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The *SCL* gene product: a positive regulator of erythroid differentiation

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The *SCL* (*tal-1*, *TCL5*) gene is a member of the basic domain, helix-loop-helix (bHLH) class of putative transcription factors. We found that (i) the *SCL* promoter for exon Ia contains a potential recognition site for GATA-binding transcription factors, (ii) *SCL* mRNA is expressed in all erythroid tissues and cell lines examined, and (iii) *SCL* mRNA increases upon induced differentiation of murine erythroleukemia (MEL) cells, and inferred that *SCL* may play a physiologic role in erythroid differentiation. We used gel shift and transfection assays to demonstrate that the GATA motif in the *SCL* promoter binds GATA-1 (and GATA-2), and also mediates transcriptional transactivation. To identify a role for *SCL* in erythroid differentiation, we generated stable transfectants of MEL and K562 (a human chronic myelogenous leukemia cell line that can differentiate along the erythroid pathway) cells overexpressing wild-type, antisense or mutant *SCL* cDNA. Increasing the level of *SCL* expression in two independent MEL lines (F4-6 and C19, a 745 derivative) and K562 cells increased the rate of spontaneous (i.e. in the absence of inducer) erythroid differentiation. Conversely, induced differentiation was inhibited in MEL transfectants expressing either antisense *SCL* cDNA or a mutant *SCL* lacking the basic domain. Our experiments suggest that the *SCL* gene can be a target for the erythroid transcription factor GATA-1 and that the *SCL* gene product serves as a positive regulator of erythroid differentiation.

Key words: bHLH protein/erythroid differentiation/*SCL*/transcription factor

Introduction

The *SCL* gene was first discovered due to its disruption in a stem cell leukemia (Begley *et al.*, 1989a). *SCL* [also known as *TCL5* (Finger *et al.*, 1989) or *tal1* (Chen *et al.*, 1990)] is expressed primarily in immature hematopoietic tissues and cell lines, and encodes a basic domain helix-loop-helix (bHLH) motif (Begley *et al.*, 1989b) similar to that found in a variety of eukaryotic transcription factors, each of which appears to play a role in cell-type specific growth or development (Murre *et al.*, 1989; Benezra *et al.*, 1990). *SCL* gene disruption and resultant dysregulation is the most commonly recognized chromosomal abnormality in T-cell acute lymphoblastic leukemia (ALL), seen in as many as 30% of newly diagnosed T-cell ALL patients (Brown *et al.*, 1990; Aplan *et al.*, 1990b, 1992). However, the mechanism by which *SCL* dysregulation contributes to leukemogenesis, and the physiologic role of *SCL*, have remained obscure.

Indirect evidence has previously suggested that *SCL* may play a role in normal erythroid differentiation. The *SCL* promoter region for exon Ia (hereafter referred to as the proximal *SCL* promoter) is known to contain a potential GATA-1 binding site (Aplan *et al.*, 1990a). Furthermore, high levels of *SCL* expression have been observed in all murine and human erythroid cell lines examined (Visvader *et al.*, 1991; Begley *et al.*, 1989b). Lastly, *SCL* expression increased dramatically in the FL-F4N murine erythroleukemia (MEL) cell line when induced to differentiate with DMSO (Visvader *et al.*, 1991).

Here we have examined the binding and action of the erythroid transcription factor GATA-1 on the proximal *SCL* promoter and then investigated the effect of overexpressing or inhibiting *SCL* action in erythroid cell lines. Increased expression of *SCL* correlated with increased percentages of cells undergoing erythroid differentiation in the absence of added inducer. Conversely, inhibition of *SCL* function through expression of antisense *SCL* cDNA or expression of a mutant form lacking the basic domain inhibited inducer-mediated erythroid differentiation. These complementary findings provide strong support for a fundamental role of the *SCL* gene product in erythroid differentiation.

Results

SCL mRNA increases upon induction of F4-6 MEL cells by DMSO

F4-6 is a Friend virus-derived MEL cell line (polycythemic variant) that matures to a normoblastic stage in response to treatment with DMSO (Billelo *et al.*, 1980). Maturation is heralded by increased heme synthesis (as reflected by benzidine staining of heme and a change in cell pellet color from white to red) and α-globin mRNA expression. Upon treatment of logarithmic growth phase F4-6 cells with 1.5% DMSO for 5 days, benzidine positive cells increase from $<1\%$ to $>60\%$, with a concomitant 50-fold increase in α-globin mRNA. For the purposes of this study, we used production of the major differentiative products of the erythrocyte series, namely heme (assayed by benzidine stain), and, in selected experiments, globin (assayed by mRNA production) as markers of erythroid differentiation. In response to DMSO, *SCL* mRNA decreases transiently in F4-6 cells prior to a subsequent increase of 5-fold above baseline (data not shown). These findings are similar to those

