Transcriptional regulation of \textit{CHI3L1}, a marker gene for late stages of macrophage differentiation

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SUMMARY

The protein product of the CHI3L1 gene, human cartilage 39 kD glycoprotein (HC-gp39) is a tissue-restricted, chitin-binding lectin and member of the glycosyl hydrolase family 18. In contrast to many other monocyte/macrophage markers, its expression is absent in monocytes and strongly induced during late stages of human macrophage differentiation. To gain insights into the molecular mechanisms underlying its cell type-restricted and maturation-associated expression in macrophages, we initiated a detailed study of the proximal HC-gp39 promoter. Deletion analysis of reporter constructs in macrophage-like THP-1 cells localized a region directing high levels of macrophage-specific reporter gene expression to approximately 300 bp adjacent to the major transcriptional start site. The promoter sequence contained consensus binding sites for several known factors and specific binding of nuclear PU.1, Sp1, Sp3, USF, AML-1 and C/EBP proteins was detectable in gelshift assays. In vivo footprinting assays with DMS demonstrate that the protection of corresponding sequences was enhanced in macrophages compared to monocytes. Mutational analysis of transcription factor binding sites indicated a predominant role for a single Sp1-binding site in regulating HC-gp39 promoter activity. In addition, gelshift assays using nuclear extracts of monocytes and macrophages demonstrated that the binding of nuclear Sp1, but not Sp3, markedly increases during macrophage differentiation. Our results further highlight the important role of Sp1 in macrophage gene regulation.
INTRODUCTION

Human blood monocytes are able to differentiate into morphologically and functionally heterogeneous effector cells, including macrophages, myeloid dendritic cells, and osteoclasts (1). In contrast to the differentiation of precursor cells this development generally proceeds in the absence of proliferation. In myelomonocytic cell-types, several transcription factors have been identified that are important for lineage commitment, subsequent differentiation and cell type-specific gene regulation. These include members of the Ets-, C/EBP-, CBF-families and several other transcription factors (2,3). In particular the Ets family transcription factor PU.1 is essential for normal monocyte/macrophage differentiation and cell type-specific gene expression (4-6). The defect of PU.1−/− hematopoietic progenitors may be attributed to their failure to express the receptors for macrophage colony stimulating factor (M-CSF), granulocyte (G)-CSF and GM-CSF which are critical for progenitor cell survival and proliferation (7). PU.1 is also directly involved in the regulation of a large number of genes that are associated with myeloid and particularly with monocytic differentiation, including e.g. PU.1 itself (8), CD11b (9), CD18 (10), FcγRI (11), FcγRIII (12), c-fes (13), macrophage scavenger receptor (14), TFEC (15), or Toll-like receptor 4 (16). The acquisition of a mature macrophage phenotype is clearly distinct from monocytic lineage commitment and requires expression of a new set of genes. Also, most ‘macrophage-specific’ marker genes that have been studied previously are already expressed in peripheral blood monocytes. To gain insight into the molecular mechanisms of macrophage differentiation, we identified a number of genes that are specifically up-regulated during the differentiation of human blood monocytes into macrophages (17). Due to its unique cell-type restricted and maturation-associated expression, the gene CHI3L1 was chosen as a model to investigate late transcriptional events during macrophage differentiation.

The CHI3L1 gene product, human cartilage 39 kD glycoprotein (HC-gp39 also known as YKL-40), is a mammalian member of the glycosyl hydrolase family 18 (18). In contrast to chitotriosidase, a related active chitinase which is also expressed during late stages of macrophage differentiation (19), HC-gp39 lacks hydrolytic activity, probably due to a single amino acid substitution in the catalytic center region and acts as a chitin-specific lectin (20). In vivo, HC-gp39 levels are increased in serum, synovial fluid and
cartilage of rheumatoid arthritis patients (18;21) in various inflammatory disorders (22), in the liver of patients with alcoholic cirrhosis (23), in serum of patients with recurrent breast cancer (24) and colorectal cancer (25), and in macrophages in the atherosclerotic plaque (26). The described expression pattern suggests a putative role in inflammation, tissue remodeling and tumor metastasis. More recently, HC-gp39 was shown to stimulate proliferation of human connective-tissue cells and to activate both extracellular signal-regulated kinase- and protein kinase B-mediated signaling pathways, indicating a role as growth factor (27).

The expression of HC-gp39 is restricted to a small number of cell types (chondrocytes (18), synovial cells (18), neutrophils (28) and macrophages (17)) and shows a strict correlation with late macrophage differentiation in vitro - expression is undetectable in monocytes and only marginally in monocyte-derived dendritic cells (17). To approach the molecular mechanisms of gene expression during late stages of macrophage differentiation, we analyzed the expression of the gene for HC-gp39 (CHI3L1) at the transcriptional level. We cloned and sequenced the CHI3L1 gene (29) and initiated a detailed characterization of its macrophage-specific promoter. Several cis-elements important for HC-gp39 promoter activity in macrophages were defined and shown to bind Sp1-, C/EBP-, ETS- and CBF-family transcription factors in vitro. Interestingly, binding sites for tissue restricted transcription factors were largely dispensable for promoter reporter activity in THP-1 macrophages. Instead, a central role for Sp1 family factors was established in regulating HC-gp39 promoter activity.
MATERIALS AND METHODS

Chemicals. All chemical reagents used were purchased from Sigma-Aldrich (Berlin, Germany) unless otherwise noted. Protease inhibitors are from Roche Biochemicals. Oligonucleotides were synthesized by TIB Molbiol (Berlin, Germany). Antisera for supershift analyses were purchased from Santa Cruz.

