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Antisense SCL suppresses self-renewal and enhances spontaneous erythroid differentiation of the human leukaemic cell line K562

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The SCL gene encodes a member of the helix-loop-helix family of transcription factors that have been implicated in regulation of differentiation and development. Although SCL mRNA is not detectable in normal thymocytes or peripheral T-lymphocytes, transcriptional activation occurs in T-cell tumours. A clue to the normal function of SCL has come from demonstration of high levels of SCL mRNA in erythroid cells. To illuminate the function of SCL in the erythroid lineage, an antisense SCL construct was introduced into the human erythroleukaemia cell line, K562. Cells electroporated with a vector containing antisense SCL grew more slowly than control cells which had received vector alone. Non-specific toxicity was excluded by showing that antisense SCL did not influence growth of Raji cells, a B-cell line that does not express endogenous SCL mRNA. Suppression of K562 growth was accompanied by increased spontaneous erythroid differentiation as measured by benzidine staining. K562 cells containing antisense SCL produced smaller colonies in agar and exhibited reduced clonogenicity compared with control cells. In addition, experiments in which K562 colonies were recloned showed that antisense SCL profoundly suppressed self-renewal of K562 cells. These data provide the first evidence that SCL promotes self-renewal in an erythroid cell line and raise the possibility that SCL may function to regulate proliferation of normal erythroid cells.

Key words: helix-loop-helix/haemopoiesis/oncogene/transcription factor

Introduction

The SCL gene was originally identified by virtue of its disruption in a cell line derived from a patient with T-lymphocyte acute lymphoblastic leukaemia (T-ALL) (Begley *et al.*, 1989a). Subsequently it has been found to be rearranged in up to 25% of cases of T-ALL and is currently the most common molecular pathology associated with this disorder (Brown *et al.*, 1990).

The predicted protein product of the SCL gene contains a basic helix-loop-helix (B-HLH) motif which has been implicated in DNA binding and protein dimerization (Murre *et al.*, 1989) and which clearly identifies SCL as a member of this rapidly expanding family of transcription factors. B-HLH proteins play critical roles in the regulation of a diverse range of developmental processes including neurogenesis, germ-layer formation and sex determination in *Drosophila* (Villares and Cabrera, 1987; Caudy *et al.*, 1988; Thisse *et al.*, 1988), myogenesis in the mouse (Weintraub *et al.*, 1991) and the tissue-specific expression of many genes in species from maize to man (Murre *et al.*, 1989; Ludwig and Wessler, 1990; Cordle *et al.*, 1991; Ruezinsky *et al.*, 1991; Pongubala and Atchison, 1991). Genes in the MYC family also contain a B-HLH motif and bind to a consensus B-HLH protein recognition sequence (Blackwell *et al.*, 1990). In addition to these structural features, MYC and SCL exhibit functional similarities since both are transcriptionally activated by chromosome rearrangement in lymphoid tumours (Visvader and Begley, 1991).

Despite the involvement of SCL in T-cell tumours, SCL mRNA is not normally detected in thymus, normal peripheral T-lymphocytes or murine T-cell lines (Begley *et al.*, 1989b; Green *et al.*, 1991; Visvader *et al.*, 1991). In contrast it is readily detectable in tissues containing large numbers of erythroid cells and in erythroid cell lines such as K562 (Green *et al.*, 1991). SCL mRNA levels also undergo biphasic changes during terminal differentiation of murine erythroleukaemia (MEL) cells (Visvader *et al.*, 1991; Green,A.R., Lints,T., Visvader,J., Harvey,R. and Begley,C.G., submitted). These data suggest that SCL encodes a transcription factor that plays a role in the regulation of erythropoietic differentiation.

The K562 cell line was originally derived from the pleural effusion of a patient with chronic myeloid leukaemia in blast crisis (Lozzio and Lozzio, 1975). K562 cells express several erythroid markers including fetal and embryonic haemoglobin, both of which can be further induced by a variety of agents (Rutherford *et al.*, 1979; Benz *et al.*, 1980; Andersson *et al.*, 1981; Rowley *et al.*, 1981). As a result K562 cells have been widely used as an *in vitro* model for aspects of human erythroid development.

We have adopted an antisense strategy to study the function of SCL in K562 cells. In this paper we show that antisense SCL specifically inhibits proliferation and self-renewal of K562 cells while enhancing their spontaneous erythroid differentiation. Our results further underline the similarities between MYC and SCL and imply that the nuclear mechanisms responsible for the regulation of proliferation involve lineage-restricted as well as ubiquitous components.

