

Transcriptional Regulation of the *SCL* Locus: Identification of an Enhancer That Targets the Primitive Erythroid Lineage In Vivo

E. Delabesse,^{†‡} S. Ogilvy,[‡] M. A. Chapman, S. G. Piltz, B. Gottgens, and A. R. Green*

University of Cambridge, Department of Hematology, Cambridge Institute for Medical Research,
Hills Road, Cambridge CB2 2XY, United Kingdom

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The stem cell leukemia (*SCL*) gene, also known as *TAL-1*, encodes a basic helix-loop-helix protein that is essential for the formation of all hematopoietic lineages, including primitive erythropoiesis. Appropriate transcriptional regulation is essential for the biological functions of *SCL*, and we have previously identified five distinct enhancers which target different subdomains of the normal *SCL* expression pattern. However, it is not known whether these *SCL* enhancers also regulate neighboring genes within the *SCL* locus, and the erythroid expression of *SCL* remains unexplained. Here, we have quantitated transcripts from *SCL* and neighboring genes in multiple hematopoietic cell types. Our results show striking coexpression of *SCL* and its immediate downstream neighbor, *MAP17*, suggesting that they share regulatory elements. A systematic survey of histone H3 and H4 acetylation throughout the *SCL* locus in different hematopoietic cell types identified several peaks of histone acetylation between *SIL* and *MAP17*, all of which corresponded to previously characterized *SCL* enhancers or to the *MAP17* promoter. Downstream of *MAP17* (and 40 kb downstream of *SCL* exon 1a), an additional peak of acetylation was identified in hematopoietic cells and was found to correlate with expression of *SCL* but not other neighboring genes. This +40 region is conserved in human-dog-mouse-rat sequence comparisons, functions as an erythroid cell-restricted enhancer in vitro, and directs β -galactosidase expression to primitive, but not definitive, erythroblasts in transgenic mice. The *SCL* +40 enhancer provides a powerful tool for studying the molecular and cellular biology of the primitive erythroid lineage.

One of the central issues facing current biology concerns the molecular mechanisms whereby stem cells give rise to differentiated progeny. Hematopoiesis is the best-characterized stem cell system, and experiments with mice and lower vertebrates have identified a small number of transcription factors, including *SCL*, *LMO-2*, and *RUNX-1*, which are central to the formation and/or behavior of hematopoietic stem cells (HSCs). Tight transcriptional control of these key regulatory genes is frequently critical for their biological functions, but the mechanisms responsible are poorly understood.

The *SCL* gene encodes a basic helix-loop-helix protein and is expressed in blood, 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 reference 4). Within the hematopoietic system, *SCL* is expressed in hemangioblasts, HSCs, and a subset of hematopoietic lineages, including both primitive and definitive erythroblasts. Although not required for self-renewal of adult HSCs (9, 46), targeted mutation of the *SCL* gene has shown that it is essential for the development of all hematopoietic lineages in mice (57, 61) and during murine embryonic stem (ES) cell differentiation (13, 14, 62). However, *SCL*^{-/-} mouse embryos and ES cells both generate endothelial cells (62, 73), suggesting that *SCL* is required for lineage

commitment to blood cell formation. Consistent with this concept, ectopic expression of *SCL* during zebra fish development results in excessive formation of hemangioblasts (19). Maintenance of *SCL* expression is required for normal differentiation along erythroid and megakaryocytic lineages (32, 46), whereas failure to downregulate *SCL* transcription during T-cell differentiation is associated with T-cell acute lymphoblastic leukemia (T-ALL) (reviewed in reference 4). Current evidence therefore demonstrates that appropriate transcriptional regulation is essential for the biological functions of *SCL*, and this focuses attention on the mechanisms whereby transcription of *SCL* itself is initiated and maintained.

Several lines of work have helped define the size of the transcriptional domain necessary for the normal pattern of *SCL* transcription. A 130-kb human yeast artificial chromosome containing both flanking genes was able to completely rescue the lethal *SCL*^{-/-} phenotype in mice (66). Consistent with this result, the pufferfish *SCL* genomic locus gave rise to appropriate expression in transgenic zebra fish (3), and comparisons of *SCL* flanking genes during vertebrate evolution revealed a limited region of conserved synteny likely to contain regulatory elements responsible for the conserved pattern of *SCL* expression (21, 24). Analysis of chromatin structure, together with large-scale comparative genomic sequence analysis, has led to identification of *SCL* enhancers with activity in transfection assays (16, 25) or in transgenic mice (6, 24, 26, 64, 67). These approaches have so far revealed a panel of five enhancers, each of which targets expression to a specific subdomain of the normal pattern of *SCL* expression and two of which are active in blood and/or endothelial cells.

The +18/19 enhancer is sufficient to direct reporter gene expression to hematopoietic progenitors and endothelial cells

* Corresponding author. Mailing address: University of Cambridge, Department of Hematology, CIMR, Hills Road, Cambridge CB2 2XY, United Kingdom. Phone: 44 1223 336 820. Fax: 44 1223 762 670. E-mail: arg1000@cam.ac.uk.

[†] Present address: Laboratory of Hematology and INSERM unit E02.10, Hôpital Necker-Enfants Malades, University Paris V, 149-161, rue de Sèvres, 75743 Paris cedex 15, France.

[‡] These authors contributed equally to this work.

during development (64), to the vast majority of long-term-repopulating HSCs from adult bone marrow and fetal liver (65), and to putative hemangioblasts within frog dorsolateral plate mesoderm (26). Expression of an *SCL* cDNA under the control of this enhancer in transgenic mice selectively rescued the formation of early hematopoietic progenitors in *SCL*^{-/-} embryos (65), and transgenic mice in which β -geo is driven by this enhancer have been used to identify endoglin as a novel HSC marker (7). Fine mapping demonstrated that the +19 component was sufficient for enhancer activity in transgenic mouse embryos, and biochemical characterization has shown that the +19 element is activated by a novel multiprotein complex containing Fli-1, Elf-1, and GATA-2 (26). These data suggest that this enhancer functions as a nodal point for the integration of signals responsible for establishing the transcriptional program for blood cell development. Interestingly, ES cells in which the +18/19 element was deleted from the endogenous *SCL* locus were still capable of forming blood cells in vitro and in vivo (23). This observation led to the characterization of a second enhancer (-4 element), which targets expression to endothelial cells and hematopoietic progenitors and which is also bound by Fli-1 and Elf-1 (23).

However, several questions remain. In particular, little is known about the hematopoietic expression of other genes within the *SCL* locus, and it is unclear whether individual *SCL* enhancers also regulate the transcription of neighboring genes. Furthermore, the +18/19 and -4 elements target hematopoietic progenitors but not erythroid cells, and yet *SCL* itself is normally expressed in the primitive and definitive erythroid lineages. As a consequence, we have previously postulated the existence of a separate erythroid enhancer necessary for maintaining *SCL* expression following erythroid commitment (65). In this paper, we describe the pattern of transcription of *SCL* and neighboring genes in a panel of hematopoietic cell lines representing multiple lineages. We show that *SCL* exhibits unexpected coexpression with its downstream neighbor *MAP17*, suggesting that they share regulatory elements. A systematic survey of histone acetylation throughout the *SCL* locus resulted in identification of a novel erythroid enhancer 40 kb downstream of *SCL* exon 1a. This element functions as an erythroid-restricted enhancer in vitro; directs expression to primitive, but not definitive, erythroid cells in vivo; and provides a powerful tool for studying the poorly understood primitive erythroid lineage.