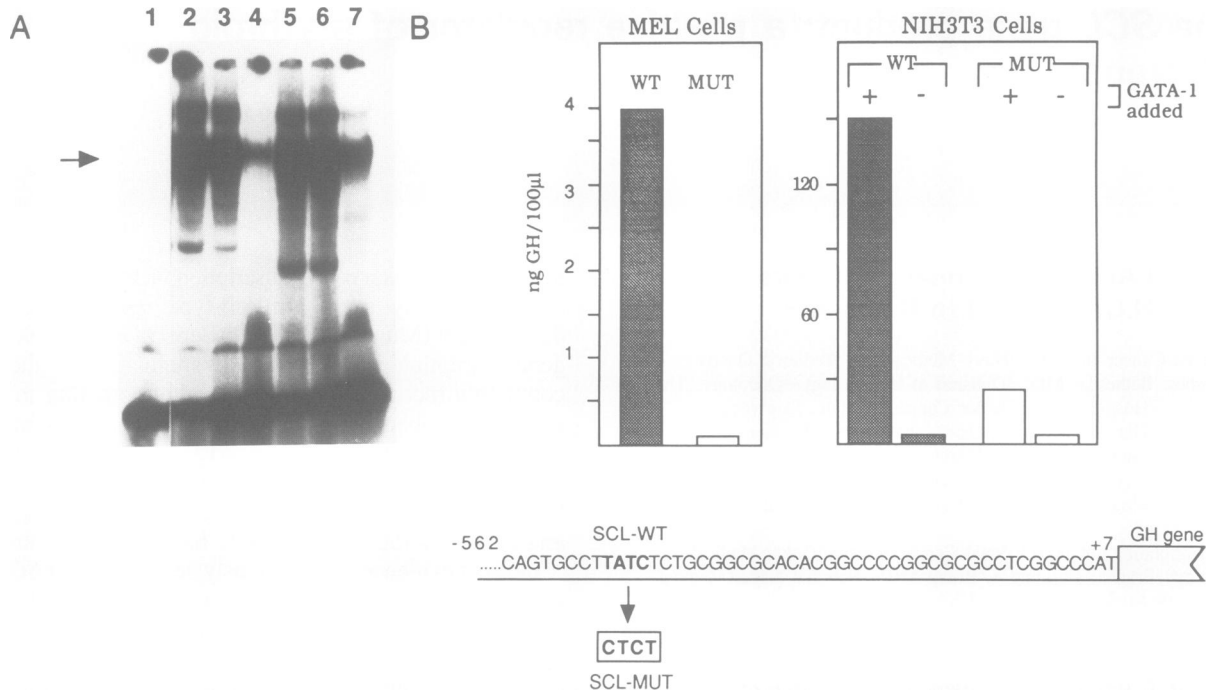

Fig. 1. A. Binding of GATA-1 and GATA-2 proteins to the proximal *SCL* promoter. COS extracts containing abundant murine GATA-1 or human GATA-2 were prepared as described in Materials and methods and employed in gel shift assays with a labeled, double stranded oligonucleotide spanning the GATA motif in the proximal *SCL* promoter. Lane 1: no added COS extract; lanes 2–4: COS extract containing murine GATA-1; lanes 5–7: COS extract containing human GATA-2. Lanes 3 and 6: heterologous competitor oligonucleotide added; lanes 4 and 7: excess oligonucleotide containing the *SCL* GATA motif added. Competition is incomplete in lanes 4 and 7 due to the abundance of GATA protein in the COS extract. **B.** The GATA motif is functional in the proximal *SCL* promoter. SCL-WT and SCL-MUT growth hormone reporter constructs, depicted at the bottom, were introduced transiently into MEL or NIH3T3 cells as described in Materials and methods. In the cotransfection NIH3T3 experiment (+) indicates expression of wild-type GATA-1 protein; (−) indicates expression of a mutant GATA-1 protein ('mini', see Martin and Orkin, 1990) which binds DNA but cannot transactivate reporters.

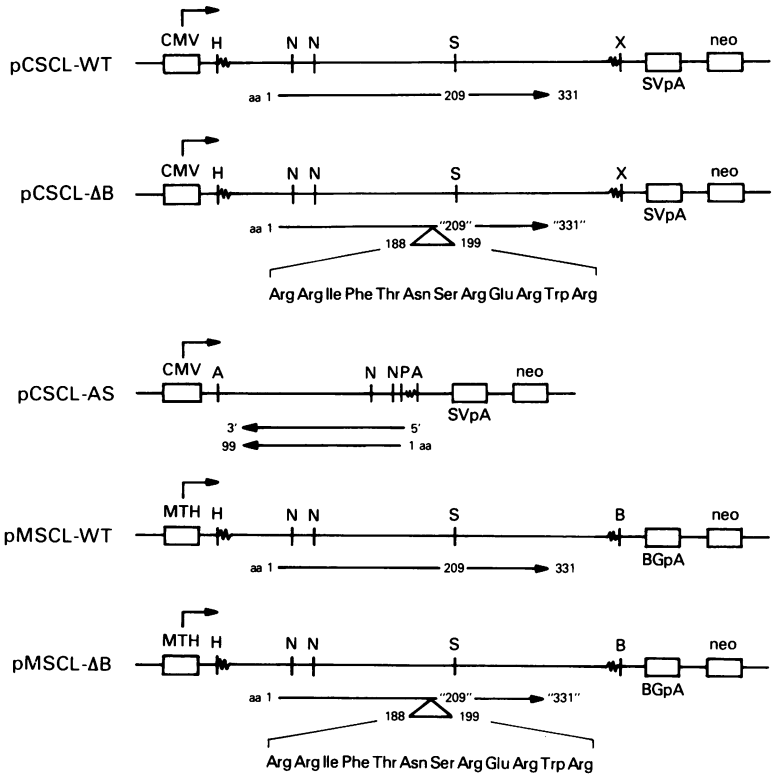

Fig. 2. Transfection vectors. The first three constructs indicated are derived from the PRCCMV (Invitrogen) vector. pCSCL-WT contains an insert encoding a full length SCL protein; pCSCL-ΔB was derived from pCSCL-WT by deleting the basic domain (amino acids 188–199), and pCSCL-AS has an insert encompassing 39 bp of 5′ untranslated region and the first 297 bp of coding sequence, placed in the antisense orientation. Two additional vectors, pMSCL-WT and pMSCL-ΔB, contain inserts identical to the pCSCL-WT and pCSCL-ΔB respectively, driven by a murine metallothionein (MTH) promoter. The CMV promoter, MTH promoter, SV40 polyadenylation signal (SVpA), β-globin polyadeylation signal (BGpA) and neomycin resistance gene are indicated.

reported by Visvader *et al.* (1991) who observed a 15-fold increase in *SCL* mRNA levels upon DMSO induction of the FL-F4N MEL cell line.

The SCL promoter is a potential target of GATA-binding transcription factors

The pattern of expression of *SCL* among hematopoietic lineages (erythroid, mast and megakaryocytic) (Begley *et al.*, 1989b; Visvader *et al.*, 1990; Elefanty and Cory, 1992) parallels that of GATA-1 (previously known as GF-1, Nf-E1 or Eryf 1), a transcription factor that binds to (T/A GATA A/G) consensus sequences (for review, see Orkin, 1990). As the proximal promoter of the *SCL* gene contains a potential GATA-binding motif (Aplan *et al.*, 1990a), we have addressed whether GATA-1 or other GATA-binding proteins, such as the related factor GATA-2 (Yamamoto *et al.*, 1990), might participate in its function. To assess protein binding at this site, we performed gel-shift assays with GATA-1 and GATA-2 proteins overexpressed in monkey kidney COS cells and an oligonucleotide containing the *SCL* GATA motif. As shown in Figure 1A, both GATA-1 and GATA-2 recognize this site. To establish a role for the GATA motif in promoter function, we introduced proximal *SCL* promoter−growth hormone (GH) reporter constructs into MEL (parental line 745) cells. The proximal *SCL* promoter−GH construct with a wild-type GATA motif (SCL-WT) was active (Figure 1B). Mutation of the GATA site (plasmid SCL-MT) greatly reduced promoter activity (Figure 1B). To ascertain whether the *SCL* promoter could be activated directly through the GATA site, we cotransfected the *SCL* promoter−GH reporter constructs and expressible GATA-1 cDNA into NIH3T3 fibroblasts (which do not express GATA-1). Although NIH3T3 cells express low levels of GATA-2, in the absence of exogenously supplied GATA-2, the level of GATA-binding proteins in NIH3T3 cells is insufficient to drive this reporter gene construct. Transactivation of the *SCL* promoter was observed through the proximal GATA motif (Figure 1B). Mutation of the GATA site or expression of the mutated, inactive form of GATA-1 blunted transactivation (Figure 2B). These data suggest that the proximal *SCL* promoter may be a direct target of GATA-binding proteins. In MEL cells, where GATA-1 is abundant and GATA-2 is present only at very low levels (Zon *et al.*, 1991), it is likely that GATA-1 is the effector protein. In other cells, such as K562, where GATA-1 and GATA-2 are coexpressed, either or both might function on the proximal *SCL* promoter. The architecture and behavior of the proximal *SCL* promoter are reminiscent of other erythroid-expressed, presumptive GATA-binding protein gene targets, such as the erythropoietin receptor (Zon *et al.*, 1991).

