Lineage-Restricted Regulation of the Murine SCL/TAL-1 Promoter


The SCL/TAL-1 gene encodes a basic helix-loop-helix transcription factor that is expressed in multipotent hematopoietic progenitors before lineage commitment. Its expression is maintained during differentiation along erythroid, mast, and megakaryocytic lineages, but is repressed after commitment to nonexpressing lineages. To begin to address the molecular mechanisms underlying this complex pattern of expression, we have studied the regulation of the murine SCL promoter in erythroid and T-cell lines. Analysis of the methylation and chromatin structure of the SCL promoter region showed that SCL mRNA expression correlated with DNase hypersensitive sites and methylation status of the promoter. Transient reporter assays showed that promoter 1a was active in erythroid cells but not in T cells. Sequences between −187 and +26 were sufficient for lineage-restricted activity of promoter 1a. A joint promoter construct containing both promoter 1a and promoter 1b also exhibited lineage-restricted activity. Conserved GATA (−37), MAZ (+242), and ETS (+264) motifs were all shown to contribute to SCL promoter activity in erythroid cells, but several other motifs were not required for full promoter activity. The pattern of complexes binding to +242 MAZ and +264 ETS sites were the same in erythroid and T cells. However, GATA-1 bound the −37 GATA site in erythroid cells, whereas in T cells GATA-3 was only able to bind weakly, if at all. Moreover, GATA-1 but not GATA-2 or GATA-3 was able to transactivate SCL promoter 1a in a T-cell environment. These results suggest that inactivity of SCL promoter 1a in T cells reflected the absence of GATA-1 rather than the presence of trans-dominant negative regulators.

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TRANSCRIPTION FACTORS play a central role in the molecular regulation of hematopoiesis. A growing body of evidence suggests that lineage commitment and differentiation are regulated by the combinatorial effects of multiple transcription factors on different target genes.1,2 The mechanisms responsible for establishing lineage-restricted patterns of transcription factor expression are therefore central to any analysis of the molecular control of hematopoiesis.

We have chosen to focus on the role of the SCL gene in hematopoiesis. The SCL gene (also known as TAL-1) encodes a protein of the basic helix-loop-helix (bHLH) family of transcription factors and is expressed predominantly in hematopoietic tissues.3,4 Studies of SCL expression in a wide variety of hematopoietic cell types have shown high levels of SCL mRNA in cells of the erythroid, megakaryocytic, and mast cell lineages5-10 as well as in endothelial cells11 and brain.12 Activation of the SCL gene by chromosomal rearrangements in up to 25% of T-cell acute lymphoblastic leukemias13-15 suggests that it acts as an oncogene in the T-cell lineage, but the precise role of SCL in other cell types in which it is normally expressed remains uncertain.16,17

Insight into the function of SCL has been provided by antisense experiments. In K562 cells, antisense SCL inhibited proliferation and self-renewal,16 whereas in murine erythroleukemia (MEL) cells it inhibited erythroid differentiation.17 In addition, constitutive expression of exogenous SCL inhibited myeloid differentiation of M1 cells18 but enhanced erythroid differentiation of MEL cells.17 These data suggest that SCL plays a positive role in erythroid differentiation and also that SCL downregulation is necessary for myeloid differentiation. Furthermore, the results suggest that the SCL protein performs distinct functions in different cell types. Interestingly, SCL protein levels do not increase during terminal erythroid differentiation, in marked contrast to SCL mRNA levels.19 SCL protein is phosphorylated19,20 and forms complexes with E2A proteins and with RhoM-2.21,22 However, although the binding site for SCL/E2A heterodimers has been characterized,23 physiologic target genes regulated by SCL remain obscure.

The human SCL gene comprises a complex transcriptional unit with two alternate 5′ exons and multiple alternately spliced transcripts.15,24 Most of the reported splice variants affect the 5′ untranslated region and do not produce an altered protein product. However, both exon 1a and exon 1b can splice directly to exon 5. These transcripts potentially encode a truncated protein lacking a putative N-terminal transactivation domain.25 The human SCL promoter 1a can be transactivated by GATA-1 in fibroblasts and Cos cells.17,26 Moreover GATA-1 and/or GATA-2 are coexpressed with SCL in various hematopoietic cell types.14,15,27 GATA proteins are therefore likely to play an important role in regulating SCL expression.

SCL mRNA levels are upregulated during erythroid differentiation of MEL cells19 and downregulated during myeloid differentiation of K562 cells.20 More recently, SCL mRNA modulation has been studied using FDCP-mix cells as a more physiologic model for hematopoietic differentiation.21 SCL was expressed in multiple different FDCP-mix cell lines before lineage commitment. These studies suggest that SCL is expressed in multipotent progenitors and that its expression is maintained during differentiation along some hematopoietic lineages but is downregulated after commitment to other nonexpressing lineages. Erythropoietin-induced erythroid differentiation of FDCP-mix A4 cells resulted in upregulation of SCL mRNA, whereas growth factor-induced granulocyte/monocyte differentiation was accompanied by a marked decrease in SCL mRNA.

