Transcription factors Sp1 and AP-2 mediate induction of acid sphingomyelinase during monocytic differentiation

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Abstract Cells from the human monocytic leukemia cell line THP-1 differentiate towards a macrophage-like phenotype when stimulated with phorbol 12-myristate-13-acetate (PMA), 1,25-dihydroxy-vitamin D_3, and various other agents. We demonstrate here that the expression of the lysosomal enzyme acid sphingomyelinase (ASM; E.C. 3.1.4.12) is induced during this process and is strongly elevated in differentiated THP-1 cells, as well as in differentiated human mononuclear phagocytes. Using Northern blotting, RNase protection assay, and nuclear run-on analyses, we show that the up-regulation of ASM expression is regulated mainly at the level of transcription and that new protein synthesis is required for enhanced ASM activity. This cell-type specific induction by PMA treatment was further investigated with respect to transcriptional control. A series of 5′ deletion derivatives of the upstream regulatory region were used in transient transfection assays to identify promoter elements required for basal and inducible gene expression. A PMA responsive element was localized to a region between −319 and −219 bp upstream of the initiation codon and co-transfections with transcription factor expression plasmids for AP-2 and Sp1 resulted in augmented ASM promoter activity, which was abolished when the binding sites for these two factors were deleted. Using electrophoretic mobility shift assays and supershift assays we demonstrate that this region is specifically bound by Sp1 and AP-2. These factors are present in nuclear extracts prepared from both induced and uninduced THP-1 cells. However, the intensity of the complex formed appeared to increase when nuclear extracts from PMA-treated cells were used. From these studies, we conclude that a concerted action of the transcription factors AP-2 and Sp1 is essential for the up-regulation of ASM expression during the process of macrophage differentiation.—Langmann, T., C. Buechler, S. Ries, A. Schaeffler, C. Aslanidis, M. Schuierer, M. Weiler, K. Sandhoff, P. J. de Jong, and G. Schmitz. **Transcription factors Sp1 and AP-2 mediate induction of acid sphingomyelinase during monocytic differentiation.** ***J. Lipid Res.*** **1999.** 40: **870–880.**

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Human acid sphingomyelinase is a lysosomal hydrolase required for the cleavage of sphingomyelin to phosphocholine and ceramide (1). The enzyme with a pH optimum of 4.5 and a molecular mass of 72 kDa was purified from human urine by Quintern et al. (2). ASM deficiency has been shown to be responsible for the autosomal recessive sphingomyelin storage disorders Niemann-Pick disease type A (NPD-A) and type B (NPD-B). These two forms are distinguished by well-defined clinical symptoms. NPD-A patients show severe neurodegenerative lesions and die within the first 2 years of life, while in NPD-B patients who have a prolonged lifespan beyond middle age, only the organs of the reticuloendothelial system are affected and minimal neurologic involvement occurs (3, 4). A valuable animal model for NPD-A has been generated recently (5, 6), with a phenotype similar to the human disease that is currently used for the evaluation of various therapeutic regimens.

Beyond its catalytic lysosomal function, ASM is discussed to contribute in cytokine signalling pathways by releasing ceramide, as a second messenger from sphingomyelin (7–10). Three distinct ASM transcripts resulting from alternative splicing were isolated and expressed in COS-1 cells, demonstrating that only the full-length mRNA form encoded a catalytically active enzyme (11). The gene is 5kb in length, consists of six exons, and maps to chromosome 11p15.1–11p15.4 (12, 13). The putative promoter region has been cloned, however, no detailed characterization of promoter elements has been reported so far (14).

Abbreviations: PMA, phorbol 12-myristate-13-acetate; ASM, acid sphingomyelinase; NPD, Niemann-Pick disease; M-CSF, macrophage-colony stimulating factor; PAC, P1 artificial chromosome; SR-A, scavenger receptor type A; GAPDH, glyceraledehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; Sp1, specificity protein 1; AP-2, activator protein 2; SIF, shift fragment.

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ASM obviously plays a major role in phagocytic tissue macrophages as is reflected by the presence of a large lysosomal compartement for intracellular digestive processes. Recently, it has been shown that ASM can be secreted by various cell types, including macrophages (15), hydrolyze atherogenic lipoproteins, and play an important role in the development of atherosclerotic lesions (16, 17). However, no data on the ASM expression in monocytic development are currently available. Therefore we became interested in the regulation of ASM during monocyte to macrophage maturation and in the more immature monocytic cell line THP-1. This cell line is considered as an excellent, legitimate in vitro model system for the analysis of processes related to monocytic differentiation due to its simple conversion to mature macrophages upon phorbol ester stimulation in cell culture (18).

The present study demonstrates that monocytic differentiation induces ASM mRNA expression mainly at the level of transcription. Furthermore, we identified DNA sequence elements and their corresponding transcription factors that are required for the PMA-mediated induction of the ASM promoter in THP-1 cells. In order to determine the *cis*-elements necessary for basal and differentiation-induced promoter activity, we used 5′ truncations and mutagenized fragments of the ASM gene fused to a luciferase reporter gene. With these constructs we were able to define PMA-responsive elements. Binding of transcription factors to specific promoter sites has been demonstrated by electrophoretic mobility shift assays and DNaseI footprinting experiments. Our studies clearly demonstrate the engagement of the transcription factors Sp1 and AP-2 in the transcriptional induction of the acid sphingomyelinase gene observed during monocyte to macrophage differentiation.

EXPERIMENTAL PROCEDURES

Isolation and culture of cells

Peripheral blood monocytes were isolated by leukapheresis and counterflow elutriation (19). To obtain fractions containing >90% CD14 positive mononuclear phagocytes, cells were pooled and cultured on plastic Petri dishes in macrophage SFM medium (Gibco BRL) containing 25 U/ml recombinant human M-CSF. THP-1 cells (20) were obtained from ATCC, cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Gibco BRL), 100 U penicillin per ml, 100 μg of streptomycin per ml, and incubated in 10% CO_2 in air at 37°C. To induce monocyte to macrophage differentiation, THP-1 cells were cultured in the presence of 160 nm PMA.

Isolation of human genomic clones for the acid sphingomyelinase gene and reporter gene construction

A human genomic PAC library in pCYPAC-1 (21) was screened with a [α-^{32}P]dCTP-labeled probe encompassing nt −203 to +44 of the ASM gene with +1 referring to the initiation codon. The probe was generated in a genomic PCR reaction using primers ASM-203 (5′-GCCCGCCACCGAGAGATCAGCTGTC-3′) and ASM+44 (5′-GACCTGGGGCAGCTCTGGCGGAGTGACGCT-3′). To generate a series of 5′ deletion mutants of the ASM promoter, DNA from positive PAC clones was used as template in a modified PCR reaction using 7-deaza-2′-deoxyguanosine (c^7dGTP) (22). A *Xho*I restriction site was created in the upstream and a *Hin*dIII restriction site in the downstream primers used in the PCR. The sequences of the upstream oligonucleotides used for generating the various constructs are as follows: p(−519/+112)Luc, 5′-CCCCTCGAGATCTGCCGCCTGCTCTCTCTACC TCCC-3′; p(−419/+112)Luc, 5′-CCCCTCTGAGGTCTTCCAC CGACCACATCACATG-3′; p(−319/+112)Luc, 5′-CCCCTCGAG CCCACCCCCGCAGCCCGTGCGCCC-3′; p(−219/+112) Luc, 5′-CCCCTCGAGCGCAGCGTTGACAGCCGCCCGCCACC-3′; p(−119/+112)Luc, 5′-CCCCTCGAGCCGACTACAGAGAAGGGTAATCG GG-3′. The sequence of the downstream oligonucleotide used for the above constructs was 5′-CCCAAGCTTCCAGCGGCCAGG CCCATCCAAAGG-3′. The PCR products were incubated with *Xho*I and *Hin*dIII restriction enzymes and subcloned into the *Xho*I/*Hin*dIII site of the pGL3-basic vector (Promega). Mutations in the AP-2 site at −289 and in the Sp1 site at −279 were generated using oligonucleotides with base substitutions (23). The identity of the subcloned DNA fragments was confirmed by DNA sequencing.