Results

Antisense SCL suppresses proliferation of K562 cells

Because of the likely role of SCL in erythroid differentiation events and its high level expression in the human erythroleukaemia K562, we decided to examine the function of SCL in K562 cells by establishing clonal cell lines containing an antisense SCL construct. A human SCL cDNA fragment was inserted in the antisense orientation into the EBOpLPP expression vector which also contains the gene

for hygromycin resistance (*Hph* gene). Plasmids were then introduced into K562 cells by electroporation and hygromycin resistant clonal cell lines were obtained. The efficiency of electroporation was equal for the parental plasmid (EBOpLPP) and the plasmid containing the antisense SCL cDNA (EBO-SCL-A/S).

All seven K562 clonal cell lines containing the antisense SCL construct (K562-SCL-A/S cell lines) derived in this manner were found to grow much more slowly than control K562 cell lines containing the parental plasmid (K562-EBO control cell lines). This effect was so marked that it was initially very difficult to derive and maintain the K562-SCL-A/S cell lines let alone obtain enough cells for biochemical analysis. No such problem was encountered with any of 12 independently derived K562-EBO control cell lines. By reducing the hygromycin concentration to 20 μg/ml it proved possible to expand the K562-SCL-A/S cell lines. However, they still grew very slowly, with doubling times approximately twice as long as those of K562-EBO control cell lines grown under the same conditions (Figure 1). Eventually we obtained seven independent antisense clonal cell lines which grew sufficiently well to allow further analysis.

The effect of antisense SCL on K562 proliferation was confirmed by measuring [^{3}H]thymidine uptake. Five independent K562-SCL-A/S and K562-EBO control cell lines were cultured in quadruplicate in 96-well trays for 24 or 48 h before addition of [^{3}H]thymidine for a further 8 or 16 h. In each of three independent experiments, K562-SCL-A/S cell lines consistently incorporated less [^{3}H]thymidine than K562-EBO control cell lines grown under the same conditions (Figure 2). Thus the difficulty experienced in establishing the K562-SCL-A/S cell lines probably reflected the marked suppression of proliferation observed in cell lines containing the antisense SCL construct.

Antisense SCL suppresses clonogenicity and self-renewal of K562 cells

The suppression of K562 cell growth observed in liquid cultures (above) was also seen in clonal agar cultures. In primary cultures, the per cent frequency of clonogenic cells present in K562-SCL-A/S cell lines was less than in K562-EBO control cell lines (Table I). Moreover, there was also a marked decrease in the number of cells per colony after 14 days (Table I). This striking difference in colony size was also evident at earlier time points. In plate mapping experiments of agar cultures, 50 consecutive clones from K562-SCL-A/S and K562-EBO control cell lines were monitored. In these experiments the onset of proliferation was observed within 24 h for clones derived from K562-EBO control cell lines. Clones from these control cell lines continued to proliferate and by 96 h had attained a size of 13.4 ± 5.8 (mean ± SD of 55 consecutive clones from two independent K562-EBO control cell lines). In contrast, clones from K562-SCL-A/S cell lines did not proliferate until at least 48 h of culture. At 96 h, clones from K562-SCL-A/S cell lines were therefore significantly smaller (4.6 ± 2.5 cells/clone; mean ± SD of 100 consecutive clones from

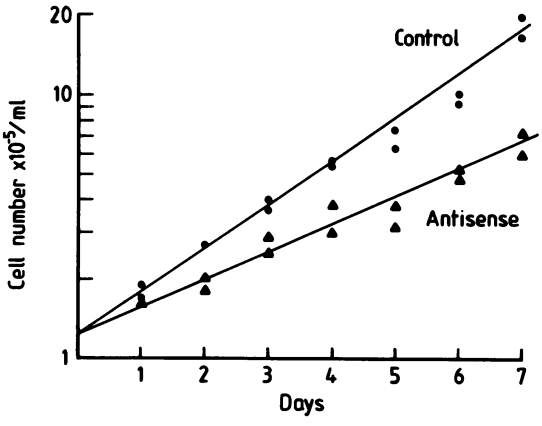

Fig. 1. Proliferation of K562-SCL-A/S (antisense) clonal cell line and K562-EBO (control) cell line. Cells in log phase were cultured in duplicate at 1 × 10^5/ml and counted using a haemocytometer. Duplicate counts for each time point are shown. All seven K562-SCL-A/S cell lines exhibited similar growth patterns.

