Sp3 Mediates Transcriptional Activation of the Leukocyte Integrin Genes CD11c and CD11b and Cooperates with c-Jun to Activate CD11c

(Received for publication, April 22, 1997, and in revised form, June 23, 1997)

John D. Noti‡

From the Guthrie Research Institute, Sayre, Pennsylvania 18840

The leukocyte integrin genes CD11c and CD11b are expressed predominantly in myelomonocytic cells. In previous experiments, the −70 to −65 and −121 to −103 regions of the CD11c promoter and the −66 to −59 region of the CD11b promoter were shown to be essential for Sp1-mediated activation of these genes. In vivo genomic footprinting had also revealed cell-specific binding of protein, presumably Sp1, to these regions. In this study, electrophoretic mobility shift analysis showed that the Sp1-related factor, Sp3, also binds at or near these same regions. Cotransfection of Sp3 along with CD11c promoter-luciferase constructs into Sp-deficient Drosophila Schneider 2 cells showed that Sp3 could activate the CD11c promoter. Deletion of both the −70 to −65 and −121 to −103 regions of the CD11c promoter resulted in the loss of activation by Sp3. Both sites showed activation by Sp3; however, the −70 to −65 region was more responsive to Sp3 than to Sp1. Similar transfection analysis of the −66 to −59 region of the CD11b promoter showed Sp3-dependent expression. Further, cotransfection analysis in Drosophila cells showed that Sp3, as was previously shown for Sp1, also synergizes with c-Jun to activate CD11c. Antisense experiments that knocked out endogenous Sp3 expression in the myelomonocytic cell line, HL60, revealed that Sp3 participates in activation of the CD11c and CD11b promoters in vivo.

Myeloid cells play a key role in a host of leukocyte-dependent functions within the immune system. A number of transcription factors including PU.1 (1–4), Spi-B (5), MZF-1 (6), Ets (7–9), GATA-2 (10, 11), and c-Jun and c-Fos (7, 12), some of which are lineage-restricted, have been shown to control expression of myeloid genes.

The leukocyte integrin genes CD11c (13) and CD11b (14), which encode the alpha subunits for the dimeric receptors p150,95 and Mac-1, respectively, are among the myeloid-specific genes that are transcriptionally activated during myeloid differentiation (15, 16). Recent reports by my laboratory and others have shown that both the CD11c (17) and CD11b genes (18) are controlled by the ubiquitous transcription factor Sp1, which binds these respective promoters in a cell-specific manner. The mechanism for cell-specific binding of Sp1 to either of these promoters is unknown. Since Sp1 binding in other gene systems is affected either directly or indirectly by other transcription factors such as Egr-1 (19), GATA (20, 21), NF-E1 (22), and Pit-1 (23), it is conceivable that binding of Sp1 to CD11c and CD11b may also be influenced by other factors.

Sp1-related genes have recently been cloned based on their sequence homology to Sp1 (24, 25). Both Sp2 and Sp3 are widely expressed; however, Sp4 expression is restricted to certain cells of the brain. Sp4 (26), like Sp1, activated Sp1-responsive promoters, whereas transfection of an Sp3 expression plasmid into various cell lines repressed the activity of the uteroglobin promoter (27), the SV40 enhancer/promoter (28), and the HIV-I promoter (29). Further, Sp1-mediated activation of promoter constructs containing the E1B TATA box fused to either two Sp1 binding sites from element II in the uteroglobin promoter (27) or the two Sp1 binding sites from the HTLV-III promoter (28) were reversed when Sp3 was cotransfected, and it was proposed that Sp3 acts by competitively binding to the Sp1 binding site.

The finding that Sp3 can act as a transcriptional repressor prompted us to examine whether cell-specific expression of CD11c and CD11b was a result of the repressor activity of Sp3 on these promoters in nonmyeloid cells. Surprisingly, Sp3 was found to activate rather than repress the promoters of both CD11c and CD11b. This study shows that intact Sp1 sites are necessary for activation of both promoters by Sp3 and that Sp3 apparently competes for binding with Sp1 for the same or overlapping sites. Further, antisense studies indicate that both endogenous Sp1 and Sp3 proteins are functionally active on these promoters in myeloid cells. Lastly, Sp3 can cooperate with c-Jun for activation of the CD11c promoter.

EXPERIMENTAL PROCEDURES

Cell Culture—HL60 (promyelocytic leukemia, ATCC CCL 240), U937 (histiocytic lymphoma, ATCC CRL 1593), and MolM cells (T cell lymphoblastic leukemia, ATCC CRL 1582) were grown in RPMI 1640 medium containing 10% fetal calf serum (Biofluids, Rockville, MD). HeLa cells (cervical epitheloid carcinoma, ATCC CCL 2) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Biofluids); Schneider’s Drosophila cells (Drosophila melanogaster embryo, ATCC CRL 1663) were grown in Schneider’s medium containing 10% insect-tested fetal calf serum (Sigma). All media contained 100 units/ml of penicillin and 100 units/ml streptomycin.

Plasmids—The −133 to −66 region of the CD11c promoter was prepared by the polymerase chain reaction (PCR)1 with oligonucleotide primers specific to this region and fused to the luciferase gene. The primers used in the PCR were initially synthesized with a HindIII restriction site for cloning of the final PCR product into the HindIII site immediately upstream of the luciferase gene in plasmid pGRL2-Basic (Promega, Madison, WI) to create a CD11c-luciferase reporter plasmid (referred to as wild type, wt). The −500 to +50 region of the CD11b promoter was prepared in a similar manner and ligated into the HindIII site immediately upstream of the luciferase gene in plasmid pGL2-Basic.