To begin to address the molecular mechanisms underlying
the complex regulation of the SCL gene during hematopoiesis, we have previously cloned a murine SCL cDNA and characterized the structure of the murine genomic locus. The murine gene consists of 7 exons spanning 20 kb and has an intron/exon structure very similar to that of the human gene. Two transcription initiation sites in alternate 5' exons have been described, together with a complex pattern of alternate splicing. We show here that the absence of detectable SCL mRNA in T cells was accompanied by transcriptional inactivity of SCL promoters 1a and 1b. We have characterized proteins binding to conserved transcription factor binding sites upstream of promoter 1a and promoter 1b and have shown that the regulatory patterns are different for full SCL promoter activity in erythroid cells. Moreover, GATA-1 but not GATA-2 or GATA-3 was able to transactivate SCL promoter 1a in a T-cell environment.

Materials and Methods

Cell Lines

The MEL cell line F4N, the murine T-cell line BW 5147, and the LTK fibroblast lines have been described previously. Cell lines were grown in Dulbecco's modified Eagle's medium (DME) plus 10% fetal calf serum.

Plasmids

SCL promoter sequences in all constructs are from a genomic clone isolated from a Balb/C genomic library. All luciferase reporter constructs were derived from a parental construct. This construct was generated by cloning a Sau3A---Sac I genomic subfragment (containing exon 1a and 1b) into the Sac I site of pGL-2 basic (Promega, Madison, WI). All nucleotide positions are relative to the exon 1a start site (see Fig 2). The reporter construct −2,000 SCL1a-Luc was obtained from the parental plasmid by digestion with BstHII and Mlu I followed by religation. Plasmids −328 SCL1a-Luc, −187 SCL1a-Luc, and −55 SCL1a-Luc were derived from −2,000 SCL1a-Luc by digestion with Age I, Esp I, and Ava I, respectively; incubation with T4 DNA polymerase to generate blunt ends; cutting with Sma I; and religating. Plasmid −2,000 SCL1a-b-Luc was obtained from the parental plasmid by digestion with Xho I and partial digestion with Eag I followed by filling in with DNA polymerase I Large (Klenow) fragment and religation. Plasmid −55 SCL1a-b-Luc was obtained from plasmid −2,000 SCL1a-Luc by digestion with Ava I and Sac I, incubation with T4 DNA polymerase to generate blunt ends, and religation. Plasmid +26 SCL1b-Luc was obtained from plasmid −2,000 SCL1a-b-Luc by digestion with Sac I and BstHII, incubation with T4 DNA polymerase to generate blunt ends, and religation. Construct +209 SCL1b-Luc was derived from the parental plasmid by digestion with Sma I and religation.

pEF-BOS lac-Z contains the lac-Z gene under the control of the pEF-promoter and was kindly provided by Dr K. Chatterjee (Department of Medicine, Cambridge University, UK).

Expression plasmids containing human GATA-1, GATA-2, and GATA-3 (pEF-BOS GATA-1, pEF-BOS GATA-2, and pEF-BOS GATA-3) were kindly provided by Dr T.H. Rabbits (MR/LMB, Cambridge, UK).

In addition, all three GATA expression plasmids contained the 3' in frame nucleotide sequence AGAGGACCTGGATCTCCCGAGGAGACCTGAACTCAG that encodes the EKQLISEEDLN MYC-tag--specific epitope. This MYC-tag epitope is specifically recognized by the 9E10 monoclonal antibody, which was kindly provided by Dr T.H. Rabbits.

Site-Directed Mutagenesis

Site-directed mutants were created using a mutagenesis kit (Clontech, Palo Alto, CA) or the Kunkel method. The following oligonucleotides were used as primers in the mutagenesis: −37 GATA, 5' GCCGGCGCAGAGAGGAGGCGCC 3'; −63 GATA, 5' CCGGCCGCCCTGTGCAGCCCTGGCGCC 3'; −63 SPI, 5' CCGAGATAAGGAAGCGCTCCTGGGCGC 3'; −101 API, 5' GCCATATGGGGGCAATTGGATCTTTTATTGGCAATTTCC 3'; −112 CCAA, 5' ATTCAATTTTGAATCTGCAATTTCTCGG 3'; +242 MAZ, 5' AGTCTCCCGGCTGCGAGGCGAGAGGGGGGAGG 3'; +259 ETS, 5' GGCAGAAGGGAAAAAGGGGAGTCGGAAGAGGATTGCCC 3'.

When the Clontech mutagenesis kit was used, a pGL2−specific selection primer 5' CAAGGGGCTACGGTGACGGTACGACG 3' was also used. All mutations were subsequently confirmed by nucleotide sequence analysis. Furthermore, gel shift assays were used to show that the mutations did not introduce new protein binding sites.

Transfection and Transient Expression Conditions

A total of 2 × 10⁶ cells growing in log phase (1 to 5 × 10⁶ cells/mL) were electroporated under standard conditions (960 μF, 220 V; BioRad gene pulser; BioRad, Hemel Hempstead, Herts, UK) with 10 μg pEF-BOS lac-Z and 15 μg of the appropriate luciferase reporter constructs or with 15 μg of pEF-BOS expression plasmid, 10 μg of reporter plasmid, and 10 μg of pEF-BOS lac-Z. All plasmids were cesium-banded or prepared by purification through Quiex P-500 columns (Qiagen, Chatsworth, CA). After electroporation, the cells were resuspended in 10 mL of tissue culture medium with 10% fetal calf serum and grown for 18 to 24 hours at 37°C in 5% CO₂.