Cells. Peripheral blood mononuclear cells (MNC) were separated by leukapheresis of healthy donors, followed by density gradient centrifugation over Ficoll/Hypaque. MO were isolated from MNC by countercurrent centrifugal elutriation in a J6M-E centrifuge (Beckman, München, Germany) as previously described (17). MO were > 85 % pure as determined by morphology and expression of CD14 antigen. Isolated MO were cultured in RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with vitamins, antibiotics, pyruvate, nonessential amino acids (all from Gibco BRL, Eggenstein, Germany), 5 x 10^{-8} \text{M} \beta\text{-mercaptoethanol}, 2 \% human pooled AB-group serum on teflon foils for a period of 7 days. The human monocytic cell lines THP-1 and U937 were grown in RPMI 1640 medium supplemented with plus 10 \% fetal calf serum (Gibco BRL, Eggenstein, Germany). The human cervical carcinoma cell line HeLa and the human melanoma cell line Mel Im (a gift from Dr. A. Bosserhof) were maintained in DMEM plus 10 \% fetal calf serum. Where indicated, cells were treated with PMA (10^{-8} \text{M}) or 1,25\text{-dihydroxyvitamin D}_3 (10^{-7} \text{M}, Hoffman-La Roche). Drosophila S2 Schneider cells (a gift from Dr. W. Falk) were cultivated in DES serum free medium (Invitrogen).

RNA-preparation and Northern analysis. Total RNA was isolated from different cell lines by the guanidine thiocyanate/acid phenol method (30). Electrophoresis, Northern blotting and cDNA hybridization were carried out as described previously (17). A 1200 bp cDNA of the coding region was used as HC-gp39-specific probe.

Gene cloning, plasmid construction and purification. A genomic library constructed in \lambda\text{FIXII} (Stratagene) was screened with a 1.3 kb probe corresponding to the proximal promoter region of HC-gp39 (HC-1300) that was previously generated using Promoter Walking PCR (29). Two genomic clones covering the 5' region of the CHI3L1 gene were identified by Southern analysis and gene specific PCR. From one of these clones, a 1.0 kB fragment of the proximal promoter was amplified using the Expand
High Fidelity PCR system (Roche Biochemicals) and the primers HC1023_S and HC1_AS.Introduced restriction sites for Nhe I and Bgl II were used for subcloning into the luciferase reporter vector pGL3-B (Promega) to yield the HC-1023 reporter plasmid. Larger fragments of the HC-gp39 promoter were released from genomic λ clones by restriction digest with Nco I/Apa I, and Sal I/Apa I, sequenced and subcloned into the existing HC-1023 plasmid, generating the -4.3 kB and -3.2 kB reporter constructs. Unidirectional deletions of the HC-1023 construct were generated using the Nested deletion Kit (Pharmacia). Subclones generated in steps of about 50-150 nucleotides were sequenced and used in reporter analysis. The HC-377 plasmid was generated by PCR using the primer HC377S and HC1AS. Mutation of individual binding sites and construction of chimeric constructs and constructs with specific internal deletions was done by PCR-mediated mutagenesis using primers in Supporting Table 2. PCR-fragments with correctly introduced alterations were subcloned back into the corresponding wild-type plasmid. Drosophila expression plasmids for human Sp1-family proteins were a gift from Dr. G. Suske. For transient transfections, plasmids were isolated and purified using the EndoFree Plasmid Kit from Qiagen.

**DMS in vivo footprinting.** Genomic footprinting was performed essentially as described (31). For each footprint reaction 10^7 cells were harvested, washed with phosphate buffered saline (PBS), resuspended in 1 ml of PBS, and incubated at room temperature for 1 minute with 5 µl of dimethyl sulfate. The reaction was stopped by the addition of 5 ml DMS stop solution containing 1% bovine serum albumin and 100µM β-mercaptoethanol in PBS. Cells were washed once more in DMS stop solution and twice more with PBS. Finally, cells were resuspended in 1 ml of PBS and genomic DNA was prepared. Footprinted DNAs were subjected to piperidine treatment (32). For visualization of footprints by LMPCR, 2 µg of sequenced or footprinted DNA were analyzed as described (33;34) with modifications (31). The primers for LMPCR are listed in Supporting Table 2. The first strand primer extension reaction was done in 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl, 2 mM MgSO₄, 0.1% TritonX-100, pH 8.8 [25°C] (Vent buffer, New England Biolabs), containing 0.3 pmol of primer LM1 of each set, 240 µM each dNTP, and 1 unit of Vent (exo-) DNA polymerase (New England Biolabs) for 5 minutes at 94°C, 30 minutes at 60°C, and 10 minutes at 72°C. For ligation of the common linker, the sample was transferred to ice and 5 µl of PCR
linker mix as in Mueller and Wold (34), 2 µl of ligation buffer (660 mM Tris-HCl, 50 mM MgCl₂, 10 mM dithioerythritol, 10 mM ATP, pH 7.5 [20°C], Roche), 1 µl of T4-DNA-ligase (5U/µl, Roche), and 12 µl of water were added. After an overnight incubation at 4°C the DNA was ethanol precipitated, washed once with 75% ethanol, dried, and then resuspended in water. The PCR amplification was done in 100 µl Vent buffer containing 10 pmol each primer LM2 of each set and the longer linker primer, 240 µM each dNTP, and 3 units of Vent (exo-) DNA polymerase for 20 cycles using 1 minute at 94°C, 1.5 minutes at 60°C, and 3 minutes at 72°C. For labeling, the sample was transferred to ice, 5 pmol of T4-kinase [γ³²P]-ATP labeled primer LM3 of each set, 2.5 nmol each dNTP, 0.5 units Vent (exo-) DNA polymerase in a volume of Vent buffer not exceeding 15 µl were added. Then the sample was heated to 94°C for 1.5 minutes, subjected to 8 cycles of 2 minutes at 94°C, 2 minutes at 62°C, and 5 minutes at 72°C, and kept at 72°C for 5 more minutes. Samples were phenol/chloroform extracted, ethanol precipitated, ethanol washed, and resuspended in loading dye. One fifth of each sample was separated on a standard 5% sequencing gel, and the gels were dried and autoradiographed for 15 hours at room temperature with Kodak BioMax MR film.