DNA transfection experiments

THP-1 cells were transfected by the DEAE–dextran procedure as described previously (24) with minor modifications. Cells (2×10^7) were harvested, washed with 5 ml of TS (25 mm Tris/HCl, pH7.4; 5 mm KCl; 0.7 mm $CaCl_2$; 137 mm NaCl; 0.6 mm Na_2HPO_4; 0.5 mm $MgCl_2$) and incubated in 1 ml of TS containing 10 μg of supercoiled plasmid DNA and 50 μg/ml of DEAE–dextran (Sigma) for 15 min. Cells were then incubated with 100 μm chloroquin in RPMI 1640 medium containing 10% FCS for 30 min at 37°C. Cells were washed, resuspended in complete medium, and cultivated for 46 h in the absence or presence of PMA. In cotransfection experiments, 50 ng of transcription factor expression plasmid or pCMV plasmid as negative control was used. In each set, pGL3-basic and pGL3-promoter plasmids served as negative and positive controls, respectively. Two μg of pSVβ-galactosidase plasmid (Promega) was included in all assays to gauge transfection efficiencies.

Luciferase assays

Transfected cells were harvested, washed once in PBS, and resuspended in 500 μl of reporter lysis buffer (Promega). After pelleting of cellular debris in a microfuge, 20 μl of cell lysate was assayed for luciferase activity with 100 μl luciferase assay reagent (Promega) in a Lumat LB9501 (Berthold). β-Galactosidase activity was measured in a standard color reaction (25).

Transcription factor expression plasmids

The transcription factor expression plasmids for Sp1 (pPacSp1) driven by the actin promoter was a gift from Dr. R. Tjian, University of California, Berkeley. Plasmids for AP-2α (pCMVAP-2α), Myc (pCMVMyc), and BTEB (pCMVBTEB) driven by the CMV promoter were a gift from Dr. R. Buettner, Institute for Pathology, University of Regensburg.

Isolation of RNA and Northern blot analysis

Total cellular RNA was isolated by the guanidinium isothiocyanate–cesium chloride technique (26). Ten μg of total RNA was electrophoresed through a 1.2% agarose gel containing 6% formaldehyde and blotted onto nylon membranes (Schleicher & Schuell). After crosslinking with UV-irradiation (Stratalinker model 1800, Stratagene), the membranes were hybridized with cDNA probe P3/P4 spanning nt 950 to 1330 of the ASM cDNA (11), stripped, and subsequently hybridized with a human β-actin probe. The probes were radiolabeled with [α-^{32}P]dCTP using the Oligolabeling kit from Pharmacia. Hybridization and washing con-

ditions were performed as recommended by the manufacturer of the membrane.

Nuclear run-on transcription assay

Intact nuclei were isolated from untreated or PMA-treated (160 nm for 24 h) THP-1 cells and subjected to nuclear run-on transcription as described previously (27). Radiolabeled RNA was isolated by the RNAzol method (Biogentex, Houston TX). The P3/P4 fragment of the human ASM cDNA, a GAPDH gene fragment, and a scavenger receptor type A cDNA fragment were subcloned into pUC18 (Pharmacia). Ten μg of these plasmids was linearized with appropriate restriction enzymes, alkaline denatured, and blotted onto nylon membranes using a slotted filtration manifold (Schleicher & Schuell). Hybridization and washing of the membrane strips were performed as described by Srivastava, Cable, and Bonkovsky (27).

Ribonuclease protection assay

A 333 bp *Bam*HI-*Sac*I restriction fragment from the ASM cDNA was used to discriminate between type 1 and type 2 transcripts (11). The DNA fragment was subcloned into a pGEM4Z vector (Promega). To generate a ^{32}P-labeled riboprobe, the plasmid was linearized and in vitro transcribed using [α-^{32}P]CTP and SP6 RNA polymerase. Nuclease protection assay was performed with the RNase Protection kit (Boehringer). Ten μg of total RNA from untreated or PMA-induced THP-1 cells was hybridized with the radiolabeled riboprobe. After RNase treatment, protected fragments were analyzed on a 8% denaturing polyacrylamide gel. The gel was fixed, dried, and exposed to film.

DNaseI footprinting

A genomic DNA fragment spanning from −368 to −252 with respect to the start of translation was radiolabeled at one end with [α-^{32}P]dCTP and Klenow DNA polymerase. The probe was purified using Microspin S-200 HR columns (Pharmacia). The binding site for Sp1 was detected using the SureTrack Footprinting kit (Pharmacia) and purified Sp1 protein (Promega). Binding reactions and DNaseI digestion were performed following the manufacturer's instructions. The resulting products were separated on a 8% denaturing polyacrylamide gel. For a sequence ladder, the same probe fragment was partially cleaved at guanine and adenosine residues by the method of Maxam and Gilbert (25).

Preparation of nuclear extracts

THP-1 cells (50 × 10^6) were harvested by centrifugation, washed once with ice-cold PBS, and washed twice with wash buffer composed of 10 mm HEPES, pH 7.9, 1.5 mm $MgCl_2$, 10 mm KCl, 0.5 mm dithiothreitol (DTT), and 0.5 mm phenylmethylsulfonyl fluoride (PMSF). After resuspension in 1 ml ice-cold wash buffer, cells were centrifuged 1 min at full speed in a microfuge. Hypotonic buffer containing 0.1% NP40 was added to lyse the cell pellet. After 5 min incubation on ice, nuclei were pelleted in a microfuge for 15 min at 4°C. Nuclei were resuspended in lysis buffer containing 20 mm HEPES, pH 7.9, 0.42 m NaCl, 1.5 mm $MgCl_2$, 0.2 mm EDTA, 0.5 mm DTT, 0.5 mm PMSF, and 10% v/v glycerol, and incubated at 4°C for 15 min with gentle vortexing. Subsequently, the nuclear debris was pelleted by centrifugation at 4°C for 15 min and the supernatant was diluted 1:6 with storage buffer composed of 20 mm HEPES, pH 7.9, 0.05 m KCl, 0.2 mm EDTA, 0.5 mmDTT, 0.5 mm PMSF, and 20% v/v glycerol. The extracts were aliquoted and stored at −70°C. Protein concentration of an aliquot was determined using the Bio-Rad protein assay reagent. The integrity of the nuclear extract preparations was assessed by determining the ability of proteins to bind to a radiolabeled Oct-1 double-stranded oligonucleotide (5′-TGTCGAATGCAAATCACTAGAA-3′). Oct-1 binding was constitutive and thus served as internal control.

