Establishing the transcriptional programme for blood: the SCL stem cell enhancer is regulated by a multiprotein complex containing Ets and GATA factors

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Stem cells are a central feature of metazoan biology. Haematopoietic stem cells (HSCs) represent the best-characterized example of this phenomenon, but the molecular mechanisms responsible for their formation remain obscure. The stem cell leukaemia (SCL) gene encodes a basic helix–loop–helix (bHLH) transcription factor with an essential role in specifying HSCs. Here we have addressed the transcriptional hierarchy responsible for HSC formation by characterizing an SCL 3' enhancer that targets expression to HSCs and endothelium and their bipotential precursors, the haemangioblast. We have identified three critical motifs, which are essential for enhancer function and bind GATA-2, Fli-1 and Elf-1 *in vivo*. Our results suggest that these transcription factors are key components of an enhanceosome responsible for activating SCL transcription and establishing the transcriptional programme required for HSC formation.
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Introduction

One of the fundamental issues facing current biology concerns the molecular mechanisms whereby multipotent stem cells give rise to distinct differentiated progeny. This issue is central to the development of multicellular organisms from a single fertilized egg and also to the ability of adult stem cells to support tissue regeneration. Interest has been heightened by results from several laboratories, which suggest that adult stem cells have a much broader developmental potential than previously recognized, and that differentiated cells can regain aspects of stem cell function (for reviews see Fuchs and Segre, 2000; Blau *et al.*, 2001; Lovell-Badge, 2001; Morrison, 2001). Haematopoiesis is the best-characterized stem cell system, with functional assays and molecular markers for multiple stages of differentiation from haematopoietic stem cells (HSCs) through a plethora of progenitors to multiple mature cell types (Metcalfe, 1998; Orkin, 2000; Weissman, 2000). However, the molecular mechanisms

responsible for establishing the transcriptional programme for blood remain obscure.

In the mouse, haematopoiesis is first detected both in the yolk sac blood islands and also within the body of the post-implantation embryo, before subsequent sequential transfers to the fetal liver, thymus, spleen and bone marrow (Dzierzak and Medvinsky, 1995; Cumano and Godin, 2001; Palis and Yoder, 2001). The development of blood and endothelium is intimately linked throughout vertebrate development. The close association between the development of blood and endothelial cells in the avian and murine yolk sac gave rise to the concept of the haemangioblast, a bipotent precursor of both cell types (Sabin, 1920; Wagner, 1980). This idea has been reinforced by the observation that murine embryonic stem (ES) cells can give rise to Flk-1⁺ cells capable of generating both blood and endothelial progeny (Choi *et al.*, 1998; Nishikawa *et al.*, 1998a). Moreover, single Flk-1-positive cells from avian embryos can develop into either haematopoietic or endothelial colonies (Eichmann *et al.*, 1997), and numbers of both endothelial and haematopoietic cells are severely reduced in the zebrafish mutant *cloche* (Stainier *et al.*, 1995; Liao *et al.*, 1997). In the body of the amphibian, avian or murine embryo, blood cells arise as clusters of cells attached to the endothelium of arteries (Dieterlen-Lievre, 1975; Garcia-Porrero *et al.*, 1995; Ciau-Uitz *et al.*, 2000; de Bruijn *et al.*, 2000), and it has been suggested that differentiated endothelial cells may directly generate blood progenitors (Jaffredo *et al.*, 1998, 2000; Nishikawa *et al.*, 1998b). Interestingly, Runx1^{−/−} embryos exhibit normal primitive erythropoiesis but fail to develop both intra-arterial clusters and definitive haematopoiesis (Okuda *et al.*, 1996; Wang *et al.*, 1996; North *et al.*, 1999; Mukoyama *et al.*, 2000). Runx1 may therefore regulate production of blood progenitors from haemogenic endothelium or formation of the latter from a mesodermal precursor. In contrast, two other transcription factors, encoded by the stem cell leukaemia (*SCL*) and *LMO-2* genes, are essential for the development of both primitive erythropoiesis and definitive haematopoiesis (Warren *et al.*, 1994; Porcher *et al.*, 1996; Robb *et al.*, 1996).

The *SCL* gene (also known as *TAL-1*) encodes a basic helix–loop–helix (bHLH) protein and is normally expressed in blood progenitors, in endothelium and within specific regions of the central nervous system, a pattern of expression that is highly conserved across vertebrate species from mammals to teleost fish (reviewed in Begley and Green, 1999). Within the blood and endothelial system, *SCL* is expressed in haemangioblasts, HSCs, a subset of haematopoietic lineages and, at lower levels, in angioblasts and at least some mature endothelial cells (Green *et al.*, 1992; Mouthon *et al.*, 1993; Kallianpur *et al.*, 1994; Drake *et al.*, 1997; Gering *et al.*, 1998; Liao *et al.*,

Fig. 1. Identification of candidate core enhancer regions. (A) Restriction endonuclease accessibility assays identify three regions of accessible chromatin [+17, +18 and +19; numbering corresponds to distances in kb from the start of exon 1a in two haematopoietic progenitor cell lines (EML-C1, 416B)]. (B) Diagram of the murine *SCL* locus indicating the three regions of open chromatin above a homology profile of a human/mouse sequence alignment. All sites for *Ava*II, *Hae*III and *Hinf*I are indicated. The summary shows the three regions accessible to endonucleases (black boxes labelled +17, +18 and +19) which correspond to peaks within the homology profile.