β-Galactosidase and Luciferase Assays

β-Galactosidase (β-gal) assays were performed as described. β-gal activity was measured using a V-max microplate reader (Molecular Device, Menlo Park, CA) in the kinetic mode at a wavelength of 405 nm. For the reading, only the linear part of the curve was used. Luciferase assays were performed as follows. The transfected cells were harvested and washed twice in phosphate-buffered saline (PBS). Subsequently, the cell pellets were lysed with 135 μL of LB buffer (25 mmol/L Tris-phosphate buffer, pH 7.8, 8 mmol/L MgCl₂, 1 mmol/L 1,4-Dihio-DL-threitol [DTT], 1% Triton-X 100, 1% albumin from bovine serum [BSA] [w/vol] and 15% glycerol [v/vol]), incubated for 5 to 10 minutes at room temperature, and centrifuged for 5 seconds. One hundred microliters of each supernatant was assayed in a Berthold LB 953 luminometer (Berthold, Wildbad, Germany) for light emission after the injection of 100 μL of LB (mix). The RLU values presented are the mean of at least four independent experiments and the same pattern was also obtained using a different DNA preparation.
Nuclear extracts (typically 2 μg) were incubated on ice for 10 minutes in 5× buffer (containing 20 mmol/L 4-2-Hydroxyethyl)pi-perazine-1-propanesulfonic acid (HEPES), pH 7.9, 100 mmol/L KCl, 1 mmol/L DTT, 2.5 mmol/L MgCl₂, 0.4 mmol/L ethylenediaminetetra-acetic acid disodium salt (EDTA) and 10% glycerol) with 6 μg poly d(I-C) and 100-fold molar excess of competitor oligonucleotides. [32P]-labeled probe (8 ng) was then added and incubation continued at room temperature for 15 minutes. Antibody, when added, was preincubated with extracts for 30 minutes on ice. The protein-bound DNA complexes were separated from the free probe on 6% polyacrylamide gel electrophoresis (30 mA), dried, and exposed to a Fuji x-ray film (Fuji Film, Tokyo, Japan) at −80°C.

DNA Methylation Analysis

Genomic DNA was isolated from cell lines using standard methods. Ten micrograms of each DNA was digested with Sau3A or EcoRI, 0.2% NP-40, 5% sucrose] and 60 mmol/L KCl, 0.15 mmol/L spermine, 0.5 mmol/L spermidine, 1 mmol/L EDTA, 0.1% sodium EDTA, and 5 mmol/L NaCl. DNA was then isolated and digested with varying amounts (0.8 to 16 μg) of DNase I. The reaction was started by the addition of EcoRI and Saull, followed by 10× buffer containing 20 mmol/L 4-(2-Hydroxyethyl)pyridine (HEPES), pH 7.4, 15 mmol/L NaCl, 60 mmol/L KCl, 0.15 mmol/L spermine, 0.5 mmol/L spermidine, 1 mmol/L EDTA, 0.15 mmol/L Ethyleneglycol-0,0′-bis(2-aminoethyl)- N,N,N′,N′-tetraacetic acid (EGTA), 0.2% NP-40, and 5% sucrose and digested with varying amounts (0.8 to 16 ng) of Dnase I (Sigma-Aldrich, Poole, UK). The reaction was started by the addition of MgCl₂ and CaCl₂, continued at 37°C for 10 minutes, and stopped by the addition of EDTA and SDS. DNA was then isolated and digested with the appropriate enzymes according to the manufacturer's instructions and analyzed using Southern blotting.

RNA Extraction and Northern Blotting

Poly A⁺ RNA was isolated from cell lines and blotted as previously described. Filters were sequentially hybridized to the following probes: murine SCL, a 1.8-kb Xba I cDNA fragment; murine GATA-1, full-length cDNA; murine GATA-2, 0.7 cDNA fragment kindly provided by Prof. S.H. Orkin (Boston, MA); murine GATA-3, full-length cDNA kindly provided by Prof. H. Clevers (Utrecht, The Netherlands); murine NF-E2 p45, full-length cDNA; human GAPDH.

Western Blotting of Transiently Expressed GATA Proteins

BW 5147 cells were transiently transfected with 15 μg pEF-BOSS or pEF-BOSS GATA 1, 2, or 3 expression vector and 10 μg of the −2,000 bp SCL-Luc reporter plasmid as outlined, grown for 18 hours, washed once in PBS at room temperature, and then washed twice in ice-cold PBS. Aliquots of 0.1 × 10⁶ cells were pelleted and the supernatant was aspirated before snap freezing the pellet on dry ice. An aliquot of the same culture was tested for luciferase activity as described to confirm transfection. For Western blotting, 30 μl of extraction buffer (100 mmol/L DTT, 60 mmol/L Tris-HCl, pH 6.8, 4% SDS) was added to each snap-frozen aliquot, followed by vortexing for 5 minutes. The samples were then mixed with an equal volume of 2× loading buffer (200 mmol/L DTT, 120 mmol/L Tris-HCl, pH 6.8, 2% SDS, and 0.02% bromphenol blue) and heated at 95°C for 5 minutes before electrophoresis in a 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After wet electrophoretic transfer of proteins from the gel to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), immunodetection was performed by blocking the membrane for 1 hour in Marvel (5% milk powder, 1% calf serum in PBS) and then incubating the membrane for 90 minutes at room temperature in 9E10 hybridoma supernatant. The membrane was washed three times in 1% NP40-PBS for 15 minutes each before the secondary antibody (goat antihorse IgG, conjugated with horseradish peroxidase, diluted 1:1,000 in Marvel mix) was applied for 90 minutes, followed by three further washes in 1% NP40-PBS. The bound antibody was detected by chemiluminescence using the Amersham ECL system.

