Specification of hematopoietic and vascular development by the bHLH transcription factor SCL without direct DNA binding

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SUMMARY

Transcription factors, such as those of the basic-helix-loophelix (bHLH) and homeodomain classes, are primary regulators of cell fate decisions and differentiation. It is considered axiomatic that they control their respective developmental programs via direct binding to cognate DNA sequences in critical targets genes. Here we test this widely held paradigm by in vivo functional assay of the leukemia oncoprotein SCL, a bHLH factor that resembles myogenic and neurogenic proteins and is essential for both hematopoietic and vascular development in vertebrates. Contrary to all expectation, we find that SCL variants unable to bind DNA rescue hematopoiesis from gene-targeted SCL⁻/⁻ embryonic stem cells and complement hematopoietic and vascular deficits in the zebrafish mutant cloche. Our findings establish DNA-binding-independent functions of SCL critical for transcriptional specification, and should encourage reassessment of presumed requirements for direct DNA binding by other transcription factors during initiation of developmental programs.

Key words: SCL/tal-1, bHLH transcription factor, Hematopoiesis, Vasculogenesis, Phenotypic rescue, Zebrafish mutant, cloche

INTRODUCTION

Blood cell production in vertebrates occurs in successive waves in development, first in the extraembryonic yolk sac, later within the fetal liver of the embryo, and finally in the bone marrow (Orkin, 1996). Primitive (or embryonic) erythrocytes are formed in the yolk sac blood islands. Cells of various lineages (erythrocytes, megakaryocytes, myeloid and lymphoid cells) are produced during definitive (or adult) hematopoiesis in fetal liver and bone marrow. Throughout embryogenesis, the hematopoietic and vascular systems, both mesodermal derivatives, develop in close proximity (Dieterlen-Lievre, 1998). Within blood islands bipotential hemangioblasts (or flk-1+VE-cadherin⁺ endothelial-like cells) are believed to give rise to those primitive progenitors that produce primitive erythrocytes (Nishikawa et al., 1998). Later, definitive hematopoietic progenitors arise in selected regions, lining the floor of the dorsal aorta as well as the umbilical and vitelline arteries (Jaffredo et al., 1998). The intimate relationship between hematopoietic and vascular development is supported by in vitro culture of hemangioblasts or hemogenic endothelial cells (Choi et al., 1998), combined hematopoietic and vascular defects in mice lacking the vascular endothelial growth factor receptor flk-1 (Shalaby et al., 1997), and absence of both hematopoietic and endothelial cells in the zebrafish mutant cloche (Stainier et al., 1995).

Among the nuclear regulators critical to the proper development of hematopoietic and vascular cells is the transcription factor SCL/tal-1/tcl-5 (hereafter referred to as SCL), a member of the basic-helix-loop-helix (bHLH) family (for review, Littlewood and Evan, 1998). Normally, expression of SCL is restricted to hematopoietic progenitors and erythroid, megakaryocytic and mast cell precursors, as well as endothelial cells and the central nervous system (Porcher et al., 1996; Elefanty et al., 1998). Deregulated expression of the SCL gene due to chromosomal translocations or interstitial deletions is associated with T-cell acute lymphoblastic leukemia (T-ALL) in humans (Bash et al., 1995). Ectopic T-cell-directed expression of SCL in transgenic mice leads to lymphomas following a long latency, demonstrating its oncogenic potential (Kelliler et al., 1996). Latency is dramatically shortened by coexpression of LIM-only polypeptides, such as LMO2 (Larson et al., 1996), which physically associate with SCL in larger protein complexes (see below).

Gene targeting experiments in mice established SCL as essential for development of all blood lineages and for angiogenic remodeling of the primary capillary plexus in the yolk sac (Porcher et al., 1996; Robb et al., 1996; Visvader et al., 1998). Forced expression of SCL cDNA in wild-type zebrafish embryos expands the domains of expression of hematopoietic and vascular markers, consistent with a role in specification of the hemangioblast or expansion of committed bipotential progenitors (Gering et al., 1998). Moreover, expression of SCL cDNA in cloche embryos rescues defects in hematopoietic and vascular lineages, suggesting that SCL lies downstream of the cloche gene in a developmental cascade (Liao et al., 1998). SCL, therefore, functions at a pivotal interface in the development of these lineages.
Tissue-restricted bHLH factors, such as SCL or the prototype of this family, MyoD (and other myogenic and neurogenic factors), are believed to form heterodimers with more widely expressed bHLH polypeptides, such as E12, and activate (or repress) target gene transcription following binding to E-box DNA (CANNTG) sequences (Littlewood and Evan, 1998). Evidence in support of this paradigm in the case of SCL derives from several sources. First, DNA site selection and reporter assays reveal E-box binding of SCL/E12 heterodimers and a weak transactivation domain in the aminoterminus portion of the protein (Hsu et al., 1994; Sanchez-Garcia and Rabbitts, 1994; Wadman et al., 1994a). Second, whereas expression of full-length SCL cDNA promotes cellular maturation, a variant lacking the basic domain acts as a dominant-negative mutant with respect to maturation of cultured erythroleukemia cells (Aplan et al., 1992). Furthermore, SCL assembles in vitro as a complex with LMO2 and its interacting protein Ldb-1, GA TA-1 and E12 on a composite E-box-GA TA site (Wadman et al., 1997). Accordingly, in erythroid cells, SCL protein has the potential to be secured within this complex by binding to the E-box DNA sequence.

Thus far, SCL function has been evaluated principally in surrogate assays performed in vitro or in established cell lines. Our goal here was to define properties of the protein essential for function in normal development. Rather than relying on reporter assays, we explored the extent to which modified forms of SCL rescue developmental defects of SCL−/− embryonic stem (ES) cells in culture and in chimeric mice, and induce markers of hematopoiesis and vasculogenesis in the zebrafish mutant cloche. Our data are remarkable in revealing that direct DNA binding by SCL is dispensable for primitive erythropoiesis in ES cells and initiation of the hematopoietic and vascular programs in cloche. Though not absolutely required for the emergence of definitive hematopoietic cells, direct DNA binding by SCL is important for production of normal numbers of erythroid progenitors and for proper maturation of erythroid and megakaryocytic precursors. Our demonstration that variants of SCL unable to bind DNA are highly active in vivo is unanticipated and challenges widely held notions of how this tissue-restricted bHLH factor acts in transcriptional control. These findings have implications for the mechanism of leukemogenesis by SCL and for the function of other bHLH proteins, and possibly other transcription factors, in development.

