

# Granulocyte-Macrophage Colony-Stimulating Factor, Phorbol Ester, and Sodium Butyrate Induce the CD11c Integrin Gene Promoter Activity During Myeloid Cell Differentiation

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To analyze the activity of the CD11c promoter during myeloid differentiation without the limitations of transient expression systems, we have stably transfected the myeloid U937 cell line with the pCD11c361-Luc plasmid, in which the expression of the firefly luciferase cDNA is driven by the CD11c promoter region -361/+43, previously shown to confer myeloid specificity to reporter genes. The stable transfectants (U937-C361) retained the ability to differentiate in response to phorbol-ester (PMA), sodium butyrate (SB), granulocyte-macrophage colony-stimulating factor (GM-CSF), and other differentiating agents. U937-C361 differentiation correlated with increased cellular luciferase levels, showing the inducibility of the CD11c promoter during myeloid differentiation and establishing the U937-C361 cells as a suitable system for studying the myeloid differentiation-inducing capacity of cytokines, growth factors, and other biological response modifiers. Unexpectedly, the inducibility of the CD11c gene promoter showed distinct kinetics and

**D**URING MYELOID DIFFERENTIATION, hematopoietic stem cells become committed to myeloid precursors that later differentiate into fully functional end-stage cells, a process which is accompanied by the coordinate expression and repression of numerous genes.<sup>1</sup> Myeloid leukemic cells are arrested at distinct stages of the differentiation pathway, and a number of myeloid leukemic cell lines have been established that can be induced to acquire a more mature phenotype and, therefore, have been used to study the expression of myeloid differentiation-regulated genes.<sup>1</sup> The potential therapeutic implications of differentiation-inducing agents have been underscored by the successful application of the differentiation therapy in patients with acute promyelocytic leukemia.<sup>2</sup>

Myeloid cell adhesion and differentiation are closely inter-regulated processes. Although adhesion to extracellular matrix components and neighboring cells is required for the proper completion of the cellular differentiation program,<sup>3</sup> the pattern of expression of adhesion receptors greatly changes during myeloid differentiation.<sup>4</sup> Most cell-adhesive interactions are mediated by Integrins, a family of cell surface heterodimeric glycoproteins involved in both intercellular adhesion and in cell-extracellular matrix interactions. The members of the  $\beta 2$  integrin subfamily, ie, lymphocyte function-associated antigen-1 (LFA-1), Mac-1, and p150,95 (CD11a-c/CD18), which play a key role in myeloid cell/endothelium interactions during immune and inflammatory reactions,<sup>5</sup> are leukocyte-specific. LFA-1 is expressed on all leukocytes, whereas Mac-1 and p150,95 are almost exclusively expressed on myeloid cells.<sup>5,6</sup> The expression of the  $\beta 2$  integrins is tightly regulated during cellular differentiation.<sup>7,8</sup> Myeloid progenitor cells sequentially acquire the expression of the leukocyte integrins during the early stages of differentiation,<sup>8</sup> and terminally differentiated myeloid cells express LFA-1, Mac-1, and p150,95.<sup>5-8</sup> The expression of Mac-1 and p150,95 is induced during differentiation of the

magnitude on the PMA-, SB-, or GM-CSF-triggered differentiation. Moreover, SB synergized with either PMA or GM-CSF in enhancing both the CD11c promoter activity and the cell surface expression of p150,95 on differentiating U937 cells. Furthermore, we showed the existence of a c-Myb-binding site at -85, the importance of the -99/-61 region in the CD11c promoter inducibility during PMA- or SB-triggered differentiation, and the dependency of the GM-CSF and PMA responsiveness of the CD11c promoter on an intact AP-1-binding site located at -60. These results, together with the lack of functional effect of mutations disrupting the Sp1- and Myb-binding sites within the proximal region of the CD11c promoter, indicate that the myeloid differentiation pathways initiated by SB and phorbol esters (or GM-CSF) activate a distinct set of transcription factors and show that the myeloid differentiation-inducibility of the CD11c gene maps to the -99/-53 proximal region of the promoter.

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myeloid cell lines HL-60 and U937, because of transcriptional mechanisms acting on their respective  $\alpha$ -subunit genes.<sup>9</sup> The characterization of the CD11b<sup>10,11</sup> and CD11c<sup>12</sup> gene promoter regions has shown their involvement in the tissue-specific expression of Mac-1 and p150,95<sup>10-12</sup> and has shown the presence of initiator (Inr) sequences at their respective transcriptional start sites.<sup>10-12</sup>

U937 cells, a histiocytic lymphoma line with monoblast-like characteristics, can be induced to differentiate towards morphologically mature macrophage-like cells after treatment with agents such as phorbol myristate acetate (PMA),<sup>1,13</sup> granulocyte-macrophage colony-stimulating factor (GM-CSF),<sup>7,14</sup> or sodium butyrate (SB).<sup>15</sup> To define the molecular basis for the p150,95 integrin induction during myeloid differentiation, U937 cells were stably transfected with a CD11c promoter-based reporter gene construct (U937-C361) and were used to characterize the activity of

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the CD11c gene promoter in response to differentiation-triggering agents. Our results point out the usefulness of the derived U937-C361 cell line in determining the differentiation-inducing ability of other growth factors, cytokines, and biological response modifiers, show the inducibility of the CD11c gene promoter during the PMA-, SB-, or GM-CSF-stimulated myeloid differentiation, and identify DNA sequence elements that confer the differentiation-inducibility to the CD11c promoter.

## MATERIALS AND METHODS

**Cell culture and transfection.** U937 cells were grown in RPMI supplemented with 5% fetal calf serum, 2 mmol/L glutamine, and 50  $\mu$ g/mL gentamicin (complete medium), at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. For stable transfectants, U937 cells ( $1$  to  $2 \times 10^7$ ) were electroporated (100 V, 2,950 microfarads, 186  $\Omega$ ) in 500  $\mu$ L RPMI containing 40  $\mu$ g of BamHI-linearized pCD11C361-Luc and 10  $\mu$ g of BamHI-linearized pSV2-neo.<sup>12,16</sup> After transfection, cells were suspended in complete medium, cultured for 48 hours, and maintained in complete medium supplemented with G418 (1.5 mg/mL). Once cell growth was evident, cells were harvested, and the G418-selected population (U937-C361) was subjected to cloning by limiting dilution. For induction of differentiation, U937 or U937C-361 cells were resuspended in fresh complete medium ( $5 \times 10^5$ /mL), cultured for 9/12 hours and then treated with PMA (10 ng/mL), 1-oleoyl-2-acetyl-glycerol (OAG; 20  $\mu$ g/mL), GM-CSF (100 U/mL), 1,25 dihydroxyvitamin D3 ( $10^{-8}$  mol/L; kindly provided by Dr M. Uskokovic, Hoffmann-La Roche), dimethyl sulfoxide (DMSO; 1.4%),  $\gamma$ -interferon (200 U/mL), etoposide (VP-16,  $2 \times 10^{-7}$  mol/L), amsacrine (AMSA;  $10^{-7}$  mol/L), or SB (1 mmol/L) for up to 5 days. At the indicated time points, cells were harvested, washed, counted, and lysed. Unless otherwise indicated, luciferase determination was usually performed on  $10^5$  cells from each culture condition using a Berthold Lumat LB 9501 luminometer, as described,<sup>12</sup> and measuring light emission for 20 seconds. Output was quantitated as relative light units (RLU).