MATERIALS AND METHODS

RT-PCR in human and mouse cells. Quantitative real-time reverse transcription (RT)-PCR was performed using a 7700 sequence detection system (Applied Biosystems, Foster City, CA) as described by Huntly et al. (34). Primers were located as follows: *SIL*, from exon 15 to exon 16 in both human and mouse; *SCL*, from exon 4 to exon 6 for human and from exon 5 to exon 6 for mouse; *MAP17*, from exon 2 to exon 3 for both human and mouse; *SMDR*, from exon 1 to exon 2; *CYP4A11*, from exon 9 to exon 11; *Cyp4x1*, from exon 8 to exon 9. The sequences of the primers are available from the authors.

The cDNA quality was checked using two control genes: β 2-microglobulin (β 2M) and porphobilinogen deaminase (*PBGD*), as described by Huntly et al. (34). Threshold cycle (C_T) values were extracted using the same ΔR_n fluorescent threshold of 0.2. In human and mouse cell types, the highest transcript level was obtained for β 2M in the EM2 ($C_T = 14.61$) and F4N ($C_T = 17.53$) cell lines. These values were defined as 100% for the human and mouse series of cell lines (Fig. 1B and D), and a C_T value of 34 was defined as 0%, that is, no expression detectable (Fig. 1B and D). Transcript levels for other genes and/or cell lines

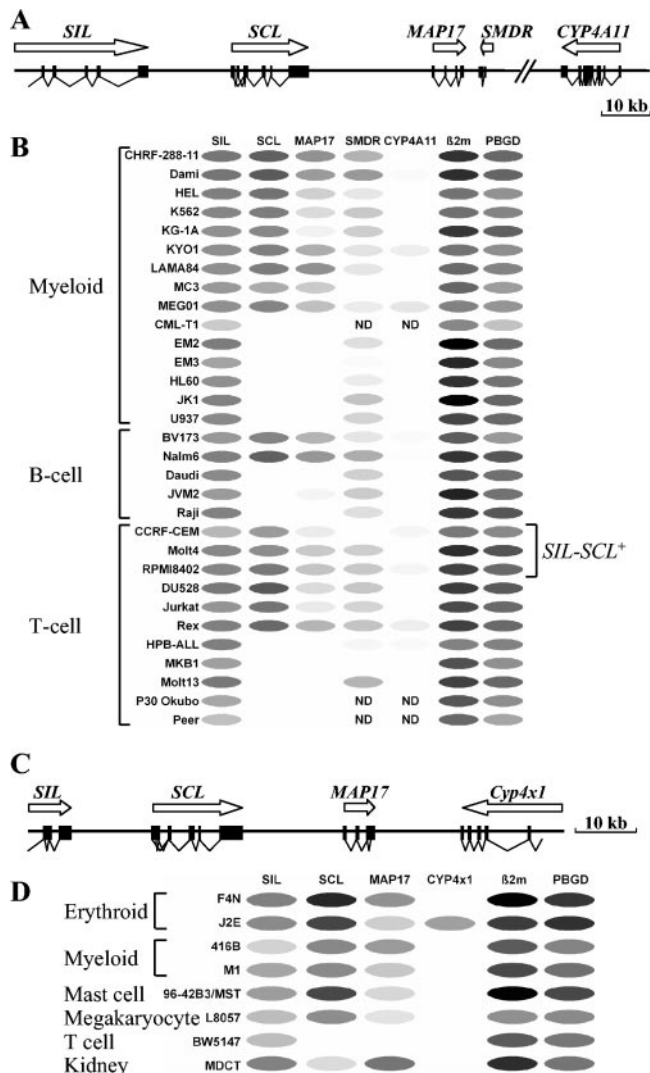


FIG. 1. Coexpression of *SCL* and *MAP17* in hematopoietic cell lines. (A) Diagram of the human *SCL* locus. (B) Relative levels of expression of the human *SCL* and neighboring genes as assessed by quantitative RT-PCR. Expression of β 2M in the EM2 cell line is defined as 100% (black oval). Other transcript levels were normalized and are shown as various shades of grey, with white ovals representing 0%. Note the striking correlation between expression of *SCL* and *MAP17*. ND, not done. (C) Diagram of the murine *SCL* locus. (D) Relative levels of expression of murine *SCL* and neighboring genes. Expression of β 2M in F4N is defined as 100% (black oval). Other transcript levels were normalized and are shown as various shades of grey, with white ovals representing 0%.

were normalized to these values. The increase was calculated using ΔC_T , the difference in C_T values between the two genes, and the yield of each PCR primer set was calculated using the following formula: increase (n -fold) = $(1 + \text{PCR yield})^{\Delta C_T}$.

RT-PCR was performed using an annealing temperature of 56°C for 35 cycles in a 25- μ l volume, which contained 0.2 mM deoxynucleoside triphosphate, 1 μ M forward primer, 1 μ M reverse primer, 10% dimethyl sulfoxide, 1.5 mM MgCl₂, and 1 U of AmpliTaq Gold (Applied Biosystems). Details of primers are available on request.

Cell lines and primary tissues. Cell lines were as described in the DSMZ German cell line bank (<http://www.dsmz.de> and reference 10), by Green (27), or as follows: CHRF-288-11 (18), Dami (29), KYO-1 (50), MC3 (52), JVM-2 (45), DU.528 (41), Rex (1), MKB-1 (43), P30 Ohkubo (33), J2E (59), L8057 (35), BW5147 (58), RM26 (42), and MDCT (20). All mouse and human cell lines were

grown in RPMI 1640 with L-glutamine (Invitrogen, Carlsbad, CA), 10% calf serum (Sigma, St. Louis, MO), and 50 U/ml penicillin-50 µg/ml streptomycin (Invitrogen). Mast cells were cultured from the spleens of (C57BL/6J × CBA/CaJ)_{F1} adult mice as previously described (28). Cell suspensions were prepared from the thymuses of 6-week-old (C57BL/6J × CBA/CaJ)_{F1} mice and embryonic day 14.5 (E14.5) livers from (C57BL/6J × CBA/CaJ)_{F1} embryos by drawing them through an 18-gauge needle.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed essentially as described previously (17) using anti-diacetylated histone H3, anti-tetra-acetylated histone H4, or nonspecific rabbit immunoglobulin G (IgG) (catalogue numbers 06-599, 06-866, and 12-370, respectively; Upstate, Lake Placid, NY). Detailed protocols are available on request.

Quantitative SyBR green PCR. Immunoprecipitates were quantified for the genomic content of the *SCL* locus using SyBR green quantitative PCRs. Primers covered for the mouse a region encompassing the 3' part of the *SIL* locus, the full *SCL* locus, the full *MAP17* locus, and a 5' part of the *Cyp4x1* locus. Similarly, a human region encompassing the 3' part of the *SIL* locus, the full *SCL* locus, the full *MAP17* locus, and a 5' part of the *CYP4A11* locus was investigated. Primers were tested to reach a sensitivity of 1 pg. The final primer sets are available from the authors. For each final primer set, the yield was calculated. The specificity of the PCR amplification was confirmed by agarose gel electrophoresis. Each mouse primer set was separated on average by 1,012 bp. The minimum and maximum distances between two murine sets were 263 bp and 3,349 bp, respectively. Human primer sets were separated on average by 1,440 bp. The minimum and maximum distances between two human sets were 370 bp and 6,682 bp, respectively. SyBR green PCR was performed on a 7700 sequence detection system (Applied Biosystems), and the C_T values were extracted. A ΔC_T value was calculated as the difference between the C_T value of the rabbit IgG immunoprecipitate and that of the acetylated histone H3 or H4 immunoprecipitate. The enrichment of the immunoprecipitated DNA fragment was calculated using the following formula: increase (n -fold) = $(1 + \text{PCR yield})^{\Delta C_T}$.