MATERIALS AND METHODS

Plasmid constructs

Numbering corresponds to amino acid residues of murine SCL protein. cDNAs were subcloned as Pfu-polymerase-generated PCR fragments between BgIII and EcoRI sites in a murine stem cell viral vector harboring a puromycin-resistance gene (MSCV-pac; (Hawley et al., 1994)). Unless otherwise specified, the template used for PCR reactions was wild-type SCL cDNA. The wild-type SCL cDNA construct was generated with primers 1 and 2 (GAA GATCTATG-

Retroviral infection of SCL−/− ES cells

Transient transfection of the packaging cell line BOSC23 and infection of SCL−/− ES cells (clone 1.2A see (Porcher et al., 1996) were performed as described previously. Populations of infected cells were expanded on gelatin-treated plates (Weiss et al., 1994) and used for further experiments.

Rabbit antisera

GST-fusion proteins containing the N-terminal 121 or the C-terminal 94 residues of SCL (Hsu et al., 1994) were expressed from pGEX-2TK plasmids and purified (Smith and Johnson, 1988). The proteins were used to produce two polyclonal SCL-specific rabbit antisera.
designed N-terminal and C-terminal antisera, respectively (Charles River Laboratories).

**Western Blot analysis, gel shift assays and coimmunoprecipitations**

Whole cell lysates were prepared from transiently transfected BOSC23 cells using a low-stringency lysis buffer (10 mM Hepes pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, protease inhibitor cocktail) (Hsu et al., 1994). For western blot analyses, 20 μg of cell lysates were submitted to electrophoresis on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Filters were incubated with affinity-purified SCL antisera (1:1000), followed by HRP-coupled secondary antibodies and developed by ECL (Amersham). For coimmunoprecipitation analyses, 40 μl of cell lysates were incubated for 2 hours on ice with 0.5 μl of SCL C-terminal antisera or 1 μl of E12 (H-208) polyclonal antibody (Santa Cruz Biotechnology). Complexes were precipitated with protein A Sepharose, washed four times with low-stringency buffer (see above) and resolved by 10% SDS-PAGE. Western blot analyses were performed as described above, with incubation of the filters with either the SCL C-terminal antisera or E12 (V-18) antibody at 0.1 μg/ml (Santa Cruz Technology). For gel shift assays, nuclear extracts were prepared from BOSC23 cells co-transfected with MSCV plasmids carrying a mutated SCL cDNA and an E12 cDNA, as described (Crossey et al., 1995). To prepare radiolabeled DNA, 100 ng of the oligonucleotide ACCTGAACAGA TGGTCGGCT containing SCL-preferred E-box motif (Hsu et al., 1994) was terminally labeled with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-32P]ATP, purified, mixed with 300 ng of the complementary oligonucleotide, heated at 90°C for 2 minutes and slowly cooled to room temperature to allow annealing. Gel shift assays were performed with 8 μg of nuclear extracts incubated at room temperature for 15 min in binding buffer (20 mM Hepes pH 7.6, 50 mM KCl, 1 mM DTT, 1mM EDTA, 5% glycerol) in presence of 1 mg/ml of poly(dI-dC) (Sigma) and 50 000 cts/minute of the probe. When indicated, extracts were preincubated for 10 minutes at room temperature with either a 50-fold excess of cold competitor or 1 μl of SCL N-terminal antiserum or E12 (H-208) antibody at 0.1 μg/ml (Santa Cruz Technology). For gel shift assays, nuclear extracts were prepared from BOSC23 cells co-transfected with MSCV plasmids carrying a mutated SCL cDNA and an E12 cDNA, as described (Crossey et al., 1995). To prepare radiolabeled DNA, 100 ng of the oligonucleotide ACCTGAACAGA TGGTCGGCT containing SCL-preferred E-box motif (Hsu et al., 1994) was terminally labeled with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-32P]ATP, purified, mixed with 300 ng of the complementary oligonucleotide, heated at 90°C for 2 minutes and slowly cooled to room temperature to allow annealing. Gel shift assays were performed with 8 μg of nuclear extracts incubated at room temperature for 15 min in binding buffer (20 mM Hepes pH 7.6, 50 mM KCl, 1 mM DTT, 1mM EDTA, 5% glycerol) in presence of 1 mg/ml of poly(dI-dC) (Sigma) and 50 000 cts/minute of the probe. When indicated, extracts were preincubated for 10 minutes at room temperature with either a 50-fold excess of cold competitor or 1 μl of SCL N-terminal antiserum or E12 (V-18) antibody at 0.1 μg/ml (Santa Cruz Technology). Electrophoresis were performed at 13 mA for 2 hours.

**In vitro ES cell hematopoietic differentiation**

In vitro hematopoietic differentiation was performed as described (Keller et al., 1993; Porcher et al., 1996). Potentially rescued ES cell populations were permitted to form EBs (one-step differentiation), then disaggregated and replated at day 6 or 10 in erythropoietin (Epo), or Epo and kit-ligand (KL), to give rise to primitive and definitive erythroid colonies, respectively (two-step differentiation). May-Grunwald-Giemsa staining was performed after cytocentrifugation of hematopoietic colonies, according to standard protocols.

**Semi-quantitative RT-PCR**

Expression of various hematopoietic markers was quantitated on RNA isolated from day 7 rescued EBs as described (Porcher et al., 1996) and from pure definitive erythroid colonies. In the latter case, RNAs were prepared using the RNeasy kit (Quiagen), cDNAs synthesized and PCR carried out as described (Porcher et al., 1996). Aliquots were removed after cycles 14, 16, 18, 20 for amplification of βmajor-globin sequences and 18, 20, 22, 24 for amplification of other sequences. Sequence of the oligonucleotides used in the PCR reactions have been reported elsewhere (Keller et al., 1993; Weiss et al., 1994).

