Distinct Mechanisms Direct SCL/tal-1 Expression in Erythroid Cells and CD34 Positive Primitive Myeloid Cells*

(Received for publication, October 3, 1996, and in revised form, December 19, 1996)

Ernst-Otto Bockamp, Fiona McLaughlin, Berthold Göttgens, Adelle M. Murrell, Andrew G. Elefanty$$, and Anthony R. Green¶

From the University of Cambridge, Department of Haematology, Medical Research Council Centre, Hills Road, Cambridge CB2 2QH, United Kingdom and the ¶The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3052, Australia

The SCL/tal-1 gene (hereafter designated SCL) encodes a basic helix-loop-helix transcription factor which is pivotal for the normal development of all hematopoietic lineages and which is expressed in committed erythroid, mast, and megakaryocytic cells as well as in hematopoietic stem cells. The molecular basis for expression of SCL in stem cells and its subsequent modulation during lineage commitment is of fundamental importance for understanding how early “decisions” are made during hematopoiesis.

We now compare the activity of SCL promoters 1a and 1b in erythroid cells and in CD34 positive primitive myeloid cells. SCL mRNA expression in CD34 positive myeloid cells did not require GATA-1. Promoter 1a activity was weak or absent in CD34 positive myeloid cells and appeared to correlate with the presence or absence of low levels of GATA-1. However, promoter 1b, which was silent in committed erythroid cells, was strongly active in transient assays using CD34 positive myeloid cells, and functioned in a GATA-independent manner. Interestingly, RNase protection assays demonstrated that endogenous promoter 1b was active in both erythroid and CD34 positive myeloid cells. These results demonstrate that fundamentally different mechanisms regulate the SCL promoter region in committed erythroid cells and in CD34 positive myeloid cells. Moreover these observations suggest that in erythroid, but not in CD34 positive myeloid cells, promoter 1b required integration in chromatin and/or additional sequences for its activity. Stable transfection experiments showed that both core promoters were silent following integration in erythroid or CD34 positive myeloid cells. Our data therefore indicate that additional regulatory elements were necessary for both SCL promoters to overcome chromatin-mediated repression.

One of the principal issues facing modern developmental biology concerns the molecular mechanisms whereby a multipotent stem cell gives rise to a variety of phenotypically distinct differentiated progeny. Hematopoiesis, the process of blood cell formation, provides a powerful experimental system for studying this process. Since lineage commitment and differentiation involve alterations in patterns of gene expression, the function and regulation of lineage-restricted transcription factors are of central importance for the behavior and subsequent fate of hematopoietic stem cells (1, 2).

The SCL/tal-1 gene (hereafter termed SCL) encodes a lineage-restricted basic helix-loop-helix transcription factor with a pivotal role in the regulation of hematopoiesis. Mice lacking SCL protein die by embryonic day 10 and exhibit a complete absence of all hematopoietic cells (3–6). Previous antisense experiments have suggested that SCL may perform different functions in distinct hematopoietic cell types. Introduction of antisense constructs into a multipotent cell line resulted in reduced proliferation and self-renewal (7), whereas similar experiments inhibited erythroid differentiation of a committed erythroid cell line (8). In addition, loss of SCL function increased apoptosis of a T cell line following serum starvation (9). However, despite its implicit involvement in several physiological processes, target genes for SCL have not yet been identified.

SCL is expressed predominantly in hematopoietic cells, although SCL mRNA and/or protein have also been detected in adult and developing brain together with endothelial cells (10–13). Within the hematopoietic system SCL is expressed in committed erythroid, mast and megakaryocytic cells as well as in interleukin-3 dependent cell lines (10, 12–16). Erythroid differentiation of committed erythroid cell lines is accompanied by up-regulation of SCL mRNA although protein levels actually fall (10, 17). SCL is also expressed in multipotent progenitors prior to lineage commitment. The use of growth factors to induce erythroid differentiation of a multipotent progenitor cell line was accompanied by up-regulation of SCL mRNA (but not protein), whereas induced granulocyte/monocyte differentiation resulted in extinction of SCL expression (18). Indeed, down-regulation of SCL expression not only accompanies, but may actually be required for normal myeloid differentiation, since over-expression of exogenous SCL impaired macrophage differentiation of M1 cells (19).

The mechanisms that regulate this complex pattern of expression are almost completely unknown. Studies of Drosophila embryogenesis have shown that complex patterns of gene expression are frequently specified by autonomous regulatory elements directing expression to distinct cell types (20, 21). Similar mechanisms may operate in vertebrate stem cell systems. However, direct experimental data are lacking and several aspects of Drosophila embryogenesis are very different from post-zygotic vertebrate development. We have therefore chosen to analyze the molecular mechanisms responsible for SCL expression in stem cells and its subsequent modulation.
during lineage commitment. Such studies should also shed light on the fundamental question of lineage determination during hematopoiesis. Moreover, by studying in detail the regulation of this one gene, we will gain insight into the molecular mechanisms necessary to direct exogenous gene expression in stem cells as well as in specific differentiated hematopoietic cell types.