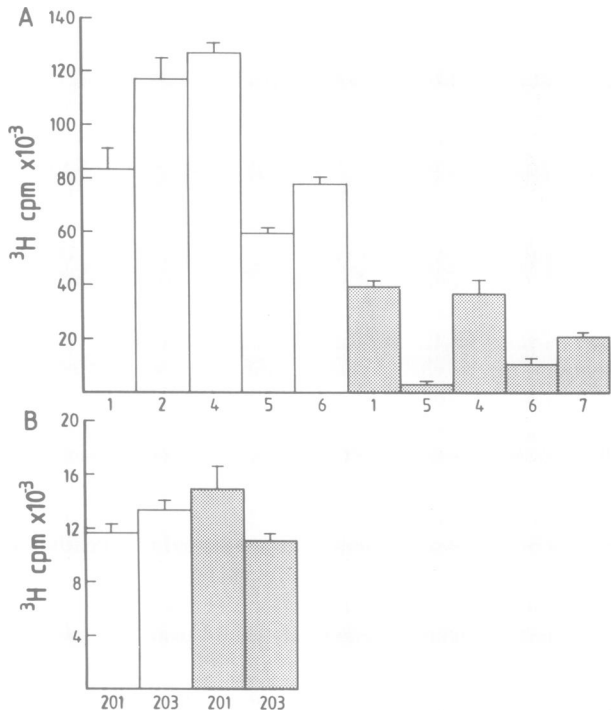

Fig. 2. [^{3}H]Thymidine incorporation by K562 (**A**) and Raji cells (**B**) expressing EBO-SCL-A/S (shaded bars) and EBOpLPP (open bars). Numbers refer to independently derived cell lines. In this experiment cells were incubated for 48 h before 0.5 μCi [^{3}H]thymidine was added to each well and incubation was continued for a further 8 h. Results are mean ± SD of quadruplicate cultures. These results were confirmed in three separate experiments.

Table I. Number and size of colonies formed by cells from K562 antisense and control cell lines

Cell line[a]	Number of colonies	Mean colony size
E 11	208 ± 24	822
E 12	219 ± 31	400
AS 8	51 ± 12	70
AS 9	70 ± 7	75

300 viable cells were cultured per ml and the total number of colonies was scored at 14 days. Number of colonies (mean ± SD) for replicate cultures is shown. Mean colony size was obtained by counting the number of cells present in 100 consecutive pooled colonies and dividing by 100. Results obtained in this experiment have been confirmed in two additional independent experiments.
[a]E, K562-EBO control cell lines; AS, K562-SCL-A/S cell lines.

three independent K562-SCL-A/S cell lines). Regular inspection of the clones did not reveal any evidence of cell death. Thus the plate mapping experiments taken together with the results of liquid cultures (Figure 1) demonstrated that antisense SCL acted to prolong the cell cycle time of K562 cells.

In addition to the effects on cell cycle duration, the growth suppression imposed by antisense SCL on K562 cells could also be based on a reduction in the proportion of cell divisions that produce clonogenic progeny. In order to address this possibility, the self-renewal potential of K562-SCL-A/S and K562-EBO clonogenic cells was investigated.

Antisense SCL markedly suppressed the self-renewal of K562 cells. In three experiments using four independent K562-SCL-A/S cell lines, primary colonies were picked off and replated in agar. The absolute number of clonogenic cells present in antisense colonies was found to be 50-fold less than the numbers of clonogenic cells in control colonies (Figure 3). Thus in addition to the effect of antisense SCL on cell cycle duration, antisense SCL profoundly decreased self-renewal in this erythroid cell line.

The antisense construct is not non-specifically toxic

To exclude the possibility that the EBO-SCL-A/S construct was non-specifically toxic for recipient cells, both the parental plasmid (EBOpLPP) and EBO-SCL-A/S were introduced into Raji cells. The Raji cell line is a human B-lymphoid cell line which does not express endogenous SCL (Green *et al.*, 1991). Raji-SCL-A/S cell lines expressed high levels of antisense SCL mRNA and contained levels of the transcript for hygromycin resistance (*Hph*) similar to those found in Raji-EBO control cell lines (data not shown). However, Raji-SCL-A/S cell lines showed no change in morphology, cytochemistry or proliferation rate when compared with Raji-EBO control cell lines. Moreover in three separate experiments, ^{3}H incorporation by Raji-SCL-A/S cell lines was no different from Raji-EBO control cell lines (Figure 2B). These results demonstrate that the SCL antisense construct was not non-specifically toxic.