Establishment of stable transfectants expressing SCL wild-type or mutant protein

To alter *SCL* function artificially in F4-6 MEL cells, we transfected the expression vectors indicated in Figure 2, and assayed for expression of wild-type *SCL*, basic domain deleted (ΔB) *SCL*, or antisense *SCL* in G418 resistant clones by Northern hybridization (Figure 3). The stable transfectants demonstrated a wide range of exogenous *SCL* expression, and several clones were selected for DMSO induction. Figure 3 (bottom) shows that SCL full length and SCL-ΔB proteins can be immunoprecipitated from stable

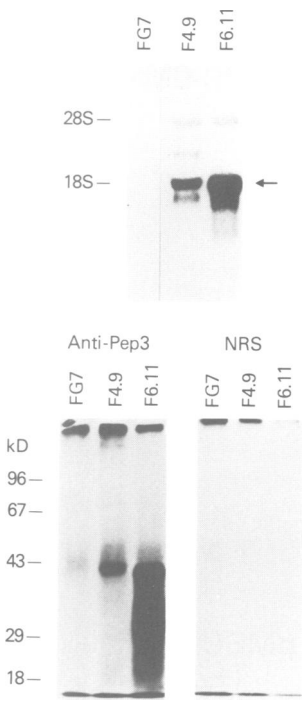

Fig. 3. Northern blot and immunoprecipitation of wild-type and mutant SCL proteins from stable transfectants. **Above:** Northern blot of RNA from clones harboring pRC-CMV (FG7), pMSCL-WT (F4.9) or pCSCL-ΔB (F6.11), hybridized to an *SCL* cDNA probe (58RS). The ribosomal RNA bands are indicated; an arrow indicates expression of the exogenous *SCL* constructs. **Below**: stable transfectants expressing either wild-type (clone F4.9) or ΔB *SCL* (clone F6.11) were metabolically labeled with [^{35}S]methionine and extracts were immunoprecipitated with antisera directed against the SCL carboxy terminus (anti-pep3) or normal rabbit serum (NRS). Size standards shown are in kilodaltons. The autoradiograph shown represents a 4 day exposure; on a briefer (4 h) exposure, the F6.11 lane shows a single band of ~40 kDa.

transfectants overexpressing *SCL* wild-type or ΔB mRNA, whereas a neomycin resistant (neo^R) clone (FG7), transfected with the pRC-CMV parent vector alone, expresses relatively low levels of *SCL* mRNA and undetectable levels of SCL protein. Growth curves of clones expressing wild-type, ΔB, or antisense *SCL* mRNA demonstrated doubling times of 18−19 h, which were similar to that of the F4-6 parent line (doubling time of 16 h).

Forced expression of the SCL gene product promotes erythroid differentiation

When F4-6 transfectants expressing wild-type *SCL* under control of the murine metallothionein promoter (Figure 2) were examined, the percentage of benzidine positive cells in the absence of inducer (DMSO) was more than that of the parent cell line or a control transfectant harboring the pRC-CMV vector alone (Figure 4A and B). Addition of zinc to the culture medium enhanced transcription of *SCL* mRNA from the metallothionein promoter by 3-fold and led to a corresponding increase in benzidine staining (Figure 4B). The correlation between increased level of exogenous *SCL* mRNA and increased spontaneous erythroid differentiation was confirmed in four additional F4-6 stable transfectants expressing exogenous wild-type *SCL*, which spontaneously demonstrated up to 13% benzidine positive cells, in the absence of the inducing agent DMSO.