**DNA sequence analysis.** The cDNA sequencing was done by Dye Deoxy Terminator Cycle Sequencing (Applied Biosystem) according to the manufacturer's instructions and sequences were analyzed on the Applied Biosystems DNA Sequencing System (model 373A).

**Transient DNA transfections.** THP-1 cells were transfected using DEAE-dextran as described earlier (16). The two cell lines HeLa and Mel Im were transfected using Lipofectamine reagent (Gibco) according to the manufacturers instructions. Briefly, 4 x 10⁵ HeLa cells were transfected using 7 µl Lipofectamine and 1 µg total DNA (including 0.05 µg renilla control vector) and incubated for 5h before the addition of complete medium. 2.7 x 10⁵ Mel Im cells were transfected using 5 µl Lipofectamine and 1 µg total DNA (including 0.05 µg renilla control vector) and incubated for 6h before the addition of complete medium. The transfection mixture was removed after 24 h and substituted with complete medium. The transfected cell lines were cultivated for 48 h, harvested and cell lysates assayed for firefly and renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) on a Lumat LB9501 (Berthold). Firefly luciferase activity of individual transfections was normalized against renilla
luciferase activity. *Drosophila* S2 Schneider cells were transfected using Effectene reagent (Qiagen) according to the manufacturers instructions. Briefly, 4 x 10^6 Schneider cells were co-transfected using 10 µl Effectene reagent and 1.5 µg total DNA (1 µg of reporter plasmid, 0.5 µg of individual expression plasmids). Duplicate transfections were harvested after 48h and cell lysates assayed for firefly luciferase activity using the Luciferase Reporter Assay System (Promega). Firefly luciferase activity of individual transfections was normalized against protein concentration measured using a BCA assay (Sigma).

**Nuclear extracts and electrophoretic mobility shift assay.** Nuclear extracts from HeLa cells, THP-1 or in vitro differentiated human macrophages were prepared using NP-40 lysis as described (15). Nuclear extracts from freshly isolated human monocytes were prepared using identical buffers except that nuclei were released without detergent by shearing with 15-20 passages through a 26-gauge needle mounted in a 1 ml syringe. All buffers used contained 1 mM Na₃VO₄ and a cocktail of protease inhibitors (2 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 µg/ml pepstatin, 20 µg/ml bestatin, 5 µg/ml E46, 50 µg/ml antipain, 100 µg/ml chymostatin). Oligonucleotides were end-labeled with \[\gamma^{32}P\]-ATP using T4 polynucleotide kinase or with \[\alpha^{32}P\]dNTP using Klenow DNA polymerase. The binding reaction contained 2.5 µg of nuclear extract protein or 0.5 - 2 µl of the in vitro translation reaction, 0.5 µg of poly d(I/C), 20 mM HEPES pH 7.9, 60 mM KCl, 1mM DTT, 1mM EDTA, pH 8.0, 5% glycerol and 20 nmol of probe DNA in a final volume of 10 µl. Antisera used in supershift analyses were added after 15 min and samples were loaded onto polyacrylamide gels after standing at room temperature for a total of 30 min. Buffers and running conditions used have been described (15). Gels were fixed in 5% acetic acid, dried and autoradiographed.
RESULTS

Expression of HC-gp39 in human macrophages and myeloid cell lines. We recently identified HC-gp39 as a marker for late stages of macrophage maturation (17). To enable studies of the HC-gp39 promoter, several myeloid leukemia cell lines were analyzed for HC-gp39 expression. The myeloid cell lines were either left untreated or induced to differentiate into macrophage-like cells with 1,25-Vitamin D3 or PMA. Figure 1 shows a Northern analysis of the myeloid cell lines U937, THP-1 and MonoMac6 as compared to human blood monocytes and in vitro differentiated macrophages. No HC-gp39 expression was detectable in treated or untreated U937 cells. HC-gp39 was also undetectable in untreated MonoMac6, but was markedly induced by PMA. THP-1 cells expressed HC-gp39 without treatment, and expression was further increased by the addition of 1,25 Vit.-D3 or PMA. HC-gp39 mRNA expression in primary cells and cell lines also correlated with protein expression determined by Western-blot analysis (data not shown).