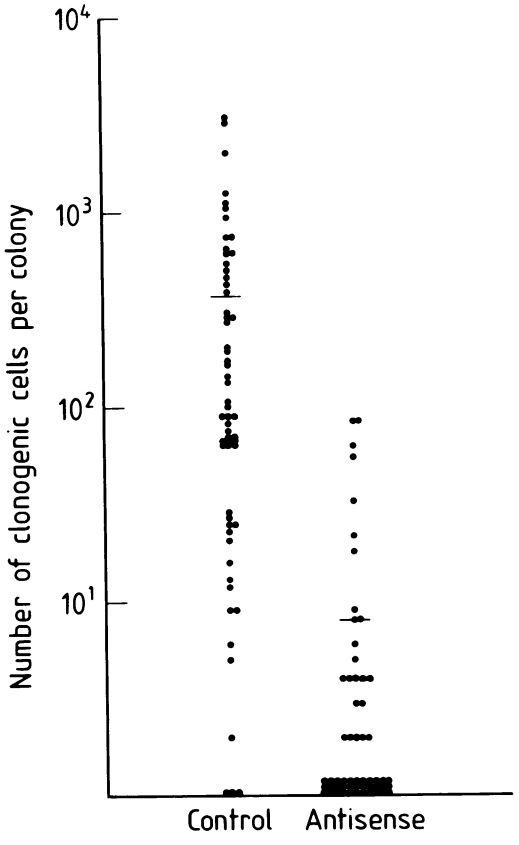

Fig. 3. Number of clonogenic cells present in primary K562-SCL-A/S (antisense) and K562-EBO (control) colonies. Consecutive colonies from each of four K562-SCL-A/S (antisense) and two K562-EBO (control) cell lines were replated in agar. There was no significant difference between the behaviour of the four different antisense cell lines. The data represent the pooled results of three independent experiments.

Reduced proliferation of K562 cells does not reflect down-modulation of Hph transcription

K562-SCL-A/S cell lines and K562-EBO control cell lines were maintained continuously in hygromycin. Their differing characteristics therefore raised the possibility that the EBO-SCL-A/S construct might express less *Hph* mRNA than EBOpLPP and therefore the antisense cell lines might be more susceptible to the toxic effect of hygromycin. Indeed two transcriptional units present in the same vector can interfere with the activity of each other (Cullen *et al.*, 1984; Emerman and Temin, 1984). However, this explanation seems untenable since Northern analysis demonstrated that all K562-SCL-A/S cell lines contained *Hph* mRNA levels at least as high as those present in K562-EBO control cell lines (Figure 4). Thus the reduced proliferation of K562-SCL-A/S cell lines cannot be attributed to reduced expression of the *Hph* gene.

Figure 4 also illustrates the high levels of exogenous SCL-A/S transcripts. It is interesting to note that, as expected, the endogenous SCL transcript levels are not obviously reduced in the K562-SCL-A/S cell lines. This suggests that the antisense transcripts do not inhibit transcription, but may instead exert their effect by interfering

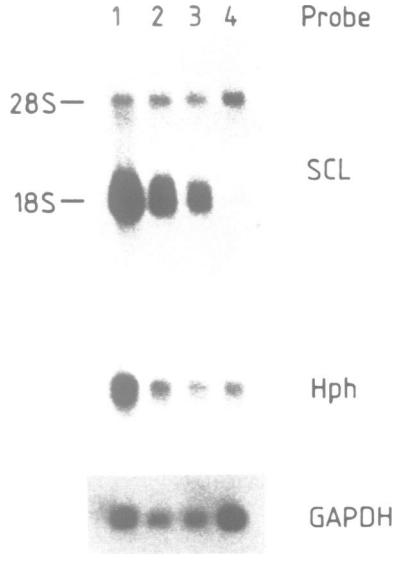

Fig. 4. Northern analysis of SCL and *Hph* expression in K562-SCL-A/S (antisense) and K562-EBO (control) cell lines. Approximately 3 μg poly(A)$^+$ RNA was used for each lane. The filter was sequentially hybridized with a human SCL probe, an *Hph* probe and a human GAPDH probe (see Materials and methods). The major endogenous SCL transcript migrates with 28S ribosomal RNA and the exogenous transcripts with 18S ribosomal RNA. Lanes 1, 2 and 3, three K562-SCL-A/S cell lines; lane 4, a K562-EBO control cell line.

with mRNA transfer to the cytoplasm or by interfering with translation.