Sequence Alignments

Human and mouse SCL genomic sequences have been published and were aligned using the GAP-program from the
UWGCG software package running on an Alliant matrix 2800 computer (Alliant Computers, Littleton, MA).

RESULTS

Steady-State SCL mRNA Correlates With Methylation and Chromatin Structure in Erythroid and T-Cell Lines

In accordance with previous studies, SCL mRNA was detected in MEL cells (F4N) but not in the T-cell line BW 5147 (Fig 1A). The SCL promoter is CpG rich in both human and murine SCL genes. Methylation of CpG-rich promoters is usually associated with transcriptional inactivity of the associated gene. Furthermore, although CpG-rich promoters are unmethylated in nonexpressing normal tissues, they are frequently methylated in nonexpressing cell lines.

We therefore investigated the methylation status of the SCL promoter region in F4N cells, BW 5147 cells, and LTK- fibroblasts. The two alternate promoters of the murine SCL gene are contained within a 0.8-kb Sau3A fragment (Fig 1B). DNA derived from MEL, fibroblast, and T-cell lines was digested with Sau3A with or without Hpa II and hybridized to a probe from the promoter region (Fig 1B). Sau3A digestion alone produced a 0.8-kb band in all three cell lines. The addition of Hpa II had no effect on the 0.8-kb fragment in DNA from fibroblast and T-cell lines. However, Hpa II completely digested the 0.8-kb fragment in DNA from the MEL cells. These results show that the SCL promoter was hypomethylated in MEL cells that expressed SCL mRNA. By contrast, the SCL promoter was methylated, and therefore presumably inactive, in both fibroblast and T-cell lines.

Further evidence that the SCL promoter was indeed transcriptionally inactive in the T-cell line was obtained from studies of DNase I hypersensitive sites within the SCL promoter region. DNase I hypersensitive sites represent regions of open chromatin in which positive and negative regulatory factors can interact with DNA. Transcriptionally active genes contain DNase I hypersensitive sites within their promoter regions. DNA from MEL and T-cell lines was therefore treated with varying concentrations of DNase I. Three hypersensitive sites were identified in MEL cells but not in T cells (Fig 1C). Moreover, the hypersensitive sites mapped to the region immediately upstream of promoter 1a and promoter 1b. These results clearly suggest that transcription factors could access the SCL promoter in an erythroid but not in a T-cell line. Taken together, the methylation studies and DNase I hypersensitive site analysis both suggest that the SCL gene is likely to be regulated at the level of transcription in erythroid and T-cell lines.

SCL Promoter 1a Exhibited Lineage-Restricted Activity

To analyze the 5’ flanking region upstream of exon 1a for regulatory elements, approximately 2 kb of upstream sequence was linked to a luciferase reporter gene and 5’ deletion mutants were subsequently generated. MEL cells (F4N) and T cells (BW 5147) were electroporated with SCL reporter plasmids together with a control plasmid in which expression of β-gal was driven by the promoter of the housekeeping gene, EF-1 α. To control for variation in DNA uptake, luciferase values were normalized against corresponding β-gal values. In addition, two independent preparations of plasmid DNA were used.

In MEL cells, luciferase activity produced by -2,000 SCL1a-Luc was approximately 11-fold greater than background values obtained using pGL-2 Basic (Fig 2A). Deletion of sequences between -2,000 and -187 did not decrease promoter activity, but further deletion to -55 resulted in a significant decrease in promoter activity. By contrast, in T cells, all the SCL 1a reporter constructs were inactive (Fig 2A). These results show that sequences between -187 and +26 were sufficient for lineage-restricted activity of promoter 1a.

Fig 1. SCL mRNA expression reflects transcriptional regulation. (A) Northern analysis of erythroid and T-cell lines. Poly A+ RNA from F4N(E) and BW 5147 cells (T) was hybridized to the probes as indicated. (B) Methylation of the murine SCL promoters. Below, diagram of promoter region. Sau3A 0.8-kb probe (III), Sau3A sites (Sa), Hpa II sites (H), exon 1a (1a), and exon 1b (1b). Above, Southern blot analysis of genomic DNA from F4N (E), BW 5147 (T), and LTK- cells (F). DNA was digested with Sau3A or with Sau3A and Hpa II and was hybridized to the Sau3A 0.8-kb probe. Left arrow, 0.8-kb Sau3A genomic fragment; right arrow, smaller fragments resulting from Hpa II digestion. (C) Lineage-restricted DNase I hypersensitive sites within the murine SCL promoter. Below, diagram of promoter region indicating 1.0-kb Smal (Sm)-Sac I (S) fragment used as a probe. Above, nuclei from erythroid (F4N) and T cells (BW 5147) were digested with varying amounts of DNase I before DNA extraction, digestion with Sac I, and hybridization to the probe (III). Open arrow, germline band; closed arrows, DNase I hypersensitive sites.
Analysis of SCL Promoter 1b Activity

Analysis of cDNA clones and mapping of transcription start sites have previously shown that both promoter 1a and promoter 1b are active in MEL cells. To study the regulation of promoter 1b, an additional series of SCL reporter constructs was generated. The parental construct (~2,000 SCL1a1b-Luc) contained exon 1a, intron 1, and promoter 1b, with 2 kb of sequence upstream of exon 1a. Convenient restriction sites were then used to generate a series of 5’ deletion constructs.