The deletion mutants from the CD11c promoter pCD11C361-Luc, pCD11C253-Luc, and pCD11C160-Luc, containing the regions -361/+43, -253/+43, or -160/+43, respectively, have been previously described.<sup>12</sup> Additional deletion constructs pCD11C99-Luc, pCD11C61-Luc, and pCD11C53-Luc, including the regions -99/+43, -61/+43, and -53/+43, were constructed after polymerase chain reaction (PCR) amplification of the corresponding fragments and ligation into the pXP2 plasmid. Site-directed mutagenesis of the Sp1-70 site into a non-Sp1-binding sequence was performed by standard procedures<sup>17</sup> and will be described elsewhere (C. López-Rodríguez, A.L. Corbí, manuscript submitted). The reporter plasmid containing a mutation at Sp1-70 was termed pCD11C160(-70MUT)-Luc. The pCD11C361(-85MUT)-Luc plasmid, was derived from pCD11C361-Luc after mutation of the Myb-binding site at -85 by site-directed mutagenesis<sup>17</sup> using an oligonucleotide later used for electrophoretic mobility shift assay (EMSA) studies (mBoxD, see below). pCD11C160(-85)-Luc was constructed by PCR on pCD11C361(-85)-Luc, using oligonucleotides (-160) 5'-CTTGGATCCAAGCCAAGTCATCTGATGAGAG-3' (-138) (sense, CD11c pXP2 -160) and (+43) 5'-GATCTCGAGCTCCTGGCCG-3' (+28) (antisense, CD11c pXP2 +43) as PCR primers to amplify the whole CD11c promoter-derived insert and using further cloning into pXP2. The pXP2-derived pCD11A100-Luc plasmid, in which the expression of the luciferase cDNA is driven by the -100/+83 fragment from the CD11a promoter,<sup>16</sup> was used as additional control in some experiments. All mutations and constructs were confirmed by sequencing and were electroporated in U937 cells, as described.<sup>12</sup> After transfection, cells were split into 4 aliquots and

either were left untreated or were cultured in the presence of PMA, GM-CSF, or SB. Fourteen hours after transfection, luciferase activities were determined. For comparative purposes, the "Promoter Activity Induction" of the differentiating agents on each construct was defined as the ratio of RLU in treated transfected cells to RLU in untreated transfected cells, after background subtraction and normalizing for cell number in each culture condition. A similar calculation was used to determine the effect of the differentiation-inducing agents on the luciferase activity in U937-C361 cells. When necessary, transfection efficiencies were normalized by including 20  $\mu$ g of the pSV $\beta$ gal plasmid in the electroporation mixture, and luciferase activities were determined on cell extracts containing identical  $\beta$ -galactosidase activity. In all cases, at least two distinct plasmid preparations of each deletion construct were tested by transfection.

**Immunofluorescence analysis.** U937 cells or U937-C361 transfectants were subjected to treatment with differentiation agents as indicated, harvested, washed in ice-cold phosphate-buffered saline, and resuspended in 100  $\mu$ L of the following hybridoma culture supernatants: Bear-1 (anti-CD11b), HC1/1 (anti-CD11c), and TS1/18 (anti-CD18). After washing, bound antibodies were detected using fluorescein-conjugated F(ab')<sub>2</sub> rabbit antimouse Ig, and cells were analyzed on a FACScan cytofluorometer. All incubations were performed in the presence of 1% human AB serum to prevent binding through the Fc portion of the antibodies. The supernatant from the myeloma P3X63 was included as negative control.

**Southern and Northern blot.** Genomic DNA was isolated from the selected U937-C361 clones, and 10- $\mu$ g aliquots were digested with EcoRI and BamHI. After electrophoresis and transfer onto nylon membranes using 10 $\times$  saline sodium citrate (SSC), filters were pre-hybridized and hybridized as described,<sup>16</sup> using a 616-bp Xho I-EcoRI fragment from the luciferase cDNA and a 404-bp BamHI-Xho I fragment from the CD11c promoter as probes. Both DNA fragments are contiguous in the pCD11C361-Luc plasmid used to establish the stable transfectants.<sup>12</sup> Membranes were sequentially washed in 2 $\times$  SSC and 0.5% sodium dodecyl sulfate at room temperature (for 45 minutes) and in 0.3 $\times$  SSC and 0.5% sodium dodecyl sulfate at 37°C (for 15 minutes) and 65°C (10 minutes), and were exposed at -70°C with an intensifying screen. Northern blot was performed on total RNA isolated from untreated and PMA-, GM-CSF-, or SB-treated U937-C361 cells,<sup>9</sup> using a 0.8-kb Pst I fragment from the CD11c cDNA, a 1.2-kb EcoRI-HindIII fragment from the c-myc cDNA, and a 1-kb Pst I fragment from the  $\beta$ -actin cDNA,<sup>9</sup> as probes.

**EMSA.** A total of 50 ng of the CD11c promoter-based double-stranded oligonucleotides BoxD, mBoxD, or 2xMyb oligo<sup>18</sup> were labeled using avian myeloblastosis virus (AMV) reverse transcriptase (7 U) and 50  $\mu$ Ci of either <sup>32</sup>P-deoxyguanosine triphosphate or <sup>32</sup>P-deoxycytidine triphosphate (specific activities,  $\approx 10^8$  cpm/ $\mu$ g). A total of 0.5 ng of probe was then incubated at 4°C with 2  $\mu$ L of bacterial extracts containing c-Myb or with control bacterial extracts in 20  $\mu$ L containing 28 mmol/L EDTA, 15 mmol/L KCl, 6 mmol/L MgCl<sub>2</sub>, 7 mmol/L HEPES (pH 7.9 at 4°C), 7% glycerol, 1 mmol/L dithiothreitol, and 2.5  $\mu$ g poly (dI-dC). Unlabeled competitor oligonucleotides were added at 500 molar excess in competition assays and were incubated at 4°C for 15 minutes before the addition of the radioactive probe. Binding reactions were incubated for 20 minutes at 4°C, and 1.5  $\mu$ L of a 10 $\times$  loading buffer (10 mmol/L HEPES, 10% glycerol, 0.01% bromophenol blue) was added to the reaction. A total of 12  $\mu$ L of each reaction was separated by electrophoresis at 15 V/cm and 4°C on 4% to 5% polyacrylamide gels containing 0.4 $\times$  TBE (45 mmol/L Tris base, 45 mmol/L boric acid, 1 mmol/L EDTA) until free probe was close to the bottom of the gel, and with buffer recirculation every 15 minutes. The oligonucleotides used for EMSA were the CD11c promoter-derived BoxD (-94 CCTCGGATCAGTTG CGTACTCTGCC -70); mBoxD (5'-CCTCGGATGTC-

GACCGTACTCTGCC-3'),<sup>12</sup> which has mutated the putative Myb- or bHLH-binding site CANNTG; 2xMyb (5'-TAGAATAACGG AAGCAATAACGG AA-3'), which includes two Myb-binding sites; and m(2xMyb) (5'-TAGAATCCAGGAAGCAATCCAGG AA-3'), which has both Myb-binding sites mutated.<sup>18</sup> Extracts from bacteria transformed with the c-Myb cDNA in pFLAG-1 or the control empty vector (kindly provided by Drs T. Bellon and B. Calabretta; Jefferson Cancer Institute, Philadelphia, PA) were prepared by sonication of bacterial cultures grown in the presence of isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) for 5 hours.