K562 cells expressing antisense SCL show increased spontaneous erythroid differentiation

Comparison of the cells of K562-SCL-A/S cell lines and K562-EBO control cell lines showed no difference in size, morphology (as assessed by staining with May–Grünwald–Giemsa) or cytochemical staining characteristics (myeloperoxidase, dual esterase and periodic acid–Schiff). Similarly no consistent differences were seen in the staining obtained with a panel of monoclonal antibodies with specificities for several myeloid antigens (CD 11a, 11b, 11c, 13, 14, 15, 33, 34 and 54), three megakaryocytic antigens (CD 36, 41 and 42), glycophorin A and HLA-A, B and C (data not shown).

However, as shown in Figure 5, K562-SCL-A/S cell lines contained a significantly higher percentage of benzidine positive cells than K562-EBO control cell lines (14.7 ± 8.9 compared with 4.4 ± 2.8; $P = 0.014$, Student's t-test). This increased spontaneous erythroid differentiation was intriguing and differentiation of parental K562 cells was therefore induced with cytosine arabinoside as described by Rowley *et al.* (1981). SCL mRNA levels did not change despite a clear increase in the percentage of benzidine positive cells and in α globin expression (Figure 6). However, the significance of this result is unclear as induced differentiation of K562 cells cannot be regarded as mimicking a normal programme of erythroid differentiation (Horton *et al.*, 1981). Cytosine arabinoside was also used to stimulate further erythroid differentiation of K562-SCL-A/S cell lines and K562-EBO control cell lines. After 72 h the percentage of benzidine positive cells present in five K562-SCL-A/S cell lines was 40.6 ± 11.9 (mean ± SD) compared with 21.2 ± 11.3 in five K562-EBO control cell lines. These results suggest therefore that an increased tendency for spontaneous erythroid differentiation occurred concomitant with the suppression of proliferation.

Discussion

The data presented here represent the first direct evidence that SCL may normally influence cell proliferation and self-renewal. K562 cells that expressed an antisense SCL construct proliferated more slowly than control cell lines that contained the vector alone (Figures 1 and 2, Table I). This effect was not a non-specific cytotoxic effect of the SCL-A/S construct since Raji cells that do not express endogenous SCL mRNA were not affected by the introduction of EBO-SCL-A/S (Figure 2). Moreover it did not result from transcriptional interference between the SCL-A/S and *Hph* transcriptional units (Figure 4). These results therefore suggest that interference with endogenous SCL either at the level of translation or transfer of mRNA to the cytoplasm resulted in reduced proliferation of K562 cells. The decreased proliferation arose, at least in part, from both a reduction in the self-renewal of clonogenic K562 cells and a prolongation of cell cycle time (Figures 1 and 3). The

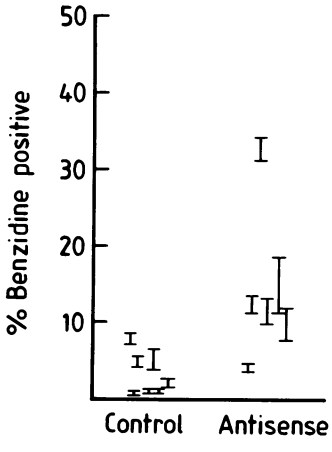

Fig. 5. Benzidine staining of K562-SCL-A/S (antisense) and K562-EBO (control) cell lines. Points represent the mean (±SD) of triplicate independent counts performed on different days. A minimum of 200 cells was scored for each count.