1 The abbreviations used are: PCR, polymerase chain reaction; wt, wild type; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift analysis; pPaO, Drosophila actin promoter expression plasmid; Rb, retinoblastoma protein.
jun sites were incorporated into the PCR primers to facilitate cloning of

reporter plasmids and a CD11b-luciferase reporter plasmid containing specific deletions of these sites (refer to figure legends for details). The plasmid pPacSp1, which expresses Sp1 from the Drosophila actin promoter, and the plasmid pPacO containing only the Drosophila actin promoter was generously provided by Dr. R. Tjian. To construct plasmids that express Sp2 and Sp3 from the actin promoter (plasmids pPacSp2 and pPacSp3, respectively), DNA clones for Sp2 and Sp3 (Ref. 24; ATCC 95507 and ATCC 95506, respectively) were obtained from ATCC and used as templates in the PCR to generate DNAs containing the complete coding sequences for these factors. The primers contained XhoI restriction sites that were used to clone the Sp2 and Sp3 DNAs into the XhoI site of pPacO. The plasmid pCMV-HrB (30), which expresses the human retinoblastoma gene from the cytomegalovirus early promoter, was generously provided by Dr. J. M. Horowitz. To construct plasmid pPacJun, which expresses c-Jun from the Drosophila actin promoter, the plasmid pCMV-e-Jun, kindly provided by Dr. R. Tjian, was used as a template in the PCR to generate DNA corresponding to the complete coding sequence for c-Jun. XhoI sites were incorporated into the PCR primers to facilitate cloning of c-jun into the XhoI site of pPacO. To construct plasmid pPac-luciferase, which expresses luciferase from the Drosophila actin promoter, gPL3-Basic was used as a template in the PCR to generate DNA corresponding to the complete coding sequence for luciferase that was subsequently cloned into the pCMV plasmid. The integrity of all constructs was verified by DNA sequence analysis.

Transfections and Reporter Assays—Transfections of human cells were performed by electroporation as described previously (31). Approximately 2 \times 10^6 cells were transfected with 15 \mu g of reporter plasmid and either 0.5 or 5 \mu g of each expression plasmid when used (see figure legends for specific details). The total volume of the plasmid transfection mix was adjusted to 30 \mu g with the control plasmid, pPacO. Electroporated cells were transferred to tissue culture dishes containing 15 ml of medium, and phorbol 12-myristate 13-acetate (PMA, 10 ng/ml final concentration) was added 24 h later. The cells were harvested 72 h post-transfection, and luciferase activity was assayed using a kit supplied by Promega. HL60 cells treated with Sp3 antisense oligonucleotides received PMA immediately after electroporation for a total of 24 h. Luciferase light output was measured in a Beckman scintillation counter and normalized against the total protein concentration in the cellular extract.

DNA was introduced into Drosophila cells by calcium phosphate-mediated transfection as described previously (17). Approximately 3 \times 10^6 Drosophila cells were transfected with 10 \mu g of reporter plasmid and either 0.5 or 5 \mu g of each expression plasmid (see figure legends for specific details). The total volume of the plasmid transfection mix was adjusted to 30 \mu g with pPacO. The calcium phosphate-DNA precipitates were left on the cells for 48 h before harvesting and assaying for luciferase activity.

Most transfections were performed in triplicate and repeated four to five times to ensure reproducibility and to monitor for transfection efficiency. Statistical analysis was performed using Microsoft Excel (Microsoft Corp., Roselle, IL). Data from individual experiments were adjusted to 30 \mu g with pPacO. The calcium phosphate-DNA precipitates were left on the cells for 48 h before harvesting and assaying for luciferase activity.

Electrophoretic Mobility Shift Analysis (EMSA)—EMSA was performed as described previously (7). Nuclear extracts for use in the EMSA were prepared from all untransfected human cell lines as described previously (7). Nuclear extracts were prepared from Drosophila cells transfected with pPacSp1, pPacSp2, or pPacSp3 as described by Andrews and Faller (32) with the following modifications. Forty-eight hours after transfection of 3 \times 10^6 Drosophila cells, the cells were harvested and pelleted for 10 min at 248 \times g in a bench-top centrifuge. The cells were resuspended in cold phosphate-buffered saline, transferred to a 2-ml Eppendorf tube, and resuspended in 5 ml of 75 \times g. The cells were resuspended in 400 ml of cold Buffer A (10 mM HEPES-KOH, pH 7.9, at 4 °C, 1.5 mM MgCl_2, 10 mM KOH, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min. The cells were vortexed at top speed for 10 s and pelleted at top speed in an Eppendorf centrifuge for 10 s. The pelleted cells were resuspended in 100 ml of chilled Buffer B (20 mM HEPES-KOH, pH 7.9, 1 mM MgCl_2, 1 mM KOH, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride), and 150 ml of high salt buffer (low salt buffer but with 1.6 M KCl) were slowly added. The nuclei were gently mixed on a rocker shaker for 30 min at 4 °C to extract nuclear proteins then pelleted at top speed in an Eppendorf centrifuge for 2 min at 4 °C, and the supernatant fraction was stored at −80 °C.

The following double-stranded oligonucleotide probes were used: 5' GTACTCTGGCCCGCCCTCCCT 3', which corresponds to the −79 to −62 region of the CD11c promoter (this probe contains site D (shown in bold; see Fig. 1 and Ref. 17), which shows homology to the Sp1 consensus binding sequence); 5' CTCCGGTTTGGG GTGGGGCGG 3', which corresponds to the −135 to −99 region of the CD11b promoter (this probe contains site C (shown in bold; see Fig. 1 and Ref. 17), which show homology to the Sp1 consensus binding sequence). The following double-stranded oligonucleotide probes were used: 5' TGGTCTCACTGA CCCCTCCCTTC TTGGA 3', which corresponds to the −80 to −48 region of the CD11b promoter (this probe contains a Sp1 binding site (shown in bold; Ref. 18)). The probes were labeled with [γ-32P]ATP to a specific activity of 2-4 \times 10^9 cpm/\mu g. Approximately 10 \mu l of each probe were incubated with 20 ml at room temperature with either 10 \mu g of nuclear extract prepared from untransfected PMA-stimulated HL60 cells or 10 \mu g of nuclear extract prepared from Drosophila cells transfected with pPacSp1, pPacSp2, or pPacSp3 as described previously (7). For supershift analysis, 1 \mu l of antibody specific for Sp1, Sp3, or Sp4 (Santa Cruz Biotechnology, Santa Cruz, CA) was added for 1 h after incubation of the probe with protein. The reaction products were analyzed by acrylamide gel electrophoresis as described (7).