In view of the powerful experimental tools available for studying hematopoiesis in the mouse, we have focused on the murine SCL gene. The structure of the murine gene is very similar to that of the human gene (22–24). Transcription of both genes is driven by two promoters in alternate 5’ exons. SCL promoter 1a directs lineage-restricted expression in erythroid cells and is regulated by GATA-1 (8, 25, 26), but the molecular basis for SCL expression in hematopoietic stem cells or in other specific cell types is not known. Several lines of evidence suggest that GATA-1 and GATA-2 regulate both overlapping and unique sets of target genes (27). In addition, a number of observations have implied that SCL may be regulated by GATA-2 in primitive hematopoietic cells, a role that is subsequently assumed by GATA-1 following commitment to the erythroid lineage. SCL is almost invariably co-expressed with GATA-1 and/or GATA-2 in hematopoietic cells (10, 15, 18) and a critical GATA motif in the SCL promoter has been shown to bind GATA-2 (8). In addition, the ratio of GATA-2 to GATA-1 mRNA is high in multipotent cells but reversed during erythroid differentiation (18). Finally, proerythroblasts from GATA-1 null mice express SCL at normal levels but GATA-2 mRNA levels were markedly up-regulated (27).

Taken together these data suggest that GATA-1 and GATA-2 may act through the same regulatory elements to direct SCL expression in distinct human cell types. To address this issue, we have compared the mechanisms responsible for SCL expression in erythroid cells with those operating in primitive myeloid cells which express the stem cell antigen, CD34. Our results show that very different mechanisms are responsible for transcriptional activity of the SCL promoter in CD34 primitive myeloid cells compared with erythroid cells. Moreover, in the CD34 positive cells, transcriptional activity of the SCL promoter region is independent of GATA motifs. This suggests that GATA-2 does not direct SCL promoter activity in primitive hematopoietic cells, an observation with important implications for the hierarchy of transcription factors operating in hematopoietic stem cells.

MATERIALS AND METHODS

Cell Lines—The murine CD34 positive myeloid cell lines M1 and 41B, the murine T-cell line BW 5147, and the murine erythroid cell line J2E have been described previously (25, 28–30). Cell lines M1, 41B (kindly provided by Dr. T. Eaves, LRP Center, Institute of Cancer Research, London, United Kingdom), and J2E (kindly provided by Dr. P. Klinken, Biochemistry Department, Royal Perth Hospital, Western Australia) were grown in RPMI 1640 plus 10% fetal calf serum. 416B, the murine T-cell line BW 5147, and the murine erythroid cell line 9 (kindly provided by Dr. S. Cory, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and a 10-fold molar excess of the linearized luciferase plasmid. After 24 h, electroporated cells were transferred into selective medium containing 2 µg/ml (F4N) or 10 µg/ml (M1) puromycin (Sigma). Cells electroporated with SCL reporter constructs were maintained as three independent pools.

Puromycin resistant pools were derived 2–4 weeks following electroporation and luciferase assays were performed using extracts derived from 10^6 cells for each assay point as described above. For each experiment, assays were performed in duplicate on each pool and a positive control (a pool of cells transfected with pA3RSVluc) and a negative control (a pool of cells transfected with pGL-2 basic) were included in each experiment. This experiment was repeated on three separate occasions for each SCL construct. Results were expressed as fold elevation over the negative control. In addition, this relative luciferase activity was normalized for luciferase DNA content by Southern blot analysis of BamHI and HindIII digested DNA. Filters were probed with a nonrepetitive sequence from the mouse vav gene locus to normalize DNA loading and with the HindIII/BamHI fragment of the luciferase gene from pGL-2 basic. Relative luciferase values were also corrected by normalizing for luciferase DNA content of test pools compared with the control pool carrying pGL-2 basic.

Electrophoretic Mobility Shift Assays—Conditions for the band shift assays were as described (25). Oligonucleotides used in the band shift assays are depicted in Table II. The N6 anti-GATA-1 antibody (34) was kindly provided by Dr. G. Partington, Randall Institute, London, United Kingdom.

RNA Extraction and Northern Blotting—Poly(A)^+ RNA was isolated from cell lines and blotted as described previously (10). Filters were sequentially hybridized to the following probes: murine SCL, a 1.8-kb XhoI cDNA fragment (35); murine GATA-1, full-length cDNA (36); murine GATA-2, 0.7 kb cDNA fragment kindly provided by Prof. S. H. Orkin (Howard Hughes Medical Institute Boston, MA); murine GATA-3, full-length cDNA kindly provided by Prof. H. Cleurs (Utrecht University, The Netherlands); murine CD34 cDNA kindly provided by Dr. T. Eaves (Institute for Cancer Research, London, United Kingdom); and human GAPDH (37).

RNAase Protection Assays—Total cellular RNA was prepared using the
RNAzol method according to the manufacturer's protocol (BioGene, Bolnhurst, United Kingdom). Genomic murine SCL fragments encompassing nucleotides 760–1156 (24) were inserted in antisense orientation into the pT7 vector (38) to generate pT7 SCL 1b. Radiolabeled antisense transcripts were generated by transcription in vitro using T7 RNA polymerase (Promega). Ribonuclease protection assays were performed using the Ambion RPA II kit (Ambion, Austin, TX) and 40 μg of total RNA for each protection. The amount and integrity of each RNA preparation was confirmed using the pTRI-b-actin mouse plasmid (Ambion) which contains a 250-bp mouse b-actin gene fragment in antisense orientation and gives rise to a 304-nucleotide transcript. The size of the corresponding protected fragments was determined by running a sequencing reaction alongside the protected fragments.