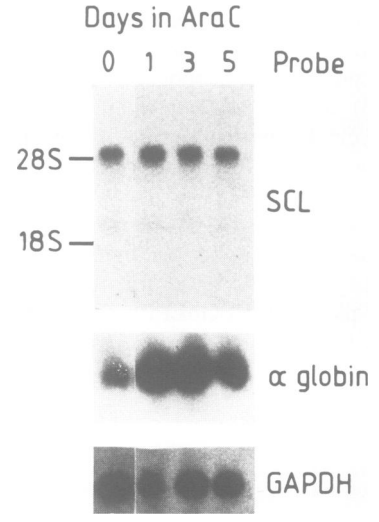

Fig. 6. Northern analysis of SCL mRNA expression in K562 cells during cytosine arabinoside induced erythroid differentiation. Approximately 3 μg poly(A)$^+$ RNA was used for each time point. The filter was sequentially hybridized with a human SCL probe, a human α globin probe and a human GAPDH probe.

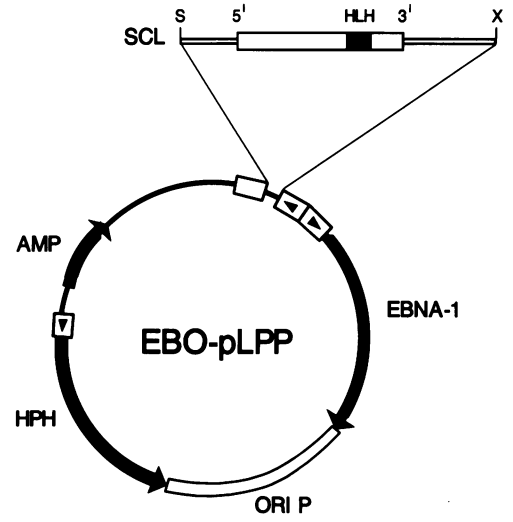

Fig. 7. Construction of SCL antisense vector EBO-SCL-A/S. A 1.8 kb *Sal*I–*Xba*I SCL cDNA fragment encompassing the entire protein coding region (open box) was inserted into the corresponding sites of the expression vector EBOpLPP (Canfield *et al.*, 1990). S, *Sal*I (present in polylinker of original SCL plasmid); X, *Xba*I; HLH, basic helix–loop–helix domain. Clockwise arrows, SV40 early promoter. anticlockwise arrow, SV40 late promoter; Ori P, Epstein–Barr virus (EBV) origin of replication; EBNA-1, EBV nuclear antigen 1.

inhibition of proliferation produced by antisense SCL was coupled with increased spontaneous differentiation as demonstrated by significantly increased levels of benzidine positive cells in K562-SCL-A/S cell lines (Figure 5). These findings are all the more striking when it is remembered that the observed phenotypes would have been continually selected against during growth of the K562-SCL-A/S cell lines. Taken together, these results suggest that SCL plays an important role in the regulation of a proliferation/differentiation switch in K562 cells.

The precise role of SCL may vary with developmental stage even within the erythroid lineage (Green *et al.* 1991), and this may explain certain apparently paradoxical results. We have recently shown that HMBA-induced erythroid differentiation of MEL cells produced a transient early fall followed by a sustained and marked rise in SCL mRNA levels. Although no early fall in SCL mRNA levels was observed in K562 cells during induced differentiation it is possible that either the SCL protein was inactivated by a negative regulator of the HLH class (Benezra *et al.*, 1990) or that the SCL mRNA was no longer translated. The sustained increase in SCL mRNA levels in MEL cells appeared to be a bona fide part of the erythroid differentiation programme (Green,A.R., Lints,T., Visvader,J., Harvey,R. and Begley,C.G., submitted). These data contrast with the K562 antisense experiments which suggest that down-regulation of SCL inhibited proliferation and increased spontaneous differentiation. These contrasting results may reflect intrinsic differences between MEL and K562 cells. MEL cells are derived by post-natal infection of mice with the Friend retroviral complex, express adult globins and seem to represent an erythropoietin-responsive stage of adult erythroid differentiation somewhere between the BFU-E and CFU-E (Friend *et al.*, 1971; Marks and Rifkind, 1978). K562 cells more closely resemble fetal or embryonic erythroblasts since they express fetal and embryonic globin, a fetal/embryonic LDH isoenzyme pattern and the i antigen (Benz *et al.*, 1980). Furthermore, whereas HMBA induced erythroid differentiation of MEL cells, it promoted K562 cell differentiation along a non-erythroid lineage (Green,A.A., Rockman,S., DeLuca,E. and Begley,C.G., submitted). Together with our previous suggestion that SCL transcript patterns may differ in distinct erythroid subpopulations (Green *et al.*, 1991) our results raise the possibility that both the function and regulation of SCL may vary in distinct populations of erythroid precursors.