Western Blotting of Extracts from Transfected Drosophila Cells—Transfected Drosophila cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 2.5 mM EDTA, 1 mM dithiothreitol, 0.5% SDS) and boiled for 5 min, and the lysate from 3 \times 10^6 cells was loaded onto each lane of an SDS-polyacrylamide gel. Western blotting was performed as described (33). Proteins were transferred to polyvinylidene difluoride membrane and probed with Sp1, Sp3, and c-Jun antibodies (Santa Cruz) at a final concentration of either 1 or 2 \mu g/ml for 1.5 h at 4 °C. The filter was washed then incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulins diluted 1:4000, and bound antibody was visualized with an ECL Western blotting kit (Amer sham Corp.).

Sp3 Knock-out with Antisense Oligonucleotides—The following phosphorothioate-modified nucleotides (34) were prepared and HPLC-puri

fied: 5’ AAGTACGACACTGAGAATCTGAC, a Sp3-specific antisense oligonucleotide that overlaps the Ile translational initiation codon of the Sp3 mRNA (24) and 5’ ATGTCATCTACGCGTTATTACCT, a nonsense oligonucleotide. HL60 cells were incubated in complete medium with either nonsense or antisense oligonucleotide at a final concentration of 20 \mu M for 48 h (fresh oligonucleotides were added after 24 h), and Northern blotting was performed as described previously (15) and analyzed on a Storm phosphorimager to determine the extent of down-regulation of mRNAs for Sp3, CD11c, and CD11b. Western blotting was performed to determine the effect of these oligonucleotides on expression of Sp3 and Sp1 protein.

RESULTS

Sp3 Binds to the CD11c and CD11b Promoters—My laboratory has recently shown using in vitro DNase I footprinting that purified Sp1 can protect the −132 to −104 and −72 to −63 regions of the CD11c promoter (Fig. 1A) and that sites within both regions are essential for promoter activation (17). EMSA of these two regions of the CD11c promoter with nuclear extract protein from HL60 cells revealed multiple DNA-protein complexes (Ref. 17 and Fig. 2). Of the three complexes formed using a probe that spans the −72 to −63 region (CD11c probe −79 to −62), complex 1 was supershifted with anti-Sp1 antibody (Fig. 2, compare lanes 2 and 3). Similarly, of the four complexes formed using a probe that spans the −132 to −104 region (CD11c probe −135 to −99), supershift analysis with anti-Sp1 antibody showed that Sp1 was bound to complex 1 (Fig. 2, compare lanes 7 and 8). The same complex formations and supershifts with anti-Sp1 antibody were seen when nuclear extract protein from nonmyeloid cell lines, Mol4 and HeLa, were analyzed (data not shown). Since Sp1-related proteins have recently been isolated, analysis of these complexes with antibodies to Sp3 and Sp4 was performed to determine whether these factors were present in any of the other complexes. When anti-Sp3 antibody was included in an EMSA using CD11c probe −79 to −62, formation of complex 3 was inhibited (rather than supershifted) when HL60 nuclear extract protein was assayed (Fig. 2, compare lanes 2 and 4). Similarly, formation of complex 4 was inhibited when the CD11c probe −135 to −99 was...
Sp3 Activates Leukocyte Integrin Genes CD11c and CD11b

...was used (Fig. 2, compare lanes 7 and 9). The same complex formations and inhibition of specific complex formations by anti-Sp3 antibody were seen when Molt4 and HeLa nuclear extract protein were analyzed (data not shown). These results also indicate that Sp1 and Sp3 are not co-bound on the same DNA molecule, since anti-Sp3 antibody did not affect the formation of the Sp1-specific complexes and anti-Sp1 antibody did not supershift the Sp3-specific complexes. Previous results showed that an Sp1-specific oligonucleotide could effectively compete with the formation of all complexes, including the Sp3-specific ones (17). Taken together, these results suggest that Sp3 competes with Sp1 for binding to the same or overlapping sites in the CD11c promoter. In contrast, anti-Sp4 antibody had no effect on any complex formation, indicating that Sp4 does not bind to these regions of the CD11c promoter (Fig. 2, lanes 5 and 10).

Chen et al. (18) show that the −66 to −59 region of the CD11b promoter was essential for Sp1-specific promoter activity (Fig. 1B). EMSA using nuclear extract protein from HL60 cells and a DNA probe spanning this region (CD11b probe −80 to −48) revealed three DNAprotein complexes (Fig. 2). Similar to what was seen in the analysis of the CD11c promoter, anti-Sp1 antibody supershifted complex 1 (Fig. 2, compare lanes 12 and 13), and inclusion of anti-Sp3 antibody in the EMSA inhibited formation of complex 3 (Fig. 2, compare lanes 12 and 14), indicating the presence of bound Sp3 to this or an overlapping region of the CD11b promoter. Complex formations and reactions to anti-Sp1 and anti-Sp3 antibodies were similar when nuclear extract protein from Molt4 and HeLa cells were used (data not shown). Anti-Sp4 antibody had no effect on complex formation and, thus, Sp4 does not appear to interact within this region (Fig. 2, lane 15).