1998; Mead *et al.*, 1998; Sinclair *et al.*, 1999; Akashi *et al.*, 2000; Ciau-Uitz *et al.*, 2000; Robertson *et al.*, 2000). Targeted mutation of the *SCL* gene has shown that it is essential for the development of all haematopoietic lineages (Porcher *et al.*, 1996; Robb *et al.*, 1996). Although *SCL* is expressed in haemangioblasts found in frog and zebrafish systems (Gering *et al.*, 1998; Ciau-Uitz *et al.*, 2000) or generated during murine ES cell differentiation (Robertson *et al.*, 2000), *SCL*^{-/-} mouse embryos and ES cells both generate endothelial cells (Visvader *et al.*, 1998; Robertson *et al.*, 2000), suggesting that *SCL* is required for lineage commitment to blood cell formation. Consistent with this concept, ectopic expression of *SCL* during zebrafish development results in excessive formation of haemangioblasts and blood cells (Gering *et al.*, 1998). The defective remodelling of primary vascular networks observed in *SCL*^{-/-} embryos may reflect a distinct later function of *SCL* (Visvader *et al.*, 1998) or may represent a consequence of the absence of haematopoietic progenitors (Takakura *et al.*, 2000).

Current evidence therefore demonstrates that *SCL* plays a pivotal role in the normal development of both blood and endothelium. This focuses attention on the mechanisms whereby transcription of *SCL* itself is initiated and maintained, and our laboratory has undertaken a systematic analysis of the transcriptional regulation of the murine *SCL* locus. Both human and murine *SCL* are transcribed from two lineage-specific promoters (Aplan *et al.*, 1990; Begley *et al.*, 1994; Leconte *et al.*, 1994; Bockamp *et al.*, 1995, 1997, 1998). A survey of the chromatin structure surrounding the murine *SCL* gene has revealed a panel of DNase I-hypersensitive sites associated with enhancer or silencer activity in transfection assays (Göttgens *et al.*, 1997). Transgenic reporter assays subsequently identified five independent enhancers, each of which targets expression to a specific subdomain of the normal *SCL* expression pattern (Sanchez *et al.*, 1999; Sinclair *et al.*, 1999; Göttgens *et al.*, 2000).

A 3' enhancer contained within a 5.5 kb fragment displayed particularly striking properties. It was active in the region of E7.5 extraembryonic mesoderm that gives rise to the yolk sac, and subsequently directed reporter gene expression to endothelial and blood cells within yolk sac blood islands of E8 embryos (Sanchez *et al.*, 1999). Within the embryo proper, the enhancer was active in endothelial cells and also in haematopoietic progenitors at multiple sites and times, including E8 para-aortic splanchnopleura, E11 AGM region and E11 fetal liver (Sanchez *et al.*, 1999). The 3' enhancer targeted expression to the vast majority of long-term repopulating HSCs from adult bone marrow and fetal liver (Sanchez *et al.*, 2001). Moreover, expression of *SCL* under control of this stem cell enhancer in transgenic mice selectively rescued the formation of early haematopoietic progenitors in *SCL*^{-/-} embryos (Sanchez *et al.*, 2001). These data suggest that the *SCL* 3' enhancer functions as a nodal point for the integration of signals responsible for establishing the transcriptional programme for blood cell development.

Here we define a 641 bp conserved core enhancer responsible for targeting expression to blood progenitors and endothelium in transgenic mice, and to dorsal lateral plate mesoderm in transgenic frogs. Three critical transcription factor-binding sites are identified, each of which is essential for enhancer function *in vitro* and *in vivo*. We demonstrate that GATA-2, Fli-1 and Elf-1 bind to the critical motifs *in vitro* and within intact haematopoietic progenitor cells. Finally, we show that the three transcription factors are capable of forming a complex, that the three corresponding motifs all lie on one face of the DNA helix and that this arrangement is essential for enhancer function. These data strongly suggest that GATA-2, Fli-1 and Elf-1 are key components of an enhanceosome responsible for directing expression to haemangioblasts together with their haematopoietic and endothelial progeny.

Fig. 2. The +19 region is both necessary and sufficient for SCL stem cell enhancer activity in transgenic mouse embryos. Top: transgenic reporter constructs in relation to the mouse *SCL* locus. (A–H) Representative E11.5 transgenic embryos for the constructs indicated in the top part of the figure. (I) Sections of fetal liver (fl) and dorsal aorta (da) of the embryos shown in (A), (B), (E) and (G).

Results

Chromatin structure of the *SCL* stem cell enhancer

Our previous analysis of the regulation of the murine *SCL* gene identified a 5.5 kb fragment 3' to the *SCL*-coding region in which we mapped the approximate positions of two regions of DNase I hypersensitivity (Göttgens *et al.*, 1997). Transgenic analysis showed that this fragment directed expression of a *lacZ* reporter gene to endothelium and blood progenitors throughout ontogeny (Sanchez *et al.*, 1999), as well as to the vast majority of long-term repopulating HSCs from fetal liver and adult bone marrow (Sanchez *et al.*, 2001). In order to refine the position of the enhancer, we mapped regions of open chromatin in two *SCL*-expressing multipotent haematopoietic cell lines, EML-C1 and 416B (Figure 1), using a restriction endonuclease accessibility assay with a resolution of ~100 bp (Göttgens *et al.*, 2001). EML-C1 cells contained

three regions of accessible chromatin (+17, +18 and +19; numbers refer to the distance in kb from the *SCL* promoter), two of which (+18 and +19) were also identified in 416B cells.