In MEL cells, ~2,000 SCL1a1b-Luc and ~55 SCL1a1b-Luc produced lineage-restricted luciferase activity that was, respectively, 12-fold and eightfold greater than background values obtained using pGL-2 Basic (Fig 2B). Removal of sequences between ~55 and +26 reduced luciferase activity to background levels. By contrast, ~2,000 SCL 1a1b-Luc was inactive in T cells. However, progressive 5’ deletion resulted in low-level activity of promoter 1b in T cells.

These results show that a joint promoter construct containing both exon 1a and promoter 1b was regulated in a lineage-restricted manner. Moreover, the data suggest that the conserved region immediately upstream of promoter 1b was insufficient to direct promoter 1b activity in MEL cells.

GATA-1, Sp1, and AP-1 Bound to the Core Promoter Region Upstream of Exon 1a

Comparison of the human and murine nucleotide sequence showed a highly conserved region immediately upstream of exon 1a. Several conserved transcription factor binding sites were present (Fig 3B): an AP-1 site was present at ~101, an Sp1 site at ~63, and GATA consensus sites at ~37 and ~69. Gel shift assays were used to characterize the proteins binding to these motifs in MEL cells and T cells (for oligonucleotide sequences, see Table 1).

AP-1 site. Oligonucleotide A6, which contained the ~101 AP-1 site, bound three complexes in MEL cells (Fig 4A, solid arrows). Binding was competed by cold probe (lane 4) but not by an unrelated oligonucleotide (lane 7). MEL cells expressed NF-E2 (Fig 1A), a bZIP transcription factor that exhibits a lineage-restricted pattern of expression similar to SCL. NF-E2 is therefore a potential regulator of SCL and is also known to bind to an extended AP-1 site.
strongly suggests that the complexes binding to the −101 AP-1 site did not contain NF-E2.

T-cell extracts contained a different pattern of complexes that bound to oligonucleotide A6 (Fig 4A). The prominent complex found in erythroid cells was also present in T cells, albeit with a lower abundance. However, the lowest erythroid complex was absent in T-cell extracts and was replaced by a more rapidly migrating complex (open arrow).

Sp1. Oligonucleotide A3, which contains the −63 Sp1 and −69 GATA sites, bound three complexes in MEL cell extracts (Fig 4B). Binding of all three was competed by cold probe (lane 3) but not by an unrelated oligonucleotide (lane 4). Several lines of evidence suggest that the upper complex contained an Sp1 family member: (1) this complex could be competed by an Sp1 consensus oligonucleotide (compare lanes 2 and 6); (2) mutation of the Sp1 site prevented competition by cold probe (compare lanes 21 and 22); and (3) the complex comigrated with recombinant Sp1 protein (compare lanes 23 and 24). A similar pattern of Sp1 binding was observed in T-cell extracts. The apparent partial competition by oligonucleotide B1 was not reproducible.

GATA sites. In addition to binding Sp1 or a related protein, oligonucleotide A3 bound two additional complexes in MEL cell extracts. Several lines of evidence suggest that the middle complex contained GATA-1 (Fig 4B): (1) the middle complex comigrated with GATA-1 bound to oligonucleotide A1 (data not shown); (2) the complex was competed by a GATA consensus oligonucleotide known to bind GATA-1 (lane 7); (3) mutation of the −69 GATA site abolished competition by cold probe for the middle complex (compare lanes 3 and 5); and (4) the N6 monoclonal anti-GATA-1 antibody supershifted the middle complex (lane 8). Because the supershifted complex comigrated with the Sp1 complex, the presence of a supershift was also confirmed by including oligonucleotide A5, which competed for the Sp1 complex (compare lanes 5 and 9).

A specific GATA-binding protein that comigrated with GATA-1 was also detected in T-cell extracts (Fig 4B). However, this protein was not supershifted by the N6 antibody (compare lanes 10 and 16 and 13 and 17). This protein is likely to represent GATA-3, although we cannot exclude the possibility that it represents a novel GATA protein.

Oligonucleotide A1 contained the −37 GATA site and bound two complexes in MEL cell extracts (Fig 4C). Binding of both was competed by cold probe but not by an unrelated oligonucleotide. Several lines of evidence suggest that the upper complex contained GATA-1: (1) it was competed by a GATA-1 consensus oligonucleotide (lane 5); (2) mutation of the −37 GATA site prevented competition by cold probe (compare lanes 2 and 4); and (3) addition of a monoclonal anti-GATA-1 antibody (N6) supershifted the upper complex (lane 7). The nature of the lower complex is not clear. Its size appeared to be too small to represent a DNA binding fragment of GATA-1 that has lost the epitope recognized by the N6 monoclonal antibody.