RESULTS

SCL mRNA Expression in CD34 Positive Primitive Myeloid Cells Did Not Require GATA-1—SCL mRNA is expressed in committed erythroid, mast, and megakaryocytic cells as well as in multipotent stem cells (10, 14–16, 18). Since it is difficult to obtain sufficient numbers of normal stem cells for biochemical experiments, we have chosen to study two primitive myeloid cell lines. Both of these cell lines expressed mRNA for the hematopoietic “stem cell” antigen CD34 (Fig. 1). 416B and M1 cells also expressed SCL mRNA, as did two erythroid lines (F4N and J2E). The two erythroid lines expressed high levels of both GATA-1 and GATA-2 mRNA. By contrast, GATA-1 mRNA was undetectable in M1 cells and only very low levels were observed in 416B cells. These results are in accord with previous data (14, 16, 39) and suggest that SCL mRNA expression in primitive myeloid cells may not require detectable GATA-1 expression.

SCL Promoter 1a Activity in Erythroid and CD34 Positive Primitive Myeloid Cells—We have previously demonstrated that SCL promoter 1a is active in a murine erythroleukemia cell line but not in a murine T cell line (25). Since murine erythroleukemia cell lines cannot be equated with normal erythroid cells, it was important to confirm the generality of our results using an alternative erythroid line, which had been generated in a different manner. For this purpose it was decided to study the J2E cell line, which was derived in vitro by infection of fetal liver with a retrovirus containing myc and raf oncogenes (30). J2E cells are growth factor independent but undergo terminal differentiation in response to erythropoietin. Luciferase reporter constructs containing SCL promoter 1a were introduced into J2E and a murine T cell line (BW 5147) in transient assays. A β-galactosidase control plasmid was also included to control for variation in DNA uptake, and luciferase values were normalized against the corresponding β-galactosidase values. A construct, containing approximately 2 kb of exon 1a upstream sequence, was active in J2E cells (34-fold stimulation over background) but not in BW 5147 (Fig. 2A).

![Fig. 1. SCL mRNA expression in CD34 positive primitive myeloid cells is not GATA-1 dependent. Probes were hybridized as indicated to poly(A)+ RNA from the following cell lines: J2E (erythroid); F4N (erythroid); 416B (primitive myeloid); M1 (primitive myeloid); BW 5147 (T cell).](image1)

![Fig. 2. Lineage-restricted activity of SCL promoter 1a and 1b in erythroid and CD34 positive primitive myeloid cells. Transient transfections were performed using J2E, 416B, M1, and BW 5147 (BW) as indicated. The left half of the figure shows a schematic representation of the different deletion constructs with exons depicted as black boxes. Luciferase values are the mean (+S.D.) of at least four independent electroporations. Relative light units (RLU) represents luciferase activity (normalized against β-galactosidase values) relative to background luciferase activity obtained using the promoterless pGL-2 basic plasmid. A, promoter 1a constructs. B, joint promoter constructs containing promoter 1a with promoter 1b or promoter 1b alone.](image2)
The activity of this construct (−2000 1a, Fig. 2A) was similar to that observed with an analogous construct containing promoter 1a, was active in J2E cells but not in BW 5147 cells. Very different results were obtained in M1 and 416B cells (Fig. 2B). In both cell lines, −2000 1a1b exhibited much higher luciferase levels than −2000 1a (40–95-fold over background compared with 5–12-fold over background). Moreover, deletion of part or all of promoter 1a did not reduce the activity. Indeed the +209 1b construct, which only contained 128 bp upstream of exon 1b, was still fully active. Deletion of promoter 1a removed the GATA sites that are situated upstream of exon 1a. Since neither +26 1b nor +209 1b constructs contain any additional GATA motifs, these data demonstrate that the activity of promoter 1b in myeloid cells was independent of GATA motifs.

Transcriptional Activity of Endogenous SCL Promoters in Erythroid and Primitive Myeloid Cells—The transient transfection assays described above suggest that promoter 1a is active in erythroid cells and promoter 1b in primitive myeloid cells. However, such experiments do not take into account the additional effects of distant regulatory elements, methylation status, or chromatin structure. It was therefore important to study the activity of the endogenous SCL promoter region in erythroid and primitive myeloid cells.