We have previously reported the complete sequence of the mouse and human *SCL* loci (Göttgens *et al.*, 2000), thus allowing the precise position of accessible restriction endonuclease sites to be placed on the mouse sequence and compared with a human/mouse homology profile. This analysis revealed two major peaks of high sequence homology corresponding to the +18 and +19 regions of open chromatin, as well as several minor peaks, one of which corresponded to the +17 region. These results therefore identified the +17, +18 and +19 regions as prime candidates for the location of the *SCL* stem cell enhancer.

The +19 region is both necessary and sufficient for *SCL* stem cell enhancer activity in transgenic mouse embryos

The position of the stem cell enhancer was refined by comparing expression of *lacZ* reporter constructs in F_0 transgenic embryos. Embryos were analysed at E11.5, which allowed enhancer activity to be assessed in yolk sac, fetal liver and the AGM region at a single time point. We have demonstrated previously that the 5.5 kb *SCL* 3' fragment within the SV/lac/Bg5.5 construct confers stem cell enhancer activity on otherwise inert minimal promoters in F_0 transgenic assays (Sanchez *et al.*, 1999). We now tested a 2.5 kb fragment containing the +17, +18 and +19 sites, which gave rise to X-gal staining in yolk sac, fetal liver and endothelium in a pattern that was the same as that produced by the original 5.5 kb enhancer fragment (Figure 2, compare A and B; Table I). Histological sections revealed that both constructs targeted haematopoietic and endothelial cells in fetal liver and dorsal aorta, including clusters of round cells attached to the ventral floor of the aorta which are thought to represent haematopoietic stem cells (Peault, 1996) (Figure 2I).

The activity of the 2.5 kb fragment was not altered by deletion of the +17 or +18 regions (Figure 2, compare B with C and D; Table I), but deletion of the +19 region resulted in complete loss of specific X-gal staining (Figure 2, compare B with E; Table I). Constructs containing both the +18 and +19 regions or the +19 region alone displayed the same activity as the 2.5 kb fragment in both whole-mount analysis (Figure 2, compare B with F and G; Table I) and histological sections (Figure 2I). Taken together, these results demonstrate that a 641 bp region containing the +19 site was both necessary and sufficient for activity of the stem cell enhancer.

Sequences containing conserved Myb, Ets and GATA sites are essential for enhancer activity in transgenic mice

The activity of the 641 bp core enhancer was abolished by a 3' deletion of 227 bp (Figure 2H; Table I). Human/mouse sequence comparisons revealed several blocks of homology within this critical 3' region, one containing conserved Myb and Ets family-binding sites, a second containing a conserved Ets site and a third containing a conserved GATA site (Figure 3A, regions 1–3, respectively). Mutations were generated in each region and their effect

Fig. 3. Identification of three critical regions in the +19 enhancer. (A) Human/mouse sequence alignment of the +19 enhancer. Highlighted regions 1–3 correspond to the three homology blocks that were mutated (mut1, mut2 and mut3 mutant sequences shown under each homology block). The arrowhead indicates the position of the deletion removing all three homology blocks (SA0.4 fragment, see Figure 2). (B) Representative E11.5 transgenic embryos for the constructs indicated below each panel. SV/lac/SH0.6 contains the wild-type 641 bp core enhancer; SV/lac/SH0.6 mut1, mut2 and mut3 contain core enhancers with mutation of regions 1, 2 and 3, respectively. The arrow in the right hand panel indicates the endothelial staining in the vitelline vessel.

Table I. Activity of SCL stem cell enhancer constructs in transgenic mouse embryos

Construct	Transgenic F ₀ embryos	LacZ expression			
		Fetal liver	Endothelium	Ectopic only	None
SV/lac/H2.5	6	5	5	0	1
SV/lac/H2.5Δ17	3	3	3	0	0
SV/lac/H2.5Δ18	6	5	5	1	0
SV/lac/H2.5Δ19	9	0	0	3	6
SV/lac/NH1.1	2	2	2	0	0
SV/lac/SH0.6	7	5	5	0	2
SV/lac/SA0.4	8	0	0	0	8
SV/lac/SH0.6mut1	8	0	0	1	7
SV/lac/SH0.6mut2	11	0	0	8	3
SV/lac/SH0.6mut3	9	0	5 ^a	3	1

LacZ reporter constructs described in Figures 2 and 3 were used to generate transgenic embryos. F₀ embryos were collected at E11.5 and stained overnight with X-gal for lacZ activity.

^aEndothelial staining for SV/lac/SH0.6mut3 embryos was generally weak and mainly confined to the vitelline vessels (see Figure 3B).

on enhancer activity was assessed at E11.5 in F₀ transgenic embryos.

A mutation in region 1 that altered both the Myb and Ets consensus binding sites completely abolished enhancer activity, as did a mutation altering the Ets site of region 2 (Figure 3B; Table I). A mutation of region 3 that altered the conserved GATA site also abolished X-gal staining in

fetal liver and yolk sac, and markedly reduced endothelial staining. Residual weak X-gal staining was evident in the endothelium of the vitelline and some intraembryonic vessels but was absent from yolk sac blood island endothelium (Figure 3B; Table I). Only a minority of dorsal aorta endothelial cells were positive, and we did not observe any staining in clusters of cells attached to the