A less abundant complex of the same size as GATA-1 was observed using T-cell extracts and oligonucleotide A1 (Fig 4C, lane 8). The complex was not supershifted by the N6 antibody (lane 13). Binding of the complex was competed by an unrelated oligonucleotide and by cold probe containing

It was therefore important to discern whether the complexes that bound to the −101 AP-1 site contained NF-E2. To address this question we took advantage of AP-1 and NF-E2 oligonucleotides that were used previously to clone NF-E2 and are known to bind differentially to AP-1 and NF-E2.41 If the complexes contained AP-1, they were predicted to bind to both oligonucleotides. However, if the complexes contained NF-E2, they were predicted to bind to the NF-E2 but not the AP-1 oligonucleotide. The complex that bound the −101 AP-1 site in the SCL promoter was competed more efficiently than the NF-E2 oligonucleotide (Fig 4A, compare lanes 5 and 6). This result
Fig 4. Gel shift analysis of promoter 1a using nuclear extracts from erythroid (F4N) and T cells (BW 5147). (A) –101 AP-1 site. Oligonucleotide A6 was used as a probe. Unlabeled competitor oligonucleotides were included as indicated. Closed arrows, three complexes found in erythroid cells; open arrow, complex found in T cells but not erythroid cells. (B) –63 Sp1 and –69 GATA sites. Oligonucleotides A3 or A8 were used as probes. Unlabeled oligonucleotide competitors and N6 anti-GATA-1 antibody were included as shown. Arrows indicate complexes containing Sp1 or GATA proteins. (C) –37 GATA site. Oligonucleotide A1 was used as a probe. Unlabeled oligonucleotide competitors and N6 anti-GATA-1 antibody were included as indicated. Closed arrow, complex containing GATA-1; open arrow, supershift obtained with N6 anti-GATA-1 antibody.

Comparison of human and murine sequences showed a highly conserved region immediately upstream of promoter 1b. This contained a MAZ binding site at +242, a completely conserved sequence of 19 pyrimidines starting at +259, and a potential C/EBP site at +269. Gel shift assays were used to characterize the proteins binding to these motifs.

MAZ site. Oligonucleotide B5, which contained the MAZ motif, bound three complexes in both MEL and T-cell extracts (Fig 5A). Binding was competed by cold probe but not by an unrelated oligonucleotide. Several lines of evidence suggest that GATA-3 either did not bind or only bound weakly to the –37 GATA site in T cells.

ETS motif. Oligonucleotide B3, which contained the conserved sequence of 19 pyrimidines, bound four main complexes in both MEL cells and T-cell extracts (Fig 5B). Binding was competed by cold probe (lane 3) but not by an unrelated oligonucleotide (lane 4). The sequence of this region contains three tandem repeats of TTCC, a potential ETS binding site. Mutation of 6 bases that altered two of the three mutated GATA site, although, in both cases, a faint complex was observed after prolonged exposure of the autoradiograph (data not shown). These observations therefore suggest that GATA-3 either did not bind or only bound weakly to the –37 GATA site in T cells.

Characterization of Proteins Binding to MAZ and ETS Motifs Upstream of Promoter 1b

Comparison of human and murine sequences showed a highly conserved region immediately upstream of promoter 1b. This contained a MAZ binding site at +242, a completely conserved sequence of 19 pyrimidines starting at +259, and a potential C/EBP site at +269. Gel shift assays were used to characterize the proteins binding to these motifs.

MAZ site. Oligonucleotide B5, which contained the MAZ motif, bound three complexes in both MEL and T-cell extracts (Fig 5A). Binding was competed by cold probe but not by an unrelated oligonucleotide. Several lines of evidence suggest that MAZ or related proteins were present in these complexes: (1) all three complexes could be competed by a known MAZ binding site (MElal) from the MYC promoter (lane 6); (2) mutation of the MAZ motif abolished competition by cold probe for all three complexes (compare lanes 4 and 5); and (3) the same three complexes were observed when ME1al was used as a probe (compare lanes 1 through 11 with lanes 12 through 22).

ETS motif. Oligonucleotide B3, which contained the conserved sequence of 19 pyrimidines, bound four main complexes in both MEL cells and T-cell extracts (Fig 5B). Binding was competed by cold probe (lane 3) but not by an unrelated oligonucleotide (lane 4). The sequence of this region contains three tandem repeats of TTCC, a potential ETS binding site. Mutation of 6 bases that altered two of the three
Fig 5. Gel shift analysis of promoter lb using nuclear extracts from erythroid (F4N) or T cells (BW 5147). (A) +242 MAZ site. Oligonucleotide B5 (SCL promoter) or ME1a1 (MYC promoter) were used as probes. Unlabeled oligonucleotide competitors were included as shown. Arrows indicate three complexes detected using either oligonucleotide B5 or ME1a1. (B) +264 ETS site. Oligonucleotide B3 was used as a probe. Unlabeled oligonucleotide competitors were included as indicated. Closed arrows, complex not competed by cold oligonucleotide 84; open arrow, complexes competed by oligonucleotide B4.

potential ETS binding sites completely abolished competition by cold probe for the lower two complexes (solid arrows) but not the upper two complexes (open arrows; compare lanes 3 and 5). These results suggest that the lower two complexes bound the ETS motifs and may contain one or more members of the ETS family.

C/EBP site. Oligonucleotide B1, which contains the +269 C/EBP site, was also studied in gel shift assays. However, it only gave rise to nonspecific protein binding using erythroid and T-cell extracts, despite the use of several different reaction conditions (data not shown).