The SCL promoter locus is CpG-rich in both human and murine SCL genes (22–24). Furthermore, in cell lines (but not in primary cells) the methylation status of CpG-rich promoters correlates with their transcriptional activity (40, 41). It was therefore important to see whether differential promoter usage in distinct cell types reflected cell-specific differences in pro-
SCL/tal-1 Regulation in Erythroid and CD34 Positive Myeloid Cells

Stably transfected erythroid (F4N) and CD34 positive myeloid cell (M1) were obtained by co-transfection of PGKpuroPa plasmid in conjunction with either pGL-2 basic (negative control), pA3RSVluc (positive control), −187 SCL 1a, or +209 SCL 1b as indicated. Luciferase activity was normalized against pGL-2 and also corrected for copy number as described under “Materials and Methods.” For the SCL constructs three independent pools were assayed in duplicate in three separate experiments. Numbers represent the mean (± S.D.) for each experiment. For the control constructs (pGL-2 basic, pA3RSVluc) each number represents the mean of duplicate assays of the same pool which was included in all experiments.

| Cell line | Experiment | pGL-2 basic | pA3RSVluc | −187 SCL 1a | +209 SCL 1b |
|-----------|------------|-------------|-----------|-------------|-------------|
| F4N       | 1          | 1           | 73        | 1.5 ± 0.2   |             |
|           | 2          | 1           | 45        | 1.2 ± 0.1   |             |
|           | 3          | 1           | 56        | 1.2 ± 0.1   |             |
| M1        | 1          | 1           | 152       | 1.2 ± 0.1   |             |
|           | 2          | 1           | 94        | 1.2 ± 0.1   |             |
|           | 3          | 1           | 82        | 1.1 ± 0.1   |             |
|           | 1          | 1           | 30        | 1.9 ± 1.0   |             |
|           | 2          | 1           | 47        | 1.6 ± 0.7   |             |
|           | 3          | 1           | 65        | 2.4 ± 1.2   |             |
|           | 1          | 1           | 59        | 3.3 ± 2.8   |             |
|           | 2          | 1           | 80        | 3.3 ± 2.7   |             |
|           | 3          | 1           | 53        | 1.6 ± 0.7   |             |
SCL/tal-1 Regulation in Erythroid and CD34 Positive Myeloid Cells

**Fig. 5. Activity of SCL promoter mutants in erythroid and CD34 positive myeloid cells.** Transient transfections were performed using wild type SCL promoter constructs or the same constructs in which individual transcription factor-binding sites were abolished by site-directed mutagenesis. The left half of the figure shows a schematic representation of the constructs indicating the position of each mutation. Luciferase values were calculated as in Fig. 2A. A, activity of SCL promoter 1a mutants in J2E erythroid cells. B, activity of SCL promoter 1b mutants in 416B and M1 primitive myeloid cells.

The lower complex contained GATA-1: binding was competed by two different oligonucleotides each containing consensus GATA motifs (lanes 12 and 13), but not by an unrelated oligonucleotide (lane 10) or by an oligonucleotide in which the −69 GATA site was mutated (lane 15). Furthermore, the N6 GATA-1 monoclonal antibody supershifted this complex (compare lanes 14 and 15). Since the GATA-1 specific supershift co-migrated with the upper complex, the supershift was performed in the presence of cold oligonucleotide, containing a mutated SCL-1a site but an intact Sp1 site, to compete the upper Sp1 complex.

A more rapidly migrating complex was evident in the presence of the N6 antibody (lanes 6 and 14). This complex was also found in the absence of antibody or competitor oligonucleotide in lane 8 and, after prolonged exposure of the autoradiograph, in lane 2. This complex has been observed previously (36, 42). Its nature was not specifically addressed in our experiments, but it is likely to contain either a smaller proteolytic product of GATA-1 or the product of an alternative AUG start codon, as recently reported by Calligaris and colleagues (43). Both products will have lost the binding site for N6 and will therefore not supershift in our band shift assay.

The low/absent activity of promoter 1a in 416B and M1 cells correlated with the low/absent expression of GATA-1 mRNA. However, both 416B and M1 cells expressed GATA-2 (Fig. 1) and it has been suggested that GATA-2 may be able to substitute for GATA-1 in directing SCL expression in primitive hematopoietic progenitors (18, 27). Moreover, recombinant GATA-2 protein has been reported to bind to the SCL promoter (8). Inactivity of promoter 1a in 416B and M1 cells could therefore reflect either absence of GATA-2 binding to the SCL promoter or failure of bound GATA-2 to transactivate the SCL promoter. Band shift analysis using oligonucleotides containing the −37 or the −69 GATA site revealed no specific binding to either GATA site in M1 cells (data not shown), thus suggest-
SCL/tal-1 Regulation in Erythroid and CD34 Positive Myeloid Cells

Fig. 6. Band shift analysis of proteins binding to SCL promoter 1a in J2E cells. Nuclear extracts from J2E cells were incubated with an oligonucleotide probe containing either the -37 GATA site (left hand panel) or a probe containing both the -63 Sp1 site together with the -69 GATA site (right hand panel). Unlabeled competitor oligonucleotides (see Table II) and N6 anti-GATA-1 monoclonal antibody were included as shown. Specific complexes containing GATA-1 (lower complex) or Sp1 (upper complex) or Sp11 (lower complex) or Sp1 (upper complex) are indicated as closed arrows, GATA-1 specific supershift obtained with the N6 anti-GATA-1 antibody is indicated by an open arrow.

Fig. 7. Transactivation of the SCL promoter 1a by GATA-1 in M1 cells. The -2000 1a SCL luciferase reporter construct was cotransfected with empty expression plasmid pCDNA 3 or with a GATA-1 specific expression vector (pEF-BOS GATA-1). Luciferase values are the mean (±S.D.) of at least four independent electroporations. For relative light units (RLU) see Fig. 2, legend.