Fig. 4. The +19 core enhancer is active in transgenic frogs and murine haematopoietic progenitor cells and critically depends on regions 1, 2 and 3. (A) An example of the variable ectopic expression patterns seen in a minority of transgenic stage 32 embryos with the SV/GFP construct (ectopic expression was never seen in the DLP region). (B) SV/GFP/H2.5 transgenic embryo showing reporter gene expression in the DLP region (arrow). (C–E) Stage 27 SV/GFP/H2.5 transgenic embryo stained for GFP (BCIP, turquoise) and endogenous SCL (BM Purple). d/e indicates the plane of the section shown in (D) and (E) which demonstrate that the purple staining (endogenous SCL) is contained within the area of transgene expression (turquoise) in the DLP (arrow). (F) Summary of transgenic frog experiments using wild-type and mutant versions of the SH0.6 core enhancer. DLP, embryos showing DLP staining with or without ectopic expression; ectopic, embryos showing ectopic expression only. The number in each bar represents the number of GFP-expressing embryos for each category. (G) The SV/luc/SH0.6 construct was active in 416B but not BW5147 cells in stable transfection assays. (H) Stable transfection assays in 416B cells demonstrate that the SH0.6 enhancer activity critically depends on the 227 bp 3' fragment deleted in the SA0.4 construct, and that the three conserved regions mutated in the mut1, mut2 and mut3 constructs are each critical for enhancer function.

floor of the aorta wall (data not shown). These results therefore demonstrate that all three regions are essential for activity of the +19 core enhancer *in vivo*.

The +19 enhancer directs expression to dorsal lateral plate mesoderm in transgenic frogs

Expression of the +19 enhancer in blood and endothelial tissues raises the possibility that it may be active in the putative bipotential progenitor of these tissues, the so-called haemangioblast. Haemangioblasts have not been described in mouse embryos, but lineage and gene expression analyses in *Xenopus* embryos have identified a population of cells in the dorsal lateral plate (DLP) mesoderm which gives rise to adult embryonic blood and the major vessels (Ciau-Uitz *et al.*, 2000). We therefore tested the activity of the mouse +19 enhancer in the DLP region of transgenic frog embryos. An SV40 minimal promoter driving green fluorescent protein (GFP) gave rise to a variable pattern of expression in the somites, head or ventral regions of stage 26–32 embryos (for example see Figure 4A). Inclusion of the murine 2.5 kb enhancer fragment (containing the +17, +18 and +19 regions) resulted in consistent expression in the region of the DLP (Figure 4, compare A with B, arrow). DLP staining was also obtained when the 2.5 kb enhancer fragment was included in GFP reporter constructs with the HSV-TK or *Xenopus* cytoskeletal actin minimal promoters (data not shown), and so, as in transgenic mice (Sanchez *et al.*, 1999), specificity was provided by the enhancer.

Sequential *in situ* hybridization demonstrated that GFP (turquoise) was co-expressed with endogenous SCL (purple) in the region of the DLP in stage 26 embryos (Figure 4C–E), a time point at which the DLP is known to contain cells co-expressing SCL and endothelial genes, i.e. putative haemangioblasts (Mead *et al.*, 1998; Ciau-Uitz *et al.*, 2000).

As shown in Figure 4F, the 641 bp fragment containing the +19 region was sufficient to target expression to the DLP in 14 of 20 (70%) GFP-expressing stage 28–32 embryos. Enhancer activity in the DLP was abolished by mutation of regions 1 or 2, and was observed in only one of 47 GFP-expressing embryos after mutation of region 3. These data therefore demonstrate that the SCL +19 enhancer can target expression to haemangioblasts in frogs and that the same three regions are critical.

The +19 region functions as a chromatin-dependent enhancer in haematopoietic progenitor cell lines

In order to perform biochemical analysis of the +19 enhancer, it was important to develop a cellular assay for enhancer function. Luciferase reporter assays were therefore established using 416B cells, an SCL-expressing cell line derived from a multipotent haematopoietic progenitor, and BW5147 cells, a T-cell line that does not express SCL and has no open chromatin in the +19 region (Göttgens *et al.*, 1997). In transient transfection experiments, inclusion of the 641 bp +19 core enhancer only provided a

Fig. 5. GATA-2, Fli-1 and Elf-1 bind to the +19 core enhancer. (A) Fli-1 and Elf-1 bind to region 1 in band shift analysis using 416B nuclear extracts. I and II correspond to complexes containing Fli-1 and Elf-1 as demonstrated by supershift analysis (compare lane 2 with lanes 7 and 11). Oligo sequences used are shown below the autoradiograph. Antibodies and competitor oligos are as indicated (con, IgG control antibody; wt, wild-type; 1, mut1; 1m, mut1m; 1e, mut1e). (B) Elf-1 binds to region 2 in band shift analysis using 416B nuclear extracts. I, II and III correspond to complexes specifically binding to region 2. Complex III was shown to contain Elf-1 by supershift analysis (compare lanes 5 and 6). Oligo sequences are shown below the autoradiograph. Antibodies and competitor oligos are as indicated (wt, wild-type; 2, mut2; 2e, mut2e). (C) GATA proteins bind to region 3 in band shift analysis using 416B nuclear extracts. I, II and III correspond to complexes binding specifically to the GATA motif present in region 3. Oligo sequences are shown below the autoradiograph (wt, wild-type; 3, mut3; Gc, GATA consensus sequence; see Bockamp *et al.*, 1995). (D) GATA-2, Fli-1 and Elf-1 bind to the +19 core enhancer but not to exon 6 sequences *in vivo*. Chromatin immunoprecipitation was performed using the indicated antibodies. The DNA content of the immunoprecipitates was analysed by real-time PCR using primers for the +19 core enhancer or for an untranslated region of exon 6. The +19 core enhancer, but not exon 6 sequences, were enriched in immunoprecipitates obtained with antibodies to GATA-2, Fli-1 and Elf-1 (Input, positive control; Rabbit IgG, negative control).

marginal increase in the activity of a luciferase reporter driven by the SV40 minimal promoter (data not shown). Stable transfection studies were then performed since the activity of some enhancers is only revealed following integration into chromatin (Tapscott *et al.*, 1992; May and Enver, 1995). Compared with an enhancerless control construct, the 641 bp +19 enhancer resulted in a 21-fold increase in luciferase activity in 416B cells but was inactive in BW5147 cells (Figure 4G). These experiments demonstrate that the +19 region functions as an integration-dependent and cell type-specific enhancer in transfection assays.