**Chimera generation**

Rescued SCL−/− ES cell populations were injected into wild-type blastocysts, as described (Porcher et al., 1996). Chimerism of the embryos was assessed by Southern Blot analysis (Shivdasani et al., 1995) after extraction of DNA from the tail of 13.5 dpc embryos.

**Hematopoietic progenitor assays**

Fetal liver progenitor assays were performed as described (Wang et al., 1998). 1-2×10^5 cells were replated into semisolid medium (Methocult, Stem Cell Technology) supplemented with various growth factors (Porcher et al., 1996) either in the presence (1.5 mg/ml) or absence of G418 (Wang et al., 1998). After 4 to 8 days, colonies were counted and harvested for May-Grunwald-Giemsa staining.

**Zebrafish strains and maintenance**

Zebrafish were raised and maintained as described (Westerfield, 1995), and staged as described (Kimmel et al., 1995). Embryos raised to time points beyond 24 hours postfertilization (hpf) were transferred to E3 embryo medium with 0.003% phenylthiourea (PTU), to prevent melanization (Sigma, Inc.). The spontaneous cloche allele, clom^39 (Stainier et al., 1995), was obtained from Mark Fishman (Cambridge, MA). Ten heterozygote mating pairs were used to produce embryos for microinjection experiments, where the embryos are uniform among different mating pairs.

**RNA in situ hybridization and o-dianisidine staining.**

In situ hybridization and riboprobe synthesis were performed as described (Schulte-Merker et al., 1992), with modifications: proteinase K digestion was extended to 30 minutes in 20 μg/ml, and hybridization steps were carried out at 65°C. The ala/s-e (Brownlie et al., 1998), fjk-1 and tie-1 (Thompson et al., 1998) probes were prepared as previously described. o-dianisidine staining of hemoglobin was done as previously described (Detrich et al., 1995). In brief, unfixed embryos were dechorionated and stained for 15 minutes in the dark, with a solution consisting of o-dianisidine (0.6 mg/ml), 0.01 M sodium acetate (pH 4.5), 0.65% hydrogen peroxide and 40% (vol/vol) ethanol.

**cDNA expression constructs and microinjection**

BglII-EcoRI restriction fragments purified from the following MSCV constructs: SCL, Ani-SCL, 165-SCL-ACl, SCL-RER, SCL-Ab, SCL-FL and SES were subcloned into pCS2+ CMV expression vector (D. Turner, Fred Hutchinson Cancer Research Center, Seattle, WA) and R. Rupp (Friedrich-Miescher Laboratorium, Tubingen, Germany) at BamHI-EcoRI sites. Plasmid DNA was purified (Qiagen Inc.), quantified spectrophotometrically and diluted to 100 ng/μl in sterile ddH2O. Microinjection was performed as described (Westerfield, 1995), utilizing Nikon pico-injector and Narishige micromanipulator. Approximately 30-50 pg of DNA was injected per embryo. The injected embryos (and un.injected controls) were raised to 48 hpf, scored for the cloche phenotype and prepared for RNA in situ analysis.

**RESULTS**

Deletion, and other variant, forms of SCL were constructed (Fig. 1A). All were expressed as stable polypeptides, as evaluated by Western blot analysis of retroviral producer cells (Fig. 1B). We examined the extent to which these SCL cDNAs could restore hematopoietic development in gene targeted SCL−/− ES cells (Porcher et al., 1996) and the expression of hematopoietic and vascular markers in zebrafish cloche (Liao et al., 1998).

**The majority of SCL protein outside the bHLH region is dispensable for rescue of primitive erythropoiesis from SCL−/− ES cells**

Upon in vitro differentiation SCL−/− ES cells fail to generate any hematopoietic cells (Porcher et al., 1996; Robb et al., 1996). Reintroduction of wild-type SCL cDNA by retroviral
gene transfer restores hematopoiesis (Porcher et al., 1996). These observations form the basis of the ES cell rescue assay. As shown previously by semiquantitative RT-PCR, transgene expression approximates that of the endogenous gene and increases somewhat during in vitro differentiation of SCL-/- ES cells into embryoid bodies (EBs) and hematopoietic colonies (Porcher et al., 1996). In the present study, all mutants were expressed from the same retroviral vector as previously for wild-type cDNA. In initial tests of biological activity, ES cells transduced with each variant were allowed to form embryoid bodies (EBs) (Keller et al., 1993) and generation of primitive erythroid cells was assessed by inspection. Examples of EBs lacking erythroid cells and rescued for primitive erythropoiesis are shown in Fig. 1C.

Deletion of the N or C termini, or both, failed to prevent SCL’s ability to rescue primitive erythropoiesis in EBs generated from retrovirally transduced ES cells (Fig. 1A). Indeed, a construct containing the minimal bHLH region and 25 additional amino acid residues (165-SCL-ΔCt) was fully active, demonstrating that the majority of the SCL polypeptide is dispensable for function.

To assess functional specificity of the bHLH region, we assayed two variants in which SCL bHLH sequences were replaced by those of lyl-1 or E12. lyl-1 is highly homologous in its bHLH region to SCL (87% amino acid identity) and also associated with T-ALL (Mellentin et al., 1989). E12 is more divergent in its bHLH sequence (Murre et al., 1989). SCL containing the lyl-1 bHLH (construct SLS) was fully functional, whereas that with E12 sequences (construct SES) was inactive (Fig. 1A).

The minimal SCL bHLH construct (165-SCL-ΔCt) contains one of two serines (ser172) phosphorylated in response to erythropoietin (Epo) signalling (Wadman et al., 1994b; Prasad and Brandt, 1997). To test a requirement for phosphorylation at this site, alanine replacement variants, either in the context of the intact protein (SCL-S172A) or the minimal construct...
DNA-binding-independent activity of SCL

(165-S172A-ΔCt), were assayed (Fig. 1A). Both were active, providing evidence that phosphorylation of serine 172 is dispensable for primitive erythropoiesis.