## RESULTS

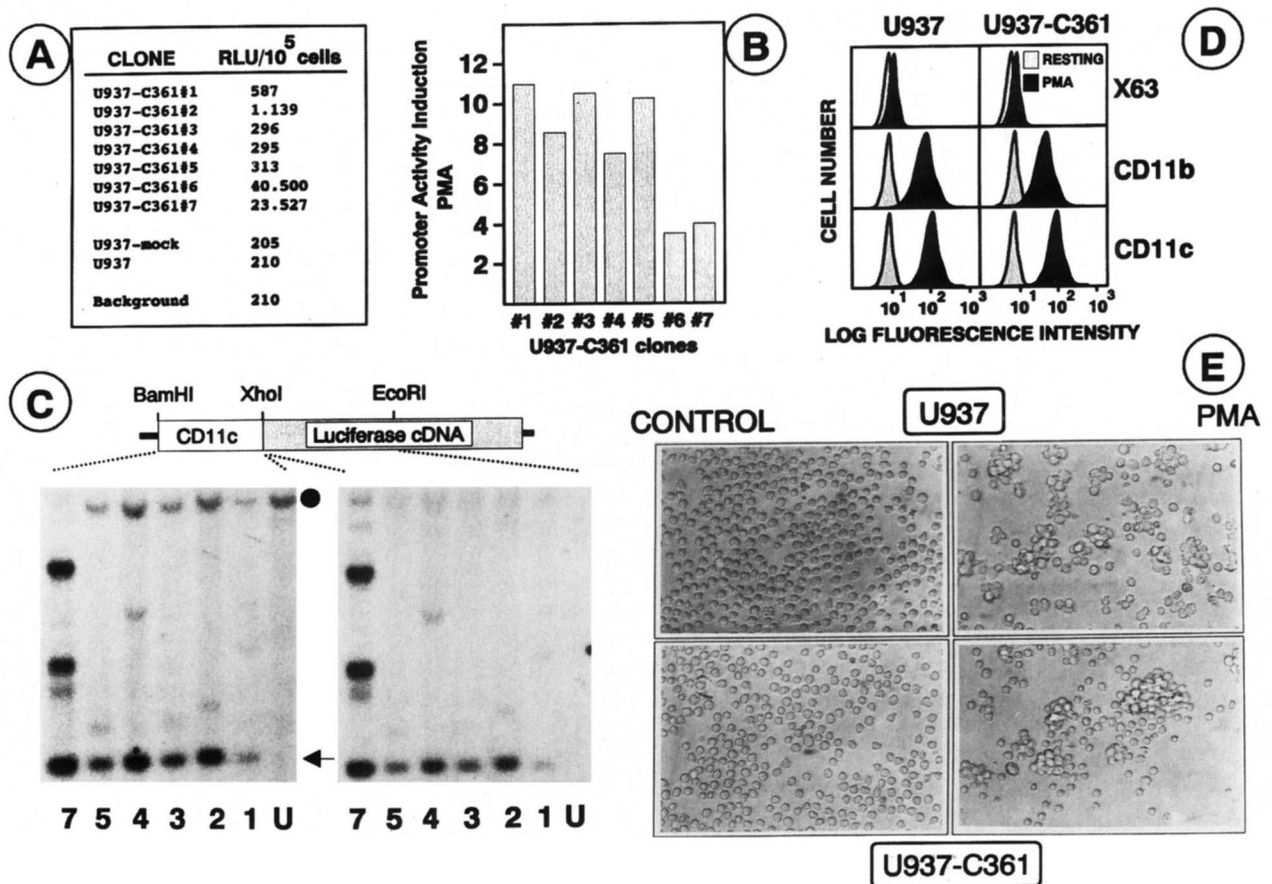
**Stable transfection of U937 cells with a CD11c promoter/luciferase reporter construct.** We have previously isolated and functionally characterized the CD11c gene promoter and shown its tissue-specific activity in myeloid and CD11c<sup>+</sup> B-lymphoblastoid cells.<sup>12</sup> To analyze the activity of the CD11c promoter during myeloid differentiation, U937 cells were transfected with the pCD11C361-Luc and the pSV2-neo plasmids. The G418-selected cell population (U937-C361) showed the same doubling time as untreated U937 cells and showed a measurable level of basal luciferase activity that increased on PMA-triggered differentiation (data not shown). Limiting dilution cloning led to the isolation of a number of clones whose levels of background luciferase activity were constant during long-term culture and ranged between 300 and 40,000 RLU/10<sup>5</sup> cells (Fig 1A). More importantly, PMA-stimulated cell differentiation led to a 4- to 12-fold increase in the endogenous luciferase activity of the isolated clones, an induction of similar magnitude to that previously reported for the pCD11C361-Luc plasmid on transient expression in U937 cells (Fig 1B).<sup>12</sup> Hybridization with a luciferase cDNA probe showed that the copy number of the construct in the distinct U937-C361-derived clones was variable and correlated with their basal level of luciferase (Fig 1C). Probing of a similar blot with the -361/+43 fragment from the CD11c promoter showed that the insertion had not affected the structure of the construct because both probes hybridized to a common set of bands (Fig 1C). Given the colinearity of the CD11c promoter and the luciferase cDNA in the transfected construct,<sup>12</sup> the PMA-mediated increase of the luciferase level in the isolated clones reflects the differentiation-inducibility of the CD11c promoter and is not caused by positional effects. The U937-C361 clone no. 2 (U937-C361#2) was chosen for further analysis based on its low but significant luciferase level under normal culture conditions (see Figs 1A and B).

Immunofluorescence analysis showed that, as previously described for U937 cells,<sup>1,7,9</sup> the isolated U937-C361 clones lacked Mac-1 and p150,95 cell surface expression, which were detected only on PMA-stimulated differentiation (Fig 1D). PMA addition also promoted LFA-1/intercellular adhesion molecule-1-mediated homotypic aggregation with similar kinetics in both U937 and U937-C361 cells (Fig 1E). As a whole, the differentiation-associated phenotypic changes in U937-C361 cells were indistinguishable from those observed in U937 cells, indicating that the differentiation capacity of the parental cell line had not been altered by the insertion of the pCD11C361-Luc construct.