Enhancer activity in 416B cells was abolished by the 227 bp deletion, which removes conserved regions 1, 2 and 3 (Figure 4H, SV/luc/SA0.4). Moreover, mutation of any one of the three conserved regions also abrogated enhancer function (Figure 4H). Thus, as in mouse and frog embryos, the activity of the +19 core enhancer was critically dependent on the integrity of regions 1, 2 and 3.

Elf-1, Fli-1 and GATA-2 bind to the +19 enhancer *in vitro* and *in vivo*

In order to identify transcription factors capable of binding to the +19 core enhancer, gel shift assays using 416B

Fig. 6. Formation of a multiprotein complex on the +19 core enhancer. **(A)** GATA-2, Fli-1 and Elf-1 proteins interact with Elf-1. *In vitro* translated GATA-2, Fli-1 and Elf-1 are bound specifically by GST-Elf-1 in pull-down experiments (1, input; 2, adsorption to GST alone; 3, adsorption to GST-Elf-1). **(B)** GATA-2, Fli-1 and Elf-1 proteins interact with GATA-2. *In vitro* translated GATA-2, Fli-1 and Elf-1 are bound specifically by GST-GATA-2 in pull-down experiments (1, input; 2, adsorption to GST alone; 3, adsorption to GST-GATA-2). **(C)** The activity of the +19 enhancer requires the binding sites for GATA-2, Fli-1 and Elf-1 to be on the same surface of the DNA helix. A 6 bp insertion between regions 1 and 2 (SV/luc/SH0.6mut4) or between regions 2 and 3 (SV/luc/SH0.6mut5) severely reduces enhancer activity in stable transfection assays in 416B cells. **(D)** A model for the role of the +19 enhancosome in the development of HSCs and blood. It is anticipated that different GATA and Ets family members may participate in distinct cell types or in response to specific signals. SCL expression is essential for lineage commitment to blood but not endothelium.

extracts were performed. Region 1 contains conserved *myb* (TAACAG) and *ets* (GGAT) sites. A region 1 oligo was bound by two specific complexes (Figure 5A, I and II), which could be competed by a wild-type oligo (Figure 5A, compare lanes 2 and 3) but not by a mutant oligo with altered *myb* and *ets* sites (Figure 5A, compare lanes 2 and 4). Binding of both complexes I and II was reduced by competition with an oligo containing only an altered *myb* site (Figure 5A, compare lane 4 with lane 5), but not by an oligo containing only an altered *ets* site (Figure 5A, compare lane 4 with lane 6). These results suggest that complexes I and II contained Ets family transcription factors.

RT-PCR was used to assess expression of 19 Ets family members in 416B cells. Ten Ets family members were found to be expressed (Ets1, Ets2, Fli-1, Pu.1, Elf-1, Tel, GABP α , Erg, Elk4 and Mef/Elf4), but nine others were not detected (SpiB, Elk1, Er71, Erm, Er81, Pea3, Elf2/Nerf, Ehf and Elf3/Esx) (data not shown). Antibodies to Ets1, Erg, Elf1, Fli-1, Pu.1 and GABP α were used in supershift experiments. Ets1, Erg, Pu.1 and GABP α antibodies did not alter complex I or II (Figure 5A, lanes 9 and 10; data not shown). However, a Fli-1 antibody gave rise to a low mobility complex and a concomitant loss of complex II (Figure 5A, compare lane 2 with lane 7). Moreover, an antibody specific to Elf-1 generated a low mobility complex accompanied by a weakening of complex I (Figure 5A, compare lane 2 with lane 11). These data therefore strongly suggest that complexes I and II contain Elf-1 and Fli-1, respectively.

Region 2 contained a conserved *ets* motif (GGAA). Specific mutation of the *ets* site (Figure 5B, mut2e) abolished enhancer activity as effectively as the original more extensive mutation (Figure 5B, mut2) in stable reporter assays performed in 416B cells, thus demonstrat-

ing the essential role of the Ets motif (data not shown). Gel shift assays performed using a region 2 oligo identified three complexes, all of which were bound by a wild-type competitor oligo (Figure 5B, compare lanes 2 and 3). A competition oligo containing the extensive mut2 mutation was able to bind complex I, but not complexes II and III (Figure 5B, compare lanes 2 and 4). Only complex III was unable to bind to a competitor oligo containing a specific mutation of the *ets* site (Figure 5B, compare lanes 2 and 5), thus demonstrating that complex III binds to the *ets* site. Antibodies to Ets1, Erg, Fli-1, Pu.1 and GABP α did not alter complex III (Figure 5B, compare lanes 5 and 7; data not shown). In contrast, inclusion of an antibody to Elf-1 generated a low mobility complex with weakening of complex III (Figure 5B, compare lanes 5 and 6). These results suggest that complex III contains Elf-1.

Region 3 contains a conserved GATA motif. Previous northern blotting analysis had shown that our 416B cells express readily detectable levels of GATA-2, but low or undetectable levels of GATA-1 and GATA-3 (Bockamp *et al.*, 1997). Expression of GATA-4, GATA-5 and GATA-6 was not detectable by RT-PCR (data not shown). Gel shift analysis using conventional nuclear extracts failed to detect a specific complex binding to a region 3 oligo (data not shown). However, three complexes were identified using a rapid nuclear extract protocol (see Materials and methods). All three were bound by wild-type competitor oligo and an oligo containing a GATA consensus site (Gc), but not by an oligo with the region 3 mutation (Figure 5C, compare lane 2 with lanes 3, 4 and 5). These results therefore suggest that a GATA protein (probably GATA-2) binds to the GATA site in region 3.