Gel mobility shift assay

Nuclear extracts (10 μg of protein) or purified transcription factors (1 footprinting unit) were incubated with 2 μg poly (dI-dC) (Pharmacia) in 20 μl binding buffer containing 50 mm HEPES, pH 7.9, 6 mm $MgCl_2$, 50 mm KCl, 5 mm DTT, 100 μg/ml BSA, and 0.01% NP40. [^{32}P] end-labeled probes (30,000 cpm) were added and the reaction mixture was incubated for 20 min at room temperature. In competition experiments, nuclear extracts were preincubated for 10 min with a 50-fold molar excess of nonlabeled competitor. Supershift assays were performed with antibodies purchased from Santa Cruz Biotechnology; Sp1, catalog number sc-59x, rabbit polyclonal against residues 436–454 of human Sp1; AP-2α, catalog number sc-184x, rabbit polyclonal against residues 420–437 of human AP-2α; Ets-1, catalog number sc-112x, rabbit polyclonal against residues 362–364 of human Ets-1; PU.1, catalog number sc-352x, rabbit polyclonal against residues 420–437 of mouse PU.1. The DNA-protein complexes were analyzed on a 8% polyacrylamide gel at 80 V using 0.25 × TBE (25) as electrophoresis buffer. Gels were dried and exposed to Kodak X-Omat AR films overnight at −70°C. For the electrophoretic mobility shift experiments the following oligonucleotides annealed to their respective complementary oligonucleotides have been used: SIF3 (5′-AGCCCCGCAGCCCGTGCGCCCGGGGCAGGGCGGGGGCAGGGA-3′); SIF3MI (5′-AGCCCCGCAGCCCGTGCGAACGGAACAGGGCGGGGGCAGGGA-3′); SIF4 (5′-AGGAGGGGGCGGAATCGGGGCGGTCCCGGGAGCGCCCCGCCC-3′); SIF6 (5′-AGGATCAGCTGTCAGAGATCAGAGGAAGAGGAAGGGGCGGAG-3′).

Electrophoresis and immunoblotting

SDS-polyacrylamide gelelectrophoresis was performed as described previously (28). Proteins were transferred to Immobilon as reported. Transfer was confirmed by Coomassie Blue staining of the gel after the electroblot. Polyclonal affinity-purified antibodies (Santa Cruz Biotechnology) against Sp1, PU.1, and c-Jun were used at a 5 μg/ml dilution in 1% nonfat dry milk, 0.02% sodium azide in PBS and incubated overnight at 4°C. Detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham Corp.).

RESULTS

Changes in ASM mRNA expression associated with monocytic differentiation

Northern analysis was used to quantitate the relative amounts of ASM mRNA in THP-1 cells at different time points after PMA treatment, in monocytes and in monocyte-derived macrophages (**Fig. 1**). In undifferentiated THP-1 cells (Fig. 1, lane 1) no measureable amount of ASM mRNA was present. Steady-state levels of the ASM message increased successively after PMA-induced differentiation, reaching maximum levels after 24 h (Fig. 1, lanes 2–6). To compare these results from the THP-1 cell line with results from human blood monocytes, ASM mRNA abundance in total RNA from human peripheral blood monocytes (Mo) and from monocyte-derived macrophages after 7 days in culture (7dMNP) were analyzed. mRNA levels for ASM similarly increased during the differentiation process of native monocytes (Fig. 1, lanes 7–

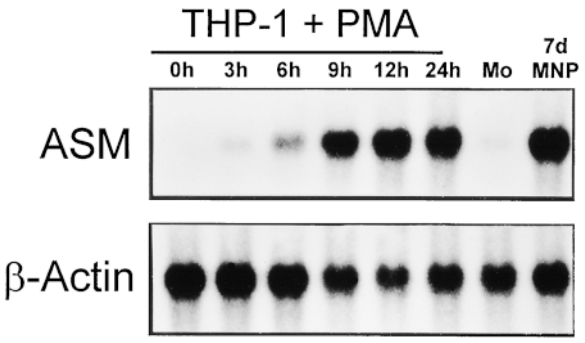

Fig. 1. ASM mRNA is induced during monocyte to macrophage differentiation. Total RNAs were isolated from THP-1 cells cultured in the presence of PMA for 0, 3, 6, 9, 12, 24 h, from freshly isolated monocytes (Mo) and from monocyte-derived macrophages (7dMNP). Each lane was loaded with 10 μg of total RNA. The blot was hybridized with the ^{32}P-labeled ASM cDNA fragment P3/P4. The lower panel displays the hybridization analysis of the same blot performed with a human β-actin probe as control.

8). To confirm these results on the level of protein expression, immunoblots were carried out and were found to produce similar results (data not shown).

Expression of type 1 and type 2 ASM transcripts in differentiating THP-1 cells

RNase protection analysis was performed to determine the relative levels of type 1 and type 2 ASM transcripts in total RNA of THP-1 cells (**Fig. 2A**). Hybridization of the 333bp antisense probe (12) with the ASM mRNA resulted in two protected fragments of 333bp and 266 bp, originating from the type 1 and type 2 ASM transcripts, respectively. The expression of both transcripts was up-regulated during the monocytic differentiation process (Fig. 2A), with type 2 transcripts representing only a minor part of the ASM message.

Measurement of ASM transcription rate during PMA-induced differentiation of THP-1 cells

In order to elucidate whether the up-regulation of the steady-state level of ASM mRNA was due to changes in the rate of transcription or post-transcriptional mechanisms, nuclear run-on analysis was carried out using nuclei isolated either from undifferentiated cells or cells treated with PMA for 24 h. Radiolabeled transcripts were allowed to hybridize to cDNA probes for ASM, scavenger receptor type A (SR-A), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or the insertless pUC18 cloning vector on nylon membranes (Fig. 2B). The transcriptional induction of scavenger receptor type A after stimulation of THP-1 cells by PMA is well characterized and was thus used as positive control (29, 30). Our experiments clearly demonstrate that the increase of ASM mRNA amount during monocytic differentiation was largely at the level of transcription. The transcription rate of the GAPDH gene was up-regulated to a much lesser extent than that of the ASM gene after PMA induction of THP-1 cells, and hybridization to the pUC18 vector produced no signal (data not shown). Simultaneous incubation of THP-1 cells with