**Effect of protein kinase C (PKC)-activation on the luciferase**

**ase levels in U937-C361#2.** Although PMA and OAG activate PKC, OAG can neither promote U937 differentiation nor induce the expression of p150,95 (Calabretta<sup>19</sup> and our unpublished results). PMA or OAG treatment of U937-C361#2 cells caused a small transient increase in luciferase activity, that returned to basal levels 6 to 9 hours after treatment (Fig 2A), an effect also observed on addition of fresh serum or replacement of the culture medium (data not shown) and which is consistent with the presence of an AP-1 sequence within the transfected construct.<sup>12</sup> However, at later time points, OAG-treated cells showed basal levels of luciferase activity, whereas the luciferase level in PMA-treated cells constantly increased up to 5 days after the phorbol-ester addition (Fig 2A), which is in agreement with the previously described activity of the CD11c promoter in PMA-treated U937 cells.<sup>9,12</sup> Therefore, as with U937 differentiation, the induction of luciferase activity in U937-C361#2 requires the continuous activation of PKC and supports the lack of differentiation-inducing ability of OAG in U937 cells.<sup>19</sup> Furthermore, whereas PMA differentiation led to the appearance of CD11c on the cell surface, OAG had no effect on the p150,95 expression (data not shown). These results show that U937-C361#2 cells respond to PKC activators in the same manner as the parental U937 cell line and, more importantly, that the luciferase activity in U937-C361#2 cells reflects the differentiation state of the cells.

**Effect of SB and GM-CSF on the luciferase activity in U937-C361 cells.** U937 cells have been previously reported to differentiate in response to SB<sup>15</sup> and GM-CSF,<sup>7,14</sup> and, in both cases, the expression of CD11c/CD18 is induced during the process.<sup>7,15</sup> As shown in Fig 2B, luciferase level augmented during both SB- or GM-CSF-driven differentiation, although with different kinetics (Fig 2B), whereas agents that do not alter the differentiation state of U937 cells ( $\gamma$ -interferon at 200 or 2,000 U/mL) left the luciferase levels unaltered (data not shown). During SB differentiation, luciferase activity increased greater than 25-fold at 24 hours, thus correlating with the appearance of CD11c on the cell surface (see Fig 3C), and almost returned to basal levels after 72 hours (Fig 2B). Following a similar kinetic profile as that used during PMA-triggered differentiation, the increase was considerably slower during GM-CSF differentiation, with a 2- to 4-fold increase between 24 and 72 hours and a maximal induction after 5 days (5-fold; see Fig 2B), which is in agreement with the later appearance of CD11c on the membrane (Fig 3C). Northern blot showed the presence of CD11c mRNA 24 hours after the addition of each differentiation agent, a time at which *c-myc* mRNA had been already down-regulated (Fig 2C). At all time points analyzed, PMA induced higher levels of CD11c mRNA than did SB or GM-CSF, a fact probably related to the slight *c-myc* mRNA reinduction observed 48 to 72 hours after GM-CSF or SB treatment (Fig 2C). Altogether, and allowing for some quantitative differences because of posttranscriptional mechanisms, luciferase activity levels correlate with both the expression of the endogenous CD11c gene and the *c-myc* mRNA downregulation and, therefore, may be considered as a differentiation marker in U937-C361 cells.



**Fig 1.** Characterization of the U937 cells stably transfected with the pCD11C361-Luc plasmid is shown. (A) Basal activity of the CD11c promoter by seven U937-C361 independent clones during normal in vitro culture conditions, as determined by luciferase activity. (B) The increase in luciferase activity caused by the PMA-mediated differentiation of the seven isolated U937-C361 clones was determined by measuring the luciferase activity 24 hours after PMA treatment. Luciferase determinations were performed on  $10^5$  cells, and the background level of the luminometer was always lower than 220 RLU. Promoter activity induction was calculated as determined under Materials and Methods. (C) Detection of the pCD11C361-Luc plasmid DNA in U937-C361-derived clones was performed by means of Southern blot on genomic DNA from the U937-C361-derived clones (lanes 1-7) or from untreated U937 cells (lane U). Two identical membranes were hybridized with either an *Xho*I-*Eco*RI luciferase cDNA probe (right) or the *Bam*HI-*Xho*I CD11c promoter fragment (left). A schematic representation of the pCD11C361-Luc plasmid, with the relative position of each probe, is shown at the top. The black circle indicates the position of the genomic CD11c promoter, and the arrow signals the *Bam*HI-*Eco*RI fragment that includes both probes. (D) Comparative differentiation capacity of U937 cells and the U937-C361#2 transfectant as measured by the expression of Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18) on untreated and PMA-differentiated cells. (E) Comparison of the homotypic aggregation on PMA-differentiated U937 and U937-C361#2 cells is shown.

*SB- and PMA-triggered differentiation synergistically induce the CD11c gene promoter activity and p150,95 cell surface expression.* The distinct behavior of the CD11c promoter during the SB- and PMA (or GM-CSF)-triggered differentiation prompted us to analyze whether their effects were additive. As shown in Fig 3, the luciferase activity induced during the PMA- or GM-CSF-triggered differentiation was further augmented in the presence of SB, giving rise to promoter activity induction values that ranged between 50- and 100-fold. The presence of OAG during the SB-triggered differentiation had no effect on the activity of the CD11c promoter (data not shown), showing that transient PKC activation is not sufficient for the potentiating effect of SB. At the same time, p150,95 cell surface expression during the GM-CSF- or PMA-triggered differentiation was considerably higher in the presence of SB than was the ex-

pression induced by either agent alone, especially at early time points. At 24 hours, the mean fluorescence intensity (MFI) for CD11c was 27 in the case of SB differentiation, 20 for the GM-CSF differentiation, and 49 in the presence of both agents, and the same synergistic effect was observed at 48 hours (MFI of 49, 17, and 67 with both agents; see Fig 3C). In addition, a similar effect could be observed when SB was present during the PMA-stimulated differentiation (MFI of 49 with SB, of 79 with PMA, and of 103 with both differentiating agents; see Fig 3C). Altogether, the above results indicate that SB-triggered differentiation induces the CD11c promoter activity with distinct kinetics than PMA or GM-CSF, and possibly through distinct signaling pathways.