Findings based on inspection of EBs were corroborated by quantitation of embryonic-specific βH1-globin transcripts by semiquantitative RT-PCR and by morphological examination of pure primitive erythroid colonies generated upon replating EB cells in Epo-containing methylcellulose. As shown in Fig. 2A, βH1 transcripts are present in SCL+/− EBs, absent in SCL−/− EBs, and restored to normal levels in SCL−/− EBs rescued with wild-type cDNA (panels 1-3, respectively). βH1 transcripts were expressed at normal, or above normal, levels in EBs generated from ES cells transduced with the 165-SCL-ΔCt, SLS and 165-S172A-ΔCt variants (panels 4, 5, 7), whereas transcripts were absent in EBs with the SES variant (panel 6). The quality of erythroid maturation was assessed by morphological examination of pure colonies obtained upon replating of EB cells (Fig. 2B). Full primitive maturation was observed with wild-type, 165-SCL-ΔCt and 165-S172A-ΔCt variants.

Thus, primitive erythroid development is similar to wild type, both qualitatively and quantitatively, in the absence of substantial portions of SCL protein outside the bHLH region. In addition, phosphorylation of serine 172 is not required for in vivo function. In addition to delimiting the principal domain of biological activity of SCL to the bHLH region, our findings establish functional specificity of this region. SCL and lyl-1 bHLH sequences are active, whereas the E12 bHLH is non-functional, even within the context of the otherwise intact SCL polypeptide.

**Primitive erythropoiesis from SCL−/− ES cells does not require SCL DNA-binding activity**

To identify properties of the bHLH region required for function, we tested three different mutants in the context of the full-length SCL polypeptide (Fig. 1A). Two basic region mutants were designed that ablate DNA binding by SCL heterodimers (or homodimers). In SCL-Ab, the entire basic region was deleted. In SCL-RER, three highly conserved residues (195-197, RER), which are required for DNA binding by MyoD and E12 proteins (Davis et al., 1990; Ma et al., 1994), were substituted by alanines. In a third construct (SCL-FL, Fig. 1A), dimerization of SCL with other bHLH polypeptides was prevented by alanine substitution of two hydrophobic residues (phenylalanine 207, leucine 210) in helix 1. These residues facilitate contact between bHLH partners at the dimerization interface (Shirakata et al., 1993; Ellenberger et al., 1994).

The consequences of these mutations for DNA binding and heterodimer formation were verified by gel-shift and coimmunoprecipitation experiments (Fig. 3). SCL cDNAs were cotransfected with E12 cDNA into BOSC23 cells. Nuclear extracts were prepared and subjected to gel-shift analysis using an SCL consensus E-box motif (CAGA TG)-containing probe (Hsu et al., 1994) (Fig. 3A). As expected, the complex corresponding to the SCL/E12 heterodimer (lane 7) failed to form with SCL-RER or SCL-Ab (lanes 12, 16). However, the mutant proteins assemble as heterodimers with E12, as shown by coimmunoprecipitation experiments using whole-cell lysates (Fig. 3B, lanes 8-11). In contrast, the dimerization mutant (SCL-FL) was unable to associate with E12 (Fig. 3B, lanes 12, 13), and cannot bind DNA (Fig. 3A, lane 20).

Given the prevailing view that tissue-specific bHLH factors require integrity of the bHLH region for in vivo function, rescue of primitive erythropoiesis by SCL-Ab and SCL-RER mutants was completely unanticipated (Fig. 1). These findings, derived from inspection of EBs, were corroborated by...
detection of βH1 globin transcripts (Fig. 2A, panels 8,9). Remarkably, SCL-RER was fully active in promoting primitive erythroblast development, as assessed either by measurement of βH1 transcripts (Fig. 2A; panel 9) or by morphological examination of pure primitive erythroid colonies (Fig. 2B). As expected, SCL-FL was inactive (Fig. 2A, panel 10). We conclude from these findings that DNA binding by SCL is not required for rescue of primitive erythropoiesis, and infer that heterodimer formation by SCL is necessary. Our data are the first to suggest that a tissue-specific bHLH transcription factor, such as SCL, may exert powerful effects on developmental decisions without binding directly to DNA.

**DNA binding by SCL is also dispensable to initiate primitive erythropoiesis and vasculogenesis in zebrafish mutant cloche**

The above findings were entirely unanticipated. Therefore, we proceeded to test SCL cDNA constructs (Fig. 1A) for rescue of expression of hematopoietic and vascular marker genes in zebrafish cloche. In prior experiments, we showed that expression of zebrafish SCL cDNA partially rescues cloche phenotypes, characterized by the absence of hematopoiesis and vasculogenesis (Liao et al., 1998). In this setting, rescue of combined hematopoietic and vascular defects reflects a cascade of events triggered by SCL lying downstream of cloche. In view of the conservation of mouse and zebrafish SCLs, we first asked if expression of mouse cDNA would induce hematopoietic and vascular markers in injected cloche embryos. Mouse cDNA was highly active in this assay (Table 1), and equivalent to zebrafish cDNA (not shown). Blood cells detected by o-dianisidine staining and alas-e (δ-aminolevulinate synthetase-erythroid) RNA in situ (Brownlie et al., 1998) were abundant in the dorsal aorta of the trunk and large vessels of the tail in 48 hours postfertilization (hpf) embryos (arrows Fig. 4A, B). For assessment of vascular rescue, we focused on flk-1 expression in small vessels of the head and tie-1 expression in the endocardium (Liao et al., 1997, 1998). Mouse SCL-injected embryos displayed flk-1 staining in the choroid plexus and projecting retinal arteries (arrow, Fig. 4C) and tie-1 expression in the cardiac chamber (arrow, Fig. 4D). Cloche lacks endocardium and has no endothelial tissue in the cardiac chamber that can be detected with tie-1 (Stainier et al., 1995; Liao et al., 1997, 1998). Cloche embryos rescued for tie-1 expression had severe cardiac edema characteristic of the cloche phenotype, suggesting that the endothelial cells are rescued but fail to form a competent endocardium. Embryos with tie-1 staining were scored correctly for the cloche phenotype, as shown by the lack of tie-1 staining in the reduced heads (compare tie-1 staining pattern in the head between wild type and cloche, Fig. 4D).