DISCUSSION

The SCL gene is essential for the development of all hematopoietic lineages and is expressed in hematopoietic stem cells

7, and 16). Three lines of evidence suggest that either MAZ or a MAZ related protein are present in these complexes. First, all complexes were competed with an excess of an oligonucleotide containing the known ME1a1 MAZ-binding site (44) from the myc promoter (lanes 6, 11, and 20). Second, all three complexes required an intact MAZ site since an oligonucleotide containing a mutated MAZ site could not compete for binding of any of the complexes (lanes 4, 9, and 18). Third, the three complexes co-migrated with complexes obtained using a known MAZ-binding site (ME1a1) as a probe (data not shown).

Band shift assays were also performed to study the proteins binding to the ETS motif. The oligonucleotide used as a probe for this experiment contained three overlapping TTCC motifs. Using J2E, 416B, and M1 nuclear extracts, three main complexes were detected in all three cell lines (Fig. 8B, lanes 2, 6, and 10). Addition of excess cold probe abolished binding of all complexes (lanes 3, 7, and 11), whereas an unrelated oligonucleotide did not compete for binding (lanes 5, 9, and 13). An oligonucleotide in which two of the three ETS binding motifs were mutated still competed for binding of the upper complex (lanes 4, 8, and 12) but not for binding of the lower two complexes. During these experiments it was noted that the addition of an excess of unlabeled oligonucleotide seemed to enhance the binding of the middle complex (asterisk) in all cell lines. We did not further investigate this finding, but one possible explanation would be the presence of an inhibitor which reduces binding of the complex which is sequestered by the addition of excess cold oligonucleotide. These results suggest that the lower two complexes bound to the TTCCTT sequence that was mutated in the mETS oligonucleotide (Table II), but that the upper complex bound elsewhere within the oligonucleotide.

Taken together these data demonstrate that the MAZ and ETS sites were important for full activity of promoter 1b in primitive myeloid cells. However, a similar pattern of complexes was observed in J2E cells in which transient reporter assays showed promoter 1b to be silent. These observations suggest that the MAZ and ETS sites are necessary but not sufficient for promoter 1b activity, and that additional lineage-restricted transcription factors may be needed for promoter 1b activity in primitive myeloid cells. Nevertheless, our data do not exclude the possibility that transcription factors binding to the MAZ and ETS sites may undergo lineage-restricted post-translational modifications which are not detectable by band shift analysis, or may represent co-migrating but functionally distinct transcription factor family members.

Previous sequence comparison of the human and murine promoter 1b has revealed a highly conserved region upstream of exon 1b (25). Furthermore, a MAZ-binding site at +242 and overlapping tandem ETS motifs at +264 were necessary for full transcriptional activity of constructs containing both promoter 1a and promoter 1b in F4N cells (25). Since promoter 1b was independently active in the primitive myeloid cell lines, mutations of the MAZ and ETS sites were introduced into the +209 1b construct. As shown in Fig. 5B, both mutations markedly reduced activity of promoter 1b in 416B cells and M1 cells. This effect is unlikely to represent the introduction of novel protein-binding sites since nuclear extracts from J2E, 416B, or M1 cells did not give rise to complexes after incubation with labeled mutant oligonucleotide (Fig. 8A, lanes 13, 14, and 22).

Band shift analysis was then performed to characterize the proteins binding to the MAZ binding ets motifs. Using an oligonucleotide containing the MAZ site as a probe, specific binding of three major complexes was observed (Fig. 8A). The same three complexes were seen in J2E, 416B, and M1 cells (lanes 2, 7, and 16). Three lines of evidence suggest that either MAZ or a MAZ related protein are present in these complexes. First, all complexes were competed with an excess of an oligonucleotide containing the known ME1a1 MAZ-binding site (44) from the myc promoter (lanes 6, 11, and 20). Second, all three complexes required an intact MAZ site since an oligonucleotide containing a mutated MAZ site could not compete for binding of any of the complexes (lanes 4, 9, and 18). Third, the three complexes co-migrated with complexes obtained using a known MAZ-binding site (ME1a1) as a probe (data not shown).

Band shift assays were also performed to study the proteins binding to the ETS motif. The oligonucleotide used as a probe for this experiment contained three overlapping TTCC motifs. Using J2E, 416B, and M1 nuclear extracts, three main complexes were detected in all three cell lines (Fig. 8B, lanes 2, 6, and 10). Addition of excess cold probe abolished binding of all complexes (lanes 3, 7, and 11), whereas an unrelated oligonucleotide did not compete for binding (lanes 5, 9, and 13). An oligonucleotide in which two of the three ETS binding motifs were mutated still competed for binding of the upper complex (lanes 4, 8, and 12) but not for binding of the lower two complexes. During these experiments it was noted that the addition of an excess of unlabeled oligonucleotide seemed to enhance the binding of the middle complex (asterisk) in all cell lines. We did not further investigate this finding, but one possible explanation would be the presence of an inhibitor which reduces binding of the complex which is sequestered by the addition of excess cold oligonucleotide. These results suggest that the lower two complexes bound to the TTCCTT sequence that was mutated in the mETS oligonucleotide (Table II), but that the upper complex bound elsewhere within the oligonucleotide.

