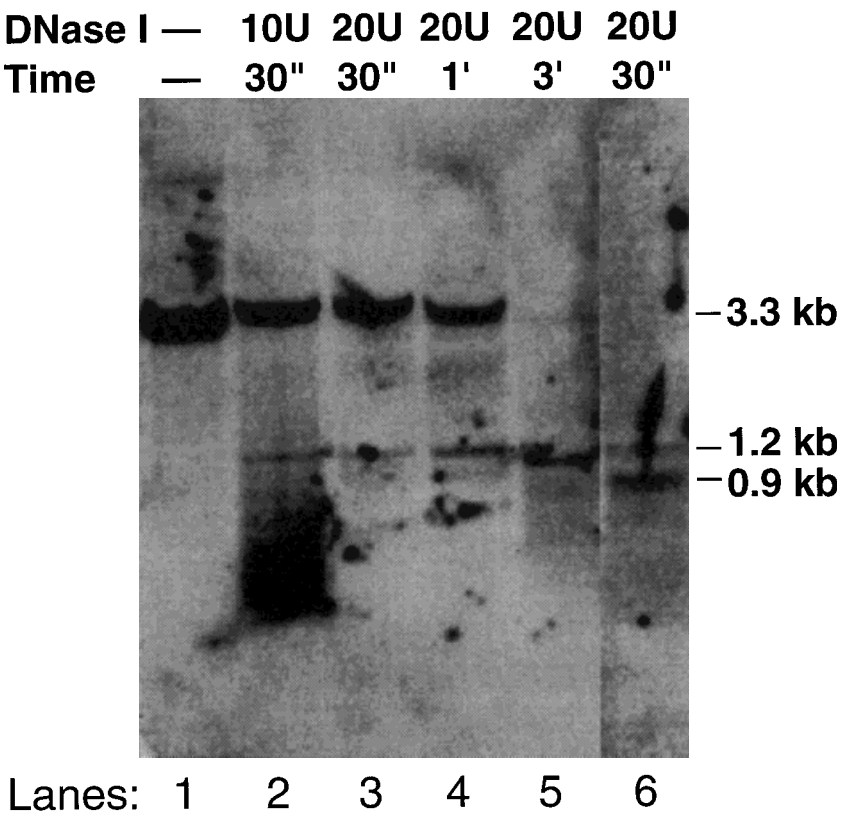


Fig. 5. Identification of DNase I hypersensitive sites in the MNDA gene. Nuclei (2×10^7) were isolated from MNDA non-expressing (K562, *Lanes 1–5*) as well as from MNDA expressing cells (U937, *Lane 6*) and treated with either 10 or 20 units of DNase I for the times indicated. DNA was purified and digested with *Xba* I. Southern blots were hybridized with probe 1 (Fig. 2, nt −849 to −207). Numbers to the right of the blot indicate the initial *Xba* I fragment (3.3 kb) and the DNase I generated subfragments (1.2 kb and 0.9 kb).

The Sp1 Transcription Factor Binds the GC Box Region

An oligonucleotide encoding the MNDA Sp1 sequence containing the GC box produced mobility shifts when incubated with nuclear extract from U937 cells (Fig. 8, lane 2). The shifted bands were competed by adding cold-specific oligonucleotide (Fig. 8, lanes 3 and 4), but were not affected by the addition of a non-specfic competitor (Fig. 8, lanes 5 and 6). To identify the protein binding the GC box region of the MNDA promoter, a supershift experiment was performed with a specific Sp1 antibody (Santa Cruz Biotechnology, Inc.). This antiserum was raised against an epitope corresponding to amino acid residues 436–454 of human Sp1 recognizing both p95 and p106 Sp1 proteins. The antibody does not crossreact with Sp2, Sp3, or Sp4. The Sp1 antibody generated a supershift band (Fig. 8, lane 9). No supershift is observed when the Sp1 antibody was replaced with antibody against MNDA (Fig. 8, lane 11). Although the antibody did not completely supershift the bands, the results indicate that Sp1 is one of the proteins that binds the GC box within the MNDA gene. Confirmation of antibody specificity and ability to supershift Sp1 in EMSA was obtained by substituting purified Sp1 protein in place of nuclear extract in the reaction both with and without Sp1 antibody (Fig. 8, lanes 7 and 8).