Stable transfection assays. The simian virus 40 (SV40) "promoter alone" luciferase reporter construct (SV40/luc) was the pGL2-Promoter plasmid (Promega, Southampton, United Kingdom). The thymidine kinase (TK) "promoter alone" luciferase construct was made by inserting the human herpesvirus TK minimal promoter (equivalent to bases 121 to 252 in GenBank sequence V00470) as a BglII-to-HindIII fragment into the pGL2-Basic plasmid (Promega). The *SCL* +40 region was amplified by PCR from a genomic DNA template extracted from mouse strain 129-derived ES cells using a proofreading polymerase (Extensor Hi-Fidelity Mix; ABgene, Epsom, United Kingdom). Restriction sites were engineered on either end (BglII and SalI for the SV40 and TK promoter plasmids; SalI and KpnI for the -0.9E3 luciferase plasmid). In each case, the +40 region (equivalent to bases 98649 to 102310 of AJ297131) was inserted into the BamHI/SalI sites situated 3' of the luciferase gene.

The -0.9E3 "promoter alone" construct (-0.9E3/luc) contained a 3.8-kb mouse genomic DNA fragment extending from 0.9 kb upstream of the *SCL* transcription start site at exon 1a to exon 3 (corresponding to bases 59449 to 63258 of AJ297131) (64). This fragment was inserted into the BamHI/NheI sites of the pBluescriptII plasmid (Stratagene, La Jolla, CA), with the NheI-to-SalI fragment of pGL2-Basic containing the luciferase gene coding region and SV40 poly(A) (Promega) fused downstream of exon 3 (64). In this case, the +40 region was inserted into the SalI/KpnI sites situated 3' of the SV40 poly(A) sequence to create the -0.9E3/luc/+40 plasmid.

For in vitro transfection assays of enhancer activity, 10^7 log-phase growing cells were electroporated (220 V; 900 µF) with 10 µg of linearized luciferase test plasmid and 2 µg of linearized pPGK-neo pA selection plasmid (kindly provided by Lorraine Robb, The Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia). Immediately after electroporation, the cells were divided evenly into four independent pools. After 24 h, the neomycin analogue G418 (Invitrogen, Paisley, United Kingdom) was added to a final concentration of 0.3 mg/ml (F4N and J2E) or 0.8 mg/ml (416B and BW5147). Neomycin-resistant pools were derived 1 to 2 weeks following electroporation, and luciferase assays were performed on extracts from 10^6 cells per pool essentially as described previously (5). Each experiment was repeated at least three times per cell line. The results are expressed as luciferase activity (relative light units), where the mean of the four pools of the relevant "promoter alone" transfected cells was normalized to 1 and the activities in each of the four pools transfected with the +40-containing plasmid were compared.

Generation and analysis of transgenic mice. The SV/lac/+40 transgene was constructed from the -0.9E3/lacZ plasmid (64) in the pBluescriptII backbone. It contains the SV40 minimal promoter from pGL2-Promoter (inserted as a BglII/HindIII fragment, after removal of the -0.9E3 promoter as a SpeI/NheI fragment and religation of the remaining vector) and the *lacZ* coding region and

SV40 poly(A), both derived from the -0.9E3/lacZ plasmid. As with the -0.9E3/luc/+40 plasmid, the 3.7-kb +40 region was inserted into the SalI/KpnI sites 3' of the SV40 poly(A) to create the SV/lac/+40 transgene vector. The SV intron/lac/+40 transgene is equivalent to this, with the exception that it also contained the rabbit β -globin intron from the pSG5 vector (Stratagene; equivalent to bases 395 to 967 of GenBank sequence AF013258) inserted between the SV40 minimal promoter and the *lacZ* coding region.

The transgene fragment was separated from the plasmid vector by electrophoresis of the NotI-digested plasmid and purified using a QIAquick gel extraction kit (QIAGEN, Crawley, United Kingdom). Transgene DNA was diluted in the supplied EB buffer at 4 µg/ml and spun through an Ultrafree MC sterile centrifuge filter unit (Millipore, Watford, United Kingdom) before injection into the pronuclei of (C57BL/6J × CBA/CaJ)_{F1} fertilized mouse embryos. The injected embryos were transferred into the oviducts of C57BL/6J pseudopregnant recipient females. The resulting transgenic founders were bred to nontransgenic (C57BL/6J × CBA/CaJ)_{F1} mice to generate progeny lines. For analysis of embryos or tissues, timed matings between transgenic males and nontransgenic (C57BL/6J × CBA/CaJ)_{F1} females were performed. The morning on which a vaginal plug was observed was designated E0.5 of gestation. PCR-based genotyping of mice and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) staining of whole-mount embryos were performed as described previously (47).

Flow cytometric analysis of β -galactosidase activity. Single-cell suspensions of embryonic peripheral blood and fetal liver were prepared and analyzed for β -galactosidase activity as described previously (49), except that no red cell lysis was done. The data acquisition and analysis were carried out on a FACScalibur using CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, CA). Phycoerythrin-conjugated rat anti-mouse Ter119 and rat IgG2b isotype control antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA). A forward scatter gate excluded debris, whereas dead cells were excluded by propidium iodide (Sigma, St. Louis, MO) uptake. For peripheral blood analyses, between 0.5×10^4 and 2×10^4 viable cells were examined. Similarly, between 2×10^4 and 10×10^4 viable fetal liver cells were analyzed.

RESULTS

Coexpression of *SCL* and *MAP17* in hematopoietic cells.

The genomic organizations of the human and mouse *SCL* loci are shown in Fig. 1A and C. *SCL* is flanked by *SIL* and *MAP17* in both species and also in chickens (22, 26). A novel gene called *SMDR* (*SCL*-*MAP17* downstream RNA) was identified by expressed sequence tag analysis downstream of *MAP17* in the human, but not the mouse, sequence. *SMDR* consists of two exons (225 bp and 472 bp) separated by an intron of 429 bp. No major open reading frame is present, suggesting that *SMDR* may encode a regulatory or structural RNA. A chromosome p450 gene cluster lies telomeric to *SMDR* in humans and to *MAP17* in mice. However, the first gene in the human cluster (*CYP4A11*) is not orthologous to the first gene in the murine cluster (*Cyp4x1*), an observation that reflects genomic rearrangement within the gene cluster following divergence of humans and mice (24).

In order to clarify the transcriptional activities of genes within the *SCL* locus in different hematopoietic lineages, quantitative RT-PCR was used to measure transcript levels of *SCL* and its neighboring genes, together with $\beta 2M$ and *PBGD* as widely expressed control genes. The results obtained using a large panel of human hematopoietic cell lines are shown in Fig. 1B. *SIL* was expressed in all cell lines, and there was a significant correlation between the levels of *SIL* and *PBGD* ($r = 0.77$; $n = 31$). *SMDR* was also widely expressed, with transcripts detectable in 25 of 28 lines tested. By contrast, *CYP4A11* was expressed only at very low levels in 10 out of 29 cell lines. These results are consistent with previous data suggesting that *SIL* is widely expressed (8, 36) and that *CYP4A11* expression is largely restricted to the liver and kidney (2).