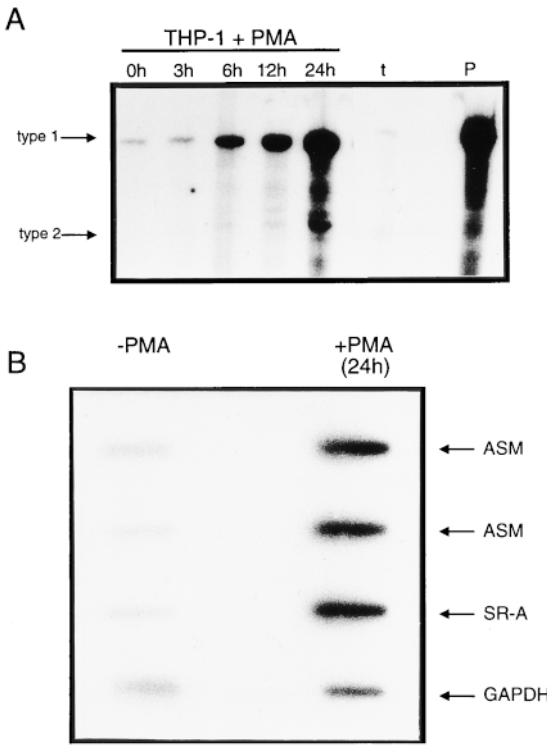

Fig. 2. (A) RNase protection analysis of ASM transcripts in differentiating THP-1 cells. The 333 bp antisense RNA probe was designed to discriminate between type 1 and type 2 ASM transcripts. The protected fragments for type 1 and type 2 transcripts were 333 bp and 266 bp in length, respectively. Lanes 1–5 contain RNA from THP-1 cells incubated with PMA for the indicated times; lane t, mock control with tRNA; lane P, free probe. The arrows indicate the positions of type 1 and type 2 protected fragments. (B) Induction of ASM mRNA depends on new transcription. Nuclei were isolated from control cells (−PMA) and cells treated with PMA for 24 h (+PMA). Nascent transcripts were labeled with [α-^{32}P]UTP and hybridized to cDNA probes for scavenger receptor type A (SR-A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ASM cDNA fragment P3/P4 bound to nylon membranes.

actinomycin D and PMA did not result in enhanced ASM mRNA levels, suggesting that ASM mRNA stability is not prolonged (data not shown).

5′-Deletional analysis of the ASM promoter

To identify sequences important for promoter activity, human genomic clones containing the ASM gene were isolated by screening a PAC library with a probe encompassing nt −203 to +44 of the ASM gene. Five promoter constructs with successive 5′ truncations were generated by PCR using the isolated PAC clones as template. As shown in **Fig. 3A**, the deletions ranged from −519 bp to −119 bp proximal to the initiation codon. Two minor transcription start sites at nt −251 and nt −117 and one major transcription start site at nt −160 have been identified using primer extension analysis by Newrzella and Stoffel (14). The promoter activities of the constructs are shown in Fig. 3B. The promoterless pGL3basic vector served as negative control and displayed no promoter activity. By and large, the following results are evident from

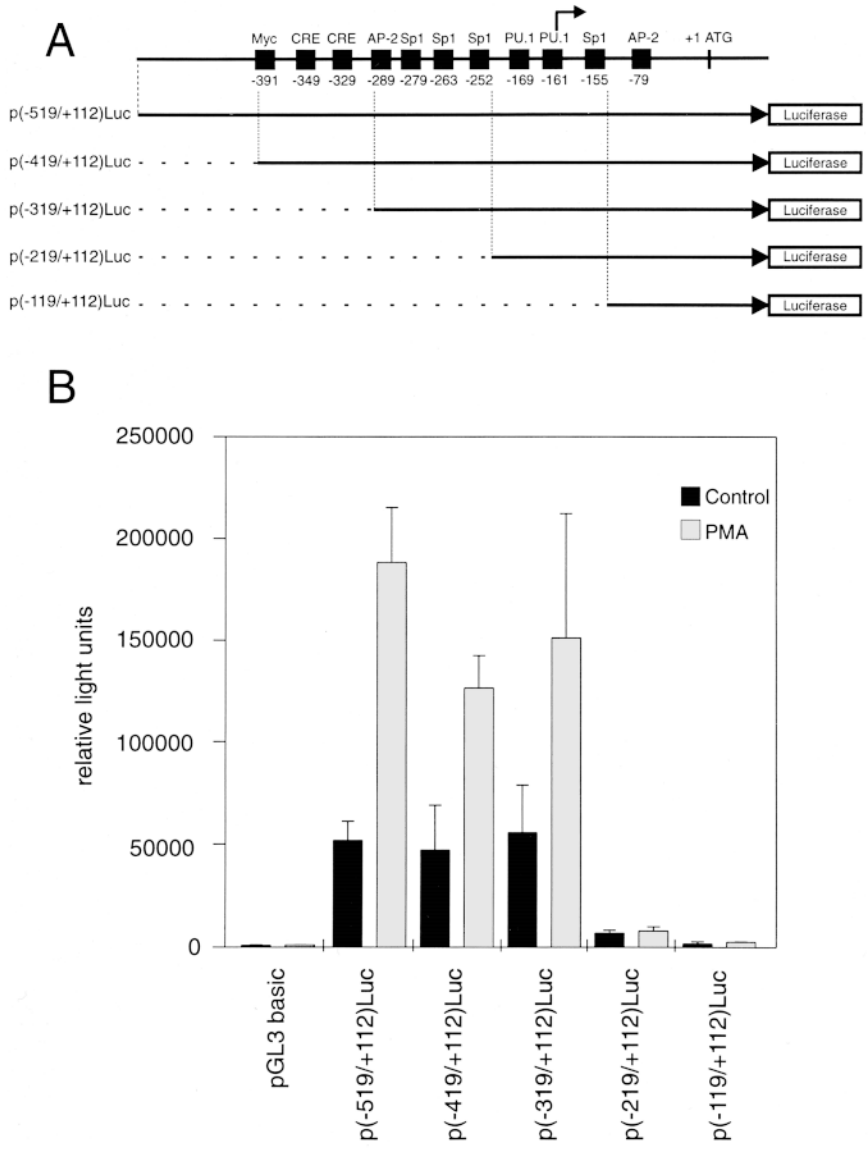

Fig. 3. (A) ASM promoter region with putative regulatory elements. Putative *cis*-acting consensus sequences are denoted by boxes and numbers identify their positions as the distance to the ASM start codon. The major transcription start site is indicated by an arrow. The extent of the 5′-truncations of the ASM promoter is shown. The promoter fragments were ligated to the luciferase reporter gene in the forward orientation. (B) ASM promoter activity lies within the first 319 bp of the 5′-UTR. THP-1 cells were transfected with 10 μg of each reporter gene construct, or the promoterless pGL3basic vector and 2 μg of pSVβ-gal plasmid and cultured for 46 h in the presence or absence of PMA (160 nm), at which time cell lysates were assayed for luciferase activity. Assays were performed in triplicate and results shown are normalized to the β-galactosidase expression. The results are representative of five independent experiments. Black bars show basal (uninduced) activity of each promoter construct and grey bars indicate PMA-induced levels of activity.

these experiments. *1*) Similar basal promoter activities in THP-1 cells can be detected with constructs p(−519/+112)Luc, p(−419/+112)Luc, and p(−319/+112)Luc. *2*) The shorter promoter fragments p(−219/+112)Luc and p(−119/+112)Luc show a significant decrease of basal promoter activity (Fig. 3, solid bars). *3*) In transfected THP-1 cells incubated in the presence of PMA, luciferase activity increased between 2.7- and 3.6-fold in each of the constructs analyzed, except for the two shortest fragments (Fig. 3B, grey bars). These promoter activities correlate well with the enhanced transcription found in the nuclear run-on analysis (see Fig. 2B). Therefore, it is assumed that the response elements for PMA inducibility are located within −319 bp and −219 bp. However, the presence of additional upstream enhancer elements cannot be ruled out.