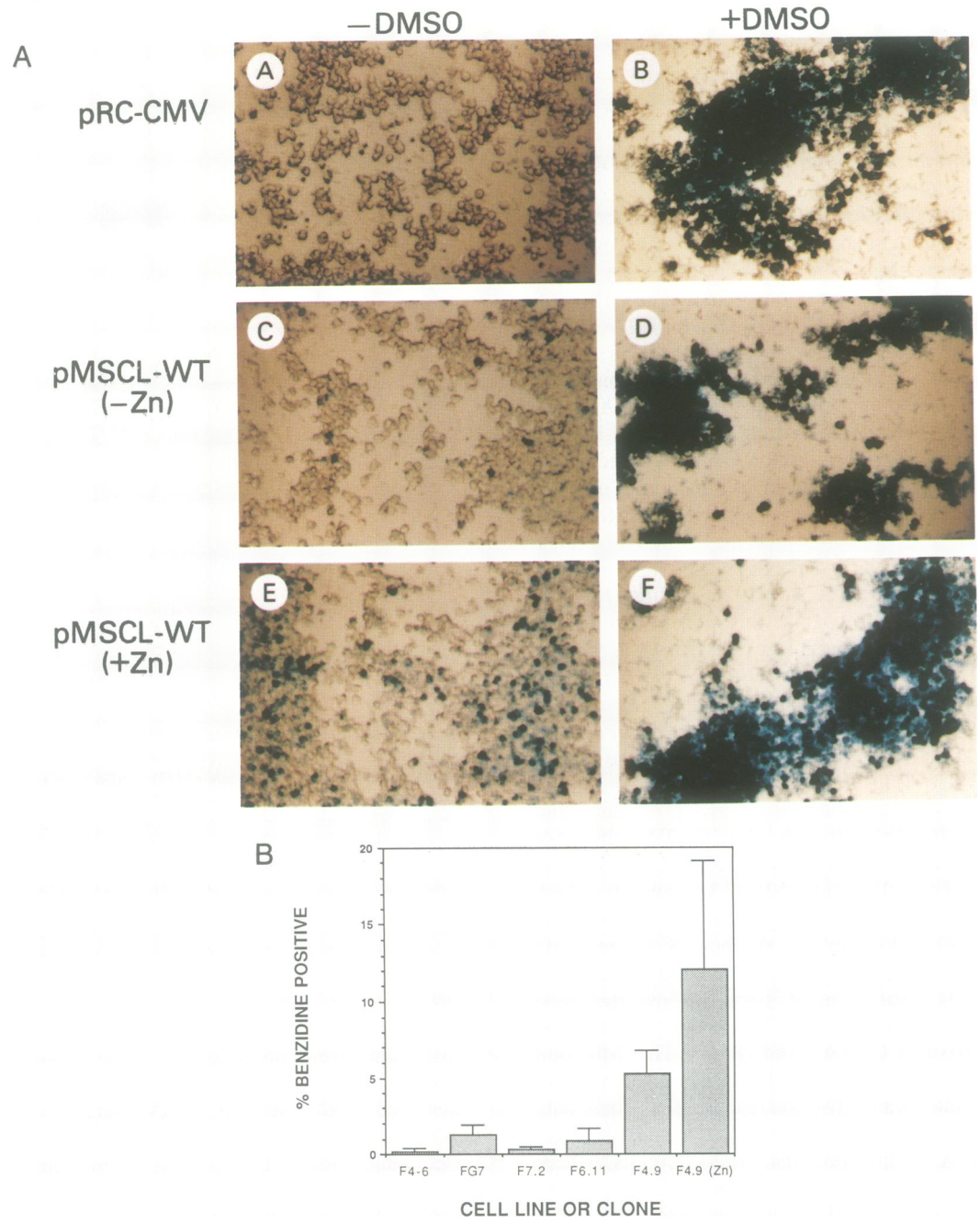

Fig. 4. A. Benzidine staining of wild-type *SCL* transfectants. A clone transfected with the wild-type *SCL* (PMSCL-WT) vector, driven by a murine metallothionein promoter, was studied with and without zinc and DMSO. A stable neo^R transfectant, obtained by transfection with the pRC-CMV parent vector, was examined as a control. Panels A and B, a neo^R transfectant (FG7) without or with 1.5% DMSO, respectively; C and D, a pMSCL-WT transfectant (F4.9) grown in the absence of zinc, without and with 1.5% DMSO respectively; E and F, the same pMSCL-WT transfectant (F4.9) grown in the presence of zinc, without and with DMSO respectively. The F4.9 transfectant shows an increase in benzidine positive cells without the addition of inducer (DMSO); this increase is magnified by increasing expression of the exogenous *SCL* through the addition of zinc. **B.** Spontaneous differentiation of transfectants expressing wild-type *SCL* mRNA. The indicated cell lines or transfectants (F4-6, parent cell line; FG7, neo^R control transfectant; F7.2, *SCL* antisense transfectant; F6.11, *SCL* ΔB transfectant; and F4.9, *SCL* wild-type transfectant, under control of the murine metalothionein promoter) were assayed for spontaneous differentiation, in the absence of inducer. The F4.9 transfectant was assayed with and without 100 μM Zn in the growth medium. Values represent mean ± standard deviation.

A functional SCL gene product is required for efficient DMSO-induced differentiation of F4-6

Stable transfectants expressing high levels of either antisense *SCL* or ΔB *SCL* mRNA were identified and induced with 1.5% DMSO. Figure 5A demonstrates two clones from this study, an *SCL* antisense clone (F7.2) and an *SCL* ΔB clone (F6.11). In both clones, induced erythroid differentiation was dramatically inhibited as compared with the parent cell line (F4-6) and a clone (FG7) harboring the pRC-CMV vector (Figure 5B). Previous studies using MEL cell differentiation as a model system have suggested that a decline in c-myc protein levels is crucial for MEL cell erythroid differentiation. F4-6 stable transfectants expressing antisense *SCL* mRNA and blocked in their ability to differentiate

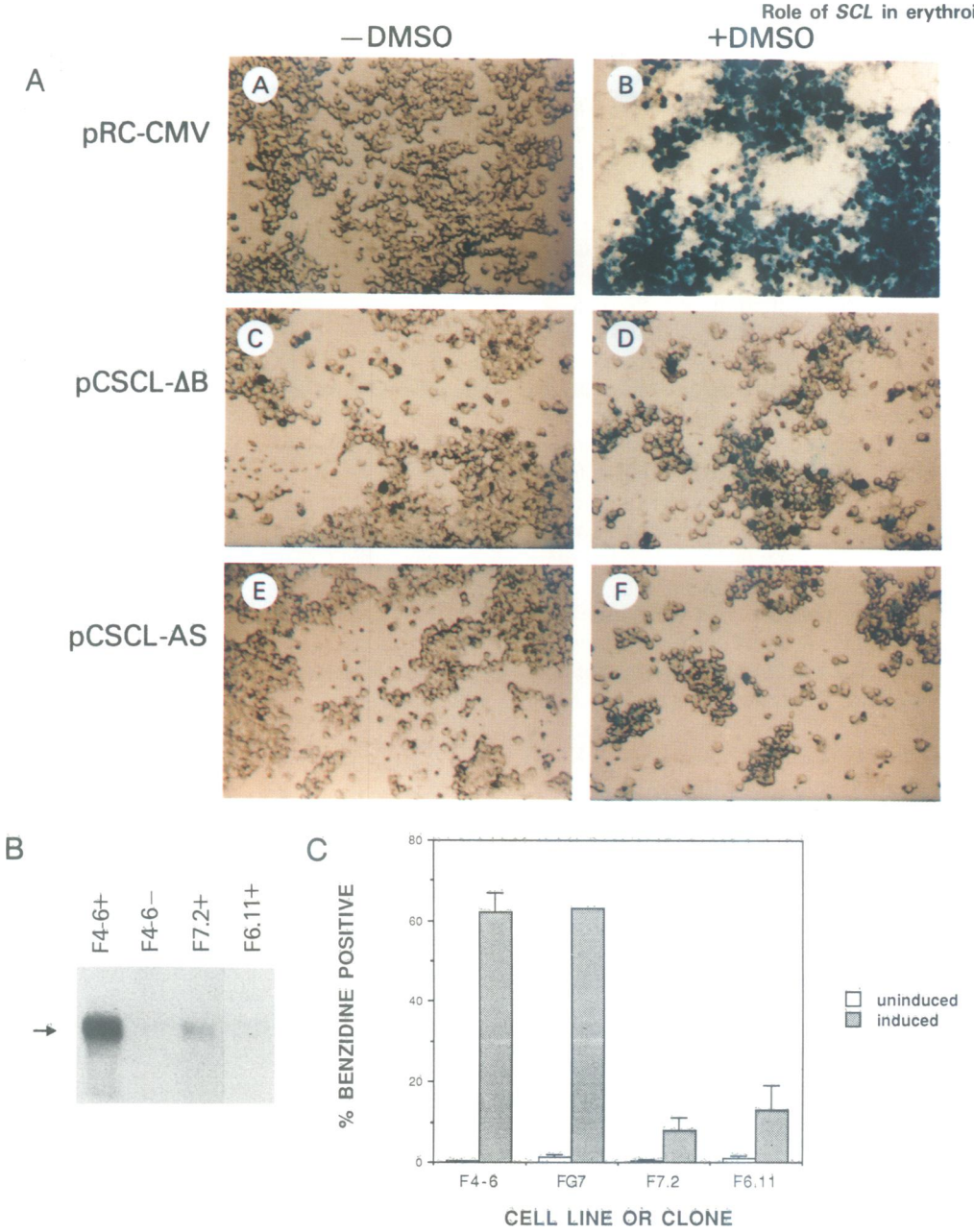

Fig. 5. A. Benzidine staining of antisense and ΔB clones. Three stable transfectants were stained with benzidine after 5 days' passage with or without 1.5% DMSO. Panels A, C and E represent stable transfectants harboring pRC-CMV, pCSCL-ΔB or pCSCL-AS vectors respectively, without DMSO. Panels B, D and F represent stable transfectants harboring the pRC-CMV, PCSCL-ΔB, or pCSCL-AS vectors respectively, with DMSO. **B.** Globin hybridization. The parent cell line, F4-6, and stable transfectants F7.2 (expressing antisense *SCL* mRNA) and F6.11 (expressing ΔB *SCL* mRNA) were treated for 5 days with 1.5% DMSO. Total RNA was extracted and Northern blots were hybridized to a murine α-globin probe. Lane 1, F4-6, + DMSO; 2, F4-6, no DMSO; 3, F7.2, + DMSO; 4, F6.11, +DMSO. The globin signal is indicated by an arrow. **C.** Effect of *SCL* on erythroid differentiation. The indicated cell lines (F4-6, parent cell line; FG7, neo^R control transfectant; F7.2, *SCL* antisense transfectant; F6.11, *SCL* ΔB transfectant) were assayed for benzidine positivity after 5 days' induction with 1.5% DMSO. Values represent mean ± standard deviation.

showed a decline in c-*myc* mRNA levels similar to controls not expressing antisense *SCL* mRNA (data not shown).