Transfection of Sp-deficient Drosophila Cells Shows That Sp3 Is Functional—To determine whether transfected Sp3 can bind to and activate the CD11c and CD11b promoters, Drosophila cells, which are deficient in Sp-related proteins (26, 35), were initially cotransfected with 5 μg of pPacSp3 along with either the wt CD11c promoter-luciferase plasmid (containing the −133 to +66 region of CD11c) or the wt CD11b promoter-luciferase plasmid (containing the −500 to +50 region of CD11b) (Fig. 3). For comparison, the role of transfected Sp1 and Sp2 from expression constructs pPacSp1 and pPacSp2, respectively, was similarly analyzed. EMSA revealed that Sp3 in these transfected Drosophila cells was expressed and could bind efficiently to CD11c probes −79 to −62 and −135 to −99 and CD11b probe −80 to −48 (Fig. 3, lanes 5, 12, and 19).
Transfected Sp1 in these Drosophila cells could also bind these three probes (Fig. 3, lanes 3, 10, and 17). When pPacSp1 and pPacSp3 were transfected together, both Sp1 and Sp3 were expressed and could bind CD11c probes −79 to −62 and −135 to −99 (Fig. 3, lanes 7 and 14) and CD11b probe −80 to −48 (data not shown). As expected, transfected Sp1 bound to the CD11b probe was supershifted with anti-Sp1 antibody (Fig. 3, compare lanes 17 and 18), and binding of transfected Sp3 to the CD11b probe was blocked with anti-Sp3 antibody (Fig. 3, compare lanes 19 and 20). In contrast, transfected Sp2 did not bind either the CD11c probes (Fig. 3, lanes 4 and 11) or the CD11b probe (data not shown).

Sp3-dependent luciferase activity from the CD11c promoter was shown to increase linearly with up to 1 μg of cotransfected pPacSp3 (Fig. 4A). As was previously shown (17), CD11c promoter activity was also stimulated by Sp1, and this activity was also increased linearly with up to 1 μg of cotransfected pPacSp1 (Fig. 4A). Maximal CD11c promoter activity was attainable with either 10 μg of pPacSp3 (23-fold induction) or 5 μg of pPacSp4 (27-fold induction). A similar response of the CD11b promoter to Sp3 and Sp1 was seen (Fig. 4B). CD11b promoter activity increased linearly with up to 1 μg of either pPacSp3 or pPacSp1, and maximal activity was reached with either 5 μg of pPacSp3 (28-fold induction) or 5 μg of pPacSp1 (35-fold induction).

Since antibodies to Sp1 and Sp3 affected formation of different complexes in EMSA using either the CD11c or CD11b probes (Fig. 2), this indicated that Sp1 and Sp3 are not co-bound to the same DNA molecule. Instead, this result indicated that Sp1 and Sp3 compete for the same or overlapping sites when interacting either on the CD11c or CD11b promoter. To confirm this, Drosophila cells were transfected with the appropriate reporter construct and either 1 or 5 μg each of pPacSp1 and pPacSp3. If Sp1 and Sp3 act at distinctly different sites on either promoter, then cotransfection of pPacSp1 and pPacSp3 should result in higher promoter activity than that obtained when either expression construct was transfected separately. Luciferase activity from either the wt CD11c-luciferase plasmid (Fig. 4A) or the wt CD11b-luciferase plasmid (Fig. 4B) was not increased above that seen in the presence of only pPacSp1. This indicates that Sp1 and Sp3 compete for the same sites, and as subsequent rounds of transcription initiate from these promoters, either Sp1 or Sp3 is bound at each site, depending on relative binding affinities.

Sequence analysis of the −135 to −99 and −79 to −62 regions of the CD11c promoter previously revealed four putative Sp1 binding sites (referred to as sites A, B, C, and D; Fig. 1A and Ref. 17). My laboratory reported that deletion of either the −121 to −103 (containing sites B and C) or the −70 to −65 (containing site D) region led to loss of PMA-inducibility of the CD11c promoter in HL60 cells and that Sp1 interacted at these sites (17). To confirm that Sp3 interacts at the same sites as Sp1 in these promoters, CD11c-luciferase plasmids containing deletions (Δ) of either the −121 to −103 (ΔBC) or −70 to −65 region (ΔD) or both (ΔBCD) were cotransfected into Drosophila cells along with pPacSp1 or pPacSp3 (Fig. 5A). Consistent with previous results, deletions of either site resulted in significantly lowered Sp1-mediated CD11c promoter activity. In contrast, although deletion of either site led to significantly decreased Sp3-mediated promoter activity, deletion of the −70 to −65 region had a much greater effect on activation of the CD11c promoter by Sp3. Compared with the induction of the wt CD11c promoter by Sp3 and Sp1 (18.1- and 23.2-fold, respectively), the induction by Sp3 was disproportionately lower than that by Sp1 when the −70 to −65 region was deleted (2.1-fold versus 10.2-fold, respectively). Further, interaction of Sp3 at each site is noncooperative, with each site responsible for a portion of the intact promoter activity. Analysis of the CD11b promoter indicated that neither Sp3 nor Sp1 was able to activate the CD11b promoter-luciferase plasmid containing a deletion of the −64 to −58 region (ΔSp), showing that this site can be occupied by either factor (Fig. 5B).