Tissue specific reporter activity of the 5’-proximal HC-gp39 promoter. To test whether the HC-gp39 promoter could confer cell type-specific activity in reporter assays, we cloned a 1.0 kb fragment of the 5’-proximal promoter region of the human CHI3L1 gene into a luciferase reporter plasmid (HC-1023). Transient transfection analysis was performed in the myeloid cell lines THP-1 and U937, and two non-myeloid cell lines, Mel Im (melanoma) and HeLa (cervical carcinoma), which do not express HC-gp39 mRNA (data not shown). Luciferase activities were normalized for transfection efficiency by co-transfection with a renilla construct and results for individual cell lines were compared relative to the activity of a positive control (a CMV-driven luciferase construct). As shown in Table 1, strong reporter activity was detectable only in THP-1 cells, which is consistent with endogenous HC-gp39 expression in this cell line. Similar results were obtained using a shorter (377 bp) fragment of the proximal promoter (s. Table 1).

Deletion analysis of the 5’-flanking HC-gp39 sequence region. To narrow down regions that are important for HC-gp39 promoter activity in macrophages, multiple deletion constructs were generated. λ-clones containing the promoter of the human CHI3L1 gene were isolated by hybridization screening. 4.4 kb of the proximal 5’-flanking region were subcloned into the pGL3 vector and sequenced. Deletions of
the 4.4 kb promoter were constructed by restriction digest or nested deletion. Transient transfection analysis was performed with reporter constructs of different length in the monocytic cell line THP-1 to identify regulatory sequences in the 5’-flanking HC-gp39 region. As shown in Figure 2, the two longest promoter constructs showed a relatively low reporter activity, indicating the presence of repressive transcriptional elements more than 1.3 kB 5’ of the transcriptional start site. Maximal reporter activity was observed with the -1.3 kb promoter fragment. Further deletion of the promoter to -433 bp caused a strong decrease in promoter activity. The activity was largely restored after the removal of a further 56 nucleotides, indicating another repressor element in this region. Deletion down to -144 bp resulted in a strong loss of activity. These results suggested that cis-elements important for the macrophage specific activity of the proximal promoter were located between base pairs -377 and –144.

**Structure of the proximal HC-gp39 promoter.** The initial deletion analysis localized a region directing strong macrophage specific reporter gene expression to approximately 300 bp proximal to the major transcriptional start site that was determined by primer extension in a previous study (29). In contrast to many other myeloid-specific genes the HC-gp39 promoter contains a TATA-box and a TATA-like CATA-element, both approximately 30 bp upstream of the two transcriptional initiation sites (sequence is shown in Supporting Figure 10). Within the proximal promoter region, several putative binding sites for ubiquitous transcription factors (Sp1, AP-1, AP-2, basic helix-loop-helix) and tissue restricted factors (e.g. C/EBP, AML-1) were identified by computer analysis using the MatInspector database (Genomatix). In addition we identified several purine-rich elements that might be bound by members of the Ets-family, including the myeloid and B-cell-specific transcription factor PU.1. To compare human and murine promoters, the corresponding promoter region of the putative mouse homologue brp39 was cloned. Sequence comparison with the human HC-gp39 promoter revealed a marked degree of conservation between the species man and mouse (Supporting Figure 10).

**Deletion analysis of the proximal HC-gp39 promoter.** To further localize cis-acting elements in the proximal HC-gp39 promoter, a more detailed deletion analysis was performed. A schematic representation of the luciferase constructs tested by transient transfection in THP-1 cells is shown in Fig. 3A. The region between bp -377 and -217 was largely dispensable for transient reporter gene expression.
Luciferase activity only dropped approximately 12% if this region was removed from the 5'-end. However, strong decreases in reporter activity were observed after deletion of the putative Sp1 (S) sequence and a putative binding site for bHLH-ZIP (B) transcription factors. Internal deletions were generated within the HC-377 construct which covered three sets of neighboring putative binding sites. Deletion of the putative C/EBP-AML-1 region (HC-377_ExAC construct) resulted in the loss of about 20% reporter activity, removal of the Sp1-bHLH-ZIP region (HC-377_ExSB construct) showed approximately 50% decreased reporter activity and deletion of the region between CATA and TATA box (HC-377_ExZT construct) had no impact on the reporter activity (Figure 3B).

**DNA-protein interactions in the proximal HC-gp39 promoter.** To analyze for protein binding sites within the proximal HC-gp39 promoter, *in vivo* footprinting with DMS was performed using freshly isolated monocytes, in vitro differentiated macrophages and THP-1 cells. The results for upper and lower strands are summarized in Figure 4. Both macrophages and THP-1 cells showed strong DMS-protected sites as well as several DMS-hypersensitive sites. Hypersensitive sites between the putative –206 Sp1-element, the –187 bHLH-element and upstream of the –137 ZF-site were undetectable in monocytes. Macrophage-specific DMS-protected sites corresponded to the putative –206 Sp1-, the –187 bHLH- as well as the -240 C/EBP element. In monocytes, protection of putative binding sites was mostly weak, although detectable at several sites. E.g. the –137 ZF-region located between the two TATA-boxes was partially protected in all three cell types. Genomic footprintings of THP-1 cells and macrophages were essentially the same, validating the use of THP-1 as a model for late macrophage expression of HC-gp39.