SCL mutants ΔNt-SCL, 165-SCL-ΔCt, SCL-RER and SCL-Δb (see Fig. 1) were able to partially rescue blood and vascular markers in cloche to the same degree and pattern as that observed for wild-type SCL (Table 1; Fig. 4A-D). SCL-FL and SES cDNAs were inactive with respect to rescue of hematopoietic and vascular markers.

We also examined the level of endogenous zebrafish SCL expression induced in response to wild-type and variant mouse SCL cDNAs (Table 1, Fig. 4E). In SCL- and SCL-Δb-injected wild-type embryos, not only was the domain of endogenous SCL expression expanded, comparable to that observed by Gering et al. (1998), but endogenous SCL expression was maintained inappropriately at high levels in embryos at 48 hpf in circulating blood (Fig. 4E, and data not shown), when SCL...
expression should normally be nearly undetectable (Gering et al., 1998; Liao et al., 1998). In SCL and SCL-Δb-injected cloche mutants, endogenous SCL transcripts were also detected at 24 hpf, and maintained to 48 hpf in the posterior tail (Fig. 4E and data not shown). Forced SES expression failed to elicit endogenous SCL expression. Though these results suggest that induction of endogenous SCL may contribute to the observed partial rescue of blood and vascular markers, it is clear, nonetheless, that the injected constructs function to overcome the cloche developmental block. Taken together, our data demonstrate that the DNA-binding activity and the putative activation domain of SCL are dispensable for the initiation (or specification) of primitive erythropoiesis and vasculogenesis in cloche embryos.

**DNA-binding-independent and -dependent roles of SCL in definitive hematopoiesis from SCL<sup>-/-</sup> ES cells**

The above experiments in ES cells and zebrafish address the earliest stages of hematopoiesis during embryogenesis, but do not provide insight into properties of the SCL protein required for definitive (or adult) hematopoiesis. We and others demonstrated that SCL function is essential for development of definitive progenitors, and inferred to serve important roles in maturing erythroid precursors (Aplan et al., 1992; Porcher et al., 1996; Robb et al., 1996). The potential of SCL cDNAs to support rescue of definitive hematopoiesis was assessed at the fetal liver stage (13.5 dpc) in mouse chimeras created by injecting transduced SCL<sup>-/-</sup> ES cells into wild-type blastocysts. Fetal liver cells were plated in methylcellulose medium with combinations of growth factors, either with or without G418. As cells harboring a targeted allele are resistant to G418, colonies grown in G418 are of ES origin. The percentage of fetal liver hematopoietic colonies resistant to G418 provides a measure of rescue potential for a given cDNA construct. Erythroid (CFU-E, BFU-E), myeloid (CFU-GM) and megakaryocyte (CFU-Mk)-derived colonies were enumerated. The overall extent of chimerism in the embryo was assessed by Southern blot analysis of tail DNA of each chimera.

Previously we showed that retroviral transfer of wild-type SCL cDNA fully restored definitive hematopoiesis in chimeras (Porcher et al., 1996). As shown in Table 2, the minimal construct (165-SCL-ΔCt) is equivalent to full-length cDNA for the rescue of CFU-E (G418-resistant colonies: 19±4.9% versus 18.6±9.4%, *P*<0.01), BFU-E (5.1±3.5% versus 10.5±3.8%, *P*<0.01), CFU-GM (14±6.6% versus 18±5.9%, *P*<0.01), and CFU-Mk (14±10% versus 28±5.3%, *P*<0.01). In contrast, SCL<sup>-/-</sup> ES cells transduced with SCL-RER cDNA exhibited poor contribution to erythroid progenitors; BFU-Es were undetectable, whereas CFU-Es were much less frequent than wild type (2.6±1.9% versus 18.6±9.4%, *P*<0.001). Contribution to myeloid progenitors was less affected (10±7±7.3% versus 18±5.3%, *P*<0.1); megakaryocyte colonies were also somewhat reduced in number, though not to a statistically significant level (10±6.4% versus 28±5.3%, *P*<0.1). Of 11 chimeras generated with ES cells transduced with the RER cDNA, only one showed significant contribution to progenitors (Table 2). Even in this exceptional chimera, G418-resistant BFU-Es were not detected. These data indicate that DNA binding by SCL is required to achieve complete restoration of erythroid progenitor numbers. The data also suggest, but do not conclusively demonstrate, that full rescue of CFU-GM and CFU-Mk numbers may depend on SCL DNA binding. Nonetheless, these findings show that SCL DNA binding is not absolutely essential for the generation of definitive hematopoietic progenitors.

In order to assess a possible requirement for DNA binding by SCL in maturation of precursor cells, we examined the morphology of fetal liver hematopoietic colonies of chimeras. The minimal construct (165-SCL-ΔCt) was as effective as wild-type cDNA in promoting full maturation of adult erythroid, myeloid or megakaryocytic cells (see Fig. 5). In contrast, differentiation was incomplete in precursors rescued with SCL-RER. A paucity of terminally mature, enucleated erythrocytes and an abundance of immature erythroblasts and dying cells (indicated by arrow, Fig. 5) were seen within these colonies. In SCL-RER-derived myeloid colonies, in addition to morphologically normal granulocytes, we observed numerous cells of unknown lineage, characterized by intensely eosinophilic cytoplasmic granules (indicated by arrow, Fig. 5). Furthermore, SCL-RER-derived megakaryocyte colonies contained a high proportion of immature, basophilic cells with few cytoplasmic granules and decreased nuclear lobulation (Fig. 5).

To validate these observations, we also examined definitive erythroid colonies obtained by two-step in vitro differentiation of ES cells. As with colonies cultured from fetal livers of chimeras, erythroid cells derived from ES cells rescued with SCL-RER were less mature and remained nucleated, whereas those expressing the 165-SCL-ΔCt variant were indistinguishable from wild type (Fig. 6A). RT-PCR analysis demonstrated expression of adult β-major globin, Epo-receptor (EpoR), EKLF and GATA-1 in erythroid cells of all genotypes (Fig. 6B). Thus, DNA binding by SCL is not essential for expression of these genes.