Sp3 Activates Leukocyte Integrin Genes CD11c and CD11b

Sp3 Regulates the CD11c and CD11b Promoters In Myeloid Cells—Recent in vivo genomic footprinting of the leukocyte integrin promoters in my lab (17) and in others (18) revealed that the −121 to −103 and −70 to −65 regions of the CD11c promoter and the −66 to −59 region of the CD11b promoter are occupied by protein, specifically in myeloid but not nonmyeloid cells. Since transfection studies in Drosophila cells revealed that Sp1 (expressed from pPacSp1) could activate both integrin promoters, it was presumed that Sp1 was the protein that occupied these promoters in myeloid cells. Transfection of pPacSp1 or pPacSp3 into myeloid cells, however, did not activate these promoters, (which was not surprising as Sp proteins
Sp3 Activates Leukocyte Integrin Genes CD11c and CD11b

FIG. 6. Down-regulation of CD11c and CD11b mRNAs with Sp3 antisense DNA. HL60 cells were incubated for 48 h with either antisense Sp3 oligonucleotides or nonsense oligonucleotides. A, total RNA was then isolated and hybridized with Sp3, Sp1, and actin gene probes. The corresponding RNA gel is shown. Total protein from antisense- and nonsense-treated cells was subjected to Western blot analysis with anti-Sp3 and anti-Sp1 antibodies. B, PMA was added for 24 h to induce transcription of CD11c and CD11b in HL60 treated 48 h with either antisense or nonsense oligonucleotides. Antisense or nonsense oligonucleotides were present during the induction with PMA. Total RNA was then isolated and hybridized with CD11c, CD11b, and actin gene probes.

FIG. 5. Effect of deleting the Sp binding sites on CD11c and CD11b promoter expression. Deletions were introduced into the wt CD11c-luciferase reporter plasmid (containing the −133 to +66 region of CD11c) (A) and wt CD11b-luciferase reporter plasmid (containing the −500 to +50 region of CD11b) (B). The promoter deletion plasmids were cotransfected with either 5 μg of pPacSp1 or 5 μg of pPacSp3 into Drosophila cells. The schematics summarize the regions deleted, and sites B, C, D, and Sp refer to consensus Sp1 binding sequences within these regions. The specific deletions (Δ) are as follows: ΔB, (−121 to −103 of CD11c deleted); ΔD, (−70 to −65 of CD11c deleted); ΔBCD, (−121 to −103 and −70 to −65 of CD11c deleted); and ΔSp, (−64 to −58 of CD11b deleted). The Fold Induction is as described in Fig. 4. The standard deviation of the mean is indicated with an error bar.

are ubiquitously expressed and already present) and, as such, did not provide further insight (data not shown). To determine whether Sp3 regulates the CD11c and CD11b promoters in myeloid cells, antisense oligonucleotides to the 5’ portion of the coding region for Sp3 were added to unstimulated HL60 cells for 48 h to down-regulate Sp3 expression. Northern blot analysis showed that the level of Sp3 mRNA in antisense-treated HL60 cells was undetectable as compared with that in cells treated with a nonsense oligonucleotide (Fig. 6A). Further, the level of Sp1 mRNA was unaffected by the Sp3 antisense oligonucleotide. Western blotting revealed that the amount of Sp3 protein was significantly reduced to 3.5% that of the normal level, and Sp1 levels were unaffected (Fig. 6A). Upon stimulation of the antisense and nonsense oligonucleotide-treated HL60 cells with PMA for 24 h, both CD11c and CD11b mRNAs were induced to approximately the same levels. In contrast, the levels of CD11c and CD11b mRNAs were induced to only 56 and 61%, respectively, of those levels detected in HL60 cells treated with the nonsense oligonucleotide (Fig. 6B).

In a parallel experiment, the reporter constructs wt CD11c luciferase, wt CD11b luciferase, or the Drosophila actin promoter luciferase (pPac-luciferase) were transfected into antisense-treated and PMA-induced HL60 cells (Fig. 7). PMA-induced CD11c promoter activity in the antisense-treated cells was reduced to 55% that of the nonsense-treated cells (Fig. 7A). Cotransfection of 5 μg of pPacSp3 along with the reporter plasmid partially restored CD11c promoter activity to 87% that of the nonsense-treated cells. A similar reduction in CD11b promoter activity (62% that of the nonsense-treated cells) was obtained in antisense-treated cells (Fig. 7B). Cotransfection of 5 μg of pPacSp3 along with the reporter plasmid essentially restored CD11b promoter activity to 93% that of the nonsense-treated cells. The Drosophila actin promoter, which lacks binding sites for Sp proteins, was unaffected (Fig. 7C). Taken together, these results indicate that Sp3 regulates these promoters and is most probably one of the Sp-type proteins that occupies these promoters in vivo. Attempts to lower the endogenous Sp1 level in HL60 cells in an analogous manner, however, were not successful.

Sp3 Cooperates with c-Jun to Induce the CD11c Promoter—A previous study in my laboratory (17) showed that Sp1 cooperates with c-Jun for activation of the CD11c promoter. In that study it was shown that the Sp1 and AP1 (see Fig. 1A) binding sites in the CD11c promoter can interact and that both Sp1 sites are needed for full cooperativity. To determine whether Sp3 also cooperates with c-Jun, Drosophila cells were cotransfected with the wt CD11c-luciferase plasmid along with either pPacSp3 or pPacJun or both (Fig. 8). The amount of pPacSp3 transfected along with pPacJun was reduced to 0.5 μg to ensure that the CD11c promoter was not already fully activated and that any cooperative interactions with c-Jun would not be obscured. Luciferase expression from the CD11c promoter increased 4.3-fold in the presence of transfected pPacSp3. No significant increase in luciferase expression was detected when only 5 μg of pPacJun was transfected with the CD11c-luciferase plasmid. EMSA confirmed that significant amounts of c-Jun was expressed from pPacJun (data not shown). In contrast, CD11c promoter activity increased to 13.4-fold when both pPacSp3 and pPacJun were cotransfected. This enhancement was dependent on the presence of the two contiguous AP1 sites in the CD11c promoter, as little enhancement was detected when pPacSp3 and pPacJun were cotransfected with a reporter construct containing a deletion of both AP1 sites (data not shown).
shown). As previously shown, Sp1 also cooperated with c-Jun to activate the CD11c promoter. In contrast, Sp2 (expressed from pPacSp2) did not show cooperativity with c-Jun.