Gel shift analysis identifies proteins capable of binding to a target sequence *in vitro*, but the results do not

necessarily reflect the pattern of binding within intact cells. To address this issue, chromatin immunoprecipitation (ChIP) studies were performed. Briefly, proteins bound to DNA were cross-linked using formaldehyde and, after shearing by sonication, protein–DNA complexes were immunoprecipitated using control antibodies and antibodies to specific transcription factors. The DNA content of immunoprecipitates was then analysed by quantitative real-time PCR (Figure 5D). As a negative control, primers were designed for an untranslated region of exon 6 of the mouse *SCL* gene, a region not thought to play a role in transcriptional regulation. This region of exon 6 was not enriched in immunoprecipitates obtained using antibodies to Fli-1, Elf-1, GATA-2 or Pu.1 relative to those obtained using rabbit IgG. In contrast, the +19 core enhancer was clearly enriched in immunoprecipitates obtained with antibodies to Fli-1, Elf-1 and GATA-2 (but not Pu.1) relative to immunoprecipitates obtained using rabbit IgG. We therefore conclude that Fli-1, Elf-1 and GATA-2 all bind to the +19 core enhancer *in vitro* and also within intact 416B cells.

A haematopoietic stem cell enhanceosome

Our results identify three critical transcription factor-binding sites in the +19 core enhancer. Mutation of each individual site abolishes enhancer activity, suggesting that the corresponding transcription factors participate in a multimeric complex necessary for enhancer function. To investigate whether the three proteins identified in the previous section were capable of interacting, GST pull-down experiments were performed. *In vitro* translated Elf-1, Fli-1 and GATA-2 were all capable of binding GST–Elf-1 and GST–GATA-2 fusion proteins (Figure 6A and B, compare lanes 2 and 3).

The concept of a multimeric protein complex is also consistent with the spacing between regions 1, 2 and 3 (18 and 21 bp), which suggests that transcription factors binding to these regions would all lie on the same face of the DNA helix. To assess whether this arrangement was critical for activity of the +19 core enhancer, we generated two new reporter constructs with a 6 bp insertion between regions 1 and 2 or between regions 2 and 3 (SV/luc/SH0.6mut4 and SV/luc/SH0.6mut5, respectively). Both constructs showed greatly diminished activity when assayed in 416B cells (Figure 6C). Taken together, our results therefore suggest a model in which Fli-1, Elf-1 and GATA-2 form three DNA-binding components of an enhanceosome responsible for establishing the transcriptional programme necessary for HSC and blood cell development (Figure 6D).

Discussion

Activation of *SCL* expression is essential for the initiation of haematopoiesis and the formation of HSCs, but not for the specification of endothelial cells. The *SCL* stem cell enhancer is active in haemangioblasts together with their haematopoietic and endothelial progeny, and expression of *SCL* under control of the stem cell enhancer results in selective rescue of early haematopoietic progenitors in *SCL*^{−/−} embryos. This enhancer therefore functions as a pivotal element responsible for integrating signals necessary for lineage commitment to HSCs and blood cell

formation. Our results identify a core +19 enhancer containing three key motifs, each of which is essential for enhancer function in haemangioblasts, blood progenitors and endothelial cells. In addition, our data identify GATA-2, Fli-1 and Elf-1 as critical components of a novel multiprotein complex with a central role in establishing the transcriptional programme for blood.

Formation of a multiprotein complex on the +19 enhancer is essential for targeting expression to blood progenitors and endothelium

Several aspects of our data point to the assembly on the +19 enhancer of a multiprotein complex with a central role in blood and endothelial development. Mutation of any one of three critical binding sites resulted in marked loss of enhancer function, an observation which is reminiscent of the interferon- γ (IFN- β) enhanceosome (reviewed in Carey, 1998; Merika and Thanos, 2001). The intact IFN- β enhancer displays a highly specific response to viral infection, a property which cannot be reproduced by individual components of the enhancer. Its biological function therefore represents more than the sum of its parts and involves the integration of multiple regulatory inputs in a precise way. Our results also demonstrate that activity of the +19 enhancer requires all three critical transcription factor-binding sites to be on the same face of the DNA helix, analogous to the prototypic IFN- β and T-cell receptor α (TCR α) enhanceosomes. In both of these cases, an architectural protein (HMG-I or LEF-1, respectively) bends the DNA and thereby reduces the energetic threshold for complex assembly (Giese *et al.*, 1995; Kim and Maniatis, 1997). It may therefore be relevant that Elf-1 has been shown to interact with HMG-I(Y) (John *et al.*, 1995).