To assess whether our results reflected true inhibition of erythroid differentiation, or merely clonal variation, we induced additional *SCL* antisense, ΔB and neo^R transfectants. Six neo^R transfectants were indistinguishable from the parent cell line in their response to DMSO-mediated induction (data not shown). In contrast, *SCL* antisense and ΔB clones showed an inhibition to differentiation in rough proportion to the level of *SCL* antisense or ΔB mRNA expression (Figure 6). These findings, together with the observation that transfectants with undetectable levels of *SCL* antisense or ΔB mRNA (data not shown) are not inhibited, suggest that the block to differentiation seen in these transfectants is a consequence of *SCL* antisense or ΔB mRNA expression.

The effects of SCL on erythroid differentiation are confirmed in other cell lines

As a further test of the significance of these findings, we examined the effect of overexpressing wild-type, antisense and ΔB *SCL* cDNAs in an independent MEL line, C19, which is a derivative of the 745 MEL cell line (anemic variant) (Friend *et al.*, 1971). In parallel, a transfection with the pRCCMV (no insert) vector alone was performed as a control. Stable transfectants were selected and induced with

HMBA, as the C19 cell line is relatively insensitive to DMSO as an inducing agent (data not shown). Table I demonstrates results similar to those observed with F4-6, including the 'dose−response' phenomenon discussed above for the F4-6 antisense clones.

There is no known human counterpart to the MEL system. However, the K562 chronic myelogenous leukemia (CML) cell line (Lozzio and Lozzio, 1975) exhibits a low level of spontaneous erythroid differentiation which can be augmented with several agents, including sodium butyrate (for review, see Koeffler, 1986). Stable transfectants expressing wild-type *SCL* mRNA were isolated and analyzed in the absence of inducer. As indicated in Table II, clones expressing wild-type *SCL* showed a high rate of spontaneous differentiation (up to 22% as assayed by benzidine staining) in the absence of inducer. There was a synergistic effect of exogenous wild-type *SCL* with the chemical inducer sodium butyrate. Whereas 54% of parental K562 cells were benzidine positive after 5 days' treatment with sodium butyrate, ≥90% benzidine positive cells were seen with several induced transfectants. A direct correlation between expression of exogenous *SCL* mRNA and high rates of both spontaneous and sodium butyrate induced erythroid differentiation was observed. Clones expressing negligible levels of exogenous wild-type *SCL* mRNA (such as K4.9) were indistinguishable from either the parent K562 cell line or neo^R stable transfectants obtained by transfecting the pRCCMV vector alone.

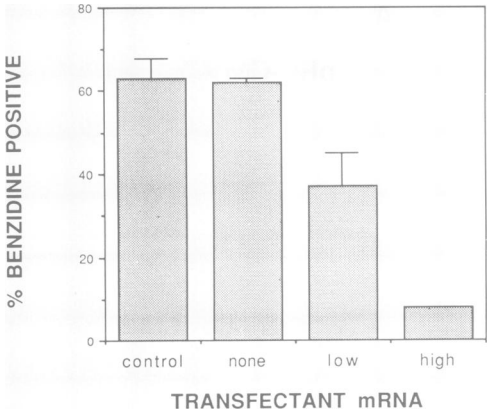

Fig. 6. Inhibition of F4-6 maturation by *SCL* antisense mRNA is correlated with *SCL* antisense mRNA expression. F4-6 transfectants were examined for expression of the antisense mRNA, induced to differentiate with 1.5% DMSO, and assayed for benzidine positivity. Control transfectants represent six independent neo^R clones transfected with the pRCCMV vector. The antisense clones were separated into three categories based on quantitative analysis of mRNA expression on Northern blots using a Betagen image acquisition system, either high (ratio of antisense mRNA to cross-hybridizing 28S rRNA > 3.0; one clone), low (ratio between 0.1 and 3.0; four clones) or none (undetectable by Northern blotting). Values shown represent the mean ± standard deviation of percent benzidine positive cells after 5 days' induction.

Discussion

We have sought to determine whether the product of the *SCL* gene, a member of the bHLH family of transcription factors, plays a role in erythroid cell differentiation. Though *SCL* gene expression is associated with T-cell ALL in the context of chromosomal translocations and deletions, its expression in three hematopoietic lineages (erythroid, mast and megakaryocytic) and increased mRNA expression following DMSO treatment of MEL cells indirectly suggest an involvement of the *SCL* gene product in some aspect of hematopoietic differentiation.

Transient expression and transactivation experiments implicating GATA-binding proteins in the function of the proximal *SCL* promoter provide a direct link between the *SCL* gene product and erythroid development. Given the abundance of GATA-1 protein in maturing erythroid cells, the data interpreted in the simplest fashion suggest that the proximal *SCL* promoter may be a direct target of GATA-1. Although this view places *SCL* downstream of GATA-1, an essential transcription factor for erythroid development (Pevny *et al.*, 1991), we cannot exclude a role for other GATA-binding proteins (e.g. GATA-2) in earlier progenitors or involvement of *SCL* in the control of GATA-1 (or related proteins). In MEL cells, GATA-1 is abundant and related GATA-binding proteins are expressed at low or undetectable levels. In these cells, it is possible that *SCL* is one effector of GATA-1 action in promoting erythroid development.

The effects of overexpressing wild-type *SCL* in two MEL cell lines (a polycythemic variant, F4-6, and an anemic variant, C19) and the K562 CML cell line provide more direct support for the participation of *SCL* in erythroid differentiation. Each cell line shows a low (<1%) basal rate of spontaneous erythroid differentiation, in the absence of a chemical inducing agent (e.g. DMSO, HMBA or sodium butyrate). Transfectants overexpressing the wild-type *SCL* mRNA exhibited elevated rates of spontaneous erythroid differentiation (Tables I and II), up to 20-fold higher than either the parent cell line or neo^R control transfectants.