It was possible that the observed cooperativity between Sp3 and c-Jun was simply the result of one factor up-regulating the other. Since Drosophila lacks Sp proteins, it seems unlikely that Sp3 was up-regulating the actin promoter of pPacJun to increase the c-Jun concentration. Also, transfection of 5 µg of pPacJun alone was not able to activate the CD11c promoter; therefore, it is unlikely that increasing the endogenous concentration of c-Jun would have any effect. Further, overexpression of c-Jun in Drosophila did not increase luciferase expression from pPacO, which contains the Drosophila actin promoter fused to luciferase (data not shown). As previously shown, Sp1 also cooperated with c-Jun to activate the CD11c promoter. In contrast, Sp2 (expressed from pPacSp2) did not show cooperativity with c-Jun.

It was possible that the observed cooperativity between Sp3 and c-Jun was simply the result of one factor up-regulating the other. Since Drosophila lacks Sp proteins, it seems unlikely that Sp3 was up-regulating the actin promoter of pPacJun to increase the c-Jun concentration. Also, transfection of 5 µg of pPacJun alone was not able to activate the CD11c promoter; therefore, it is unlikely that increasing the endogenous concentration of c-Jun would have any effect. Further, overexpression of c-Jun in Drosophila did not increase luciferase expression from pPacO, which contains the Drosophila actin promoter fused to luciferase (data not shown). To confirm that enhancement of CD11c promoter activity by cotransfected Sp3 and c-Jun was simply the result of one factor up-regulating the other, Western blotting was performed on proteins isolated from the above transfected Drosophila cells (Fig. 9). The level of c-Jun in Drosophila cells transfected with 5 µg of pPacJun (Fig. 9, lanes 5 and 6, probed with two different concentrations of anti-c-Jun antibody) was essentially the same as that detected when 0.5 µg of pPacSp3 was included in the transfected DNA (Fig. 9, lanes 9 and 10, also probed with two different concentrations of anti-c-Jun antibody). Similarly, the level of Sp3 in pPacSp3-transfected cells (Fig. 9, lanes 3 and 4, two different antibody concentrations) was approximately the same as that found in cells transfected with both pPacSp3 and pPacJun (Fig. 9, lanes 11 and 12, two different antibody concentrations).

**DISCUSSION**

Drosophila cells do not express Sp proteins and, therefore, are a useful system to study the role of such proteins in the regulation of Sp-regulated genes. However, since the leukocyte integrin genes are not present in Drosophila cells, studies of these genes in this cell type may or may not accurately reflect the actions of Sp proteins on the endogenous genes expressed in myeloid cells. This concept is underscored by the finding that Sp1 and Sp3 are ubiquitous, and Northern analysis revealed that the levels of these proteins are essentially the same in myeloid cells that do not express the CD11c and CD11b genes (unstimulated HL60 cells) and in myeloid cells that do (PMA-stimulated HL60 cells). In myeloid cells, these Sp proteins may be bound to an inhibitor that renders them nonfunctional. Chen et al. (36) identify a 20-kDa protein fraction referred to as Sp1-I from CV-1 cells (green monkey kidney cells) that inhibited binding of Sp1 to the rat c-jun promoter in vitro. Murata et al. (37) show that two proteins, 74 and 110 kDa in size, present in mouse (NIH/3T3), rat (PC12), and nine human cell lines can associate with the amino terminus of Sp1 in vitro. Drosophila cells, however, did not express the 74- and 110-kDa proteins. Further, deletion of 82 amino acids from the amino terminus of Sp1 and Sp3 are ubiquitous, and Northern analysis revealed that the levels of these proteins are essentially the same in myeloid cells that do not express the CD11c and CD11b genes (unstimulated HL60 cells) and in myeloid cells that do (PMA-stimulated HL60 cells). In myeloid cells, these Sp proteins may be bound to an inhibitor that renders them nonfunctional. Chen et al. (36) identify a 20-kDa protein fraction referred to as Sp1-I from CV-1 cells (green monkey kidney cells) that inhibited binding of Sp1 to the rat c-jun promoter in vitro. Murata et al. (37) show that two proteins, 74 and 110 kDa in size, present in mouse (NIH/3T3), rat (PC12), and nine human cell lines can associate with the amino terminus of Sp1 in vitro. Drosophila cells, however, did not express the 74- and 110-kDa proteins. Further, deletion of 82 amino acids from the amino terminus of Sp1 and Sp3 are ubiquitous, and Northern analysis revealed that the levels of these proteins are essentially the same in myeloid cells that do not express the CD11c and CD11b genes (unstimulated HL60 cells) and in myeloid cells that do (PMA-stimulated HL60 cells). In myeloid cells, these Sp proteins may be bound to an inhibitor that renders them nonfunctional. Chen et al. (36) identify a 20-kDa protein fraction referred to as Sp1-I from CV-1 cells (green monkey kidney cells) that inhibited binding of Sp1 to the rat c-jun promoter in vitro. Murata et al. (37) show that two proteins, 74 and 110 kDa in size, present in mouse (NIH/3T3), rat (PC12), and nine human cell lines can associate with the amino terminus of Sp1 in vitro. Drosophila cells, however, did not express the 74- and 110-kDa proteins.