DISCUSSION

The restricted expression of MNDA in the human myelomonocytic lineage has been demonstrated by examining its expression in more than 40 cell lines and mature blood cells [Briggs et al., 1994b,c; Cousar and Briggs, 1990; Goldberger et al., 1986; Kao et al., 1996]. The specificity of expression is now further substantiated by examination of MNDA in paraffin-embedded, fixed tissues. The immunohistochemical staining of bone marrow is consistent with MNDA expression restricted to cells within the

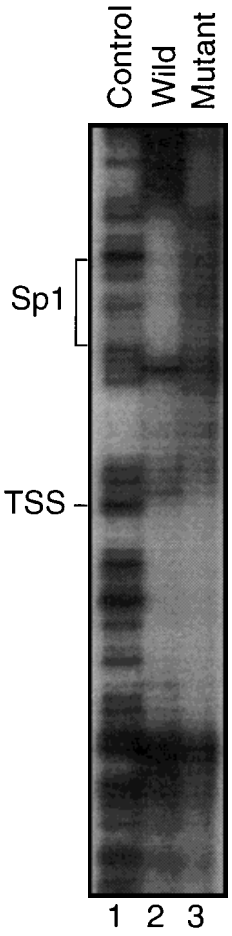
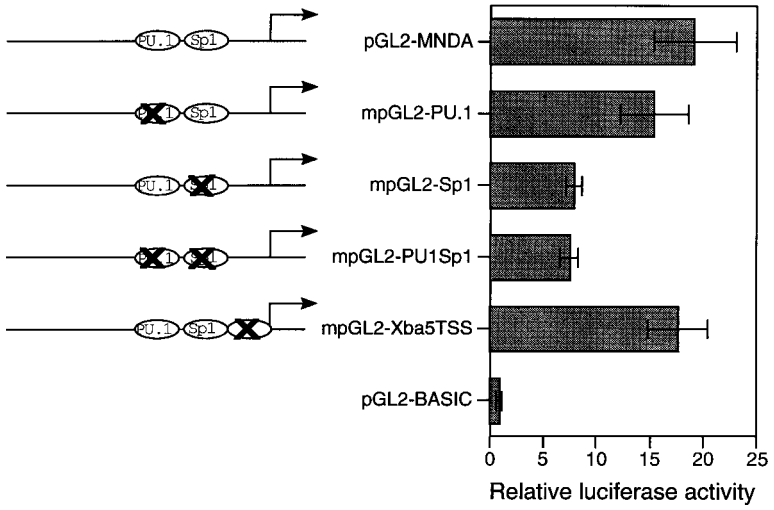


Fig. 6. DNase I footprinting analysis over the GC box region of the MNDA promoter. One DNA fragment corresponding to nt −226/+160 relative to the major transcription start site of the MNDA promoter was radiolabeled with ^{32}P on the sense strand. The DNA fragment was subjected to DNase I treatment (*lane 1*), or incubated with 80 µg U937 nuclear extract and subjected to DNase I treatment (*lane 2*). Another DNA fragment corresponding to the same region of MNDA promoter but carrying a 4-bp mutation of the GC box consensus element was radiolabeled, incubated with 80 µg of nuclear extract from U937 and subjected to DNase I digestion (*lane 3*).

myelomonocytic lineage. The granulocytes and tissue macrophages produced from the myelomonocytic lineage localized in skin were also MNDA positive.