Luciferase levels were also determined after treatment with a distinct set of differentiating agents.<sup>18-20</sup> The DNA topoisomerase II inhibitor AMSA increased luciferase activ-

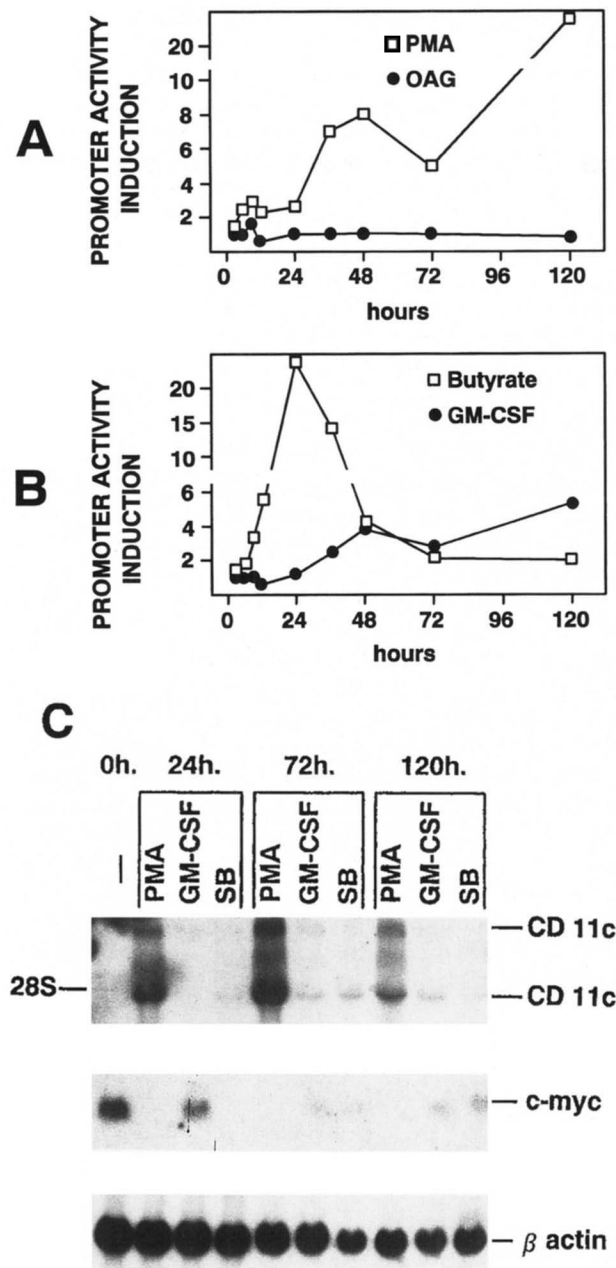


Fig 2. The effect of PKC activators and differentiation-triggering agents on the CD11c gene promoter activity is shown. (A and B) U937-C361#2 cells were either untreated or treated in the presence of PMA (10 ng/mL) or OAG (20  $\mu$ g/mL; A) or of GM-CSF (100 U/mL) or SB (1 mmol/L; B). At the indicated time points, cells were harvested, counted, and lysed, and their luciferase levels were determined. The promoter activity induction reflects the activity of the CD11c promoter and represents the fold induction of luciferase levels caused by the addition of each agent, after normalizing for cell number. Four independent experiments were performed, and a representative one is shown. (C) Determination of the steady-state level of CD11c mRNA (upper panel), *c-myc* mRNA (middle panel), and  $\beta$ -actin mRNA (lower panel) in U937-C361#2 cells at the indicated time points during PMA-, GM-CSF-, or SB-triggered differentiation is shown.

ity with similar kinetics and to similar levels as those for GM-CSF, which is in agreement with its ability to downregulate *c-myc* and to induce CD11c expression (Fig 4).<sup>20,21</sup> Interestingly, the effect of VP-16, which also inhibits DNA topoisomerase II, was somewhat different as luciferase levels peaked at 72 hours and decreased afterwards (Fig 4). On the other hand, the polar solvent DMSO<sup>22</sup> triggered luciferase levels that were maximal at 48 hours and returned to basal levels at 120 hours. Therefore, the luciferase activity in response to DMSO and SB (Fig 2B and Fig 4) returned to background levels after 120 hours, a time at which other agents showed maximal activity (GM-CSF, AMSA, PMA) or still maintained augmented levels (VP-16). As a control, 1,25 dihydroxyvitamin D3, which induces incomplete U937 differentiation,<sup>23</sup> failed to induce either the p150,95 expression or the activity of the CD11c promoter (Fig 4).

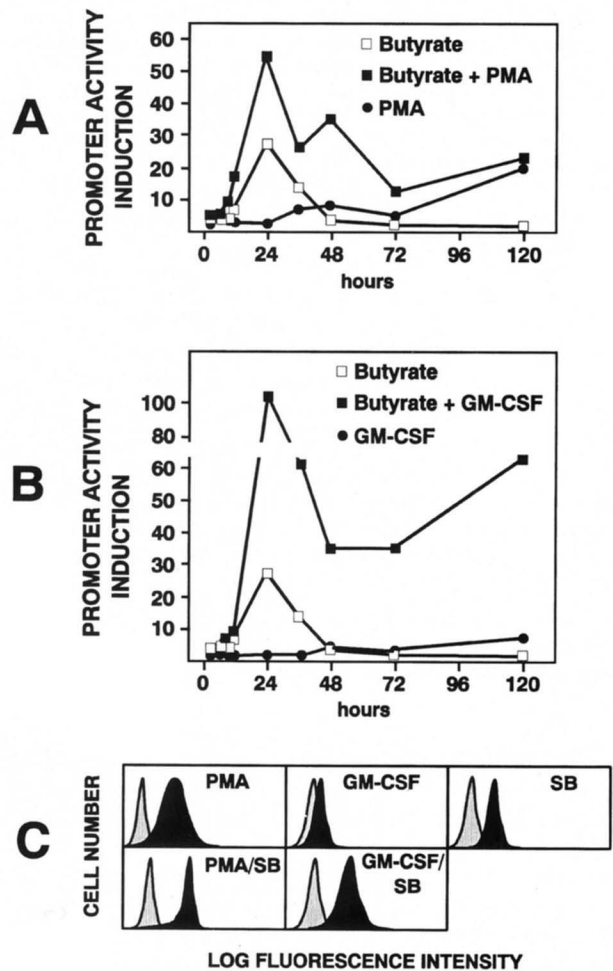
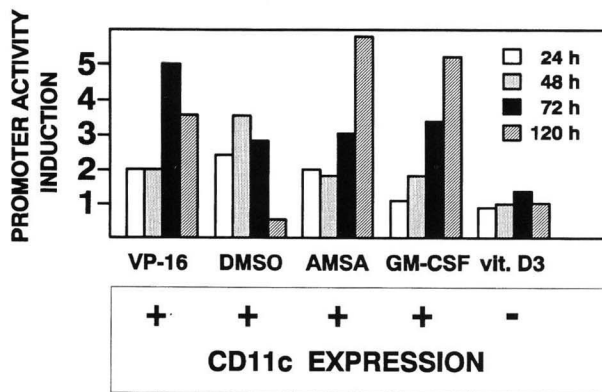


Fig 3. SB potentiates the effect of PMA- (or GM-CSF-) triggered differentiation on the CD11c promoter. U937-C361#2 cells were treated with SB and PMA (A) or with SB and GM-CSF (B), and the luciferase levels were determined at the indicated time points. Three independent experiments were performed, and a representative one is shown. (C) Cell surface expression of p150,95 48 hours after the initiation of each differentiation protocol is shown. The background immunofluorescence is shown for each culture condition and is represented by a stippled curve.