SCL Promoter la Activity in Erythroid Cells Was Dependent on the -37 GATA Site

To address the functional significance of the AP-1 site, the Sp1 site, and the two GATA motifs, site-directed mutagenesis was used to alter all four of these sites within a luciferase construct containing approximately 2 kb of sequence upstream of promoter la. All mutations were identical to the changes previously shown to abolish protein binding in gel shift assays. In addition, a fifth mutant was generated that abolished a putative CCAAT box at -112 upstream of exon la. The mutant constructs were transiently transfected into MEL cells and the resultant luciferase activity was compared with that obtained using -2,000 SCL1alb-Luc. In each case, two independent preparations of plasmid DNA were studied. To control for variation in DNA uptake, luciferase values were normalized against corresponding β-gal values.

Mutants that abolished the AP-1, Sp1, -69 GATA, and CCAAT sites did not significantly reduce promoter la activity (Fig 6A). By contrast, mutation of the -37 GATA site reduced luciferase activity to background levels. These results show that the -37 GATA site was critical for activity of SCL promoter la in erythroid cells. By contrast, the -101 AP-1, -63 Sp1, -69 GATA, and -112 CCAAT sites were all individually dispensable.

MAZ and ETS Motifs Were Required for Full SCL Promoter Activity

We have also used site-directed mutagenesis to study the functional significance of the MAZ and ETS motifs upstream of promoter 1b. The mutations shown to abolish protein binding were introduced into -2,000 SCL1alb-Luc. In addition, we have studied the effect of mutating the -37 GATA site on the activity of the joint promoter construct. Mutants and wild-type constructs were electroporated into MEL cells as described above.

Mutation of the -37 GATA site impaired activity of the joint promoter construct (Fig 6B). This result would be consistent with regulation of promoter la by the -37 GATA site. The residual activity of the mutant construct (threefold above background) suggests that the -37 GATA site was not necessary for promoter 1b activity.
Interestingly, mutation of either the ETS or MAZ motifs also impaired activity of the joint promoter construct (Fig 6B). These results suggest that protein binding to both of these sites is important for SCL promoter function. Our data would be consistent with a role for these sites in the regulation of promoter Ib or promoter la or both.

GATA-1 But Not GATA-2 or GATA-3 Transactivated SCL Promoter la in T Cells

Our results suggest that GATA-1 plays a pivotal role in the regulation of SCL promoter la in erythroid cells. However, SCL is expressed in some cell types that contain low or undetectable GATA-1 and may therefore be regulated by other GATA proteins, particularly GATA-2. Furthermore, the absence of SCL expression in T cells may merely reflect the absence of GATA-1, or it may also reflect active repression of SCL expression. To address these issues, we have tested the ability of different GATA proteins to transactivate SCL promoter la in a T-cell environment.

An SCL reporter construct (−2,000 SCL la-Luc) or the parental pGL-2 Basic luciferase vector was therefore introduced into T cells together with an MYC-tagged expression plasmid containing GATA-1, GATA-2, or GATA-3 cDNA. The expression level of all three GATA proteins was monitored by Western blotting of transiently transfected BW 5147 cells using the MYC-tag specific 9E10 monoclonal antibody. This monitoring showed high-level expression of both GATA-2 and GATA-3 and lower level expression of GATA-1 (data not shown). None of the GATA expression vectors enhanced activity of the parental pGL-2 Basic reporter plasmid. Cotransfection of GATA-1 with −2,000 SCL la-Luc resulted in a sevenfold to eightfold increase in luciferase activity (Fig 7). By contrast, neither GATA-2 nor GATA-3 enhanced activity of the SCL reporter construct. These results show that GATA-1 but not GATA-2 or GATA-3 was capable of transactivating SCL promoter la in a T-cell environment. Moreover, our data do not support active repression of SCL promoter la in T cells, but suggest that the inactivity of SCL promoter la in T cells reflects lack of GATA-1.

DISCUSSION

The SCL gene is expressed in multipotent hematopoietic progenitors before lineage commitment. Its expression is
Regulation of Murine Scl/Tal-1 Promotor

The lineage-restricted pattern of SCL mRNA expression could represent the operation of transcriptional or posttranscriptional mechanisms. This question was addressed by studying the methylation and chromatin structure of the promoter region. The SCL promoter was methylated in fibroblast and T-cell lines but hypomethylated in MEL cells. Because methylation is frequently associated with transcriptional inactivity, these observations suggest that the SCL gene is very likely to be transcriptionally silent in fibroblast cells and binds to an extended AP-1 site. Moreover, its coexpression with SCL in several different cell types would be consistent with a role for NF-E2 in the regulation of SCL. Competition experiments using oligonucleotides that bind AP-1 and NF-E2 proteins differentially suggested that none of the complexes observed in erythroid cells represented NF-E2. Site-directed mutagenesis of the −101 AP-1 site showed that it was dispensable for full promoter activity in erythroid cells.

The −63 Spl site bound an Spl family member in both erythroid and T cells. No lineage-restricted differences in complex abundance or mobility were detected. Moreover, site-directed mutagenesis of the −63 Spl site showed that it was dispensable for full promoter activity in erythroid cells. This result differed from that reported by Lecointe et al., who reported that mutation of the equivalent Spl site in the human SCL promoter produced a 40% reduction in promoter activity in K562 cells. These results may reflect differences between K562 cells, which have erythroid features but which are also multipotent (Green et al., and references therein), and MEL cells, which are committed to the erythroid lineage.