MAP17 has been reported to be expressed mainly in renal-

proximal tubules (40) and in the skin (37). However, in hematopoietic cells we observed an unexpected and striking concordance between the levels of transcripts derived from *SCL* and *MAPI7* ($r = 0.80$; $n = 31$). All 17 cell lines expressing *SCL* also expressed *MAPI7*, and 13 out of the 14 cell lines that were negative for *SCL* were also negative for *MAPI7* (Fig. 1B). The only discrepancy observed was in the B-cell line JVM-2, which was negative for *SCL* but in which very low levels of *MAPI7* were detected. Levels of *SCL* transcripts were higher than those derived from *MAPI7* in all 17 *SCL*-expressing cell lines (mean difference, 234-fold; median difference, 34-fold; range, 3- to 1,744-fold).

Similar results were obtained using a smaller panel of mouse cell lines (Fig. 1D). *SIL* was expressed in all eight cell lines, whereas *Cyp4x1* was expressed in only a single cell line (J2E). Of seven hematopoietic cell lines, six expressed *SCL* and all six also expressed *MAPI7*, albeit at a lower level. By contrast, the MDCT line (derived from renal tubule cells) expressed 162-fold more *MAPI7* transcripts than *SCL* transcripts. Thus, all 23 human or mouse hematopoietic cell lines that expressed *SCL* also expressed *MAPI7*, and in each case, *SCL* transcripts were more abundant than *MAPI7* transcripts. These results demonstrate that *SCL* and *MAPI7* lie within a common transcriptional domain and suggest the existence of regulatory elements that act on both genes in hematopoietic cells.

Identification of a putative *SCL* enhancer downstream of *MAPI7*. In order to identify additional enhancers that might contribute to the regulation of *SCL* or *MAPI7*, a systematic analysis of histone acetylation was performed using several different cell types. PCR primers were designed at approximately 1-kb intervals throughout the *SCL* locus, and ChIP was then used to assess the acetylation of histones H3 and H4 over a 90-kb region of the murine locus from *SIL* intron 14 to *Cyp4x1* intron 8. Figure 2 summarizes the results obtained with an antibody directed to diacetylated histone H3. A similar pattern of results was obtained with an antibody directed to tetra-acetylated histone H4 (data not shown).

In the thymus and the T-cell line BW5147, neither of which expresses *SCL*, acetylation was at background levels throughout the *SCL* locus (Fig. 2A and B). By contrast, a broadly similar pattern of increased histone acetylation was observed in the vicinity of the *SCL* and *MAPI7* genes in five other hematopoietic cell types (Fig. 2C to G). The *SCL* promoter region and the body of the *SCL* gene were associated with several peaks of acetylation that correspond to regions previously shown to have promoter or enhancer activity, including *SCL* promoter 1a and promoter 1b, together with regions located +3 and +7 kb downstream of the beginning of *SCL* exon 1a (25). Upstream of *SCL*, most of the hematopoietic cell lines showed acetylation peaks at -4 and -9 kb. These are likely to correspond, respectively, to a previously described endothelial or hematopoietic enhancer at -4 kb (23, 67) and to a DNase I-hypersensitive site at -9 kb, the function of which remains obscure (21, 25, 67). An acetylation peak located between *SCL* and *MAPI7* at approximately +18 kb was observed in F4N cells (erythroid), 416B cells (early myeloid), and primary mast cells (Fig. 2D to F). This peak corresponds to the +18/19 enhancer previously shown to target expression to hemangioblasts and to their hematopoietic and endothelial progeny (26, 64, 65). In addition, a peak at +32 kb colocalized with the

putative *MAPI7* promoter and was the major peak observed in the MDCT renal tubule cell line (Fig. 2H), which expresses high levels of *MAPI7*. Smaller peaks at +32 kb were seen in several hematopoietic cell types (Fig. 2C, E, F, and G), consistent with activity of the *MAPI7* promoter in some hematopoietic cell types.

These results, therefore, did not identify new candidate enhancers within the *SCL* gene or between it and its immediate flanking genes. However, downstream of *MAPI7*, two acetylation peaks were of particular interest. A peak at +40 kb was identified in all five *SCL*-expressing hematopoietic cell types, two of which also exhibited a smaller peak at +47 kb. The presence of the +40 and +47 kb peaks was not associated with *Cyp4x1* expression, since F4N, 416B, and RM26 do not express detectable levels of *Cyp4x1* transcripts (Fig. 1D and data not shown). Moreover, neither peak was necessary for *MAPI7* expression, since they were not found in the MDCT cell line. The presence of both peaks, therefore, correlated with *SCL* expression, suggesting that they might represent previously unrecognized *SCL* enhancers.

Additional ChIP experiments were performed to investigate whether similar acetylation patterns were observed downstream of *MAPI7* in human cells (Fig. 3). Experiments were performed using an *SCL*-negative T-cell line (HPB-ALL) and two primitive myeloid lines (K562 and HEL), both of which express *SCL* and exhibit erythroid potential. No peaks of acetylation were evident in HPB-ALL, whereas both K562 and HEL exhibited an acetylation peak at +50 kb, a region that corresponds to the mouse +40 region. These results are consistent with the presence of a conserved element that regulates *SCL* transcription in hematopoietic cells.

The +40 region functions as an erythroid enhancer in vitro.

A four-way comparison of the human, dog, mouse, and rat sequences downstream of *MAPI7* is shown in Fig. 4A. The positions of the mouse +40 and human +50 acetylation peaks are indicated and were found to span a region containing several peaks of high sequence homology. A 3.7-kb fragment of mouse DNA spanning the +40 acetylation peak was cloned into a luciferase reporter plasmid containing either the -0.9E3 *SCL* promoter cassette (64), an SV40 promoter, or a TK promoter. As shown in Fig. 4B, the 3.7-kb fragment increased luciferase transcription from all three promoters in the erythroid cell line F4N and from two of the three promoters in a second erythroid line, J2E. By contrast, little or no activity was observed in primitive myeloid cells (416B) or in a T-cell line (BW5147). These data demonstrate that the +40 region functions as an enhancer in erythroid cells in vitro. Interestingly, 416B cells did contain an acetylation peak at +40 (Fig. 2E), suggesting that the enhancer may be in an open chromatin configuration but that 416B cells lack one or more critical transcription factors necessary for enhancer function.

The +40 region directs expression to primitive, but not definitive, erythroid cells in vivo. In order to assess the activity of the +40 enhancer in vivo, four transgenic mouse lines were generated in which β -galactosidase activity was controlled by the 3.7-kb +40 enhancer. Three lines were generated using a construct containing an intron from the rabbit β -globin gene (SV intron/lac/+40) and one with a construct lacking this intron (SV/lac/+40) (Fig. 5A).