Taken together these data demonstrate that the MAZ and ETS sites were important for full activity of promoter 1b in primitive myeloid cells. However, a similar pattern of complexes was observed in J2E cells in which transient reporter assays showed promoter 1b to be silent. These observations suggest that the MAZ and ETS sites are necessary but not sufficient for promoter 1b activity, and that additional lineage-restricted transcription factors may be needed for promoter 1b activity in primitive myeloid cells. Nevertheless, our data do not exclude the possibility that transcription factors binding to the MAZ and ETS sites may undergo lineage-restricted post-translational modifications which are not detectable by band shift analysis, or may represent co-migrating but functionally distinct transcription factor family members.
**Table II**

**Oligonucleotides used in gel shift assays**

| Oligonucleotide | Sequence                        |
|-----------------|--------------------------------|
| −37 GATA        | 5′-AGGCCATCTTCTTATCGGC-3′      |
| m−37 GATA       | 5′-GGGCGCTCTTCTTATCGGC-3′      |
| Sp1/GATA        | 5′-GGGCGCTCTTCTTATCGGC-3′      |
| mSp1/GATA       | 5′-GGGCGCTCTTCTTATCGGC-3′      |
| ETS             | 5′-CTTCTTCCCCCTCTTGGGGGGGG-3′  |
| mETS            | 5′-CTTCTTCCCCCTCTTGGGGGGGG-3′  |
| MAZ             | 5′-CTTCTTCCCCCTCTTGGGGGGGG-3′  |
| mMAZ            | 5′-CTTCTTCCCCCTCTTGGGGGGGG-3′  |
| NR (nonrelated) | 5′-GGGCGCTCTTCTTATCGGC-3′      |
| GATA cons       | 5′-GGGCGCTCTTCTTATCGGC-3′      |
| ME1a1           | 5′-GGGCGCTCTTCTTATCGGC-3′      |

Oligonucleotide ME1a1 contains the MAZ consensus binding site of the c-myc promoter (44); GATA cons, an oligonucleotide containing a consensus GATA-binding site from the murine α1-globin promoter (42). Mutated binding sites are underlined.

as well as committed erythroid, mast, and megakaryocytic cells. Although GATA-1 has been shown to be important for transcriptional regulation of SCL in erythroid cells, the molecular basis for SCL expression in other hematopoietic cell types is unknown. In this paper we have shown that the molecular basis for SCL expression is very different in CD34 positive primitive myeloid cells compared with committed erythroid cells.

Transient reporter assays have previously been used to show that SCL promoter 1a was active in murine erythroleukemia cells but not in murine T cells and that core promoter 1b was inactive in both cell types (25). These studies, together with work from other laboratories (8, 26), have also demonstrated a critical role for GATA-1 in regulating the activity of SCL promoter 1a. Murine erythroleukemia cells cannot be equated with normal erythroid progenitors, not least because the former express ETS family members as a result of retroviral insertions. It was therefore important to confirm the pattern of SCL promoter activity in a different class of erythroid cells. Whereas murine erythroleukemia cell lines are generated by infection of mice with Friend virus, the 2E cell line was generated *in vitro* by infection of fetal liver cells with a replication incompetent virus carrying *raf* and *myc*. Analysis of SCL promoter constructs in 2E cells gave rise to the same pattern of activity (active promoter 1a and silent promoter 1b) as previously found in murine erythroleukemia cells. In addition, GATA sites within promoter 1a bound GATA-1 and were critical for promoter 1a function.

Very different results were obtained when SCL promoter activity was studied in CD34 positive primitive myeloid cell lines. In both 416B and M1 cells promoter 1a activity was weak and no specific transcription factor binding could be detected at the −37 and −69 GATA sites upstream of promoter 1a. However, the most striking observation was the presence of strong core promoter 1b activity in both 416B and M1 cells. A series of 5′ deletion constructs was used to show that removal of promoter 1a and the upstream GATA sites did not reduce activity of promoter 1b. Since the constructs with promoter 1b alone did not contain additional GATA sites, these data demonstrate that promoter 1b was functioning in a GATA-independent manner.

GATA-1 plays a central role in the regulation of SCL promoter 1a in erythroid cells. GATA-1 binds to the functionally important −37 and −69 GATA motifs and also transactivates promoter 1a in heterologous cells (8, 25, 26). However, levels of GATA-1 are low in primitive progenitors (18, 45, 46) and as, shown here, GATA-1 mRNA may be undetectable in primitive myeloid cells expressing SCL. Furthermore, in mice lacking GATA-1 protein, erythroid differentiation was blocked at the
level of the proerythroblast, but GATA-1 target genes including SCL were still expressed at relatively normal levels along with markedly raised levels of GATA-2 (27). It has therefore been suggested that GATA-2 and GATA-1 regulate both overlapping and unique sets of genes (47). According to this hypothesis, SCL would be regulated by GATA-2 in primitive hematopoietic progenitors, a role that is taken over by GATA-1 during erythroid differentiation. Several additional observations are consistent with this scenario: (i) the ratio of GATA-2 to GATA-1 is high in multipotent progenitors and reversed during erythroid differentiation (18, 45, 46); (ii) SCL and GATA-2 both regulate self-renewal in multipotent progenitor cell lines (7, 48); (iii) both SCL and GATA-2 are required for the survival and/or differentiation of pluripotent stem cells during development (3–6); (iv) GATA-2 binds to a functional GATA motif in the SCL promoter (8); (v) GATA responsive promoters can frequently be transactivated by different GATA proteins (49–52).