Sp1 and AP-2 transactivate the ASM promoter

Within the DNA sequence upstream of the ASM gene transcription start site, several putative *cis*-acting consensus sequences are present (Fig. 3A). These include binding sites for transcription factors c-Myc, CREBP, AP-2, Sp1, and PU.1 (31–35). As shown in the deletion analysis (Fig.

3B), construct p(−219/+112)Luc, which contains only the two PU.1 binding sites, shows no basal or PMA-inducible promoter activity. This indicates that PU.1 alone may not play a significant role in ASM gene expression. To further investigate the role of other transcription factors, we co-transfected THP-1 cells with the longest construct p(−519/+112)Luc and expression vectors for Myc (pCMV Myc), AP-2α (pCMVAP-2α), Sp1 (pPacSp1), and BTEB (pCMVBTEB), respectively (**Fig. 4A**). Expression of Sp1 and AP-2 caused constitutive augmentation of ASM promoter activity (1.8-fold, and 2.6-fold, respectively), which could be further enhanced by the combination of Sp1 and AP-2 (3.2-fold). c-Myc lowered luciferase expression (0.7-fold) and co-transfection of BTEB, an Sp1-related transcription factor, did not affect ASM promoter activity. To determine the importance of the Sp1 site at −279 and the AP-2 site at −289 for the induction of the ASM promoter activity, luciferase constructs with mutated binding sites were transfected in THP-1 cells. As shown in Fig. 4B, mutation of the Sp1 site, the AP-2 site, or both significantly reduced basal and inducible promoter activity; however, it did not entirely abolish activity. These data indicate that Sp1 and AP-2 are relevant and necessary for the transcriptional activity of the ASM promoter and that c-Myc may function as a negative regulator.

Characterization of proteins that bind to the ASM promoter

To identify nuclear proteins that interact with the ASM promoter region, electrophoretic mobility shift assays (EMSAs) were performed. The studies focused on three regions, SIF 3, SIF 4, and SIF 6, including binding sites for the transcription factors Sp1 and AP-2, which were shown to induce ASM promoter activity in co-transfection experiments (see Fig. 4). SIF 3 (−310/−270), which contains overlapping binding sites for AP-2 and Sp1 interacts with proteins in both control and induced nuclear extracts (**Fig. 5**, lanes 2 and 8). However, complexes appeared to be more intense when PMA-induced extracts were used. Binding was specific as indicated by the effective competition of a 50-fold molar excess of unlabeled probe (Fig. 5, lanes 3 and 9). The addition of a 50-fold molar excess of AP-2 and Sp1 consensus oligonucleotides competed for the binding activity in both extracts, whereas NFκB, AP-1,

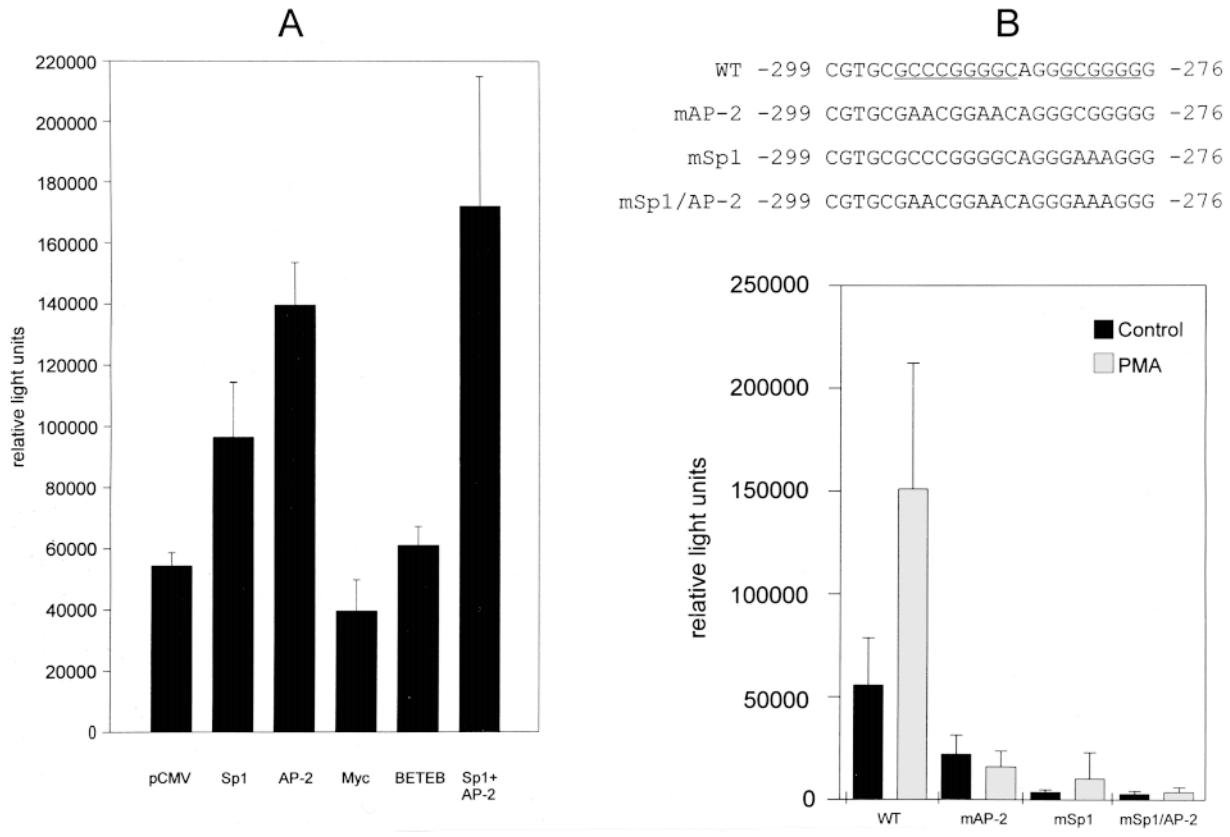

Fig. 4. (A) Sp1 and AP-2 can transactivate the ASM promoter. THP-1 cells were co-transfected with 10 μg of p(−519/+112)Luc promoter construct and 50 ng of transcription factor expression plasmids for Sp1 (pPacSp1), AP2α (pCMVAP-2α), Myc (pCMVMyc), and BTEB (pCMVBTEB) in individual experiments or in combination. pCMV was used as a control for the amount of DNA in each sample and had no effect on ASM promoter activity. (B) Mutation of the Sp1 and AP-2 site significantly reduces ASM promoter activity. The wild type construct p(−319/+112) (WT) or the constructs mAP-2, mSp1, and mSp1/AP-2 which contain base pair substitutions in the binding sites for AP-2, Sp1, or for both (upper panel) were transfected in THP-1 cells. The cells were incubated in the presence or absence of PMA for 48 h and the luciferase activity was determined. Cell lysates were assayed in triplicate for luciferase activity and results are representative of four experiments. Black bars show basal (uninduced) activity of each promoter construct and grey bars indicate PMA-induced levels of activity. Consensus binding sites for Sp1 and AP-2 are underlined.