**Fig 4.** The effect of alternative U937 differentiating agents on the CD11c promoter activity is shown. U937-C361#2 cells were treated in the presence of the indicated differentiation-triggering agents. The cells were harvested, counted, and lysed, and their luciferase levels were determined at the indicated time points. The promoter activity induction represents the fold induction of luciferase levels caused by the addition of each agent. A representative experiment is presented. The expression of p150,95 was determined in parallel by immunofluorescence analysis 48 hours after the start of each treatment. (+) indicates more than 60% of CD11c<sup>+</sup> cells, whereas (-) represents less than 5% of CD11c<sup>+</sup> cells.

*Localization of the SB and GM-CSF differentiation-responsive elements within the CD11c promoter.* Transient expression of serial deletion mutants from the -361/+43 fragment of the CD11c promoter showed that the differentiation-responsiveness of the CD11c promoter was retained in the -160/+43 region (Fig 5A). Moreover, the PMA, GM-CSF, and SB differentiation-responsiveness of additional deletion mutants (pCD11C99-Luc, pCD11C61-Luc, and pCD11C53-Luc) was not affected by deletion of the region contained between -160 and -99 (compare pCD11C160-Luc and pCD11C99-Luc; see Fig 5B). However, a further deletion reduced the SB differentiation-responsiveness by a factor of 7 (70.4- v 10.6-fold induction), showing SB-responsive elements within the region -99/-61 (Fig 5B). Removal of the consensus AP-1 sequence led to an additional threefold decrease (10.6- v 3.6-fold; see Fig 5B), indicating that the AP-1 sequence also contributes to the SB-responsiveness of the CD11c promoter. The remaining slight effect of SB on pCD11C53-Luc was also observed in other reporter plasmids, including the control CD11a-derived pCD11A100-Luc plasmid (Fig 5B),<sup>16</sup> thus suggesting a general effect on transcription. Regarding the PMA differentiation-responsiveness of the CD11c promoter, it was substantially reduced by deleting the -99/-61 fragment and was completely abolished on removal of the AP-1-binding sequence (-61) 5'-CTGACTCAT-3' (-53; see Fig 5B). The response to GM-CSF was unaffected by deletion of the -99/-61 region (Fig 5B) and was only abrogated by deletion of the -61/-53 AP-1 sequence (Fig 5B), which is in agreement with the GM-CSF ability to enhance AP-1 activity in U937 cells.<sup>14</sup> Therefore, it can be concluded that the AP-1 sequence at -61/-53 is absolutely required for the CD11c promoter responsiveness to PMA and GM-CSF differentiation and that

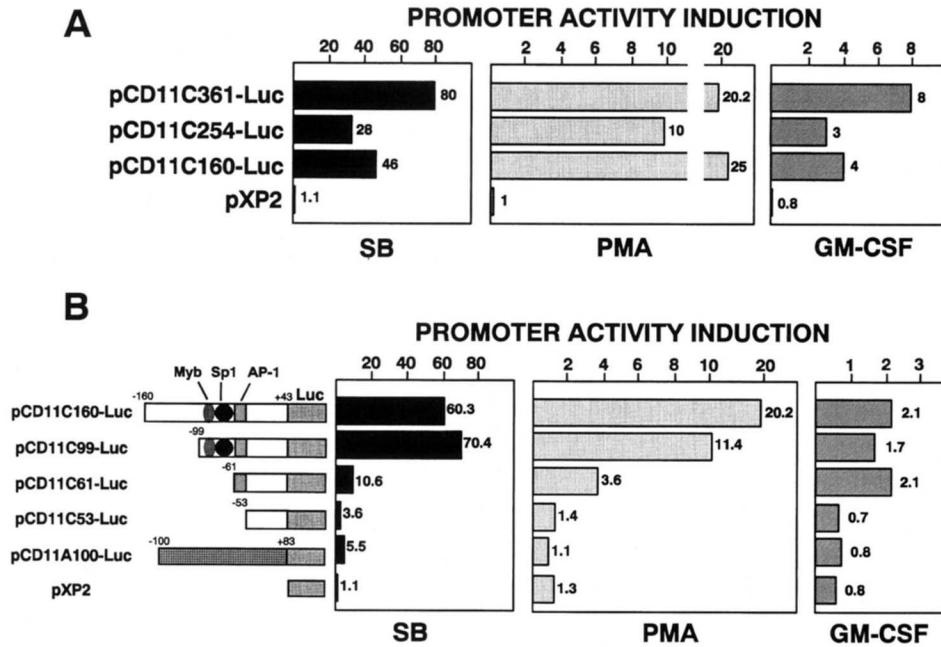
it also influences its SB-inducibility, although to a lower extent.

Structural analysis on the -99/-61 region showed a consensus Myb-binding site (PyAACG/TG) at -85 (CAACTG) that is also a consensus E-box binding site for members of the basic/helix-loop-helix (b/HLH) family of transcription factors (E box = CANNTG).<sup>12</sup> c-Myb was shown to bind to -85 (CAACTG)(Myb-85, BoxD), because c-Myb-containing bacterial extracts, which specifically recognized the 2xMyb consensus oligo, were able to retard the BoxD double-stranded oligonucleotide (spanning from -94 to -70) but did not recognize the mBoxD probe, which lacks the Myb-binding site (Fig 6A). Moreover, BoxD inhibited the binding of Myb to 2xMyb, and the latter abrogated the Myb-containing retarded species on the BoxD oligonucleotide (Fig 6A), thus clearly indicating that the CD11c proximal promoter is specifically recognized by the c-Myb transcription factor. The SB-responsiveness of the CD11c promoter was then analyzed by eliminating either a previously described Sp1-binding site (Sp1-70) which is required for optimal basal promoter activity (C. Lopez-Rodriguez and A.L. Corbi, manuscript submitted) or the E-box/Myb-binding site. Mutations that abrogated binding to Sp1-70 [pCD11C160(-70MUT)-Luc] or that simultaneously disrupted the E-box and the Myb-binding site at -85 [pCD11C160(-85MUT)-Luc] did not significantly inhibit the SB responsiveness of the CD11c promoter in the context of the -160/+43 fragment (Fig 6B) and also left unaltered the PMA differentiation inducibility of the promoter. These results showed that the SB inducibility of the CD11c promoter is mediated by the -99/-61 proximal promoter region but without the involvement of the ubiquitous Sp1, the tissue-restricted Myb transcription factor, or any E-box-binding b/HLH factor.