The activation of SCL transcription in a significant proportion of cases of T-ALL (Begley *et al.*, 1989a,b; Bernard *et al.*, 1990; Brown *et al.*, 1990; Chen *et al.*, 1990) suggests that SCL can provide a growth advantage to T-lymphocytes. In this case it is likely that the ectopic expression of SCL in a T-cell environment gives rise to unphysiological effects resulting from interaction of the SCL protein with dimerization partners or other cofactors to which it is not normally exposed. The results presented here suggest that SCL can also mediate a previously unrecognized proliferative effect in an erythroid cell line, a lineage in which SCL is normally expressed (Green *et al.*, 1991; Visvader *et al.*, 1991).

The data presented here also strengthen the analogy between SCL and MYC. Both are implicated in the pathogenesis of lymphoid malignancies by virtue of their involvement in chromosomal rearrangements mediated by sequence-specific recombination mechanisms (Visvader and Begley, 1991; Spencer and Groudine, 1991). Moreover both encode B-HLH proteins and are therefore likely to function as sequence-specific transcription factors. In the case of MYC, this possibility has been further supported by the demonstration of sequence specific DNA binding activity (Blackwell *et al.*, 1990). Our observations provide a third parallel between SCL and MYC since there exists considerable evidence that suggests a role for MYC in the regulation of cell proliferation and DNA replication (Lüscher and Eisenman, 1990). In particular, antisense MYC constructs have been shown to inhibit proliferation and promote differentiation in a human leukaemic cell line (Holt *et al.*, 1988; Wickstrom *et al.*, 1989), observations which are entirely analogous to the results described here for SCL. However, there exists at least one major difference between SCL and MYC—whereas MYC is ubiquitously expressed in all proliferating cells, SCL expression is normally restricted to a subset of haemopoietic lineages.

Our results therefore provide the first evidence that SCL can influence self-renewal, proliferation and spontaneous differentiation in erythroid cells, a lineage in which SCL is normally expressed. In addition they imply that the nuclear mechanisms responsible for the regulation of proliferation may entail lineage restricted as well as ubiquitous components, a notion that has received recent independent support (Matsushime *et al.*, 1991).

Materials and methods

Cells

K562 and Raji cells were obtained from Dr A.Boyd (this institute) and maintained in RPMI supplemented with 10% pre-selected fetal calf serum (FCS). Induction of erythroid differentiation was performed by culturing cells at 1×10^5/ml in the presence of varying concentrations of cytosine arabinoside (Upjohn).

Clonal cultures

K562 EBO-SCL-A/S cell lines and K562-EBO control cell lines were grown in agar by culturing cells in IMDM, 0.3% agar, 25% pre-selected FCS and 20 μg/ml hygromycin at 37°C for 14 days. Colonies were defined as clones containing >10 cells and were counted using a dissection microscope. The frequency of clonogenic cells in control cell lines (K562 cells electroporated with EBOpLPP) was >70%. To estimate the average size of K562 colonies, 100 consecutive colonies were picked off using a fine pipette, the cells pooled, the total number of cells counted and divided by 100. In recloning experiments, consecutive colonies were picked off with a fine pipette, single cell suspensions prepared and the cells recultured for a further 14 days. In plate mapping experiments agar cultures were examined at intervals between days 1 and 14 of culture. The position of all clones (of >2 cells) was recorded using culture plates that were marked to allow accurate realignment on a numbered reference grid.

Plasmids

The expression plasmid EBOpLPP (Canfield *et al.*, 1990) which contains the gene for hygromycin resistance (*Hph*) was obtained from Dr R.Margolskee (Roche Research Centre, Nutley, NJ 07110, USA). Since it also contains the Epstein–Barr origin of DNA replication and the EBNA-1 gene, it can replicate as an episome in human cells so long as hygromycin selection is maintained. A human SCL cDNA clone in the *Eco*RI site of pBluescript KS$^+$ was obtained as previously described (Aplan *et al.*, 1990). The SCL antisense expression vector EBO-SCL-A/S was constructed by inserting a 1.8 kb *Sal*I–*Xba*I SCL cDNA fragment (containing the entire SCL protein coding region) into the *Sal*I and *Xba*I sites of the EBOpLPP polylinker (Figure 7).