**Transcription factor binding to the proximal HC-gp39 promoter.** Gelshift experiments were performed to identify transcription factors that bind sequence elements determined by *in vivo* footprinting or consensus site analysis. As the importance of PU.1 recognition motifs for tissue-restricted expression of myeloid genes has been demonstrated in a growing number of cases, we tested the ability of PU.1 to bind elements within the human HC-gp39 promoter. EMSA was performed with double stranded oligonucleotides corresponding to two sequences containing a 5’-GGAA-3’ core sequence (hc-PU, and hc-Ets). Figure 5A and 5B show EMSAs using either in vitro translated PU.1 or nuclear extracts of THP-1 cells which specifically bound to both oligonucleotides. In both cases, complex formation was disrupted
by the addition of an excess amount of unlabelled wild-type oligonucleotide, but not by oligonucleotides with a mutated 5’-GGAA-3’ core sequence. Also, formation of both complexes was hindered in the presence of a PU.1-specific antiserum. In contrast to the hc-PU oligonucleotide which preferentially bound PU.1, several additional specific bands were detected using the hc-Ets oligonucleotide. Several antisera against possible binding proteins (STAT1, -3, and 5 as well as SRF) were used for super shift experiments - none of them altered the observed band pattern (data not shown).

The central region containing the putative Sp1 (-206) and bHLH (-187) factor binding sites were also analyzed using EMSA. As shown in Figure 5C, using cold competition and supershift analysis, complexes containing Sp1, Sp3, USF1 and USF2a were detectable, including high mobility complexes containing factors from both families. A C/EBP-AML1 motif (-240 and -247) also represented a good candidate region for myeloid specific gene regulation. EMSAs and supershift experiments performed with a corresponding double stranded oligonucleotide identified three specific complexes in THP-1 nuclear extracts containing either AML1, C/EBPα or both factors (s Figure 5D). Possible binding sites for C/EBP-factors are located both upstream and downstream of the AML-1 site. To determine which sequence(s) bind to C/EBP factors, short oligonucleotides covering either side were used as cold competitors. Only the downstream oligonucleotide effectively competed with the labelled probe, indicating that C/EBP proteins preferentially bind the downstream site.

**Mutational analysis of transcription factor binding sites.** Site-specific mutations at putative transcription factor binding sites were introduced into the HC-377 construct to determine the importance of individual sites for promoter activity in transfection assays. As shown in Figure 6 the activity of the proximal promoter in THP-1 cells was not completely abolished after mutation of any single binding site, which is consistent with the results obtained with internal deletion constructs (see Figure 3). Mutation of the central Sp1-binding site caused the greatest reduction of reporter activity to 50%. The mutated C/EBP site caused a 30% loss of reporter activity, whereas several other mutations reduced activity around 10-20%. The presence of an intact AML-1 site was not required for full reporter activity in THP-1 cells. In constructs bearing two or more mutations, the effects of individual mutations were mostly additive. After mutation of both the central Sp1 and the C/EBP site only 20% of the wild-type reporter activity was left. The presence of two TATA-like sequences as well as multiple binding sites probably allows alternative
arrangements of transcription factor complexes, still resulting in relatively strong reporter activity in THP-1 cells. Minimal constructs were generated lacking the region between CATA and TATA box and sequences upstream of the AML-1 site, reducing the proximal promoter to fewer binding sites and only one TATA-box to initiate transcription. As shown in Figure 6, luciferase activity of the minimal construct was similar to the wild-type construct. Mutation of the central Sp1-site had a markedly higher impact on reporter activity, further demonstrating the importance of this cis-element for HC-gp39 promoter activity.

**Analysis of elements required for PMA-induced activation of the proximal HC-gp39 promoter.** As shown in Figure 1, HC-gp39 expression is markedly up-regulated during PMA-induced differentiation of THP-1 cells. To test whether the observed induction was mediated through elements of the proximal promoter, transfection studies were performed comparing the activity of HC-377 constructs in THP-1 cells untreated or treated with PMA. As shown in Figure 7 the wild-type construct was induced approximately three-fold by PMA. Deletion of the C/EBP-AML-1 region had no significant impact on PMA-induced reporter activity and removal of the region between the CATA and TATA boxes resulted in a slight loss of PMA-induced activity relative to the wild-type construct. Deletion of the Sp1-bHLH region however showed a complete loss of PMA induction, indicating that cis-elements in this region are required for the observed effect of PMA. PMA induction was also completely lost with constructs bearing mutations in the Sp1 site, demonstrating its importance for the activating effects of PMA.

**Macrophage maturation-associated binding of nuclear factors to the Sp1-site.** The above findings strongly suggested an important role of the Sp1-element in the induction of HC-gp39 promoter activity in both monocyte to macrophage differentiation and PMA-induced differentiation of THP-1 cells. To compare the levels of nuclear Sp1 family proteins in cells of different maturation stages, gelshift assays were performed using a double stranded oligonucleotide corresponding to the Sp1-element. As shown in Figure 8, nuclear levels of Sp1 are markedly increased in mature macrophages as compared to freshly isolated human blood monocytes. Densitometric evaluation of band intensities revealed an approximately 4 – 5 fold increase in bound and supershifted nuclear Sp1 binding. We also noted the presence of an additional complex which was not supershifted with anti-Sp1-family antibodies, competed with Sp1/3 binding and was specific for mature macrophages. PMA-induced differentiation of THP-1 cells also
correlated with an increased binding of nuclear Sp1 and a concomitant loss of nuclear Sp3 binding (Figure 8). The additional complex observed in macrophages was absent in differentiated or undifferentiated THP-1 cells or HeLa cells.