Similar patterns of β -galactosidase staining were observed in

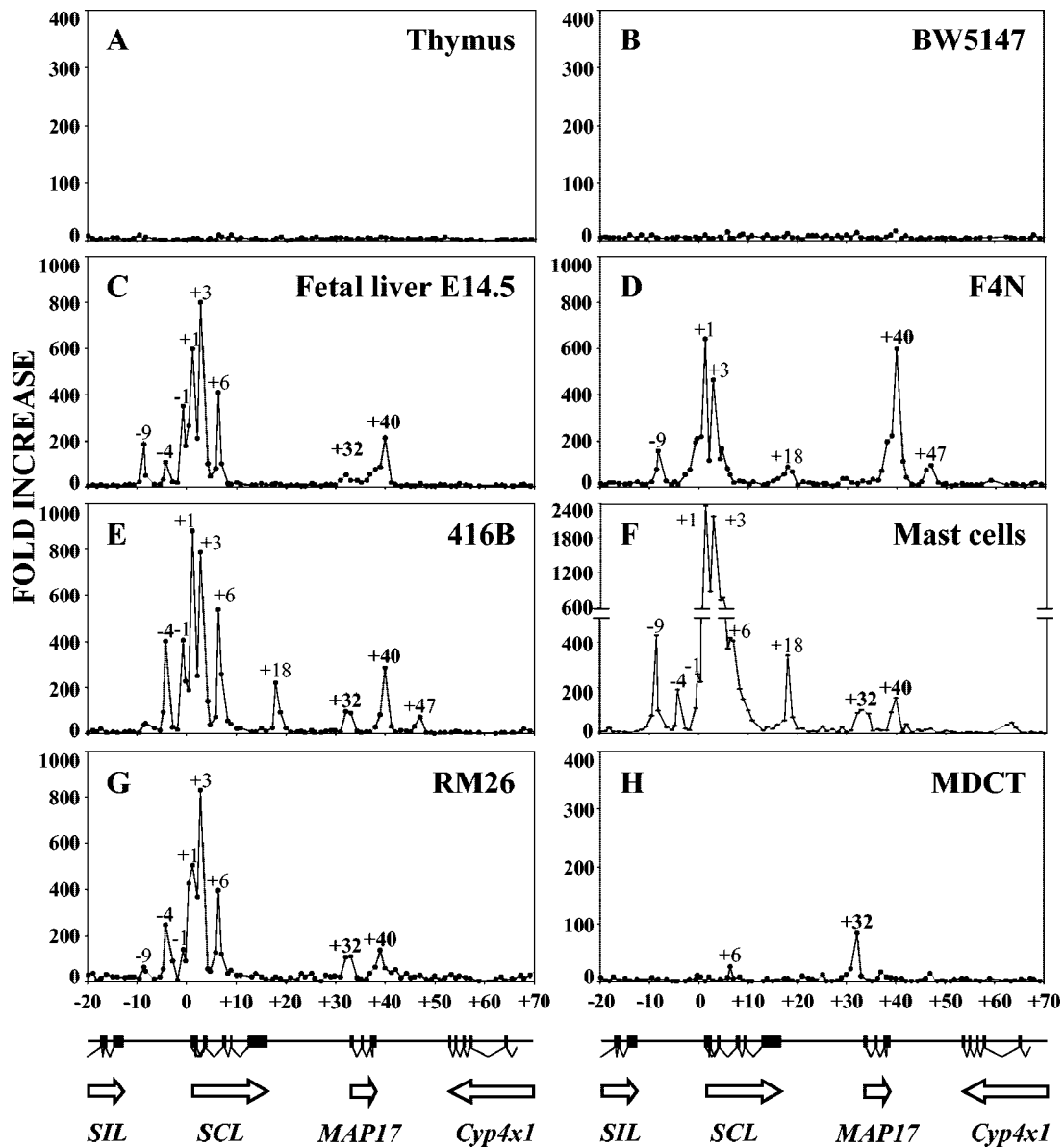


FIG. 2. Chromatin immunoprecipitation of acetylated histone H3 throughout the murine *SCL* locus is shown beneath each set of four panels. Quantitative PCR was performed using primers located approximately every 1 kb throughout a 90-kb region. The increase over background was calculated by comparison with immunoprecipitates obtained using rabbit IgG. Peaks of H3 acetylation are labeled according to their positions relative to the first nucleotide of *SCL* exon 1a. (A) Adult mouse thymus; (B) BW5147 T-cell line; (C) embryonic day 14.5 fetal liver; (D) F4N erythroid cell line; (E) 416B myeloid progenitor cell line; (F) adult spleen mast cells; (G) RM26 myeloid cell line; (H) MDCT kidney cell line.

three of the four transgenic lines (SV intron/lac/+40 lines 4092 and 4102; SV/lac/+40 line 4274), with only ectopic expression in the remaining line. In E12.5 to E13.5 embryos from these three lines, β -galactosidase activity was observed in the mid-brain and in blood vessels of the embryo (Fig. 5B, i to iii). Close inspection of whole-mount embryos suggested that the vascular staining reflected β -galactosidase expression in hematopoietic cells within the lumen of the vessels (Fig. 5B, ii). This was confirmed by analysis of histological sections, which showed that the vast majority of hematopoietic cells in the yolk sac blood islands (Fig. 5B, iv) and within the lumen of blood vessels (Fig. 5B, v) expressed β -galactosidase. By contrast,

β -galactosidase activity was detected in only a minority of cells in E12.5 livers (Fig. 5B, vi).

During mouse development, circulating erythroid cells are initially generated by a transient cohort of primitive erythroid progenitors originating in the yolk sac between E7 and E9 (55). The resultant primitive erythroid cells are large and nucleated and undergo semisynchronous differentiation, with eventual enucleation while circulating in the bloodstream (15, 39). Smaller enucleated definitive erythrocytes begin to emerge from the fetal liver at E12.5 and thereafter rapidly become the predominant cell type in the circulation.

To further investigate the ability of the +40 element to

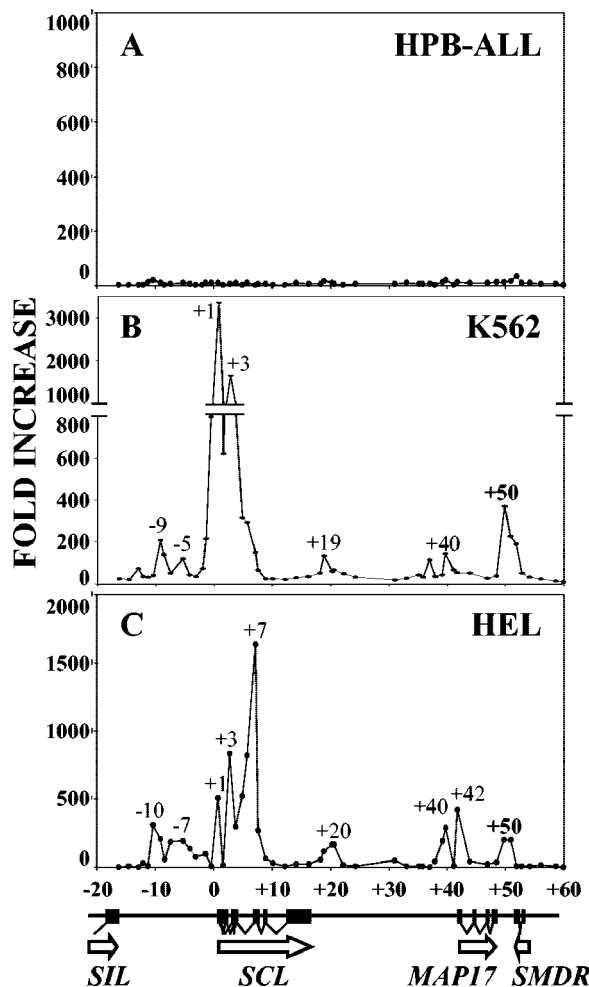


FIG. 3. Chromatin immunoprecipitation of acetylated histone H3 throughout the human *SCL* locus. A diagram of the human *SCL* locus is shown at the bottom. Quantitative PCR was performed using primers located approximately every 1 kb throughout an 80-kb region. The increase was calculated by comparison with immunoprecipitates obtained using rabbit IgG. Peaks of H3 acetylation are labeled according to their positions relative to *SCL* exon 1a. (A) HPB-ALL T-cell line; (B) K562 myeloid-erythroid progenitor cell line; (C) HEL myeloid-erythroid cell line.