### Table 1. Rescue of hematopoietic and vascular markers in zebrafish cloche

<table>
<thead>
<tr>
<th>Construct</th>
<th>Wild type</th>
<th>Mutant</th>
<th>o-dianisidine</th>
<th>alas-e</th>
<th>flk-1</th>
<th>tie-1</th>
<th>Endogenous scl</th>
<th>Total rescued</th>
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<tr>
<td>SCL</td>
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<td>167</td>
<td>21/46 (46%)</td>
<td>10/22 (45%)</td>
<td>18/30 (60%)</td>
<td>19/41 (46%)</td>
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<td>395</td>
<td>118</td>
<td>21/44 (48%)</td>
<td>10/18 (56%)</td>
<td>21/33 (64%)</td>
<td>10/19 (53%)</td>
<td>NA</td>
<td>62/118 (53%)</td>
</tr>
<tr>
<td>165-SCL-ΔCt</td>
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<td>205</td>
<td>36/83 (43%)</td>
<td>19/45 (42%)</td>
<td>12/37 (32%)</td>
<td>12/40 (30%)</td>
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<td>79/205 (39%)</td>
</tr>
<tr>
<td>SCL-RER</td>
<td>513</td>
<td>171</td>
<td>27/58 (47%)</td>
<td>9/18 (50%)</td>
<td>30/65 (46%)</td>
<td>15/30 (50%)</td>
<td>NA</td>
<td>81/171 (46%)</td>
</tr>
<tr>
<td>SCL-Δb</td>
<td>455</td>
<td>138</td>
<td>11/31 (35%)</td>
<td>9/21 (43%)</td>
<td>16/34 (47%)</td>
<td>16/27 (59%)</td>
<td>18/25 (72%)</td>
<td>70/138 (51%)</td>
</tr>
<tr>
<td>SCL-FL</td>
<td>478</td>
<td>160</td>
<td>0/48 (0%)</td>
<td>1/44 (2%)</td>
<td>0/37 (0%)</td>
<td>0/31 (0%)</td>
<td>NA</td>
<td>1/160 (0.6%)</td>
</tr>
<tr>
<td>SES</td>
<td>345</td>
<td>134</td>
<td>0/33 (0%)</td>
<td>0/24 (0%)</td>
<td>0/24 (0%)</td>
<td>0/33 (0%)</td>
<td>0/12 (0%)</td>
<td>0/134 (0%)</td>
</tr>
<tr>
<td><strong>Construct Wild type Mutant</strong></td>
<td><strong>Wild type Mutant</strong></td>
<td><strong>Wild type Mutant</strong></td>
<td><strong>Wild type Mutant</strong></td>
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<td><strong>Wild type Mutant</strong></td>
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</tbody>
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Summary of microinjection rescue assays in cloche. Expression plasmids containing murine SCL, ΔNt-SCL, 165-SCL-ΔCt, SCL-RER, SCL-Δb, SCL-FL, and SES were microinjected into 2- to 4-cell-stage embryos from a cloche heterozygote pair-wise mating. The embryos were scored at 48 hpf and stained with o-dianisidine, or fixed for RNA in situ analysis with alas-e, flk-1 and tie-1. For experiments using SCL, SCL-Δb and SES, endogenous SCL expression was also analyzed. Actual numbers of embryos analyzed for each expression construct (rows) are tabulated, with percentage of rescue detected with each molecular marker (columns) indicated within parentheses.
Fig. 4. DNA-binding domain by SCL is not required for initiation of primitive erythropoiesis and vasculogenesis in cloche. Embryos are shown in lateral views and oriented with rostral (left) and caudal (right) in A,B and E; in ventral view with rostral (top) in C; and in lateral views with rostral (top) in D. Embryos are shown at 48 hpf in A-D, and at 24 hpf in E. Wild-type embryos microinjected with full-length murine SCL are shown on the top of A-D. cloche embryos microinjected with murine SCL, 165-SCL-ΔCt, SCL-RER, SCL-Δb, SCL-FL and SES are shown in that descending order in A-D. (A) o-dianisidine staining of SCL-injected wild-type embryo revealed abundant blood cells pooling in the cardinal sinus (arrowhead) and major vessels of the trunk (arrowhead with *). SCL, 165-SCL-ΔCt, SCL-RER and SCL-Δb rescued blood in cloche, where the blood cells pooled in the trunk (arrowhead) and tail (arrowhead with *). SCL-FL and SES failed to rescue blood cells in cloche, where the trunk was devoid of blood cells and the tail contained only 5-10 cells (arrowhead with *). The 5-10 cells observed in SCL-FL and SES-injected embryos were also seen in uninjected controls (data not shown). (B) Same results of blood rescue as observed in A, except blood cells were detected by alas-e riboprobe. Rescued blood cells are indicated (arrowheads). (C) flk-1 in situ detects cranial vasculature in wild-type embryos (arrowheads with *). Rescue of choroid plexus vessels abutting the optic disc were consistently observed with injections of SCL, 165-SCL-ΔCt, SCL-RER and SCL-Δb (arrowheads). SCL-FL and SES failed to rescue endothelial cells in cloche and the embryos lacked flk-1 staining. (D) tie-1 in situ delineates cranial vasculature (arrowhead with +) and endocardium (arrowhead with *) of wild-type embryos. Microinjections with SCL, 165-SCL-ΔCt, SCL-RER and SCL-Δb rescued endocardial cells and tie-1 expression in cloche mutants (arrowheads), but did not rescue tie-1 in the cranial vasculature. SCL-FL and SES failed to rescue tie-1 in cloche. (E) Endogenous SCL expression was induced by ectopic expression of mouse SCL and SCL-Δb. The domain of endogenous SCL expression was expanded in wild-type embryos (arrowheads) and rescued in cloche mutants (arrowheads with *). SES microinjection did not induce endogenous SCL expression.
DNA-binding-independent activity of SCL

Taken together, our data demonstrate that DNA binding by SCL is not obligatory for generation of definitive hematopoietic progenitors, but is important for full differentiation of specific lineages, most notably erythroid cells and megakaryocytes. In addition, quantitatively normal production of definitive progenitors, especially those of erythroid origin, depends on integrity of the basic domain. Thus, DNA-binding-independent and -dependent activities of SCL are critical for different aspects of definitive hematopoietic development.