**Analysis of the proximal HC-gp39 promoter by co-transfections in Drosophila Schneider cells.** To further validate the role of Sp1 family transcription factors as critical regulators of HC-gp39 promoter activity, we performed co-transfections in cells lacking endogenous Sp1-activity. The HC-377 construct was transfected into Drosophila Schneider cells together with expression plasmids for Sp1 family members (pPAC-Sp1, pPAC-USp3, pPAC-Sp3, pPAC-Sp4). As shown in Figure 9, both Sp1 and the long form of Sp3 (pPAC-USp3) were able to transactivate the HC-gp39 proximal promoter. A Drosophila expression plasmid for PU.1 (pPAC-PU.1, a gift from Dr. Susanne Müller) was also co-transfected alone or in combination with Sp1 or Sp3. No transactivation of the HC-gp39 proximal promoter was detectable with PU.1 alone and no collaboration or synergism was detectable with Sp1 or Sp3 (data not shown).
DISCUSSION

In the present study, we characterize the transcriptional regulation of human cartilage gp39 (HC-gp39) during macrophage differentiation. We describe a functional analysis of the proximal HC-gp39 promoter region and the identification of cis-elements and trans-acting factors that are important for the specific expression of HC-gp39 in macrophages.

In contrast to the differentiation process of myeloid precursor cells, differentiation of human blood monocytes in vitro and in vivo is typically not accompanied by proliferation. Since a number of regulatory mechanisms controlling differentiation are believed to require cell division, other mechanisms must operate during the differentiation of monocytes to achieve the functional and morphological heterogeneity of the terminally differentiated cells which include the various types of tissue macrophages, osteoclasts and myeloid dendritic cells. However, not much is known about the factors and mechanisms controlling gene expression during terminal monocyte differentiation. To gain insight into the regulatory mechanisms during this process it is necessary to study highly regulated model genes.

We have previously isolated a number of genes that are strongly up-regulated during macrophage differentiation (17). Among those, the CHI3L1 gene encoding the glycoprotein HC-gp39 appeared to be an ideal model gene to study transcription regulation during monocyte differentiation. HC-gp39 mRNA is undetectably low in peripheral blood monocytes and highly induced during in vitro differentiation of macrophages (17). As a starting point to unveil the molecular mechanisms behind its macrophage-restricted expression pattern, we cloned and analyzed the proximal promoter of the CHI3L1 gene. The proximal promoter was sufficient to confer cell-type specific reporter activity in a human macrophage cell line (THP-1) which was also found to express high endogenous levels of HC-gp39 mRNA. Levels of HC-gp39 were further induced by treatment of THP-1 cells with phorbolester PMA, an agent which induces the differentiation of THP-1 into an adherent macrophage-like cell type.

Reporter assays cannot be performed in primary human monocytes or macrophages. Therefore we used genomic footprinting analysis to identify sequence regions that are occupied by nuclear proteins in these cell types as compared to THP-1 cells. Footprints in monocyte-derived macrophages and THP-1 cells were strikingly similar, suggesting that the same promoter architectures and regulatory mechanisms operate in both cell types. Genomic footprintings in monocytes were very weak or missing as compared
with the two strongly HC-gp39 expressing cell types, indicating that the promoter in monocytes may only in part be accessible for transcription factors. Subsequent gelshift analyses with nuclear extracts from THP-1 cells identified a number of transcription factors binding the sequences that were protected in the footprinting analysis. Interestingly, the most striking changes in footprints were observed in a region that bound two ubiquitous transcription factors of the Sp1 and USF family. Other regions bound less ubiquitous or restricted transcription factors like C/EBP, AML-1 or PU.1. The latter regulatory proteins have all been implicated in myeloid- or macrophage-specific gene regulation (2;3) and therefore represented good candidates for an important role in the macrophage-specific regulation of HC-gp39. Deletion and mutation analysis, however, underlined the predominant importance of the Sp1-binding region. Specific mutation or deletion of the (-206) Sp1 site had the most profound effect on the likewise robust promoter and resulted in approximately 50% reduction of reporter activity. Probably due to the presence of two independent transcription start sites as well as the presence of multiple transcription factor binding sites, the HC-gp39 promoter was only mildly affected when other binding sites were mutated. Removal of one of the two transcription start sites resulted in an even stronger dependence on the (-206) Sp1-site, indicating a predominant role for Sp1-family factors in controlling HC-gp39 promoter activity. These observations are in line with a number of previous transfection studies implicating Sp1 family proteins in the activation of genes during monocyte to macrophage differentiation (37-42). Although Sp1 family proteins are ubiquitously expressed, their expression levels significantly vary during cellular differentiation of monocytic cell lines. Using gelshift assays, we confirmed previous observations of increasing nuclear Sp1-levels during phorbol ester-induced differentiation of THP-1 cells (38-40). In contrast to HL-60 cells, where phorbol ester-induced differentiation is accompanied by a strong increase in Sp3 expression (42), nuclear binding of Sp3 was diminished in differentiated THP-1 cells. In addition, we also demonstrate an increase in nuclear Sp1 binding to its HC-gp39 promoter binding site during the differentiation of primary macrophages, which correlates with HC-gp39 mRNA expression and has not been demonstrated before. Interestingly, an additional DNA-binding factor was identified in macrophages but not in monocytes, THP-1 or HeLa which competed with predominant Sp1 family proteins for binding to the Sp1-element. Its identity and its role in the regulation of HC-gp39 needs to be clarified. Its absence in THP-1 cells may however indicate that its presence is not a prerequisite for HC-gp39 expression in
macrophages.