Transcriptionally active genes are characterized by the presence of open chromatin structure which facilitates digestion by DNase I. HS1 and HS2 located in the MNDA promoter (Fig. 2) were detected in both MNDA expressing U937 and HL-60 cells and in MNDA null K562 cells. However, the HS2 associated 0.9 kb fragment is much more prominent in digests of nuclei from MNDA expressing cells than from null cells (Fig. 5), suggesting that HS2 may be different in expressing and non-expressing cells. The presence of hypersensitive sites in non-expressing cells is not unusual and has been reported by others [Bonifer et al., 1991; Schanberg et al., 1991; Wotton et al., 1989]. Digestion of nuclei from MNDA null KG-1a cells did not produce either subfragment indicating that certain null cells do not have HS1 or HS2. While altered chromatin structure may be necessary for MNDA expression it appears that it is not in itself sufficient for expression. At the present time a stronger case can be made for the correlation of DNA methylation status to MNDA expression than presence of DNase I hypersensitive sites (Table I). The analysis of chromatin structure also did not provide evidence for altered chromatin structure in the region of the MNDA promoter containing a cluster of Myb sites. This is consistent with other results showing that c-Myb does not play a role in regulating constitutive MNDA transcription.

The results of functional analysis of the promoter indicated that sequence within HS2 plays an important role in regulating *MNDA* transcription in myelomonocytic cells. Further characterization of the MNDA promoter through mutagenesis of the PU.1 and Sp1 (GC box) sites provided evidence for the involvement of Sp1 but not PU.1 in MNDA transcription. The GC box region is recognized by transcription factors in the Krupple family, including Sp1, Sp2, Sp4, ELKF, BTEB2, ERG-1, and G10BP [Miller and Bieker, 1993; Skerka et al., 1995; Sogawa et al., 1993; Suzuki et al., 1995]. A monospecific antibody was used to show that Sp1 bound the GC box in the MNDA promoter. The results demonstrate that Sp1, a ubiquitous transcription factor, is one of the factors binding the Sp1 consensus site and that an interaction at this site is critical for promoter activity. The findings suggest that Sp1 plays a key role in activating the lineage-specific expression of MNDA gene. Sp1 has been implicated in the lineage- and cell-specific expression of other genes including *CD11b*, *CD11c*, *CD14*, and *TEF-1* [Boam et al., 1995; Chen et al., 1993; Lopez-Rodriguez et al., 1995; Zhang et al., 1994b]. Posttranslational modifications of Sp1 (phosphorylation and glycosylation) [Jackson and Tjian, 1988; Leggett et al., 1995; Schaufele et al., 1990] and interaction with other proteins [Merika and Orkin, 1995] as well as its relatively high level of expression in hematopoietic cells [Saffer et



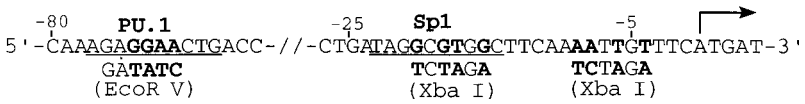


Fig. 7. A 4-bp mutation of the Sp1 site but not the PU.1 site or of a region proximal to the transcription start site significantly decreases the 1.0-kb MNDA promoter activity. A four-base mutation was introduced into the consensus sequence of PU.1 or Sp1 in pGL2-MNDA and a region between Sp1 and the transcription start site. An *Xba* I site was created by introducing the 4-bp mutation in the Sp1 DNA binding site and also through a 4-bp mutation 5 bp downstream of the Sp1 site. Forty μg of each mutant was electroporated into U937 cells. After correction for transfection efficiency by β-galactosidase activity, the promoter activity (*top right*) is reported as the luciferase activity relative to that of the pGL2-Basic construct. *Bottom panel:* The position of mutated sequences relative to the major transcription start site (sequence between nt −80 and +5) and the positions of engineered restriction sites. *Underlined sequence:* Identifies the consensus sequence for PU.1 and Sp1 sites. *Bold characters:* Nucleotide mutations within each sequence. The means ± SD of three independent experiments (triplicate samples in each) are plotted.

al., 1991] qualifies this ubiquitous factor for regulating this type of lineage-specific transcription.