target the primitive and definitive erythroid lineages, β -galactosidase expression was assessed by flow cytometric analysis (FACS) of peripheral blood cells obtained from three independent lines of transgenic mice at various time points. At E12.5 (and at earlier time points), over 90% of circulating erythroid cells are nucleated primitive erythroblasts, a figure which drops to approximately 4% by E14.5, mainly as a result of a dramatic influx of enucleated definitive erythrocytes into the circulation (39). As shown for line 4092 in Fig. 5C, at E10.5 and E12.5, most circulating erythroid cells expressed β -galactosidase, whereas by E14.5 (and E18.5), virtually all circulating erythroid cells lacked β -galactosidase expression. A similar loss of β -galactosidase expression between E12.5 and E14.5 was seen in lines 4102 and 4274 (data not shown). These results demonstrate that the +40 enhancer is active in circulating primitive erythroblasts. The lack of β -galactosidase expression in enucleated definitive erythrocytes also suggested that the

+40 enhancer was unlikely to be active in definitive erythroblasts immediately prior to their enucleation. In order to investigate whether the +40 enhancer was active in definitive erythroblasts, FACS analysis of E14.5 liver was performed. The vast majority (>90%) of liver cells at this time point are Ter119⁺ definitive erythroblasts, but virtually none of these expressed detectable β -galactosidase in multiple embryos obtained from two independent transgenic lines (lines 4092 and 4274) (Fig. 5D and data not shown). Similarly, Ter119⁺ erythroid cells in adult bone marrow also lacked detectable expression of β -galactosidase (Fig. 5D). Taken together, these data demonstrate that the +40 enhancer targets β -galactosidase expression to primitive, but not definitive, erythroblasts.

DISCUSSION

In this paper, we describe two new findings. First, we demonstrate unexpected coexpression of *SCL* and its downstream neighbor *MAP17* in hematopoietic cells and thus define a functional transcriptional domain flanked upstream by *SIL* and downstream by the cytochrome p450 locus. Second, a survey of histone acetylation across the *SCL* locus has allowed us to identify a new *SCL* enhancer that directs expression to primitive, but not definitive, erythroblasts in transgenic mice.

Coexpression of *SCL* and *MAP17*. Coexpression of *SCL* and *MAP17* in hematopoietic cells was unexpected, since *MAP17* has been reported to be expressed primarily in renal tubular epithelium and in the skin (37, 40). Coregulation of neighboring genes has been reported recently in *Caenorhabditis elegans* (63), *Drosophila* (68), and human (44, 60), but in most cases the mechanisms responsible are obscure. Shared enhancers represent one possibility (69), although in the case of the *SCL* locus none of the *SCL* enhancers identified so far are able to direct expression to the renal tubular epithelium or to the skin, tissues known to express *MAP17*. *SCL* transcripts were more abundant than *MAP17* transcripts in all 23 human or murine hematopoietic cell lines representing multiple lineages, implying that any shared enhancer would be likely to interact preferentially with the *SCL* promoter.

In addition to their coregulation in hematopoietic cells, *SCL* is expressed in specific regions of the central nervous system and *MAP17* is expressed in the renal tubular epithelium and skin. It therefore seems likely that the two genes are controlled by enhancers specific for each gene, as well as by one or more shared regulatory elements. Potential candidates for a shared hematopoietic regulatory element include the three *SCL* enhancers known to target hematopoietic cells, the +18/19 enhancer (26, 64), the *SCL* -4 enhancer (23), and the +40 enhancer described in this paper, together with three peaks of sequence homology upstream of *MAP17* (24), which may represent additional enhancers.

The boundaries of the *SCL*-*MAP17* transcriptional domain identified here are consistent with previous comparative analyses of vertebrate *SCL* loci (21). The pattern of *SCL* expression is highly conserved between mammals, chickens, and zebra fish (19). Comparative genomic sequence analysis has identified a region of conserved synteny that extends in mammals from the 3' end of the *SIL* gene to the beginning of the cytochrome p450 locus and which would be expected to contain all regulatory elements responsible for the con-