Attempts to block *SCL* gene effects through overexpression of antisense *SCL* mRNA or by expression of a mutant form of *SCL* with a deletion of its basic domain provide complementary evidence for an important role for the *SCL* gene product. In the two MEL cell lines, antisense *SCL* mRNA expression in stable transfectants was associated with a reduced ability to differentiate and produce benzidine positive cells in response to chemical inducers. The degree of inhibition was directly correlated with the level of antisense *SCL* mRNA. Prior studies on antisense plasmid vectors suggest that these vectors function either by targeting and blocking gene transcription or by forming a stable RNA−RNA duplex and preventing translation (Izant and Weintraub, 1985; Melton, 1985). The *SCL* antisense constructs most probably function at a translational level, as antisense transfectants show endogenous *SCL* mRNA levels comparable to those of controls (data not shown), suggesting that *SCL* gene transcription is proceeding normally.

Our use of the *SCL* ΔB mutant to interfere with *SCL* function was based on prior studies of the bHLH protein myoD, which demonstrated specific roles for the basic domain and HLH region in DNA-binding and dimer formation, respectively (Weintraub *et al.*, 1991). Expression of basic domain deleted myoD blocks myogeneic conversion of 10T1/2 cells, presumably by forming inactive myoD homodimers or myoD/E2A heterodimers (Davis *et al.*, 1990). The similar consequences of expressing ΔB *SCL* and antisense *SCL* cDNAs in MEL cells suggest that mutant ΔB SCL protein, indeed, acts in a dominant negative fashion in MEL cells.

Table I. HMBA induction of C19 transfectants

Cell line/clone	mRNA	Benzidine positive cells	
		Uninduced	HMBA induced
Parent cell line C19	NA	0.5	73
Clones			
Neo^R control			
CG1	NA	0.0	81
CG2	NA	0.4	66
CG3	NA	0.5	69
CG4	NA	0.0	58
Wild-type *SCL*			
1.21	8.3	4.9	80
1.4	6.1	0.8	68
1.5	1.6	0.4	52
1.10	0.2	0.8	59
ΔB *SCL*			
6.17	38	ND	48
6.20	27	ND	26
6.19	11	ND	45
6.15	0	ND	56
Antisense *SCL*			
7.19	4.0	ND	7
7.17	0.5	ND	13
7.22	0.4	ND	10
7.18	0.3	ND	14
7.21	0.2	ND	22
7.26	0	ND	84

The values for mRNA expression of the transfected clones represent the intensity of the exogenous *SCL* signal, quantified with a Betagen image acquisition system. This value was normalized to adjust for differences in RNA loading by dividing the intensity of the exogenous *SCL* signal by the intensity of the cross-hybridizing 28S ribosomal signal (see Figure 3 for an example of the cross-hybridization to the 28S ribosomal band). Clones with the CG prefix were transfected with the pRCCMV; a 1. prefix indicates transfection with pCSCL-WT, a 6. prefix indicates transfection with pCSCL-ΔB, and a 7. prefix indicates transfection with pCSCL-AS. Uninduced and HMBA-induced refer to percent benzidine positive cells after 5 days' induction with 3 mM HMBA, performed as described in Materials and methods section. NA, not applicable; ND, not done.

Table II. Sodium butyrate induction of K562 transfectants

Cell line/clone	mRNA	% Benzidine positive cells	
		Uninduced	Induced
Parent cell line K562	NA	1.2%	54%
Clones			
Neo^R control			
KG1	NA	2.6	66
KG2	NA	1.2	51
KG3	NA	0.5	65
KG4	NA	0.5	57
KG6	NA	0.5	52
KG7	NA	0.9	48
KG8	NA	2.9	52
KG9	NA	0.5	60
Wild-type *SCL*			
K4.12	28	11	94
K4.4	22	19	88
K4.1	19	16	90
K4.8	17	21	87
K4.2	14	19	94
K4.11	11	22	93
K4.5	9	17	93
K4.6	2	4.5	68
K4.7	2	ND	77
K4.10	0	1.8	52
K4.3	0	2.5	53
K4.9	0	1.6	53

mRNA quantification and inductions were carried out as described in Table I, except that 70 mM sodium butyrate was used as the inducing agent. Clones with the KG. prefix were transfected with pRCCMV; clones with the K4. prefix were transfected with pMSCL-WT. NA, not applicable; ND, not done.

Although similarities were observed in the transfection and subsequent induction experiments with all three cell lines, differences were also apparent. While stable transfectants expressing wild-type *SCL* showed an increased level of spontaneous erythroid differentiation in all three cell lines studied, the increase was most dramatic in K562. Furthermore, several (eight) K562 wild-type *SCL* clones showed a synergistic effect on erythroid differentiation with sodium butyrate. This synergistic effect, enhancing erythroid differentiation in concert with the differentiating agent, was not consistently seen in either C19 or F4-6 wild-type *SCL* clones. Stable F4-6 and C19 transfectants expressing antisense *SCL* mRNA were significantly and reproducibly blocked in their ability to differentiate in response to either DMSO (F4-6 transfectants) or HMBA (C19 transfectants). The degree of inhibition of differentiation correlated well with the level of antisense *SCL* mRNA, suggesting a dose–response relationship. However, we were unable to perform induction experiments with K562 cells transfected with the antisense *SCL* vector, as we experienced difficulty in obtaining stable transfectants that consistently expressed detectable levels of antisense *SCL* mRNA in K562. These results are similar to those described by other investigators (Green *et al.*, 1991) who also had considerable difficulty obtaining stable transfectants expressing antisense *SCL* mRNA. They found that an *SCL* antisense construct caused growth arrest in the K562 cell line and also led to an increased tendency of the growth arrested cells to accumulate hemoglobin. In contrast to the MEL transfectants, stable K562 transfectants that expressed high levels of ΔB *SCL* tended to grow slowly and gave variable results when induced with sodium butyrate (P.D.Aplan, K.Nakahara and I.R.Kirsch, unpublished results).

The observation that F4-6, K562 and C19 all express endogenous *SCL* mRNA, but exhibit low rates of spontaneous erythroid differentiation, which can be enhanced by overproduction of wild-type *SCL*, suggests that the *SCL* gene product is part of a dynamic equilibrium between molecules promoting erythroid differentiation and molecules inhibiting or limiting erythroid differentiation. Although speculative, it is possible that undifferentiated F4-6, K562 or C19 cells are in an equilibrium state, in which factors that enhance erythroid differentiation are in balance with those that serve to inhibit erythroid differentiation. By perturbing this equilibrium, and overexpressing *SCL*, which is presumably a positive regulator of erythroid differentiation, a fraction of cells may initiate an erythroid differentiative program.

Previous studies with MEL cells have indicated that levels of c-myc protein constitute a crucial variable for initiation of the erythroid program. A biphasic decline in c-myc levels is observed when F4-6 MEL cells are induced to differentiate with DMSO (Lachman *et al.*, 1984; Ramsay *et al.*, 1984; Kirsch *et al.*, 1986). Constitutive expression of c-myc blocks inducer-mediated erythroid differentiation of MEL cells (Coppola and Cole, 1986; Dmitrovsky *et al.*, 1986; Prochownik and Kukowsha, 1986). Preliminary experiments with stable *SCL* antisense transfectants derived from F4-6 suggest that transfectants that are blocked in differentiation show the same decline in c-*myc* mRNA levels as the parent F4-6 cell line (data not shown). This finding suggests that the *SCL* gene acts 'downstream' of c-*myc* in the erythroid developmental pathway.