DISCUSSION

DNA binding by SCL is dispensable for many of its in vivo functions in development

Study of tissue-restricted bHLH proteins, such as myogenic and neurogenic factors, has been particularly instructive in formulating contemporary models of transcriptional control in development. It is widely accepted these factors bind consensus E-box sequences as heterodimers with ubiquitous bHLH polypeptides, such as E12 and its relatives, and then activate (or repress) transcription (see Littlewood and Evan, 1998). SCL has been thought to act in an analogous fashion. Specifically, SCL forms heterodimers with E12, binds consensus E-box sequences and activates transcription in transient reporter systems (see Introduction). Moreover, forced expression of full-length SCL in some cells promotes maturation, whereas variants lacking a basic domain are inhibitory (Aplan et al., 1992). In this context, therefore, our findings demonstrating that mutant forms of SCL, which are unable to bind DNA due to mutations in the basic domain, remain active in rescue of development in two stringent in vivo systems are unprecedented.

The first phase of our structure-function analysis revealed that the majority of SCL protein lying outside the bHLH domain is not required for rescue of differentiation of primitive erythropoiesis or definitive hematopoiesis. Previously defined activation domains and sites of serine phosphorylation (Sanchez-Garcia and Rabbitts, 1994; Wadman et al., 1994a; Prasad and Brandt, 1997) are not

Fig. 5. Morphology of hematopoietic cells derived from chimeric mouse embryos. Erythroid (CFU-E), myeloid and megakaryocytic colonies derived from wild-type host blastocysts or SCL−/− ES cells rescued with various constructs were harvested, cytocentrifuged and cells stained with May-Grunwald-Giemsa. Note the morphological abnormalities of cells rescued with SCL-RER. Arrows identify dark and dying erythroid cells, and a myeloid cell with abnormal dark granules. MK, megakaryocyte.

Fig. 6. Analysis of definitive erythroid colonies obtained by two-step in vitro differentiation assay of rescued SCL−/− ES cells. (A) Morphology. Erythroid colonies grown in Epo and KL were harvested, cytocentrifuged and cells stained with May-Grunwald-Giemsa. (B) RNA expression. RT-PCR were performed with RNAs isolated from rescued definitive erythroid colonies. Open triangles represent increasing PCR cycles.
SCL-RER is competent to rescue primitive erythropoiesis of ES cells rescued with a minimal SCL construct and a DNA-binding mutant.

Although myogenin can transiently activate reporter gene differentiation in transfected cells (Davis et al., 1990).

The extent to which in vivo functions of other tissue-restricted bHLH factors are DNA-binding independent is unknown.

To assess to what extent SCL DNA binding is dispensable for obligatory for function. Though these findings underscore potential differences between in vivo and surrogate assays of function, they do not preclude some role for these regions during hematopoiesis or in other developmental contexts.

The in vivo relevance of DNA-binding-independent activities of SCL is established by study of the SCL-RER variant. This mutant harbors substitutions at critical residues within the basic domain, which prevent DNA recognition either as homodimers or heterodimers (Fig. 3). MyoD carrying corresponding substitutions is unable to direct muscle differentiation in transfected cells (Davis et al., 1990). Although myogenin can transiently activate reporter gene expression when tethered to DNA via interaction with MEF, it is unable to induce the myogenic program without DNA binding (Molkentin et al., 1995). Here we have shown that SCL-RER is competent to rescue primitive erythropoiesis of SCL−/− ES cells to apparently wild-type levels, partially restore definitive hematopoiesis of SCL−/− ES cells, and initiate hematopoietic and vascular development in the zebrafish cloche mutant. Induction of endogenous zebrafish SCL expression by injected mouse SCL cDNA makes it difficult to assess to what extent SCL DNA binding is dispensable for vasculogenesis per se. Rescue of defects in cloche at least reflects the initiation of the hematopoietic/vascular program in the absence of DNA binding.

The potential differences between in vivo and surrogate assays of function underscore the principle, however, it is easy to account for the dispensability of an activation domain if SCL normally functions within protein complexes containing potent transcriptional activators.

The realization that regulatory elements containing E-box sequences, either alone or in conjunction with other sites (e.g. GA TA-sites) (Cohen-Kaminsky et al., 1998; Krosl et al., 1998).
DNA-binding-independent activity of the glucocorticoid receptor have been described (Reichardt et al., 1998), though these do not include specification of cell fate. In addition, though a DNA-binding mutant of the homeodomain protein fushi tarazu has been shown to influence the development in *Drosophila* embryos, it is unable to rescue the ftz-null phenotype (Fitzpatrick et al., 1992).

**What mechanisms account for DNA-binding-independent activity of SCL?**

Two alternative models can be put forward to explain how SCL protein unable to bind DNA exhibits transcriptional effects in development. In the first model, we envision that SCL’s primary function in hemangioblasts or very early hematopoietic and vascular progenitors might be to sequester a repressor of development. Once a hypothetical repressor is sequestered in heterodimers with SCL, development of these lineages would ensue. In this context, SCL’s action resembles that proposed for Id proteins, naturally occurring basic-domain deletion polypeptides (Benezra et al., 1990). According to a second model, SCL might be tethered into larger transcriptional complexes via protein-protein interactions. The tight association of SCL with LMO2, mediated by the bHLH domain of SCL and the LIM domains of LMO2, provides one route by which tethering might occur (Valge-Archer et al., 1994; Wadman et al., 1994b). Whether this interaction alone would be sufficiently avid to obviate a requirement for DNA binding by SCL is uncertain. The identification of a composite GATA-E-box DNA element on which a pentameric complex consisting of SCL, E12, LMO2, Ldb1 and GATA-1 assembles in vitro suggests that DNA binding by SCL should be an important parameter (Wadman et al., 1997). Indeed, mutation of the E-box sequence prevents in vitro complex assembly (unpublished data). Whether it would do so in vivo is unknown. Interestingly, however, it has recently been suggested that SCL can act as a cofactor for a complex of GATA-3 and LMO2 bound to a GATA-site in T-ALL cells for activating expression of the RALDH2 gene (Ono et al., 1998). Further studies will be required to test these models for SCL function in early development.