The IFN- β and TCR α enhancers, together with the hypersensitive site 2 (HS2) of the β -globin locus control region (LCR), are all classical enhancers and are active in transient transfection assays. In contrast, the *SCL* +19 enhancer falls into the category of chromatin-dependent enhancers, which require stable integration to reveal their activity. Analogous elements include the HS3 and HS4 of the β -globin LCR (for a review see Li *et al.*, 1999) together with enhancers from the MyoD and CD34 genes (Tapscott *et al.*, 1992; May and Enver, 1995). The molecular basis for chromatin dependence is poorly understood. Part of the explanation may lie in the observation that some promoters are active in the absence of an enhancer in transient transfection assays but not in stable transfection experiments (Göttgens *et al.*, 1997; Li *et al.*, 2001). The function of a linked enhancer may be masked in the former and only become evident in stable assays. In this case, the difference between classical and chromatin-dependent enhancers may be partly quantitative, with classical enhancers being more powerful and therefore detectable in transient assays above the promoter activity. Alternatively, the two categories of enhancer may be qualitatively distinct, with chromatin-dependent enhancers requiring some aspect of chromosomal chromatin for enhanceosome assembly or function. This would be consistent with the observations that chromatin assembly on transiently transfected DNA is incomplete (Jeong and Stein, 1994) and that some enhancers exhibit distinctive chromatin-opening properties (Ellis *et al.*, 1996). In this

context, the presence of a critical GATA site in the +19 enhancer may be significant since GATA proteins interact with histone-modifying proteins (Blobel *et al.*, 1998; Wada *et al.*, 2000; Dai and Markham, 2001) and possess an evolutionarily conserved chromatin remodelling activity (Boyes and Felsenfeld, 1996; Boyes *et al.*, 1998; Muro-Pastor *et al.*, 1999).

The specificity of the SCL +19 enhancer is also striking for two reasons. First, a 641 bp core enhancer is sufficient to direct expression to blood progenitors and endothelium at multiple sites including yolk sac, para-aortic splanchnopleure/AGM region and fetal liver. This suggests either that there exists an unexpected uniformity in the signalling pathways responsible for generating blood and endothelium in the yolk sac, and at multiple sites and times within the embryo, or that the +19 enhancer is able to integrate diverse signals operating at distinct anatomical sites. Secondly, we have been unable to separate haematopoietic and endothelial activities of the +19 enhancer, since the three critical motifs within the +19 enhancer are each essential for targeting expression to both lineages. This argues that formation of a multiprotein complex on the +19 enhancer is necessary for directing expression to haemangioblasts and also for subsequent enhancer activity in both cell types. Furthermore, it is likely that the composition of the +19 enhanceosome will alter during development, with various GATA and Ets family members participating in different cell types, or in response to distinct signals.

GATA-2, Fli-1 and Elf-1 regulate the SCL +19 enhancer

The expression patterns and functions of GATA-2, Fli-1 and Elf-1, where known, are consistent with a role for all three proteins in the regulation of SCL expression during blood and endothelial development. In the mouse, GATA-2 is expressed in the yolk sac and AGM region and is known to be present in haematopoietic progenitors, mast cells, megakaryocytes and early erythroid cells (Lee *et al.*, 1991; Cross *et al.*, 1994; Minegishi *et al.*, 1999), all cell types in which SCL is also expressed. Moreover, GATA-2 and SCL are co-expressed during frog and zebrafish development in lateral mesodermal cells that give rise to blood and endothelium (Gering *et al.*, 1998; Brown *et al.*, 2000; Ciau-Uitz *et al.*, 2000). There are also several similarities in the functions of GATA-2 and SCL. GATA-2 is essential for normal levels of primitive erythropoiesis and for all lineages of definitive haematopoiesis, the latter observation suggesting a defect at the level of HSC (Tsai *et al.*, 1994a).

Haematopoietic differentiation of GATA-2^{-/-} ES cells is markedly reduced, particularly for those colony types that depend on stem cell factor (Tsai *et al.*, 1994a). Interestingly, c-kit, the receptor for stem cell factor, has been identified as a target gene of SCL (Krosl *et al.*, 1998), and both SCL and GATA-2 have been implicated in regulating the proliferation of haematopoietic progenitors (Green *et al.*, 1991; Briegel *et al.*, 1993).

Fli-1 is also co-expressed with SCL and GATA-2 in the lateral mesoderm of frog and zebrafish embryos (Meyer *et al.*, 1995; Brown *et al.*, 2000). In the mouse, Fli-1 is expressed in developing endothelial and haematopoietic cells (Truong and Ben-David, 2000; Vlaeminck-Guillem

et al., 2000). Moreover, chicken Fli-1 is expressed in endothelial cells and splanchnopleural mesoderm including haematopoietic clusters attached to the wall of the dorsal aorta (Mager *et al.*, 1998). Three targeted mutations of the Fli-1 gene have been reported. The first resulted in mice with no profound phenotype (Melet *et al.*, 1996) and subsequently was realized to represent a hypomorph (Bartel *et al.*, 2000). More recently, two other targeted alleles have been generated, one in which the Ets DNA-binding domain was replaced by lacZ (Hart *et al.*, 2000) and the other in which a C-terminal activation domain was deleted (Spyropoulos *et al.*, 2000). The latter two mutations are both embryonically lethal, fail to produce mature megakaryocytes and give rise to haemorrhage within the central nervous system, which may reflect a platelet and/or endothelial defect.

Less is known about the expression pattern and function of Elf-1. However, murine Elf-1 is widely expressed in haematopoietic cells (Davis and Roussel, 1996), and chicken Elf-1 is expressed in embryonic blood and endothelial cells (Dube *et al.*, 2001). Moreover, zebrafish Elf-1 is co-expressed with SCL, GATA-2 and Fli-1 in the region of the lateral mesoderm that gives rise to blood and endothelium (M.Gering and R.Patient, unpublished observations). No overt haematopoietic defect is seen in Elf-1^{-/-} mice (Garrett-Sinha *et al.*, 2001), but a detailed description of these mice awaits publication.