It is now well documented that the expression of a gene depends on a number of additional elements, including enhancers or silencers located upstream or downstream of the proximal promoter that control local chromatin structure, CpG methylation and accessibility of the promoter (43). The weak but detectable DNA-protein interactions at the proximal promoter suggests that the CHI3L1 gene in monocytes is in an inactive or unstable, yet primed state, which may require additional events (e.g. nucleosome remodeling) that may be initiated by additional elements upstream or downstream of the promoter. Such additional elements, yet to be discovered, likely contribute to the observed tissue-specificity of HCgp-39 expression in vivo. The tissue-restricted transcription factors that were identified to bind promoter elements in macrophages may actually serve to establish the interaction between upstream or downstream elements. Indeed, AML-1, C/EBP factors as well as PU.1 are known to interact with each other and with other factors (44-49). Further investigations will be needed to clarify these issues.

In conclusion, we have presented a thorough analysis of promoter elements controlling the expression of HC-gp39, a marker for late stages of macrophage differentiation. Our findings suggest that the ubiquitous transcription factor Sp1 is markedly up-regulated during monocyte to macrophage differentiation and is an important regulator of HC-gp39 and likely of other genes with a maturation-associated expression pattern in macrophages.

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Footnotes

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Figure Legends

Fig. 1  **Northern analysis of HC-gp39 expression in myeloid cell lines.** Total RNA was isolated from three myeloid leukemia cell lines U937, THP-1 and MonoMac6, either treated with Vitamin D3, PMA or untreated as well as from freshly isolated human blood monocytes (MO), and *in vitro* differentiated macrophages (MAK). The blots were hybridized with a $^{32}$P-labeled HC-gp39 cDNA. The ethidium bromide staining is shown as a control for mRNA loading.

Fig. 2  **Deletion analysis of the human HC-gp39 promoter.** Each deletion mutant was transiently transfected into myeloid THP-1 cells. Luciferase activity is relative to a CMV promoter-driven positive control and values are the mean ± SD obtained from at least 3 independent experiments.

Fig. 3  **Deletion analysis of the proximal human HC-gp39 (HC-377) promoter.** Deletion mutants (A.) and internal deletions (B.) of the proximal –377 bp HC-gp39 promoter were transiently transfected into myeloid THP-1 cells. (A.) A corresponding construct of the proximal brp39 promoter is shown in comparison. Luciferase activity is relative to a CMV promoter-driven positive control (A.) or to the HC-377 construct (B) and values are the mean ± SD obtained from at least 3 independent experiments.

Fig. 4  **In vivo footprinting analysis of the proximal HC-gp39 promoter.** (A) Genomic footprinting analyses of freshly isolated monocytes, *in vitro* differentiated macrophages and THP-1 cells were performed. The G-lanes show LM-PCRs of Maxam-Gilbert G reactions of naked DNA. The F-lanes show LM-PCRs of DNA from DMS-treated cells. (B) Sequence of the proximal promoter and a summary of genomic footprinting results. Circles indicate diminished DMS reactivity and rhombs indicate enhanced DMS reactivity as compared to naked DNA. Putative transcription factor binding sites and their positions relative to the start codon are indicated on the right (A) or on top of the sequence (B).

Fig. 5  **In vitro binding of nuclear proteins to HC-gp39 promoter sequences.** Labeled double-stranded oligonucleotides containing putative binding sites for (A.) PU.1 (site P (-100)), (B.) Ets/STAT
(site E (-157)), (C.) Sp1 and USF (site S (-206) and site B (-187)), and (D.) AML1 and C/EBP (site A (-247) and site C (-240)) were used in EMSA with THP-1 nuclear proteins as described in Materials and Methods. Addition of unlabeled oligonucleotides for competition analysis or antisera against several transcription factors are indicated above each lane. Specific complexes are marked with arrows, antibody supershifts with ‘SS’ and unspecific complexes with an asterisk. Question marks indicate specific complexes containing unknown nuclear proteins.

Fig. 6  **Effect of site-directed mutations on the HC-377 promoter activity in myeloid THP-1 cells.**
Site-directed mutations, were introduced in the HC-377 and the minimal –263 promoter construct using oligonucleotides listed in Supporting Table 2. A schematic representation of the reporter gene constructs is shown. Putative binding sites are indicated as boxes: site A (AML, –247), site C (C/EBP, –240), site S (Sp1, –206), site B (USF, –187), site E (Ets/STAT, –157), site Z (ZF, –127), site P (PU.1, –100). The position of the TATA-boxes as well as transcription start sites previously determined by primer extension analysis are indicated. Mutations are marked by crosses. Each site-directed mutant was transiently transfected into the myeloid cell line THP-1. Luciferase activity is relative to the HC-377 wild-type promoter (100%) and values are the mean ± SD obtained from at least 4 independent experiments.