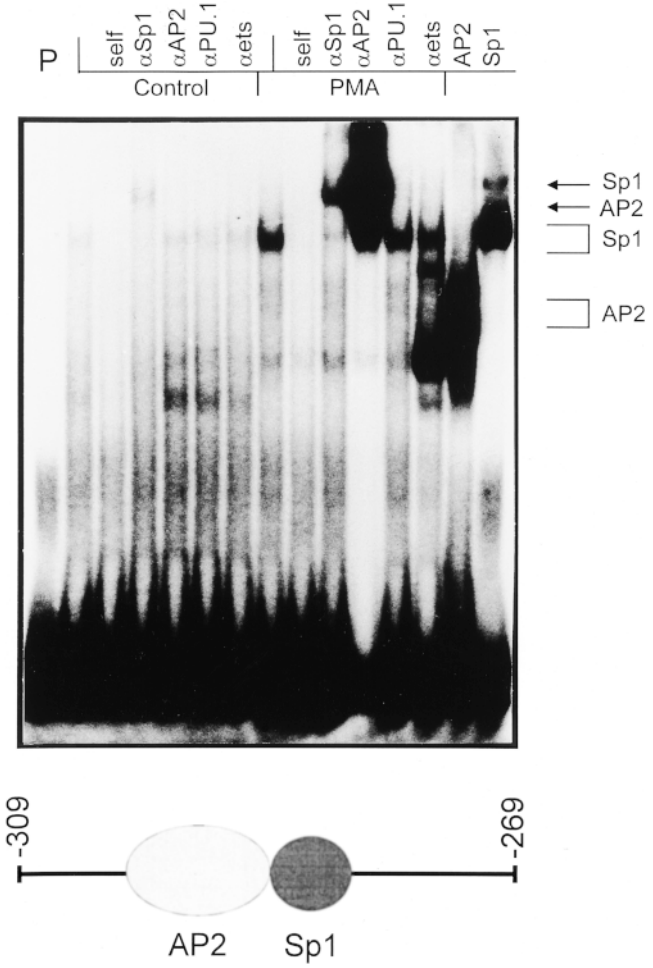

Fig. 5. Binding of Sp1 and AP-2 is enhanced in differentiated THP-1 cells. Nuclear extracts were isolated from untreated THP-1 cells (lanes 2–7), THP-1 cells treated with PMA for 24 h (lanes 8–13) or purified transcription factors (lanes 14, 15) and incubated with the radiolabeled, double-stranded oligomer SIF 3 (−310/−270), which contains adjacent binding sites for Sp1 and AP2. Lane 1 (P), free probe without nuclear extract; lane 2, extract from untreated THP-1 cells (control extract); lane 3 (self), 50-fold molar excess of unlabeled probe as competitor; lane 4, control extract plus Sp1 antibody; lane 5 control extract plus AP-2 antibody; lane 6, control extract plus PU.1 antibody, lane 7, control extract plus Ets antibody; lane 8, PMA extract; lane 9 (self), PMA extract plus 50-fold molar excess of unlabeled probe as competitor; lane 10, PMA extract plus Sp1 antibody; lane 11, PMA extract plus AP-2 antibody; lane 12, PMA extract plus PU.1 antibody, lane 13, PMA extract plus Ets antibody; lane 14 (AP-2), purified AP-2 protein; lane 15 (Sp1), purified Sp1 protein. Specific antibodies used are indicated above the lanes. Brackets indicate mobility of specific Sp1 and AP2 complexes. Arrows indicate supershifted complexes. Diagram below EMSA indicates the relative location of consensus binding sites within the probe.

or Ets consensus oligonucleotides did not (data not shown). Antibody supershift assays were performed with SIF3 to further characterize the transcription factors specifically interacting with this region. As shown in Fig. 5, proteins interacting with SIF 3 were recognized by antibodies against both the AP-2 and Sp1 transcription factors (lanes 10 and 11) but not by antibodies against PU.1 or Ets factors (lanes 12 and 13). Additional evidence for the binding of Sp1 and AP-2 comes from binding studies with purified transcription factors (Fig. 5, lanes 14 and 15) and from EMSAs using oligonucleotide SIF 3 MI which contains a mutant AP-2/Sp1 binding site (**Fig. 6**). No specific retardation bands are produced with SIF 3 MI and THP-1 nuclear extracts (Fig. 6, lanes 4 and 5), indicating that the mutant nucleotides are necessary for binding of Sp1 and AP-2. These experiments clearly demonstrate an inducible AP-2 and Sp1 binding activity in THP-1 cells treated with PMA.

To examine whether the additional consensus binding sites in the ASM promoter are functional, EMSAs with SIF 4 (−269/−229) and SIF 6 (−199/−149) were performed. SIF 4 contains two adjacent Sp1 binding sites and SIF 6 includes two neighbouring PU.1 and one Sp1 consensus sequence. Similar to SIF 3, nuclear extracts from induced THP-1 cells exhibited strong binding activity compared to uninduced extracts (**Fig. 7**) and supershift analysis demonstrated that Sp1 was present in the shifted complexes. In the presence of anti-Sp1 antibodies complex formation is reduced and a slower migrating complex is formed.

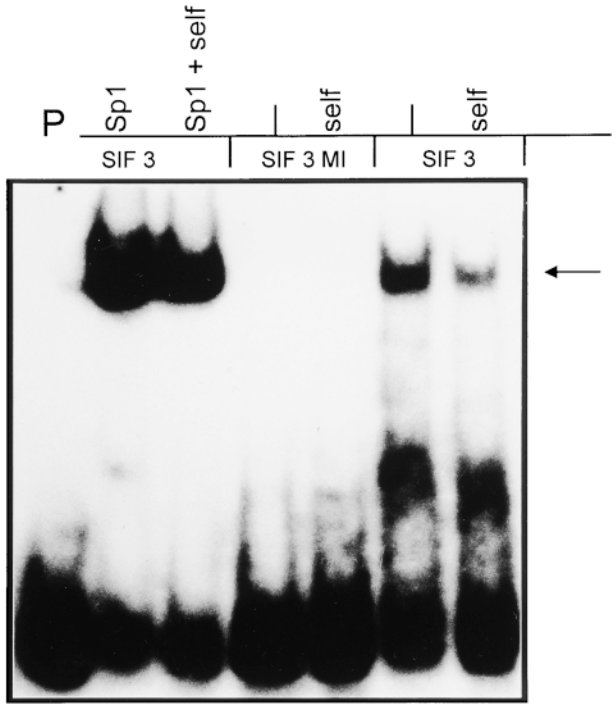

Fig. 6. Mutation of the Sp1/AP-2 site reduces specific binding of Sp1 and AP-2. Double-stranded oligonucleotides SIF 3 and SIF 3 MI, which contains a mutant AP2/Sp1 binding site, were incubated with nuclear extracts from PMA-induced THP-1 cells or purified transcription factor Sp1. Lane 1 (P), free probe without nuclear extract; lane 2 (Sp1), SIF 3 with purified Sp1; lane 3 (Sp1 self), SIF 3 with purified Sp1 plus 50-fold molar excess of unlabeled probe as competitor; lane 4, SIF 3 MI with PMA extract; lane 5 (self), SIF 3 MI with PMA extract plus 50-fold molar excess of unlabeled probe as competitor; lane 6, SIF 3 with PMA extract; lane 7 (self), SIF 3 with PMA extract plus 50-fold molar excess of unlabeled probe as competitor. Arrow to the right indicates the mobility of the specific complex.