The complete absence of primitive and definitive haematopoiesis that typifies the SCL^{-/-} phenotype is not reproduced by targeted mutation of GATA-2, Fli-1 or Elf-1, and yet alteration of the binding site for any one of these transcription factors abolishes activity of the +19 enhancer. This could reflect the existence of additional SCL regulatory elements that can substitute for the +19 enhancer. Although our data do not exclude this possibility, detailed analysis of ~55 kb surrounding the murine SCL locus and extending to the 5'- and 3'-flanking genes has not revealed any other enhancer with significant haematopoietic activity *in vivo* (Sanchez *et al.*, 1999; Sinclair *et al.*, 1999; Göttgens *et al.*, 2000). Instead, it seems more likely that altered levels of related transcription factors can compensate to varying degrees for the absence of GATA-2, Fli-1 or Elf-1. Thus, targeted mutation of GATA-1 results in a compensatory increase in levels of GATA-2 (Weiss *et al.*, 1994), and the sequences of the Ets proteins Erg and Mef/Elf-4 are very similar to those of Fli-1 and Elf-1, respectively (Miyazaki *et al.*, 1996; Baltzinger *et al.*, 1999).

A number of signalling pathways activated by growth factors or cellular stress converge on Ets and GATA transcription factors, and include the MAP kinase, phosphatidylinositol 3-kinase and phospholipase C cascades (Towatari *et al.*, 1995; Yordy and Muise-Helmericks, 2000; Liang *et al.*, 2001). Cross-talk has been reported between these pathways and the BMP pathway (reviewed in von Bubnoff and Cho, 2001), which specifies blood and endothelial lineages during embryonic development. Identification of GATA-2, Fli-1 and Elf-1 as key components of the SCL +19 enhanceosome will facilitate analysis of the signalling pathways responsible for HSC formation and thereby allow the development of novel approaches to the manipulation of stem cells.

Materials and methods

Restriction endonuclease accessibility assay

EML-C1 and 416B cells were maintained as described previously (Tsai *et al.*, 1994b; Bockamp *et al.*, 1997). The restriction endonuclease assay was performed as described (Göttgens *et al.*, 2001) using *SacI* digestion and a 400 bp *SacI*–*ScaI* fragment just 3' of exon 6 as a probe.

Transgenic analysis

Transgenic reporter constructs were generated using standard cloning procedures and details are available upon request. SV/lac/H2.5 plasmid contained a *HindIII* fragment from 76 955 to 79 480. The $\Delta 17$ deletion was generated by PCR and removed 111 bp ranging from 77 582 to 77 693; the $\Delta 18$ deletion removed a 401 bp *NcoI*–*XmnI* fragment from 78 359 to 78 760; and the $\Delta 19$ deletion removed a 292 bp *ApaI*–*HindIII* fragment from 79 188 to 79 480. The NH1.1 fragment ranged from 78 359 to 79 480, the SH0.6 fragment from 78 840 to 79 480 and the SA0.4 fragment from 78 840 to 79 188 (numbering refers to the *SCL* locus database entry AJ131017). All mutations were generated using standard procedures and verified by DNA sequencing. F₀ transgenic mouse embryos were prepared and analysed as described (Sanchez *et al.*, 1999). Transgenic *Xenopus* embryos were generated using the method of Kroll and Amaya (1996) with one modification. Sperm was frozen to improve permeability and the percentage of transgenic embryos (Sparrow *et al.*, 2000).

Whole-mount *in situ* hybridization was performed according to Bertwistle *et al.* (1996). When double *in situ* hybridization was performed, embryos were hybridized with probes differentially labelled with fluorescein or digoxigenin. The first colour was developed using BCIP (Boehringer Mannheim), then BM Purple (Boehringer Mannheim) was used for the second colour reaction. The gene with the strongest expression was chosen for the first staining.

Reporter assays and EMSA analysis

Transient/stable transfections and luciferase assays were performed as described previously (Göttgens *et al.*, 1997). For the rapid nuclear extract preparation, 2×10^6 cells were collected by centrifugation, washed in phosphate-buffered saline (PBS) and resuspended in 400 μ l of buffer A [10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)], and incubated at 4°C for 15 min. After adding 25 μ l of 10% NP-40, the sample was mixed and briefly centrifuged. The pellet was resuspended in 25 μ l of buffer C (10 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) and incubated for 30 min followed by a 5 min centrifugation. A 1 μ l aliquot of the resultant supernatant was used per track for electrophoretic mobility shift assay (EMSA) analysis. Standard nuclear extracts were prepared and EMSAs performed as described (Bockamp *et al.*, 1998). Oligonucleotides (wild-type and mutant) used for EMSA are shown in Figure 5. Antibodies for supershift analysis were purchased from Santa Cruz Biotechnology Inc.

ChIP assay

ChIP assays were performed as described (Forsberg *et al.*, 2000). Antibodies were purchased from Santa Cruz Biotechnology Inc. Precipitates were analysed by conventional and real-time PCR using the SYBR green intercalant dye according to the manufacturer's instructions (ABI7700 machine; Applied Biosystems, Foster City, CA). The primers used were: Exon6-FW, CCATACTCTTGCCAAGGC-TACC; Exon6-REV, AGCAGTCCTACATGGGCCTAAA; +19-FW, CCATACTCTTGCCAAGGCCTACC; and +19REV, AGCAGTCCTAC-ATGGGCCTAAA.

GST pull-down assay

Full-length human Elf-1 and GATA-2 were subcloned into pGEX-4T. GST fusion proteins were expressed and GST pull-down assays performed as described (Kerr *et al.*, 1993). Human Elf-1, mouse Fli-1 and human GATA-2 proteins were prepared by *in vitro* transcription/translation using the TNT system (Promega).

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