The following probes were used: human SCL, a 1.0 kb *Sac*I–*Xba*I genomic fragment (Begley *et al.*, 1989b); human α globin, a 1.5 kb *Pst*I genomic fragment from pDH7 (Dr J.M.Old, Haemoglobinopathy Reference Service, John Radcliffe Hospital, Oxford, UK); human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.2 kb *Pst*I cDNA clone in pBR322 (Tso *et al.*, 1985); *Hph*, a 1.2 kb *Bam*HI fragment from pCD-Hpfi (a gift from Dr K.Klinger) (Heinrich-Pette-Institut, Martinistrasse 52, D-2000 Hamburg 20, FRG).

RNA analysis

Poly(A)$^+$ RNA was isolated from cell lines as described previously by Gonda *et al.* (1982). RNA samples were size fractionated by electrophoresis in gels containing 0.8% agarose, 1 × MOPS (20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate), 0.22 M formaldehyde in a 1 × MOPS running buffer. The RNA was then transferred to nitrocellulose (Hybond C-extra, Amersham), baked at 80°C for 2 h, and prehybridized at 42°C for >1 h in 50% formamide, 4 × Denhardt's (0.08% Ficoll, 0.08% bovine serum albumin, 0.08% polyvinylpyrrolidione), 5 mM EDTA, 5 × SSC, 100 μg/ml denatured salmon sperm DNA. ^{32}P-Labelled probes were derived by random priming (Bresatec) and hybridization performed overnight at 42°C by adding 1–5 × 10^6 c.p.m./ml to hybridization buffer. Filters were washed at 65°C in 0.2 × SSC, 0.1% SDS and exposed to film.

Electroporation

1–2 × 10^7 cells (K562) or 4 × 10^6 cells (Raji) growing in log phase were washed with phosphate-buffered saline (PBS) and resuspended in 0.8 ml PBS. Caesium-banded plasmid (20–30 μg) was mixed with the cells in an electroporation cuvette (0.4 cm electrode width, Bio-Rad) and allowed to stand at room temperature (RT) for 5 min before electroporation with a capacitor discharge of 280 V and 960 μF using a Bio-Rad Gene Pulser. Transfected cells were kept at RT for an additional 5 min before spinning through 3 ml FCS. The cells were then resuspended in 50 ml RPMI, 10% FCS and placed into two 24-well places at 1 ml/well. After 48 h the majority of the medium was aspirated and replaced with RPMI, 10% FCS, 200 μg/ml hygromycin. This was repeated twice weekly. For K562 cells the concentration of hygromycin was subsequently reduced to 20 μg/ml. Clonal K562 cell lines were derived by limiting dilution. Clonal Raji cell lines were obtained by the same method. Clonal cell lines were subsequently maintained in RPMI, 10% FCS and hygromycin at the predetermined optimal concentrations of 20 μg/ml (K562) or 200 μg/ml (Raji) in order to select for episome retention.

[^{3}H]Thymidine incorporation

Cells were diluted to 5 × 10^4/ml in RPMI, 10% FCS and hygromycin at either 20 μg/ml (K562) or 200 μg/ml (Raji). 10^4 cells were then cultured in quadruplicate in flat-bottomed 96-well tissue culture plates. After 40–48 h incubation in a tissue culture incubator at 37°C, 0.5 μCi [^{3}H]thymidine was added to each well and incubation continued for 8–16 h. The tissue culture plates were then frozen at −20°C prior to subsequent analysis. After thawing, cells were harvested using an automatic cell harvester and [^{3}H]thymidine incorporation quantified using a Packard TriCarb 3330 liquid scintillation spectrometer.

Cytochemistry and immunophenotype

Indirect immunofluorescence staining for all surface antigen expression was performed as described by Boyd *et al.* (1989) prior to analysis using a fluorescence activated cell sorter (FACS) (Coulter Electronics Profile II, Hialeah, FL).

The following monoclonal antibodies were used: CD11b (OKMI), CD11c (150,95), CD15 (leuMI), CD14 (FMC33), CD13 (MY7), CD33 (MY9), glycophorin (10F7MN), HLA-A, B and C (W6/32), CD34 (B13C5), CD54 (WEHI-ICAMI), CD41 (FMC24), CD42 (AN51) and CD36 (IA7). Percentages of positive cells were determined by reference to non-specific isotype control antibodies. At least 10^4 cells/sample were analysed.

Cytochemical staining was performed using standard procedures (Dacie and Lewis, 1984) and benzidine staining as described by Rowley *et al.* (1981).

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