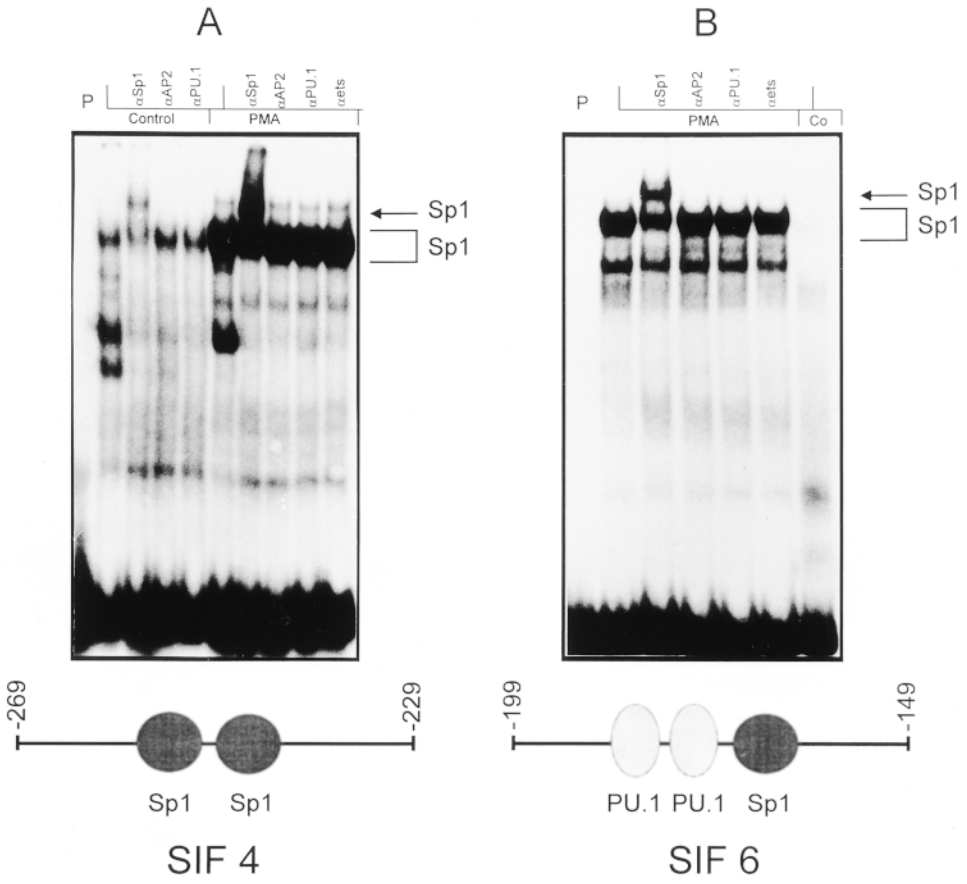

Fig. 7. Sp1 but not PU.1 binds to the putative sites in SIF 4 and SIF 6. Nuclear extracts were isolated from untreated THP-1 cells (control) or THP-1 cells treated with PMA for 24 h (PMA) and incubated with the radiolabeled, double-stranded oligomers SIF 4 (−269/−229) and SIF 6 (−199/−149). A) complexes formed with SIF 4. Lane 1 (P), free probe without nuclear extract; lane 2, extract from untreated THP-1 cells (control extract); lane 3, control extract plus Sp1 antibody; lane 4 control extract plus AP-2 antibody; lane 5, control extract plus PU.1 antibody, lane 6, PMA extract; lane 7, PMA extract plus Sp1 antibody; lane 8, PMA extract plus AP-2 antibody, lane 9, PMA extract plus PU.1 antibody; lane 10, PMA extract plus Ets antibody. B) complexes formed with SIF 6. Lane 1 (P), free probe without nuclear extract; lane 2, PMA extract; lane 3, PMA extract plus Sp1 antibody; lane 4, PMA extract plus AP-2 antibody, lane 5, PMA extract plus PU.1 antibody; lane 6, PMA extract plus Ets antibody; lane 7 (Co), extract from untreated THP-1 cells (control extract). Specific antibodies used are indicated above the lanes. Arrow indicates supershifted complex; bracket indicates specific complex. Diagram below EMSA indicates the relative location of consensus binding sites within the probe.

DNaseI footprint analysis of the ASM promoter

In order to demonstrate the binding of Sp1 to the promoter region spanning from nt −368 to nt −252, independently, DNaseI footprinting experiments were carried out. The promoter fragment was labeled at one end and either incubated with purified Sp1 protein obtained from a commercial source or HeLa nuclear extracts. As can be seen in **Fig. 8**, a convincing DNaseI protected region is detectable that colocalizes with the Sp1 binding site (GC-box) present in EMSA-fragment SIF 3. This site conforms well to the weight matrix analysis prediction of Bucher (36) for GC-boxes.

Expression of Sp1 protein is enhanced in PMA-stimulated THP-1 cells

We have found that Sp1 protein binds to the ASM promoter in differentiated THP-1 cells to a much higher extent than in undifferentiated THP-1 cells. To determine the nature of the mechanisms involved, we determined the levels of Sp1, c-Jun, and PU.1 proteins in nuclear extracts from both cell types. As shown in **Fig. 9**, the early response gene c-Jun is induced in PMA-treated THP-1 cells, whereas PU.1 levels are equal in both extracts. This is in accordance with the inducibility of c-Jun by PMA and the lack of change of PU.1 protein amount during the differentiation of monocytes to macrophages. The content of Sp1 protein in PMA-treated THP-1 cells was much greater than in uninduced THP-1 cells, in which Sp1 is nearly absent. This new finding may indicate that ASM induction in THP-1 cells is partially mediated by an increased synthesis of Sp1 and its binding to the promoter in a differentiation-dependent manner.

DISCUSSION

This study demonstrates that acid sphingomyelinase mRNA is induced in both the human monocytic leukemia