## DISCUSSION

The leukocyte integrin p150,95 (CD11c/CD18) constitutes a marker for mononuclear phagocytes<sup>24</sup> and is primarily expressed on cells of the myeloid lineage,<sup>7,25</sup> although it is also found on activated B lymphocytes and is highly expressed on hairy cell leukemia cells.<sup>26,27</sup> We have previously shown that the promoter region of the CD11c gene directs the myeloid-specific expression of p150,95 and is also active in CD11c<sup>+</sup> B lymphoblastoid cells.<sup>9,12</sup> Given the limitations of transient expression assays for performing time-course analysis in myeloid cells,<sup>28</sup> we have derived the U937-C361 stable transfectant, have shown the responsiveness of the CD11c promoter to myeloid differentiation, and have shown that the CD11c promoter activity is inducible by differentiation-triggering agents that differ in their mechanisms of action and/or target DNA elements. Moreover, we have mapped the differentiation-response elements of the CD11c gene promoter to the -99/-53 region and have shown the differential involvement of the AP-1-, Sp1-, and Myb-binding sites located within this fragment.

Comparison of the effects of three differentiation agents on the U937-C361 cells showed that PMA and GM-CSF induced the CD11c promoter activity with similar kinetics but with different strength. Transient expression experiments



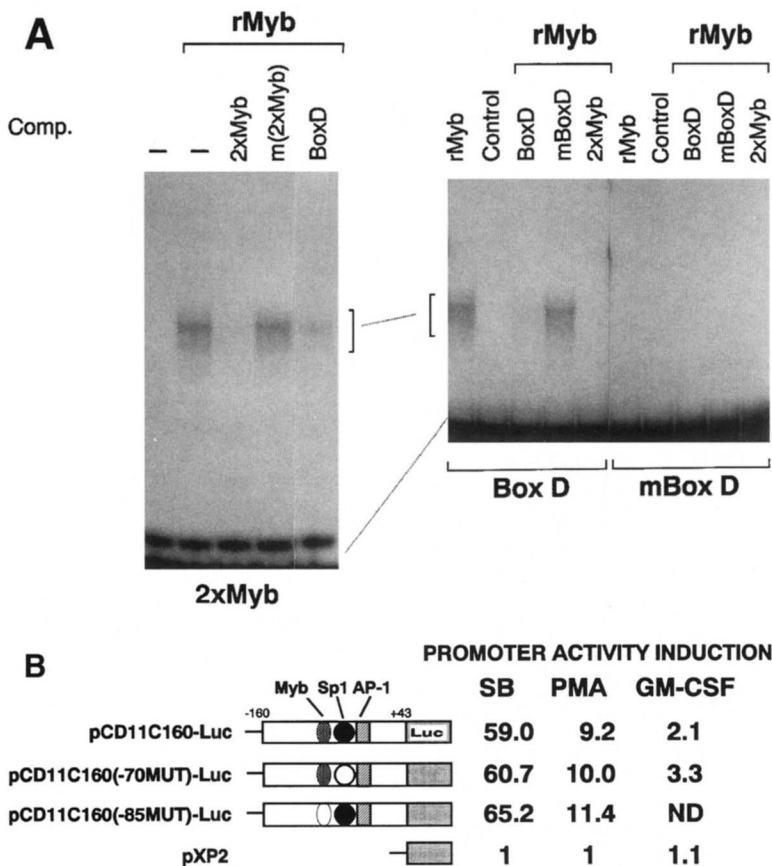
**Fig 5.** Mapping of the differentiation-inducible activity of the CD11c gene promoter is shown. The indicated CD11c promoter-based luciferase constructs were electroporated in U937 cells, and the transfected cells were split and cultured in the presence of the distinct differentiating agents or were left untreated. (A) Mapping of the differentiation-response elements to the -160/+43 region of the CD11c promoter is shown. (B) The involvement of the -61/-53 AP-1 consensus sequence and the -99/-61 fragment in the PMA, GM-CSF, and SB responsiveness of the CD11c promoter is shown. Luciferase activity was determined after 14 hours,<sup>12</sup> and identical results were obtained 24 hours posttransfection. Promoter activity induction represents the fold induction of the luciferase activity caused by each differentiating agent. In all cases, the activity of the promoterless plasmid pXP2 was similar to the background level of the luminometer (200 to 210 RLU for a 20-second measuring time) and was not altered during cell differentiation. Each construct was assayed at least four times, and a representative experiment is shown. In the experiments shown in (B), a CD11a promoter-based luciferase construct (pCD11A100-Luc)<sup>16</sup> was used as an additional control.

lend further support to the existence of a common set of factors mediating the inducibility of the promoter during PMA- or GM-CSF-triggered differentiation. Although deletion of the -99/-61 region only affected the PMA inducibility, elimination of the AP-1 consensus sequence completely abrogated the responsiveness of the CD11c promoter to both agents. This finding is in agreement with the previously reported capacity of GM-CSF to activate PKC and to enhance the AP-1 activity in U937 cells.<sup>14</sup> Therefore, PMA and GM-CSF may induce the CD11c promoter activity through a common pathway that would include activation of the AP-1 complex, because the CD11c -61/-53 sequence has been shown to be a functional AP-1 site by both in vitro footprinting and EMSAs (Lopez-Cabrera et al<sup>12</sup> and C. López-Rodríguez and A. L. Corbí, manuscript in preparation), and because maximal activation of AP-1 in U937 cells has been shown to correlate with induction of differentiation markers.<sup>29</sup> Because *c-fos* and *c-jun* are known to cooperate for transcriptional activation with members of the *ets* gene family of transcription factors,<sup>30</sup> some of which regulate the activity of the CD11b promoter,<sup>31</sup> it is possible that such a cooperation also occurs at the CD11c promoter.

The CD11c gene promoter shows an Inr element at the major transcriptional start site.<sup>12</sup> Based on physical and functional interactions between *c-myc* and transcription factor II-I, it has been recently proposed that *c-myc* may repress transcription initiation from TATA-less Inr-containing

genes.<sup>32</sup> If this is the case, the distinct magnitude of the CD11c promoter induction during the PMA- and GM-CSF-triggered differentiation might arise from their opposite effects on *c-myc* levels. PMA completely downregulates *c-myc* gene transcription in U937,<sup>33</sup> whereas the downregulation of *c-myc* by GM-CSF in U937 cells is transient (Fig 2C) and may correlate with the fact that the GM-CSF signaling pathway in other cell types leads to induction of *c-myc*.<sup>34</sup> Site-directed mutagenesis will greatly help in the determination of the role that the Inr sequence might play in the induction of p150,95 during PMA- and GM-CSF-triggered differentiation.