Taken together these previous data suggested that GATA-2 regulates SCL in primitive hematopoietic progenitors, and that this is likely to be achieved by GATA-2 interacting with the same regulatory elements that are bound by GATA-1 in erythroid cells. Our results argue against this model and demonstrate that the SCL promoter is active in a GATA-independent manner in CD34 positive primitive myeloid cells. Moreover, these data raise the possibility that initial activation of SCL transcription in pluripotent hematopoietic stem cells may be GATA independent, a speculation that accords with the recent finding that lack of SCL abolishes hematopoiesis even more effectively than lack of GATA-2 (5, 6, 53). However, our results do not address the possibility that GATA-2 may regulate SCL distal regulatory elements in primitive hematopoietic cells.

Transient reporter assays provide valuable information about the transcription factor environment within a cell. However, they do not reflect constraints imposed by chromatin structure or by distant regulatory elements, absent from the reporter constructs. An RNase protection assay was therefore established to investigate the activity of the endogenous SCL promoter in erythroid and primitive myeloid cells. Both promoter 1a and promoter 1b were active in murine erythroleukemia cells and J2E cells. This is consistent with previous reverse transcriptase-polymerase chain reaction data which revealed the presence of transcripts including exon 1a in erythroid cells (15, 24). However, a very different pattern was observed in the two CD34 positive primitive myeloid cell lines, both of which exhibited clear activity of endogenous promoter 1b but weak or absent promoter 1a activity. These results demonstrate differential usage of promoter 1a in different SCL-expressing cell types. Moreover, they suggest that promoter 1b may be the critical site of transcription initiation in stem cells that exhibit low or absent GATA-1 expression.

Our observations also suggest that the mechanisms responsible for promoter 1b activity are fundamentally different in committed erythroid cells compared with CD34 positive progenitor cells. RNase protection assays demonstrated that the endogenous SCL promoter 1b was active in both erythroid and primitive myeloid cells. However, in transient reporter assays the core promoter 1b was strongly active in primitive myeloid cell lines, but silent in erythroid cells. This observation implies that in erythroid, but not in primitive myeloid cells, the core promoter 1b required integration in chromatin and/or additional sequences for its activity.

To distinguish these possibilities stable transfections were performed. These demonstrated that integration in chromatin was not sufficient to allow activity of promoter 1b in erythroid cells. In fact both core promoters were silent following integration in erythroid or myeloid cells. Thus, although some hematopoietic promoters are active after integration into chromatin (54, 55), this is clearly not the case for either SCL promoter. Instead our results strongly suggest that additional regulatory elements are necessary for both SCL promoters to overcome chromatin-mediated suppression.

It will now be important to dissect the different mechanisms responsible for endogenous promoter 1b activity in the two cell types, not least because it may be possible to construct promoter 1b variants that target expression to hematopoietic stem cells. Mutagenesis of promoter 1b has previously demonstrated that the +242 MAZ and +264 ETS motifs were important for full activity of a reporter construct containing both promoter 1a and 1b in erythroid cells. However, it was not clear whether these effects were mediated by promoter 1a or promoter 1b (25). We have now shown that the MAZ and ETS motifs were critical for activity of the core promoter 1b in primitive myeloid cells. Nevertheless, band shift analysis revealed the same pattern of complexes binding to the MAZ and ETS motifs in erythroid and primitive myeloid cells. The simplest interpretation of these results would be that MAZ and ETS sites are necessary but not sufficient for promoter 1b activity in primitive myeloid cells. Alternatively, subtle qualitative differences may exist between the comigrating complexes in erythroid and primitive myeloid cells. Thus, closely related transcription factors or post-translational modification may give rise to lineage-restricted functional differences that are not readily detectable by band shift analysis. Distinguishing between these possibilities may allow the construction of promoter 1b variants that, together with distal regulatory elements, will direct expression specifically to hematopoietic stem cells.

Acknowledgments—We acknowledge the computing expertise of James Gilbert, the excellent technical assistance of Koula Kosmopoulos, and Lorraine Ives for typing the manuscript. We also are grateful for the reagents kindly provided by S. Orkin, S. Cory, P. Klinken, T. Enver, K. Chatterjee, G. Partington, and H. Clevers.