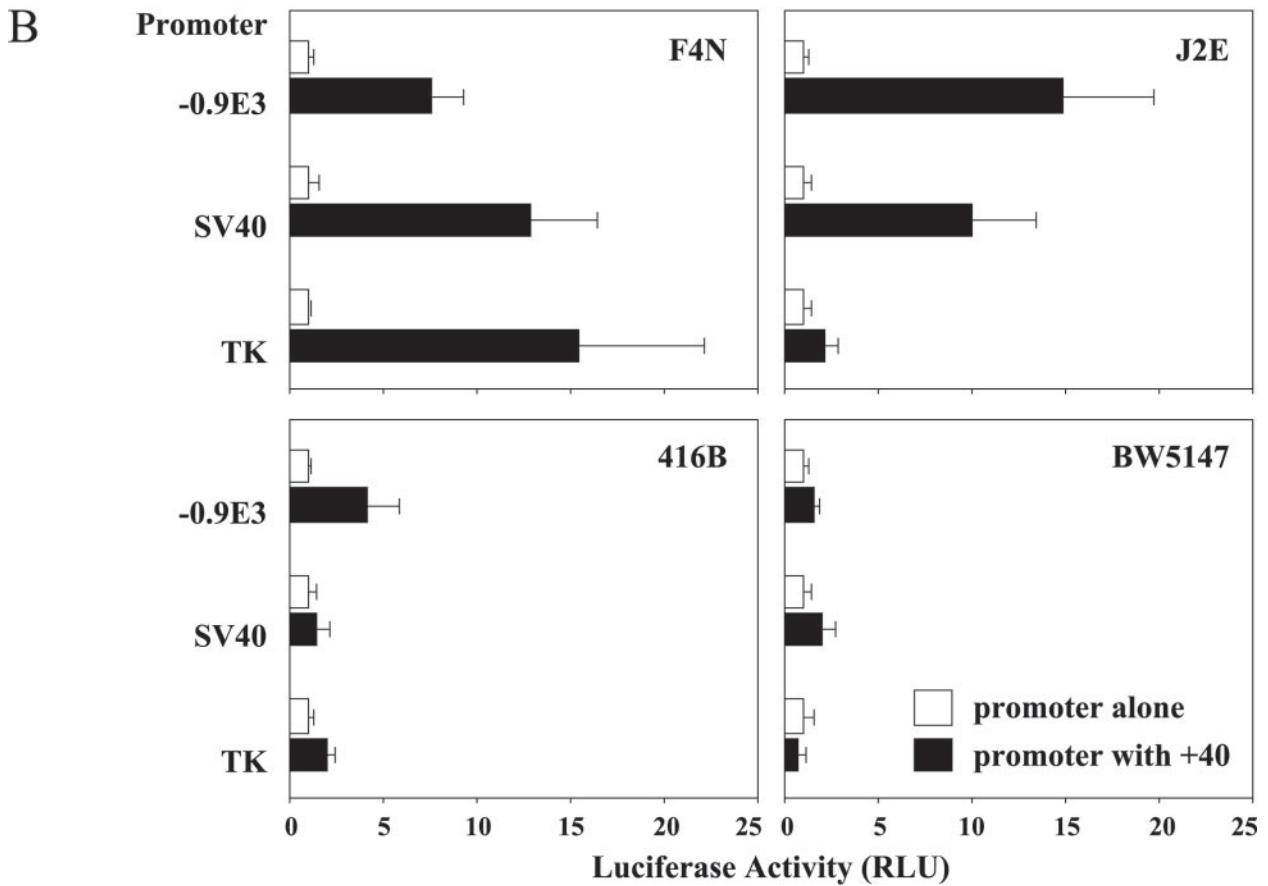
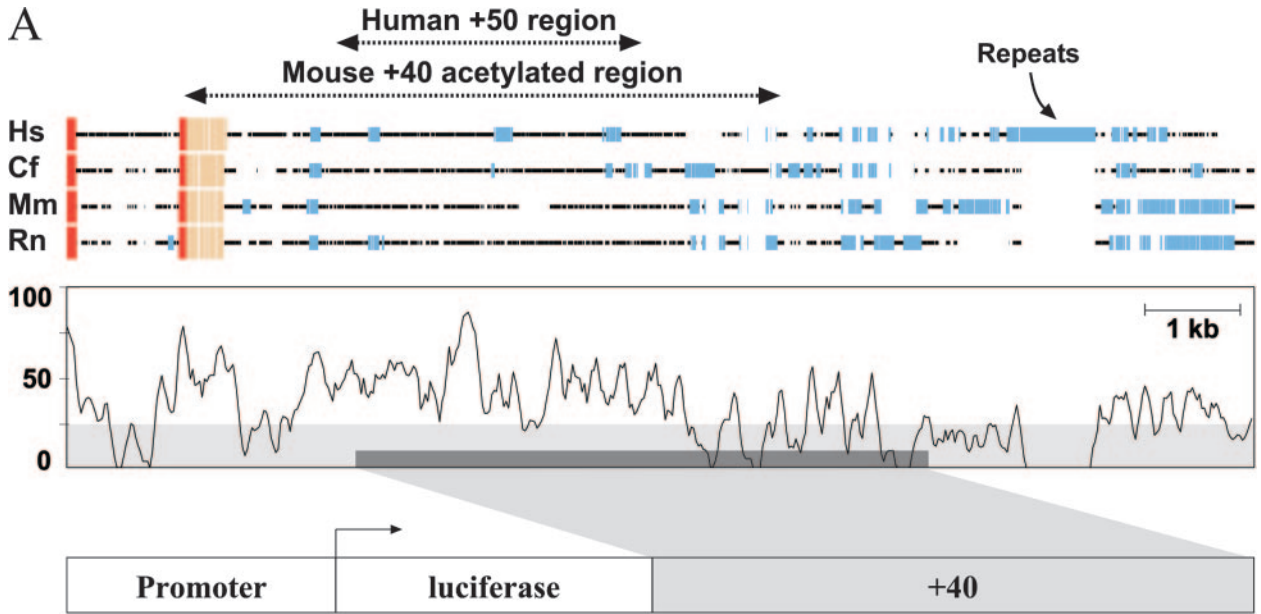


FIG. 4. The +40 region is conserved and functions as an erythroid enhancer in vitro. (A) Schematic representation of a four-way sequence alignment of the *MAP17* gene downstream region in human (Hs), dog (Cf), mouse (Mm), and rat (Rn) showing peaks of conserved sequence. Red boxes, coding exons; beige boxes, untranslated exons; blue boxes, repeat sequences. The 3.7-kb fragment incorporated into luciferase reporter constructs is shown at the bottom. (B) Stable transfection of hematopoietic cell lines with luciferase reporter constructs. Cell lines representing erythroid (F4N and J2E), myeloid progenitor (416B), or T-lymphoid (BW5147) cell types were transfected with reporter constructs containing various promoters (-0.9E3 *SCL* promoter, SV40 minimal promoter, or thymidine kinase promoter) with or without the +40 region. The histograms represent the mean (and standard deviation) luciferase activities (in relative light units [RLU]) of four independent pools normalized to the mean result obtained with a construct containing the corresponding promoter alone. The results shown were obtained in a single representative experiment. The same pattern of results was observed in at least three independent experiments for each cell line.