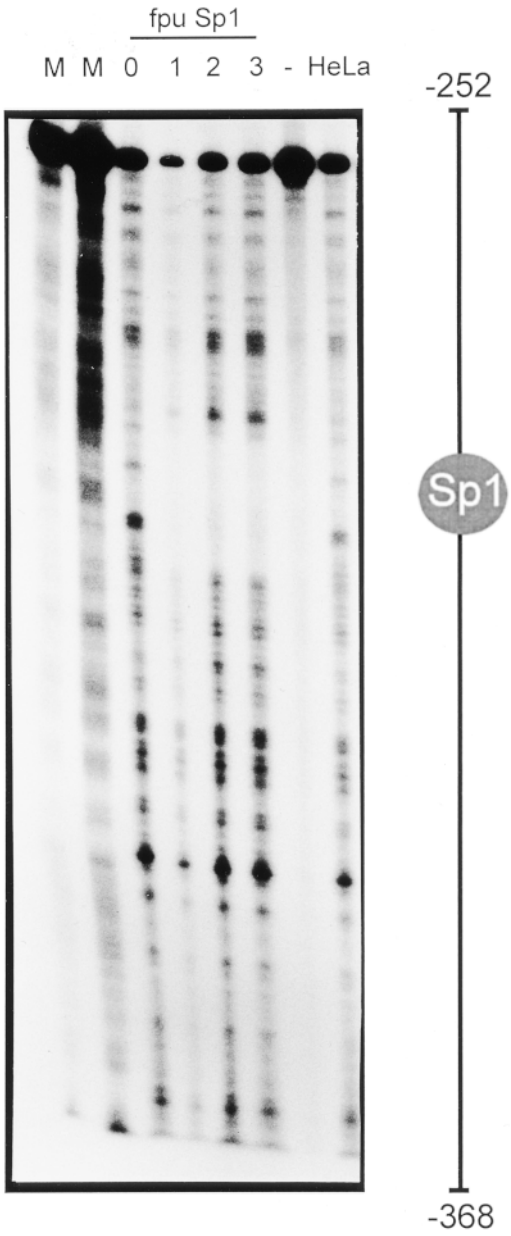

Fig. 8. DNase I footprinting identifies the binding of Sp1 to the −279 site. A DNA probe spanning from −368 to −252 was tested for its ability to bind purified Sp1 or proteins from a HeLa nuclear extract. Different Sp1 and DNase I concentrations were used as indicated. The Sp1 footprint is indicated schematically at the right. M, G + A sequencing ladder.

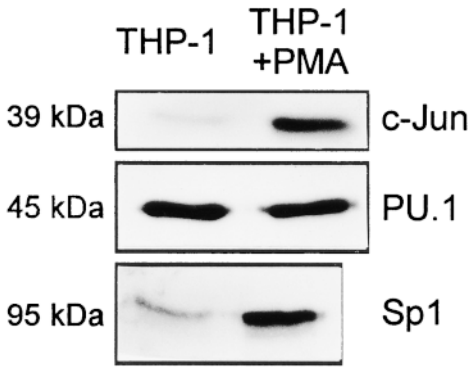

Fig. 9. Expression of Sp1 protein is induced in nuclear extracts from differentiated THP-1 cells. 20 μg of nuclear extracts from THP-1 cells or THP-1 cells treated with PMA for 24 hours were laid on the gel. After blotting, c-Jun, PU.1 or Sp1 protein was detected by incubation with a specific polyclonal antibody. A representative autoradiogram is shown.

cell line THP-1, when the cells are treated with PMA, and in differentiating mononuclear phagocytes (see Fig. 1). Nuclear run-on analysis of ASM expression (Fig. 2B) reveals that this up-regulation occurs mainly at the level of transcription. Experiments with actinomycin D provided evidence that ASM mRNA stability is not modulated during the monocytic differentiation process. ASM is expressed ubiquitously, but transcriptional induction is observed only in myelomonocytic cell lines such as U937, and THP-1, which have the ability to differentiate into macrophage-like cells upon PMA treatment, and in differentiating human peripheral blood monocytes. These results suggest that the increased ASM activity may reflect a characteristic function of mature macrophages, namely, phagocytosis of senescent cells and other particles and subsequent degradation of sphingomyelin derived from the internalized material. This provided the rationale for investigation of the regulation of ASM expression by analyzing *cis*-regulatory elements in the ASM promoter and the corresponding transcription factors. We studied the activity of five ASM promoter constructs by ligating them in front of the firefly luciferase reporter gene and used a transient transfection system in the THP-1 cell line. Promoter fragments p(−519/+112)Luc, p(−419/+112)Luc, and p(−319/+112)Luc exhibited basal and PMA-induced luciferase activity; however, all activity, including PMA-induced activity was lost using the shorter promoter fragments p(−219/+112)Luc and p(−119/+112)Luc. Thus, a promoter region that confers PMA-mediated transactivation of the ASM gene was identified. This GC-rich DNA sequence contains multiple transcription factor binding sites, including two adjacent binding sites for the transcription factors Sp1 and AP-2. These two factors are necessary for the basal and PMA-induced activation of the ASM promoter as demonstrated by transactivation studies and site-directed mutagenesis. This region was subsequently analyzed in EMSAs and DNaseI footprinting experiments. These analyses revealed a specific complex formation with Sp1 and AP-2 that is largely enhanced in nuclear extracts from differentiated THP-1 cells.

The ASM proximal promoter is GC-rich and lacks a TATA sequence motif. In the absence of a TATA box, mechanisms other than direct recruitment of TBPs have been implicated in initiating the basal transcription complex. Sp1 transcription factors have been shown to be important constituents for correct transcription initiation from TATA-less promoters (37–39). Although Sp1 is ubiquitously expressed, recent evidence suggests that Sp1 expression (40), binding affinity (41), and post-transcriptional modifications (42, 43) may be modulated to confer tissue-specific and developmental regulation on target genes (44). Sp1 expression varies greatly among tissues,

with hematopoetic cells containing very high levels of Sp1 mRNA and protein (40). Sp1 is involved in transcription of many cell-type specific genes, including the myeloid specific integrin CD11b (45), the monocytic specific gene CD14 (46), the liver specific α1 acid glycoprotein (47), and the α2 integrin gene (48). These observations appear to be valid for the ASM promoter as well, as our studies showed an increased binding affinity for Sp1 in EMSAs and enhanced promoter activity in co-transfection experiments using a Sp1 expression vector. In addition, elimination of the Sp1 binding sites in the ASM promoter resulted in an almost complete elimination of both basal and PMA-induced promoter activity. Recent studies showed that Sp1 is implicated in the PMA-induced expression of the WAF/CIP1 gene in U937 cells and the thromboxane receptor gene expression in K562 cells (49). To examine whether Sp1 protein levels increase during PMA-mediated differentiation of THP-1 cells, immunoblots with THP-1 nuclear extracts were carried out. Here, we demonstrate that the amount of Sp1 protein is markedly increased in PMA-induced THP-1 cells.

The transfection experiments and EMSAs also demonstrated that the transcription factor AP-2 is critically involved in the regulated expression of the ASM gene during monocyte to macrophage differentiation. AP-2 is an inducible nuclear factor controlling gene expression in embryonic development and cellular differentiation (50). AP-2 transactivation is subject to multiple regulatory mechanisms. Phorbol esters and cAMP-regulated signalling have been found to induce AP-2 activity independent of increased mRNA and protein levels (51). However, teratocarcinoma cells stimulated with retinoic acid show increased transcriptional activation of the AP-2 gene (52). AP-2 activity can also be down-regulated by an alternative splice product of its own gene (53, 54).

Sequence analysis of the ASM promoter revealed two binding sites for PU.1, a member of the Ets family of transcription factors, which is restricted to macrophages and B-cells (35). PU.1 was shown to be responsible for the cell-specific expression of several genes, including the macrophage scavenger receptor gene (29). Using EMSAs we provide evidence that PU.1 does not play a significant role in ASM gene expression in differentiating THP-1 cells.

In summary, we propose that the transcription factors Sp1 and AP-2 are required for the up-regulation of ASM expression during monocytic differentiation. In the future, it will be of great interest to study whether these two transcriptional activators might also regulate the expression of other genes during monocytic differentiation.

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