---

2 John D. Noti, unpublished observations.
Sp3 Activates Leukocyte Integrin Genes CD11c and CD11b

a GAL4-Sp1 expression construct resulted in a 100-fold increase in Sp1-mediated transcriptional activity in mammalian cells, which was correlated with a decrease in binding of p74 to Sp1 in vitro. In vivo, Sp1 (or a related factor) has been shown to bind the CD11c (17) and CD11b (18) promoters specifically in myeloid but not nonmyeloid cells, indicating that this transactivator controls cell-specific expression. The lack of expression of the 74- and 110-kDa inhibitor proteins in Drosophila may explain why Sp1-dependent and even Sp3-dependent activation of the myeloid-specific CD11c and CD11b promoters is seen in Drosophila. Therefore, the previously observed binding of Sp proteins to the CD11c promoter in vivo in PMA-stimulated but not unstimulated myeloid cells may depend on the release of a negative regulator protein from Sp1 and Sp3 in PMA-stimulated cells. The CD11b promoter may also be controlled by a negative regulator of Sp proteins; however, the regulation of this promoter apparently differs somewhat from that of the CD11c promoter. In vivo, Sp protein was shown to be bound to the CD11b promoter both in unstimulated as well as in PMA-stimulated myeloid cells (18). Since the CD11b promoter is inactive in unstimulated myeloid cells, the Sp protein bound to the promoter may be rendered inactive by a negative regulator.

The retinoblastoma protein (Rb) is an important transcriptional regulator of genes involved in cell growth and differentiation. Rb can mediate either gene repression or induction, and its mechanism of action is dependent on the specific gene and cell type. Transient transfection analyses of NIH3T3 cells (mouse fibroblasts) have shown that Rb represses expression of the c-fos (38), c-myc (39), and neu (40) genes. In contrast, in CCL-64 cells (mink lung epithelial cells), the c-fos, c-myc, TGF-β1 (41), and insulin-like growth factor II genes (42) are induced by Rb. Recent studies have revealed that Rb coexpression stimulates Sp1- and Sp3-mediated transcriptional activation of the c-fos, c-myc, and TGF-β1 promoters (30, 43) and that this mechanism may involve the release of a negative inhibitor from the Sp protein by Rb (36, 37). This suggests that the CD11c and CD11b promoters could conceivably be induced by Rb and would account for the cell-specific action of the Sp proteins in myeloid cells. However, neither the CD11c luciferase nor the CD11b luciferase expression constructs were induced by cotransfected Rb (expressed from pCMV-Hrb) in Drosophila cells. This lack of response by the leukocyte integrin promoters to cotransfected Rb was seen also in myeloid and nonmyeloid cells. Therefore, induction of the CD11c and CD11b promoters by Sp proteins does not appear to involve the release of a negative regulatory factor from the Sp protein by wild-type Rb. An alternative hypothesis is that wild-type Rb itself functions as a negative regulator of the CD11c and CD11b genes by complexing directly with the Sp protein or indirectly through a “tethering” molecule. PMA might then mediate the release of Rb from the Sp protein, leading to gene induction. However, although Rb has been shown to bind at least 10 distinct proteins including the transcription factors ATF-2 (44) and E2F (45), direct interaction of Rb with Sp proteins has not been reported. Since induction of the CD11c and CD11b promoters by PMA was not reduced by overexpression of wild-type Rb (which would be expected to reinforce a putative Rb-Sp complex), it seems unlikely that wild-type Rb negatively regulates the CD11c and CD11b genes. The possibility that a mutated Rb could mediate leukocyte integrin gene expression has not been tested.

Sp1-mediated activation of BCAT-2, an artificial promoter containing two Sp binding sites and a TATA box upstream of the CAT gene, was repressed by cotransfected Sp3 (28). Since an intact Sp3-DNA binding domain was required for this repression, it was concluded that Sp3 competes with Sp1 for the same site. This result suggested that Sp3 may function simply by preventing Sp1 from binding and is consistent with a passive role for this molecule in repression. Recently, however, it was shown that Sp3 contains a repression domain in that a chimeric tet-Sp3 protein (Sp3 lacking the zinc finger region but containing the tet operator DNA binding domain) tethered to the tetO sequence could repress the activating functions of various transcriptional activators bound to an adjacent GAL4 sequence (29). Whatever the mechanism of negative regulation by Sp, since both Sp1 and Sp3 are ubiquitous, it was possible that in nonmyeloid cell types Sp3 preferentially displaces Sp1 from the leukocyte integrin promoters and prevents their induction by PMA. In myeloid cells, the induction by PMA conceivably could result from the removal of bound Sp3 and the subsequent binding of Sp1 to the same site. However, in vivo genomic footprinting of the CD11c (17) and CD11b promoters (18) showed that the Sp1 binding sites were unoccupied in nonmyeloid cells. Further, although the Sp binding site in the CD11b promoter was shown to be occupied in unstimulated myeloid cells, supporting the concept that Sp3 was the bound protein, the two Sp1 sites in the CD11c promoter were not. These previous findings are consistent with the results reported in this study showing that Sp3 does not repress these promoters.

The protein structure of Sp3 is similar to that of Sp1. Both contain three zinc fingers, two glutamine-rich regions, a serine/threonine-rich region, a highly charged region, and homologous C terminal ends (24). Substitution of the regions of Sp1 involved in activation by the homologous Sp3 domains, however, did not result in activation of the BCAT-2 promoter (28). However, Kingsley and Winoto (24) cite a preliminary finding that Sp3 could activate the GT box of the TCR Vα11.1 promoter, suggesting that Sp3 may preferentially activate certain promoters. The repression domain of Sp3 apparently is functional only in conjunction with specific promoters and may be cell-specific, since activation rather than repression of the leukocyte integrin promoters was shown in this study. The presence of both repressor and activator domains in a transcription factor is not without precedent. BTEB1, which binds GC boxes, activates genes containing multiple GC sequences but represses those containing only a single GC box (46). However, Sp3 functions differently than BTEB1, as the CD11c promoter contains two Sp protein binding sites, whereas the CD11b promoter contains only one, yet both promoters are activated by Sp3. The reason why two members of the same transcription factor family bind and activate the same sites in the leukocyte integrin promoters is unclear, particularly since both factors are ubiquitous. Since cell-specific activation of CD11c and CD11b is mediated by these Sp proteins, extracellular signals that activate Sp1 and Sp3 may differ and depend on the particular cell type.