Thus, *SCL* expression promotes erythroid differentiation when expressed in MEL or K562 cell lines. However, when *SCL* is expressed at high levels in T-lymphocytes, it is associated with T-cell leukemia (Begley *et al.*, 1989b; Aplan, 1990b; Chen *et al.*, 1990). It is possible that while *SCL* expression in an erythroid cell promotes differentiation, the inappropriate expression of *SCL* in a T-cell milieu leads to interactions with factors that are not normally co-expressed with *SCL*, and that these interactions contribute to leukemogenesis.

In summary, we provide evidence that the SCL protein plays a pivotal role in erythroid differentiation. First, we have shown that the proximal *SCL* promoter is a target for GATA-binding proteins, including the essential erythroid transcription factor GATA-1. Second, forced expression of *SCL* promotes erythroid differentiation in MEL and K562 cells, whereas overexpression of antisense *SCL* or ΔB *SCL* cDNA is inhibitory. With these findings as background, it should be possible to proceed with a more refined analysis of the role of *SCL* in affecting the dynamic equilibrium of erythroid lineage development.

Materials and methods

Cell lines

The C19 cell line is a subclone of the 745 MEL line obtained from Dr Michael Kuehl (NCI/NMOB) and grown in RPMI 1640. The F4-6 cell line was supplied by Dr J.Billelo (University of Maryland) and grown in Joklik's modified MEM. The K562 cell line was obtained from frozen NCI/NMOB stocks and has been passaged continuously in RPMI 1640 for several years. All media were supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM glutamine.

Plasmid constructs

To examine the proximal *SCL* promoter (designated Ia in Aplan *et al.*, 1990b), sequences from −562 to +7 were amplified by PCR with primers containing synthetic *Bam*HI sites and subcloned into the growth hormone reporter plasmid pOGH (Selden *et al.*, 1986). Mutation of the GATA site in the proximal promoter was accomplished using a primer extending from +7 to −46 containing CTCT rather than TATC at the GATA motif core. The wild-type and mutant promoter constructs were designated SCL-WT and SCL-MUT, respectively (Figure 1B, lower panel). The *SCL* transfection vectors are shown in Figure 2. pCSCL-WT was created by subcloning a *Hin*dIII–*Xba*I cDNA fragment (nucleotides 109–1293 of Aplan *et al.*, 1990a; the *Hin*dIII and *Xba*I sites come from the plasmid polylinker) encoding a full length SCL protein, into the *Hin*dIII and *Xba*I sites of pRCCMV (Invitrogen). pCSCL-ΔB was derived using oligonucleotide mediated mutagenesis of pCSCL-WT to delete amino acids 188–199. The insert was sequenced completely to ensure that no mutations had been introduced; an SCL protein was immunoprecipitated from a ^{35}S metabolically labeled extract of a transfectant expressing *SCL* ΔB mRNA to ensure that the correct reading frame had been preserved. pCSCL-AS was created by inserting an *Apa*I–*Apa*I *SCL* cDNA fragment (nucleotides 109–449 of Aplan *et al.*, 1990a) in the antisense orientation into the *Apa*I site of pRCCMV. pMSCL-WT was derived from the pMTHfos plasmid (Holt *et al.*, 1986) by replacing the *Hin*dIII–*Bam*HI *fos* fragment with a *Hin*dIII–*Bam*HI *SCL* cDNA fragment (nucleotides 109–1293 of Aplan *et al.*, 1990a; the *Hin*dIII and *Bam*HI sites come from the plasmid polylinker) encoding a full length SCL protein. PMSCL-ΔB has an identical basic domain deleted insert as PCSCL-ΔB, inserted into the *Hin*dIII and *Bam*HI sites of the pMTHfos plasmid.

Transfections

The growth hormone reporter constructs SCL-WT and SCL-MUT were introduced into MEL cells for transient expression by electroporation, as previously described (Tsai *et al.*, 1991). Growth hormone secreted into the medium was assayed at 72 h after electroporation by radioimmunoassay (Selden *et al.*, 1986). Cotransfection transactivation experiments in NIH3T3 cells were performed as previously described (Martin and Orkin, 1990). Transfections included 1 μg reporter, 2.5 μg GATA-1 cDNA expression plasmid and 1 μg SV40-β galactosidase plasmid. Wild-type GATA-1 and

an inactive mutant ('mini', see Martin and Orkin, 1990) were expressed using the pXM vector (Tsai *et al.*, 1989). Growth hormone secreted into the medium was assayed at 72 h. β-Galactosidase activity of cell extracts was employed to assay the reproducibility of transfections into NIH3T3 cells. Stable transfectants were created by transfecting log phase C19, K562 or F4-6 cells with Lipofectin (BRL) using the manufacturer's recommended protocol. Briefly, on day zero, 2×10^7 cells were washed with serum-free Optimem (BRL), resuspended in 3 ml of Lipofectin–DNA complex (50 μg Lipofectin, 15 μg plasmid), and incubated for 6 h at 37°C/5% CO_2. Three milliliters of RPMI 1640 or Joklik's MEM (supplemented with 20% FCS, antibiotics and glutamine as above) was added and the cells were incubated overnight. On day one, serial dilutions of cells were plated in 24 well plates. On day two, G418 (Geneticin, BRL) selection was begun by the addition of G418 to a final concentration of 800 μg/ml. Wells containing single clones were selected on days 10–17 and transferred to 60 mm dishes. The stable transfectants were maintained in media supplemented with 400 μg/ml G418.

RNA analysis

RNA was isolated using an NP-40 lysis method (Sambrook *et al.*, 1989), Northern blot transfers and hybridizations to nick-translated probes were performed as described (Aplan *et al.*, 1990a). Probes used were a 1.0 kb *SstI–XbaI* human *SCL* genomic fragment (Begley *et al.*, 1989a), a 0.8 kb human *SCL* cDNA fragment (58RS, nucleotides −8 to +788 of Aplan *et al.*, 1990a), a 1.5 kb *ClaI–EcoRI* human c-*myc* fragment (Kirsch *et al.*, 1986) and a 3 kb *SstI–SstI* murine α-globin fragment (Kirsch *et al.*, 1986).

Immunoprecipitations

Immunoprecipitations were performed as previously described (Dosaka *et al.*, 1991). Briefly, 2×10^7 cells were metabolically labeled with 400 μCi of [^{35}S]methionine (specific activity >1000 Ci/mmol; Amersham) and lysed. 4×10^7 c.p.m. of [^{35}S]methionine labeled extract were precleared for 4 h with normal rabbit serum: the cleared extract was incubated overnight with normal rabbit serum or *SCL* specific antiserum (Aplan *et al.*, 1990b), precipitated with protein A–Sepharose CL-4B (Pharmacia), washed and size fractionated on a 10% stacking gel. The gel was soaked for 2 h in Autofluor (National Diagnostics), dried and exposed to X-ray film.