**DNA-binding-dependent activities of SCL in definitive hematopoietic development**

Though definitive hematopoietic progenitors were produced from *SCL<sup>-/-</sup>* ES cells expressing SCL-RER, their numbers were reduced, particularly for the erythroid lineage, and maturation of erythroid and megakaryocytic precursors was impaired. In addition, myelopoiesis appeared to be perturbed (Fig. 5), as was mast cell development (not shown). Thus, in definitive hematopoiesis DNA-binding-independent and -dependent activities for SCL exist. Erythroid precursors, and particularly BFU-E, appear most sensitive to loss of SCL’s DNA-binding capacity, a finding consistent with the upregulation of SCL expression seen during normal erythropoiesis and prior studies suggesting a role for SCL in erythroid maturation (Aplan et al., 1992). Expression of the erythroid markers that we tested, EKLF, GATA-1, EpoR or β-globin, was not demonstrably impaired in SCL-RER erythroid cells (Fig. 6B). Thus, target genes regulated by SCL in this context are obscure. This is a particularly curious finding given the presence of GATA-E-box-GATA and GATA-E-box elements in upstream enhancers of the *EKLF* and *GATA-1* genes, respectively (Anderson et al., 1998; Vyas et al., 1999).

The greater reliance of definitive erythroid and megakaryocytic development on DNA binding by SCL highlights important differences in the transcriptional programs of primitive and definitive hematopoiesis. Although several nuclear factors, such as GATA-1, FOG-1, LMO2 and GATA-2, are required for both primitive and definitive hematopoiesis, others, such as c-myb and the subunits of core-binding factor (CBF), show a differential requirement (see Shvidrasani and Orkin, 1996; Tsang et al., 1998). In these latter instances, function is essential only in definitive cells. Presumably, critical targets for these factors do not exist in primitive hematopoietic progenitors, or redundancy with other proteins present in these cells obscures any in vivo requirement. The changing requirement for DNA-binding activity during hematopoietic development points to a variety of routes by which SCL participates in transcription.

**Implications for leukemogenesis by SCL**

Deregulated expression of SCL leads to T-ALL in humans or lymphomas in mice (see Introduction). While a weak transactivation domain of SCL is not required for oncogenesis in mice (Aplan et al., 1997), it is presently unknown whether DNA binding is required. The potent DNA-binding-independent activity of SCL for initiating hematopoietic and vascular development raises the possibility that DNA binding might be dispensable for leukemogenesis. Prior evidence has been interpreted to suggest that leukemogenesis by SCL might result from sequestration of critical E-box-binding proteins, such as E12, in thymocytes (Kellihier et al., 1996; Larson et al., 1996). Indeed, loss of E12 function in gene-targeted mice is associated with the appearance of lymphomas (Bain et al., 1997). Study of the oncogenic potential of SCL DNA-binding mutants should discriminate the contributions of DNA-binding-dependent and -independent pathways to leukemia, and may provide important clues for strategies to identify relevant target genes.

**Rescue of parallel genetic systems for in vivo functional dissection of development**

The conservation of fundamental cellular programs permits parallel study of vertebrate species. For example, prior observations in avians pointed to intraembryonic origins of hematopoietic stem cells and common hematopoietic and vascular progenitors, concepts validated more recently in the mouse (Dieterlen-Lievre, 1998). Likewise, zebrafish cloche provides support for a bipotential progenitor of the hematopoietic and vascular lineages (Stainier et al., 1995). In our studies, we have used rescue of developmental defects manifest in *SCL<sup>-/-</sup>* ES cells and *cloche* to test the function of SCL variants.

This approach has several merits. First, rescue by DNA-binding mutants of SCL in both systems provides independent confirmation of our data and conclusions. Given that we did not anticipate that these mutants would be functional in vivo, similar findings in these different genetic systems strengthen confidence in their validity. Second, while the rescue of targeted ES cells has direct relevance to SCL function in mammals, the experiments are by necessity time-consuming and labor-intensive as they involve isolation of transduced ES
cells, in vitro ES cell differentiation and chimera analysis. In contrast, rescue of a zebrafish mutant phenotype provides a more rapid assay, which might be employed in the future to survey cDNA constructs prior to more definitive examination in ES cell rescue. Third, the two rescue systems illuminate different aspects of early development. In the rescue of SCL−/− ES cells, our findings relate specifically to the role of SCL in primitive and definitive hematopoiesis, but do not address potential roles in vascular development. The rescue of cloche phenotypes, on the other hand, addresses SCL’s role in initiating hematopoietic and vascular programs downstream of the cloche gene, a step presumably involving either induction or expansion of hemangioblasts in the early embryo. Given the increasing availability of targeted ES cells and the potential to screen for novel zebrafish mutants affecting virtually any developmental pathway, parallel use of these organisms for genetic rescue experiments constitutes a complementary and synergistic approach to dissecting vertebrate development.

Concluding comments

Although we have focused on a specific member of the bHLH family, there is no reason a priori to suspect that our observations are unique to SCL or the bHLH class of factors. As reported here, SCL unable to bind DNA rescues embryonic hematopoiesis in SCL−/− ES cells, substantially restores definitive hematopoiesis, and initiates the cascade that corrects deficits in zebrafish cloche. The mechanisms by which other transcription factors involved in developmental specification act in vivo need to be reevaluated in light of the findings presented here. It is commonly presumed that direct DNA binding is obligatory, yet experimental tests of this requirement in development have rarely been performed.

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REFERENCES