The −37 GATA site bound GATA-1 but not GATA-2 or GATA-3. Luciferase values are the mean (±SD) of at least four independent electroporations. For RLU, see Fig 2 legend.

Fig 7. Transactivation of SCL promoter la by GATA-1 but not GATA-2 or GATA-3. Luciferase values are the mean (±SD) of at least four independent electroporations. For RLU, see Fig 2 legend.
TAA motifs such as that found at the −37 site. Our data suggest that additional nucleotides flanking the −37 GATA site may allow binding of GATA-1 but not GATA-3. However, we cannot exclude the alternate possibility that cofactors present in T cells may inhibit binding of GATA-3 to the −37 GATA site. Mutation of the −37 GATA site abolished activity of promoter 1a in erythroid cells and markedly reduced activity of a joint promoter construct containing both promoter 1a and promoter 1b. These results show that the −37 GATA site was important for promoter 1a activity. Moreover, they are consistent with evidence that mutation of the −33 GATA site in the human SCL promoter reduced promoter 1a activity in K562 cells.

The −69 GATA site bound GATA-1 in erythroid cells. In T cells, the same site bound a complex that comigrated with GATA-1 but was not supershifted by the N6 monoclonal antibody. The GATA binding complex in T cells was likely to represent GATA-3, although our data do not exclude the possibility that the complex contains a previously unreported GATA protein. Mutation of the −69 GATA site showed that it was not necessary for full promoter 1a activity in erythroid cells (Fig 6A) or for promoter 1a silence in T cells (data not shown).

Our results therefore emphasise the importance of the −37 GATA site for the activity of promoter 1a. However, we did not find any evidence that the −63 Sp1, −69 GATA, −101 AP-1, or −112 CCAAT sites were individually important for promoter 1a activity.

We have also studied two conserved motifs upstream of promoter 1b, i.e., the +242 MAZ site and the +264 ETS motif. MAZ was originally characterized as a protein binding to the ME1a1 site upstream of MYC promoter P2. In this situation, MAZ may act both as a termination factor for P1 transcripts and as a transactivating factor for the P2 promoter. MAZ sites have also been identified between closely spaced genes, where they appear to protect the downstream promoter from transcriptional interference. The position of the +242 MAZ site between SCL promoter 1a and promoter 1b is therefore particularly pertinent. Our data suggest that MAZ or a related protein is involved in the regulation of SCL promoter 1a and/or promoter 1b. Further studies will be required to determine which promoter is regulated by MAZ and also to ascertain whether the +242 MAZ site protects promoter 1b from transcriptional interference by promoter 1a transcripts.

The +264 ETS motif bound an abundant complex in both erythroid and T cells. The binding site contained a sequence with similarities to ETS protein binding sites. In particular, it contained three conserved tandem repeats of TTCC, a core ETS binding motif. A mutation that altered two of the three TTCC motifs abolished complex binding. The same mutation also significantly reduced activity of the joint promoter construct in erythroid cells. The precise identity of the protein(s) binding to this TTCC motif is not yet known. However, our results suggest that ETS family members are likely to be important for full activity of the SCL promoter in erythroid cells.

Our data therefore show that the MAZ and ETS motifs were both necessary for full activity of the joint promoter construct containing promoter 1a and promoter 1b. Further studies will be needed to determine whether these motifs regulate promoter 1a, promoter 1b, or both.

In view of the importance of GATA binding proteins in the regulation of the SCL promoter, we have also studied the ability of GATA-1, GATA-2, and GATA-3 to transactivate SCL promoter 1a. Consistent with previous studies of the human SCL promoter, we have shown that GATA-1 is capable of transactivating the murine SCL promoter 1a. Our results also suggest that inactivity of SCL promoter 1a in T cells reflects absence of GATA-1 rather than the presence of trans-dominant negative regulators. This finding accords with those of previous studies from our laboratory, which showed that SCL mRNA expression was not extinguished in erythroid × T-cell hybrids.

Our results show that neither GATA-2 nor GATA-3 were able to transactivate SCL promoter 1a. GATA-3 was only capable of binding weakly, if at all, to the −37 GATA site. However, GATA-2 has been shown to bind to the human −33 GATA site. A role for GATA-2 in the regulation of SCL is suggested by the coexpression of SCL and GATA-2 in some cell types that expressed little or no GATA-1. Moreover, erythroid cells that were null for GATA-1 expressed normal levels of SCL mRNA together with markedly increased levels of GATA-2 mRNA. The inability of GATA-2 to transactivate SCL promoter 1a in T cells is therefore intriguing. Several explanations are possible. (1) The observation may reflect species-specific differences because we used a human GATA-2 cDNA. This explanation seems unlikely because human GATA-2 transactivated the PPET-1 promoter in murine cells. (2) GATA-2 may be prevented from trans-activating the SCL promoter in a T-cell environment. This explanation would imply the existence of cell-type–specific cofactors that could modulate the function of GATA proteins. (3) GATA-2 may regulate expression of SCL through regulatory elements outside the promoter region. (4) GATA-2 may be incapable of regulating expression of SCL. Further studies will be required to distinguish these possibilities.

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EO Bockamp, F McLaughlin, AM Murrell, B Gottgens, L Robb, CG Begley and AR Green

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