Fig. 7  **Mutational analysis of elements required for PMA-induced activation of the HC-377 promoter.** The indicated wild-type and mutant constructs of the proximal –377 bp HC-gp39 promoter were transiently transfected into myeloid THP-1 cells that were treated or untreated with PMA after transfection. Fold induction of treated and untreated cells was calculated relative to the promoterless pGL3-basic vector and values are the mean ± SD obtained from at least 4 independent experiments.

Fig. 8  **In vitro binding of nuclear proteins to the HC-gp39 –206 Sp1-element.** Labeled double-stranded oligonucleotide corresponding to the –206 Sp1-site (hc-S: 5'-AGT CAG TGG AGG CGG GGG AGT CAG-3') was used in EMSA for a comparative analysis of nuclear protein complexes from human blood monocytes (MO) and in vitro differentiated macrophages (MAK), untreated or PMA-differentiated
THP-1 cells and HeLa cells. Addition of antisera against transcription factors are indicated above each lane. Specific complexes are marked with arrows, antibody supershifts with ‘SS’ and unspecific complexes with an asterisks. Specific complexes containing the unknown nuclear factor are marked with an X.

Fig. 9  **Cotransfection analysis in *Drosophila* Schneider cells.** The indicated HC-gp39 promoter constructs were cotransfected with expression plasmids for Sp1, short and long forms of Sp3 (pPAC-Sp1 and pPACUSp3, respectively) and Sp4 into *Drosophila* Schneider cells as described in Experimental Procedures. Relative luciferase activities are relative to the control transfection (without expression plasmids) and values are the mean ± SD obtained from 3 independent experiments.
Table 1

Tissue-specific activity of the proximal HC-gp39 promoter

|        | HC -1023 (RLU) | HC -377 (RLU) |
|--------|---------------|---------------|
| THP-1  | 96.9 (± 15.0)  | 60.4 (± 4.7)  |
| U937   | 1.0 (± 0.9)    | 0.4 (± 0.2)   |
| HeLa   | 1.3 (± 0.6)    | 0.8 (± 0.2)   |
| Mel Im | 0.8 (± 0.5)    | N.D.          |

Values are % luciferase activities relative to a CMV-driven control vector and are means (± S.D.) from at least 4 independent experiments.
Figure 1
Figure 2

RLU (% CMV-luciferase)

0  20  40  60  80  100  120

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Figure 3
Figure 4

A.

lower strand

|   |   |   |   |   |   |
|---|---|---|---|---|---|
| MO | G | F | G | F | G |
|    |   |   |   |   |   |
| -156 |   |   |   |   |   |
| Ets/STAT (-157) |
| -156 |   |   |   |   |   |
| Ets (-180) |
| -156 |   |   |   |   |   |
| USF (-187) |
| -156 |   |   |   |   |   |
| Sp1 (-206) |
| -257 |   |   |   |   |   |
| C/EBP (-240) |

upper strand

|   |   |   |   |   |
|---|---|---|---|---|
| MO | G | F | G | F |
|    |   |   |   |   |
| -246 |   |   |   |   |
| AML (-247) |
| -246 |   |   |   |   |
| USF (-187) |
| -246 |   |   |   |   |
| ? |
| -112 |   |   |   |   |
| ZF (-137) |
Figure 4

B.

AML-1 C/EBP SP1 bHLH ets

STAT/ets cata ZF (SP1/AP-2) tata PU.1

GTTTTCAAAACTCTGCGTCTCAACCCTCCTCTGGTAATCCATAGTACTCCCCCGCCTCCACTTGCCAGCCTCGTGATTCCTTCATTGC
AAAAGTTTTGAGACGCCAAAGAGTTGGGAGGAGACCATTAGGTATCATGAGGGGGCGGAGGTGTTCGGTCGGAGCACTAAGGAAGTAAC

ACACATAGCTCAGTTCCCATAAAAGGGCTGGTTTGCCGCGTGGGGGAGTGGAGTGGGACAGGTATATAAAGGAAGTACAGGGCCTGGGGA
TGTGTATCGAGTCAAGGGTATTTTCCCGACCAAACGGCGCACCCCCTCACCTCACCCTGTCCATATATTTCCTTCATGTCCCGGACCCCT

by guest on March 23, 2020
Figure 5

A. hc-PU

B. hc-Ets

C. hc-SB

D. hc-CAC

[Images and figures are shown with specific DNA sequences and binding sites marked with asterisks and question marks.]

Competitor PU-Oligo
Competitor PU-M-Oligo
Antiserum

NE THP-1

[Tables with competitive binding assays and images of gel electrophoresis results showing the binding of different proteins to the DNA sequences.]
Figure 7
Figure 8

| Antiserum | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|-----------|---|---|---|---|---|---|---|---|---|----|----|
| NE MO     | - | + | - | - | + | - | + | - | - | +  | -  |
| NE MAK    | - | - | + | - | - | + | + | - | - | -  | +  |

hc-Sp (-198 – -214)

| 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|----|----|----|----|----|----|----|----|----|
| NE |  + |  + |  + |  + |  + |  + |  + |  + |

Sp1

Sp3

X

Sp1&3
Figure 9
Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation
Michael Rehli, Hans-Helmut Niller, Christoph Ammon, Sabine Langmann, Lucia Schwarzfischer, Reinhard Andreesen and Stefan W. Krause

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