The repressor function of Sp3 is dependent on binding of Sp3 to the promoter (28, 29). It is not known whether the activation domain(s) of Sp3 can function independent of DNA binding as has been shown for Sp1 by a mechanism referred to as “superactivation.” Zinc-fingerless Sp1 can physically associate with either intact Sp1 or Sp4 bound to DNA and enhance activation (26). The superactivation potential of Sp3 on leukocyte integrin expression is being investigated.

This study indicated that neither Sp2 nor Sp4 are involved in the PMA induction of the CD11c and CD11b promoters. Incubation of the –135 to –99 region of the CD11c promoter with HL60 nuclear extract protein revealed several DNA-protein complexes by EMSA (Fig. 2). Two complexes have been shown to contain either Sp1 or Sp3; however, the identities of the
proteins in the additional complexes are unknown. EMSA of this region using antibodies specific to the Sp-related proteins EGR-1 (47), EGR-2 (48), and EGR-3 (49) revealed that these proteins do not bind this region. The additional complexes may simply represent breakdown products of either Sp1 or Sp3 lacking epitopes for the antibodies used in supershift analysis or may result from additional Sp-related proteins interacting in this region.

Recently my laboratory reported that Sp1 could bind to the −121 to −103 and −70 to −65 regions of the CD11c promoter and activate transcription in Drosophila cells (17). Binding of Sp1 to these regions was shown to be independent and noncooperative in that deletion of one site did not affect Sp1 interaction at the other. Further, Sp1 was shown to cooperate with c-Jun and c-Fos to activate the CD11c promoter in Drosophila cells. In this study, Sp3 activation of the CD11c promoter was also shown to be noncooperative, although the −70 to −65 region was found to be more responsive to Sp3 than the −121 to −103 region. Clustering of GC boxes near the transcriptional start site is common, but the importance of each site varies to further increased the cooperative interaction with c-Jun and c-Fos. Through an in vitro footprinting assay, my laboratory has shown that binding of Sp1 to the CD11c promoter directly facilitates binding of c-Jun to this region. Clustering of GC boxes near the transcriptional start site is common, but the importance of each site varies to further increased the cooperative interaction with c-Jun and c-Fos. Through an in vitro footprinting assay, my laboratory has shown that binding of Sp1 to the CD11c promoter directly facilitates binding of c-Jun to this region.

Acknowledgments—The author wishes to thank Dr. R. Tjian for generously sharing the pPhcO, pPhcSp1, and pCMc-Jun plasmids; Dr. J. M. Horowitz for kindly providing pCMV-Hrb; and Dr. C. Williams for the Western blot analysis. B. C. Reinemann and M. N. Petrus provided expert technical assistance. The author is indebted to Nando Chukaya for helpful discussions.

REFERENCES

1. Perez, C., Coeffer, E., Moreau-Gachelin, F., Wietzerbin, J., and Benech, P. D. (1994) J. Biol. Chem. 269, 8296–8300
2. Zhang, D., Hetherington, C. J., Chen, H., and Tenen, D. G. (1994) J. Biol. Chem. 269, 32015–32020
3. Merika, M., and Orkin, S. A. (1995) Mol. Cell. Biol. 15, 2437–2447
4. Yu, C.-Y., Chen, J., Lin, L.-I., Tam, M., and Shen, C.-K. J. (1990) J. Biol. Chem. 265, 182–294
5. Schauhecke, P., West, B. L., and Reeder, F. D. (1991) J. Biol. Chem. 265, 17189–17196
6. Courty, A. J., and Tjian, R. (1988) Cell 55, 875–890
7. Revius, J., and Reinemann, B. C. (1995) J. Biol. Chem. 279, 27053–27059
8. Zhang, D., Hetherington, C. J., Chen, H., and Tenen, D. G. (1994) J. Biol. Chem. 269, 32015–32020
9. Fischer, K.-D., Haese, A., and Nowock, J. (1993) J. Biol. Chem. 268, 8533–8536
10. De Luca, P., Majello, B., and Lania, L. (1996) J. Biol. Chem. 271, 8533–8536
11. Udvadia, A. J., Rogers, K. T., Higgins, P. D., Martin, K. H., Humphrey, P. A., and Horowitz, J. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3655–3660
12. Rosmarin, A. G., Weil, S. C., Rosner, G. L., Griffin, J. D., Arnaout, M. A., and Tenen, D. G. (1993) Blood 82, 3893–3900
13. Patwardhan, S., Gashler, A., Siegel, M. G., Chang, L.-C., Joseph, L. J., Shows, T. B., Bollag, D. G., Clemens, K. E., and Brady, J. N. (1993) J. Biol. Chem. 268, 9122–9127
14. Ebert, S. N., and Wong, D. L. (1995) J. Biol. Chem. 270, 17299–17305
15. Noti, J. D., and Reinemann, B. C. (1995) Mol. Immunol. 32, 361–369
16. Shapiro, L. H. (1995) J. Biol. Chem. 270, 14183–14187
17. Noti, J. D., Reinemann, B. C., and Petrus, M. N. (1996) Mol. Cell. Biol. 16, 2940–2950
Sp3 Mediates Transcriptional Activation of the Leukocyte Integrin Genes \textit{CD11C} and \textit{CD11B} and Cooperates with c-Jun to Activate \textit{CD11C}

John D. Noti

\textit{J. Biol. Chem.} 1997, 272:24038-24045.
doi: 10.1074/jbc.272.38.24038

Access the most updated version of this article at http://www.jbc.org/content/272/38/24038

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 38 of which can be accessed free at http://www.jbc.org/content/272/38/24038.full.html#ref-list-1