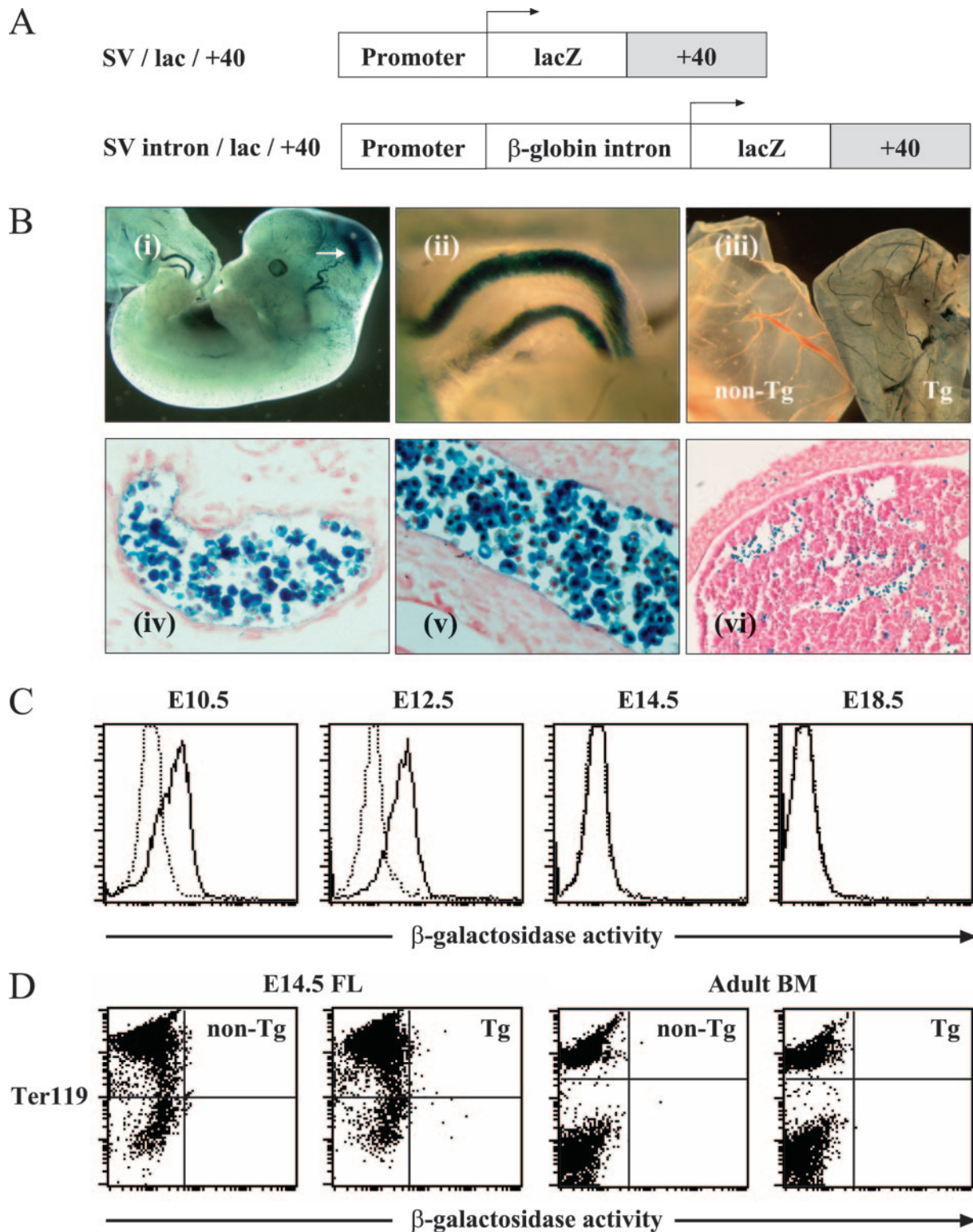


FIG. 5. The +40 region targets expression to primitive erythroblasts in vivo. (A) Constructs used to generate transgenic mice. (B) Whole-mount (i to iii) and histological (iv to vi) sections of SV intron/lac/+40 transgenic mice (line 4092). (i) Lateral view of an E12.5 embryo. Note β -galactosidase expression in blood vessels and in the midbrain (arrow). (ii) High-power view of umbilical vessels from an E12.5 embryo. (iii) Yolk sacs from E13.5 nontransgenic (non-Tg) and transgenic (Tg) embryos. (iv) Sagittal section through E12.5 yolk sac blood island. (v) E12.5 umbilical vessel. (vi) E12.5 fetal liver. (C) Flow cytometric analysis (FACS) of β -galactosidase activity in circulating erythroid cells of SV intron/lac/+40 transgenic embryos (line 4092). Data from transgenic animals (solid lines) are shown superimposed on those from nontransgenic littermates (dashed lines). Similar results were obtained in at least three mice of each genotype for each time point. Similar data were also obtained at E12.5 and E14.5 in two additional independent transgenic lines (lines 4102 and 4274). (D) FACS analysis of β -galactosidase activity and Ter119 erythroid lineage marker expression in E14.5 fetal liver and adult bone marrow from SV intron/lac/+40 transgenic (Tg) animals (line 4092) and nontransgenic (non-Tg) littermates. The fetal liver results shown are representative of at least three embryos of each genotype, whereas the bone marrow data represent experiments in which at least four mice of each genotype (lines 4092, 4102, and 4274) were analyzed.

served pattern of *SCL* expression (21). This region of conserved synteny corresponds to the *SCL-MAP17* transcriptional domain described here. In mammals, the latter is flanked upstream by the *SIL* gene, which is ubiquitously expressed, and downstream by the cytochrome p450 locus, which is not expressed in hematopoietic cells. It is not yet clear whether the functional boundaries of the *SCL-MAP17* domain reflect the presence of insulator elements, the specificity of promoters for particular enhancers, or the operation of other mechanisms.

Identification of an *SCL* erythroid enhancer. Previous studies have identified two *SCL* enhancers active in hematopoietic progenitors and endothelium, but neither enhancer was able to direct expression to primitive or definitive erythroid cells (23, 64, 67). Since *SCL* is expressed in both primitive and definitive erythroblasts (12, 28), we had postulated the existence of a distinct erythroid enhancer (65). In this paper, we describe a systematic survey of histone acetylation throughout 90 kb of the *SCL* locus, which led to the identification of a previously unrecognized enhancer with erythroid specificity *in vitro* and in transgenic mice. This enhancer is situated between *MAP17* and the cytochrome p450 gene cluster and, in the mouse sequence, lies 40 kb downstream of *SCL* exon 1a. In addition to its erythroid activity, several other lines of evidence suggest that the +40 enhancer regulates *SCL* transcription. The murine +40 acetylation peak (or the equivalent human +50 peak) was present in all seven *SCL*-expressing cell lines studied. Where tested, cell types containing the +40 (or +50) acetylation peak did not express *Cyp4x1* or *Cyp4A11*, suggesting that the +40 enhancer does not regulate these cytochrome p450 genes. In addition, our data suggest that the +40 enhancer is not necessary for *MAP17* transcription, since the +40 enhancer acetylation peak was not found in *MAP17*-expressing MDCT cells derived from renal tubule epithelium.

The activity of the +40 enhancer in primitive, but not definitive, erythroblasts was particularly striking. We considered the possibility that this pattern of expression might represent an artifact of the β -galactosidase reporter gene, since it has been reported that the β -galactosidase cassette interferes with the ability of the β -globin locus control region to confer position-independent expression (30), and it has been suggested that the β -galactosidase sequence may act as a nucleation point for the formation of heterochromatin in definitive erythroid cells (71). However, several groups have shown that a variety of erythroid enhancers, including the β -globin locus control region (30, 31), the α -globin -40 element (56, 70), and the EKLF -950 promoter (77), can direct expression of β -galactosidase to definitive erythroid cells in transgenic mice. Instead, we favor the alternative explanation that the 3.7-kb fragment containing the +40 region exhibits intrinsic specificity for the primitive erythroid lineage. This is a remarkable attribute, since most of the erythroid enhancers that have been characterized during embryonic development are active in both primitive and definitive erythroblasts (30, 70, 77). However, despite their many similarities, primitive and definitive erythropoieses differ in several characteristics, including cell size, types of hemoglobin accumulated, and location during embryonic development (39, 55). The two lineages also utilize distinct types of progenitors (38) and are differentially affected by targeted mutations of several transcription factor genes (48,

53, 72, 75, 76). These observations suggest that at least some features of the transcriptional environment are different in the two erythroid lineages and are consistent with the existence of regulatory elements displaying selective activity in one lineage but not the other. The 3.7-kb +40 region contains conserved potential binding sites for multiple transcription factors, including members of the GATA, Ets, basic helix-loop-helix, and homeobox families (data not shown). Refinement of a core enhancer region, together with subsequent detailed biochemistry and functional analyses, will begin to unravel the molecular basis for the biological activity of this unusual element.

The results presented here demonstrate that the +40 element is sufficient to direct expression in transgenic mice to primitive, but not definitive, erythroblasts. However, our data do not exclude the possibility that the +40 region may be necessary for expression in definitive erythroblasts, perhaps in collaboration with one or more additional elements. This scenario would be consistent with our observation that the +40 region is acetylated in F4N cells, which were generated by retroviral infection of neonatal mice and are therefore thought to represent definitive erythroid progenitors (11). Alternatively, expression of *SCL* in the definitive erythroid lineage may reflect the independent activity of a separate *SCL* enhancer.

It has been reported that an enhancer approximately 3.7 kb upstream of the GATA-1 erythroid promoter also directs β -galactosidase expression to primitive, but not definitive, erythroid cells (54), although a second group has found that the same element can target β -galactosidase to both cell types (74). The explanation for this discrepancy is not clear. Moreover, the reported specificity of the GATA-1 -3.7 element for the primitive erythroid lineage requires promoter-proximal sequences, as well as the -3.7 element itself (51). By contrast, the *SCL* +40 enhancer can distinguish between primitive and definitive erythroblasts in the context of a heterologous SV40 promoter.

The primitive erythroid lineage has been difficult to study because of its transient nature during embryogenesis and because the growth factor requirements of its progenitors are ill defined. The *SCL* +40 enhancer provides a powerful tool for dissecting the molecular and cellular biology of this poorly understood lineage.

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