The role of ubiquitously expressed factors in lineage-specific gene expression and development is further supported by results of targeted gene disruption in mice. The loss of those factors, including E2A, rbtn2, or c-fos, resulted in lineage-specific defects in development of B lymphoid, erythroid and osteoclastic lineages, respectively [Bain et al., 1994; Grigoriadis et al., 1994; Orkin, 1995; Warren et al., 1994; Zhuang et al., 1994]. A number of explanations have been presented for the participation of ubiquitous transcription factors in lineage-specific gene expression. In the case of the *MNDA* promoter, transcription could be controlled by limited accessibility of Sp1 to its cognate DNA binding site, as has been proposed for regulation of *CD11b* gene transcription by Sp1 [Chen et al., 1993]. Methylation status can play a role in controlling transcription [Haaf, 1995; Keshet et al., 1986; Kudo and Fukuda, 1995; Lichtenstein et al., 1994; Rhodes et al., 1994; Selker, 1990] limiting accessibility of transcription factors, e.g., Sp1, to target DNA sites [Antequera et al., 1989; Costello et al., 1994].

Sp1 is differentially glycosylated [Jackson and Tjian, 1988] and phosphorylated [Schaufele et al., 1990] and these modifications appear to be related to tissue specific function [Leggett et al., 1995]. In addition, protein-protein interactions between ubiquitous transcription factors and a tissue- or cell-specific protein could restrict function and produce a cell-specific effect [Merika and Orkin, 1995; Milos and Zaret, 1992]. In the albumin gene promoter, a ubiquitous transcription factor, NF-Y, is required for the dramatic transactivation of the gene in hepatic development which is facilitated by liver-specific C/EBP-related proteins shown to form a stable transcription complex with NF-Y in vitro [Milos and Zaret, 1992]. Characterization of erythroid-specific gene expression indicates that no single element is capable of providing lineage-specific transcription and cooperation