Gel shift

Recombinant murine GATA-1 and human GATA-2 proteins were expressed in monkey kidney COS cells transfected with pXM-mGATA-1 (Tsai *et al.*, 1989) and pMT2-hGATA-2 (Dorfman *et al.*, 1992) expression plasmids. Nuclear extracts of COS cells were prepared as described by Andrews and Faller (1991). Gel shift assays were performed as described by Martin and Orkin (1990) using 0.5 ml transfected COS cell extract and double stranded end-labeled oligonucleotide spanning the *SCL* GATA motif (AGCTTGGCAGTGCCTTATCTCTGCGGCGCG). Specificity was assessed by competition with a 100-fold excess of homologous or unrelated oligonucleotide.

Inductions

Log phase cells (stable transfectants and controls, either neo^R transfectants, the untransfected parent cell line or both) were plated at a density of 1×10^5/ml on day zero. Induction of erythroid differentiation was accomplished by treating the cells with either 1.5% DMSO (F4-6 clones), 3 mM HMBA (C19 clones) or 70 mM sodium butyrate (K562 clones). After 5 days, induced stable transfectants along with induced and uninduced controls were harvested and examined by benzidine staining (Friend *et al.*, 1971) and Northern blotting.

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References

Andrews,N.C. and Faller,D.V. (1991) *Nucleic Acids Res.*, **19**, 2499.
Aplan,P.D., Begley,C.G., Bertness,V., Nussmeier,M., Ezquerra,A., Coligan,J. and Kirsch,I.R. (1990a) *Mol. Cell. Biol.*, **10**, 6426–6435.
Aplan,P.D., Lombardi,D.P., Ginsberg,A.M., Cossman,J., Bertness,V.L. and Kirsch,I.R. (1990b) *Science*, **250**, 1426–1429.
Aplan,P.D., Lombardi,D.L., Reaman,G.H., Sather,H.N., Hammond,G.D. and Kirsch,I.R. (1992) *Blood*, **79**, 1327–1333.
Begley,C.G. *et al.* (1989a) *Proc. Natl. Acad. Sci. USA*, **86**, 2031–2035.
Begley,C.G., Aplan,P.D., Haynes,B., Waldmann,T.A. and Kirsch,I.R. (1989b) *Proc. Natl. Acad. Sci. USA*, **86**, 10128–10132.
Benezra,R., Davis,R.L., Lockshon,D., Turner,D.L. and Weintraub,H. (1990) *Cell*, **61**, 49–59.
Billelo,J.A., Kuhne,J., Warnecke,G., Koch,G. (1980) In Rossi,G.B. (ed.), *In Vivo and In Vitro Erythropoiesis: The Friend System.* Elsevier North Holland Press, New York, pp. 229–238.
Brown,L., Cheng,J.-T., Chen,Q., Siciliano,M.J., Crist,W., Buchanan,G. and Baer,R. (1990) *EMBO J.*, **9**, 3343–3351.
Chen,Q., Cheng,J.-T., Tsai,L.H., Shneider,N., Buchanan,G., Carroll,A., Crist,W., Ozanne,B., Siciliano,M.J. and Baer,R. (1990) *EMBO J.*, **9**, 415–424.
Coppola,J.A. and Cole,M.D. (1986) *Nature*, **320**, 760–763.
Davis,R.L., Cheng,P.-F., Lassar,A.B. and Weintraub,H. (1990) *Cell*, **60**, 733–746.
Dmitrovsky,E.W., Kuehl,W.M., Hollis,G., Kirsch,R., Bender,T.P. and Segal,S. (1986) *Nature*, **322**, 748–750.
Dorfman,D.M., Wilson,D.B., Bruns,G.A.P. and Orkin,S.H. (1992) *J. Biol. Chem.*, **267**, 1279–1285.
Dosaka-Akita,H., Rosenberg,R.K., Minna,J.D. and Birrer,M.J. (1991) *Oncogene*, **6**, 371–378.
Elefanty,A.G. and Cory,S. (1992) *Blood*, **79**, 1271–1281.
Finger,L.R., Kagan,G., Christopher,G., Kurtzberg,J., Hershfield,M.S., Nowell,P.C. and Croce,C.M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5039–5043.
Friend,C., Scher,W., Holland,J.G. and Sato,T. (1971) *Proc. Natl. Acad. Sci. USA*, **68**, 378–382.
Green,A.R., DeLuca,E. and Begley,C.G. (1991) *EMBO J.*, **10**, 4153–4158.
Holt.J.T., VenatGopal,T., Moulton,A.D. and Nienhuis,A.W. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4794–4798.
Izant,J.G. and Weintraub,H. (1985) *Science*, **229**, 345–352.
Kirsch,I.R., Bertness,V., Silver,J. and Hollis,G.F. (1986) *J. Cell. Biochem.*, **32**, 11–21.
Koeffler,H.P. (1986) *Semin. Hematol.*, **23**, 223–236.
Lachman,H.M., Cheng,G. and Skoultchi,A.I. (1984) *Nature*, **310**, 592–594.
Lozzio,C.B. and Lozzio,B.B. (1975) *Blood*, **45**, 321–328.
Martin,D.I.K. and Orkin,S.H. (1990) *Genes Dev.*, **4**, 1886–1898.
Melton,D.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 144–148.
Murre,C., Shonleber McCaw,P. and Baltimore,D. (1989) *Cell*, **56**, 777–783.
Orkin,S.H. (1990) *Cell*, **63**, 665–672.
Pevny,L., Simon,M.C., Robertson,E., Klein,W.H., Tsai,S.F., Dagati,V., Orkin,S.H. and Costantini,F. (1991) *Nature*, **349**, 257–260.
Prochownik,E.V. and Kukowsha,J. (1986) *Mol. Cell. Biol.*, **10**, 705–710.
Ramsay,R.G., Ikeda,K., Rifkind,R.A. and Marks,P.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6849–6853.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Selden,R.F., Burke-Howie,K., Rowe,M.E., Goodman,H.M. and Moore,D.D. (1986) *Mol. Cell. Biol.*, **6**, 3173–3179.
Tsai,S.-F., Martin,D.I.K., Zon,L.I., D'Andrea,A.D., Wong,G.G. and Orkin,S.H. (1989) *Nature*, **339**, 446–451.
Tsai,S.-F., Strauss,E. and Orkin,S.H. (1991) *Genes Dev.*, **5**, 919–931.
Visvader,J., Begley,C.G. and Adams,J.M. (1991) *Oncogene*, **6**, 195–204.
Weintraub,H. *et al.* (1991) *Science*, **251**, 761–766.
Yamamoto,M., Ko,L.J., Leonard,M.W., Beug,H., Orkin,S.H. and Engel,J.D. (1990) *Genes Dev.*, **4**, 1650–1662.
Zon,L.I., Yousoufian,H., Mather,C., Lodish,H.F. and Orkin,S.H. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10638–10641.

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