A variety of low molecular weight polar solvents, such as SB or DMSO, can induce erythroid and myeloid cell differentiation,<sup>20,35,36</sup> and SB-differentiation (and to a lower extent DMSO) produced a high and transient induction of the CD11c promoter activity. The exact mechanism of action of SB is unknown. However, mutation of the Sp1 sites within the human immunodeficiency virus-long terminal repeat (HIV-LTR) completely eliminates its SB-inducible promoter activity, and a correlation between the presence of functional Sp1 sites and SB-inducibility has been proposed.<sup>37,38</sup> Conversely, mutation of the Sp1-70 site within the CD11c promoter<sup>12</sup> had no effect on the SB-inducibility, which was greatly inhibited by deletion of the -99/-61 region, therefore suggesting distinct mechanisms for the SB-responsiveness in different genes. Sequence analysis on the -99/



**Fig 6.** The effect of mutation at Sp1-70 and Myb-85 on the differentiation responsiveness of the CD11c promoter is shown. (A) Binding of c-Myb-containing bacterial extracts to the -85 DNA element on the CD11c promoter is shown. EMSA was performed on the 2xMyb, BoxD, and mBoxD double-stranded oligonucleotides, using extracts from bacteria transformed with either c-Myb in pFLAG-1 (indicated by rMyb) or with an insertless plasmid (indicated as Control). In all cases, competitor oligonucleotides were used at 500 molar excess. The position of the Myb retarded complex is indicated by brackets. (B) The effect of mutations at Sp1-70 or Myb-85 in the myeloid differentiation-inducibility of the CD11c promoter is shown. Wild-type and mutant Sp1 and Myb sequences are depicted as filled and empty circles or ovals, respectively. In all cases, the activity of the promoterless plasmid pXP2 was similar to the background level of the luminometer (200 to 210 RLU for a 20-second measuring time) and was not altered during cell differentiation. Each construct was assayed at least three times, and a representative experiment is shown.

-61 region showed the overlapping presence of a consensus Myb-binding site (PyAACG/TG) and a consensus binding site for the members of the b/HLH family of transcription factors (CANNTG or E-box) at -85 (CAACTG).<sup>12</sup> In fact, structural studies showed that c-Myb is indeed capable of binding to the CD11c proximal promoter by recognition of the -85 CAACTG DNA element. Because both gene families (Myb and b/HLH) regulate gene expression during myeloid differentiation,<sup>39,40</sup> we tested the effect of mutations that disrupted both the E-box and the Myb-binding site (CAACTG into GTCGAC). The lack of effect of this mutation indicates that additional proteins, distinct from Myb and b/HLH factors, mediate the SB inducibility of the CD11c promoter. Conversely, deletion studies suggested that the AP-1 site slightly influences the SB-responsiveness, probably because of the positive effect that SB has on the expression of some members of the AP-1 transcription factor complex.<sup>41</sup> The identification of the specific DNA sequence mediating the SB responsiveness of the CD11c promoter requires further mutagenesis studies on the identified -99/-61 fragment.

In addition to its differentiation-inducing capacity, SB synergizes with PMA or GM-CSF in producing both a greater induction of the CD11c promoter activity and a higher cell surface expression of p150,95. Because PMA and GM-CSF activate PKC and stimulate the AP-1 activity,<sup>14,29</sup> it is possible that the SB signaling pathway is potentiated

by agents whose differentiation-inducing activity is mediated by AP-1 (Fig 7). In this sense, SB also synergizes with the antioxidant pyrrolidine dithiocarbamate, which has been recently reported to activate AP-1 (Meyer et al<sup>42</sup> and M. Rubio and A. Corbi, unpublished results). In any event, SB appears to be potentiated only by PKC activators with differentiation-inducing ability, because the SB-stimulated CD11c promoter activity was not altered by the concomitant presence of OAG. A hypothetical model for the regulation of p150,95 during myeloid differentiation, shown in Fig 7, is based on the distinct mode of action of SB and the PKC activators (GM-CSF and PMA) and their synergistic effect on the induction of the CD11c gene and assumes that the most relevant targets of their respective signaling pathways are the -99/-61 region (SB and PMA) and the AP-1 sequence (PMA, GM-CSF, and, to a lower extent, SB).

Myeloid leukemic cells are halted at distinct stages along the differentiation pathway. The potential applicability of differentiation-triggering agents is underscored by the success of both the retinoic acid differentiation therapy in the treatment of acute promyelocytic leukemia and the clinical trials of hexamethylene bisacetamide on myelodysplastic syndrome.<sup>2,43,44</sup> Our studies on the U937-C361 cells have shown the inducibility of the CD11c gene promoter activity during myeloid differentiation. The availability of a differentiation-responsive promoter will be also helpful for the construction of additional stable transfectants in other myeloid

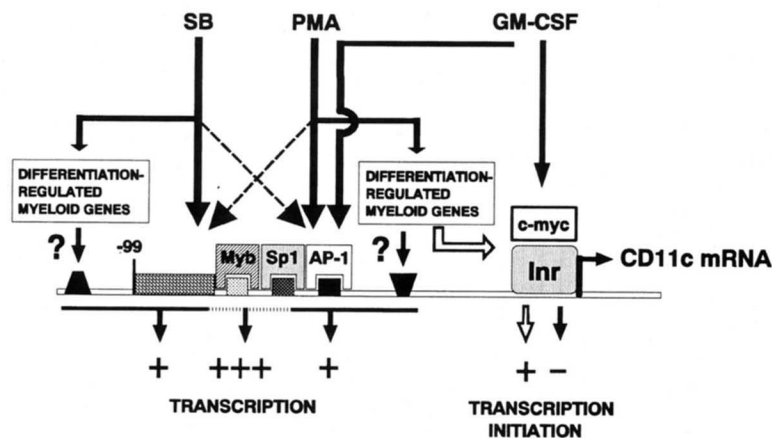


Fig 7. A hypothetical model for the induction of the CD11c gene during U937 myeloid differentiation is shown. Phorbol-esters or GM-CSF, through PKC activation, increase AP-1 activity and promote the expression of numerous genes and a transient early enhancement in the CD11c gene transcription. At later time points, AP-1 activity together with newly expressed factors could drive the myeloid-specific differentiation-regulated expression of CD11c. On the other hand, SB would act preferentially through the -99/-61 region of the promoter, in a myeloid-specific manner. The simultaneous action of both signaling cascades would be responsible for the observed synergy between SB and PMA (or GM-CSF). Dotted lines represent the contributions that -99/-61 and AP-1 sequences play in the PMA- or SB-responsiveness, respectively. At the major transcriptional start site, GM-CSF and PMA, through their opposite effects on *c-myc* levels,<sup>33,34</sup> would impair (PMA, white arrows) or facilitate (GM-CSF, black arrows) transcription factor II-I activity at the Inr, therefore lowering (GM-CSF) or increasing (PMA) the basal transcription initiation from the CD11c Inr.<sup>12,32</sup> Although not depicted, SB treatment also leads to an almost complete downregulation of *c-myc* at early time points.<sup>15</sup>

cell lines (HL-60, KG1, and THP-1) representative of different stages of the myeloid differentiation pathway,<sup>1</sup> thus allowing the identification of differentiation-inducing agents with potential therapeutic value and the dissection of the underlying molecular mechanisms. In this regard, our preliminary results on VP-16 support the proposed implication of PKC and AP-1 in the VP-16-induced U937 myeloid cell differentiation.<sup>45</sup> Furthermore, the identification of an SB-responsive regulatory region within the CD11c promoter will contribute to the knowledge of the precise mechanism of action of butyrate, a natural fatty-acid with a number of reported clinical applications.<sup>46,47</sup>

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