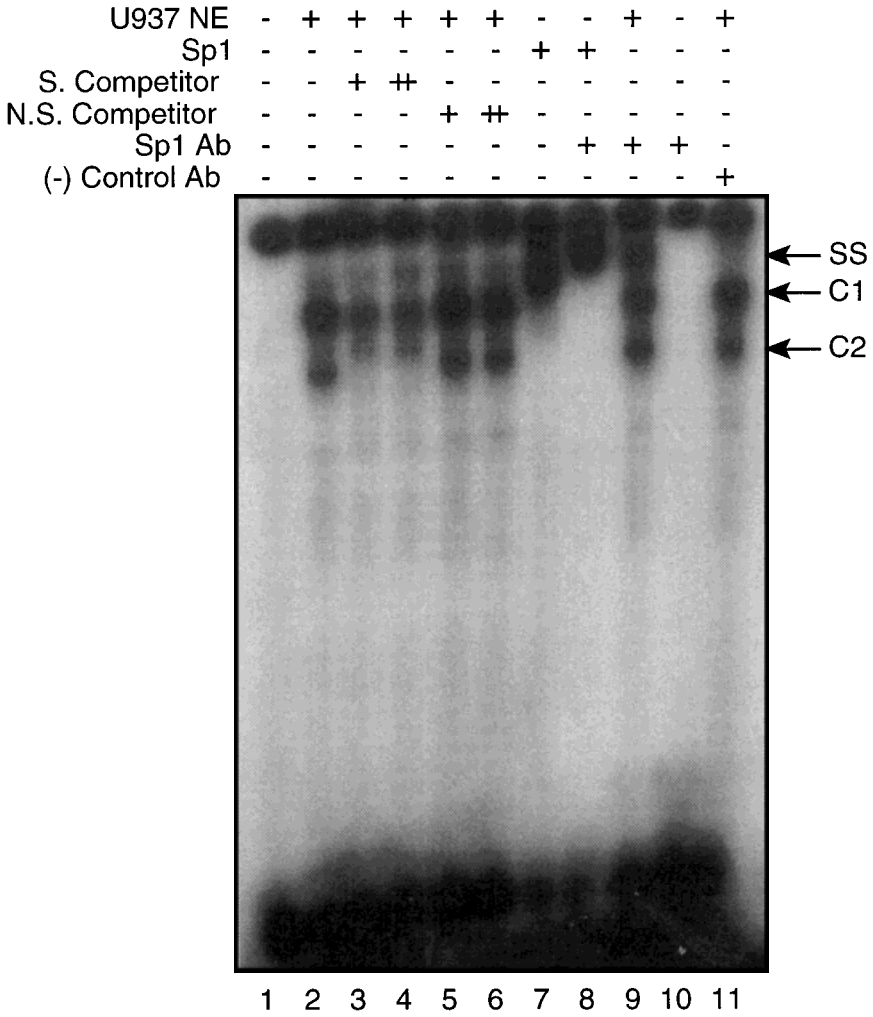


Fig. 8. Identification of Sp1 as a nuclear factor binding the GC box region of the MNDA promoter. Electrophoretic mobility shift assay (EMSA) was performed by using ^{32}P-labeled ds oligonucleotide, M-Sp1, corresponding to nt −34 to −9 of the MNDA promoter incubated with U937 cell nuclear protein extracts. The specific interaction between the oligo and nuclear proteins resulted in two shifted bands (*lane 2*), indicated by C1 and C2. The shifted bands can be competed with 50-fold (*lane 3*) and 100-fold cold (*lane 4*) M-Sp1 oligo, but not with 50-fold (*lane 5*) or 100-fold (*lane 6*) molar excess of unrelated sequence. Incubation of affinity-purified Sp1 antibody with U937 nuclear extract prior to addition of labeled M-Sp1 results in a supershifted band (*lane 9*), indicated by SS. The supershifted band is not caused by other proteins in the affinity-purified Sp1 polyclonal antibody, since no shift occurs when the nuclear extract is omitted (*lane 10*). The absence of a super-shifted band using nonspecific Ab further confirms the specificity of the Ab-protein interaction (*lane 11*). Positive controls (*lanes 7, 8*) are reactions in which purified Sp1 transcription factor (Promega) was incubated without or with Sp1 antibody before incubation with labeled M-Sp1 oligonucleotide.

between a number of cis-elements appears to be required to direct erythroid specificity and transcriptional activity [Merika and Orkin, 1995]. This conclusion is further supported from studying the lineage-specific expression of the lysozyme gene is transgenic mice [Bonifer et al., 1994]. The results showed that appropriate expression of the lysozyme gene in myeloid cells requires the concerted action of several regulatory DNA elements and deletion of a region

TABLE I. MNDA Expression, Chromatin Structure and Demethylation of the MNDA Promoter

Cell lines	Hypersensitive sites			
	MNDA	HS1	HS2	Demethylation
KG-1a	—	—	—	—
K562	—	+	±	—
U937	+	+	+	+
HL-60	+	+	+	+

containing binding sites for ubiquitous transcription factors leads to nonspecific and variable gene expression.

Both c-myb and PU.1 have been shown to be essential for the development of the myelomonocytic lineage [Mucenski et al., 1991; Scott et al., 1994] and both have been implicated in the regulation of lineage-specific transcription [Ahne and Stratling, 1994; Feinman et al., 1994; Grove and Plumb, 1993; Melotti et al., 1994; Pahl et al., 1993; Shelley et al., 1993; Zhang et al., 1994a]. However, the functional analysis of the MNDA promoter in this study showed that neither of these factors play an obvious role in regulating the transcription of the MNDA gene. On the other hand, based on results of transient transfection assays (Fig. 3) it cannot be absolutely concluded that the Sp1 regulatory site is solely responsible for the lineage-specific MNDA promoter activity. The transient transfection promoter-reporter gene analysis cannot assess the role of chromatin structure or DNA methylation. In an earlier publication [Kao et al., 1996], the *MNDA* promoter was shown to be demethylated in expressing cells in contrast to non-expressing cells. It appears that DNA methylation, chromatin structure, and possibly other factors are acting in conjunction with Sp1 to establish strict myelomonocytic lineage-specific *MNDA* transcription. Further elucidation of the importance of these mechanisms in establishing the strict lineage-specific pattern of *MNDA* expression will require use of transgenic animals combined with the embryonic stem cell system where regulation can be assessed within the context of normal chromatin structure [Faust et al., 1994; Lieschke and Dunn, 1995].

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