REFERENCES
1. Bockamp, E.-O., McLaughlin, F., Murrell, A., and Green, A. R. (1994) BioEssays 16, 481–488
2. Orkin, S. H. (1995) J. Biol. Chem. 270, 4955–4958
3. Robb, L., Lyons, I., Li, R., Hartley, L., Kountsg, P., Harvey, R. R., Metcalf, D., and Begley, C. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7075–7079
4. Shviddasani, R. A., Mayer, E. L., and Orkin, S. H. (1995) Nature 373, 432–434
5. Porcher, C., Sweat, W., Rockwell, K., Fujiwara, Y., Alt, F. W., and Orkin, S. H. (1996) Cell 86, 47–57
6. Robb, L., Elwood, N. J., Elefanty, A. G., Kountsg, P., Li, R., Barnett, L. D., and Begley, C. G. (1996) EMBO J. 15, 4123–4129
7. Green, A. R., De Luca, E., and Begley, C. G. (1991) EMBO J. 10, 4153–4158
8. Apinan, P., Nakahara, K., Orkin, S. H., and Kirsh, I. R. (1992) EMBO J. 11, 4073–4081
9. Leroy-Viard, K., Vinat, M.-A., Lecointe, N., Jouault, H., Hidere, U., Romeo, P.-H., and Mathieu-Mahul, D. (1995) EMBO J. 14, 2341–2349
10. Green, A. R., Visvader, J., Lints, T., Harvey, R., and Begley, C. G. (1992) Oncogene 7, 653–660
11. Hwang, L.-Y., Siegelman, M., Davis, L., Oppenheim-Marks, N., and Baer, R. (1993) Oncogene 8, 3043–3046
12. Kallianpur, A. R., Jordan, J. E., and Brandt, S. J. (1994) Blood 83, 1200–1208
13. Pullford, K., Lecointe, N., Leroy-Viard, K., Jones, M., Mathieu-Mahul, D., and Mason, D. Y. (1995) EMBO J. 14, 675–684
14. Green, A. R., Salvaris, E., and Begley, C. G. (1991) Oncogene 6, 475–479
15. Mouthon, M.-A., Bernard, O., Mitjavila, M.-T., Rome, P.-H., Vainchenker, W., and Mathieu-Mahul, D. (1995) Blood 81, 647–655
16. Visvader, J., Begley, C. G., and Adams, J. M. (1991) Oncogene 6, 195–204
17. Murrell, A. M., Bockamp, E.-O., Göttingen, B., Chan, Y. S., Gass, M. A., Heyworth, C. M., and Green, A. R. (1985) Oncogene 11, 131–138
18. Cross, M. A., Heyworth, B., Murrell, A. M., Bockamp, E.-O., Cohley, U. T., Dexter, T. M., and Green, A. R. (1994) Oncogene 9, 3013–3016
19. Tanigawa, T., Elwood, N., Metcalf, D., Cary, D., De Luca, E., Nicola, N. A., and Begley, C. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7864–7868
20. Bienz, M. (1992) Curr. Opin. Cell Biol. 4, 955–961
21. Bienz, M., and Muller, J. (1995) BioEssays 17, 775–784
22. Aplan, P. D., Begley, C. G., Bertness, V., Nussenzier, M. A., Esquerra, A., Coligan, J., and Kirsh, I. R. (1990) Mol. Cell. Biol. 10, 6426–6435
23. Bernard, O., Lecointe, N., Jonveaux, P., Souyri, M., Mauchauffe, M., Berger, R., Larson, C. J., and Mathieu-Mahul, D. (1991) Oncogene 6, 1477–1488
24. Begley, C. G., Robb, L., Rockman, S., Visvader, J., Bockamp, E.-O., Chen, Y.-H., and Green, A. R. (1994) Gene (Amst.) 138, 93–99
25. Bockamp, E.-O., McLaughlin, F., Murrell, A. M., Göttingen, B., Robb, L., Begley, C. G., and Green, A. R. (1995) Blood 86, 1502–1514
26. Lecointe, N., Bernard, O., Naert, K., Joulin, V., Larsen, C. J., Romeo, P. H., and Mathieu-Mahul, D. (1994) Oncogene 9, 2623–2632
27. Weiss, M. J., Keller, G., and Orkin, S. H. (1994) Genes Dev. 8, 1184
28. Dexter, T. M., Allen, T. D., Scott, D., and Teich, N. (1979) Nature 277, 471–474
29. Ichikawa, Y. (1969) J. Cell. Physiol. 74, 223–234
30. Klinken, S. P., Nicola, N. A., and Johnson, G. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8506–8510
31. Mitsushima, S., and Nagata, S. (1990) Nucleic Acids Res. 18, 5322
32. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
33. Tsai, S. F., Martin, D. I., Zon, L. I., D’Andrea, A. D., Wang, G. G., and Orkin, S. H. (1989) Nature 339, 446–451
34. Calligaris, R., Bottardi, S., Cogoi, S., Apezteguia, I., and Santoro, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11598–11602
35. Bossone, S. A., Asselin, C., Patel, A. J., and Marcu, K. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7452–7456
36. Steger, D. J., Hecht, J. H., and Mellon, P. L. (1994) Mol. Cell. Biol. 14, 5592–5602
37. Tsai, F.-Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J. Z., Rosenblatt, M., Alt, F. W., and Orkin, S. H. (1994) Nature 371, 221–226
38. Skalnik, D. G., Dorfman, D. M., Perkins, A. S., Jenkins, N. A., Copeland, N. G., and Orkin, S. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8505–8509
39. Dziennis, S., Van Eten, R. A., Pahl, H. L., Morris, D. L., Rothstein, T. L., Bloesch, C. M., Perlmutter, R. M., and Tenen, D. G. (1995) Blood 85, 319–329