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Immortalization of multipotent growth-factor dependent hemopoietic progenitors from mice transgenic for GATA-1 driven SV40 tsA58 gene

L.A.Cairns[1,2,3], S.Crotta[1,3], M.Minuzzo[1], E.Moroni[4], F.Granucci[2], S.Nicolis[1,5], R.Schiró[6], L.Pozzi[7], B.Giglioni[4], P.Ricciardi-Castagnoli[2] and S.Ottolenghi[1]

[1]Dip. di Genetica e di Biologia dei Microrganismi, Università di Milano, [2]CNR, Centro per lo studio della Farmacologia delle Infrastrutture Cellulari, Milano, [4]CNR, Centro per lo studio della Patologia Cellulare, Milano, [6]Clinica Pediatrica, Ospedale S.Gerardo, Università di Milano and [7]Dip. di Biopatologia Umana, Sezione Biologia Cellulare, Università di Roma, Italy
[5]Present address: NIMR, Mill Hill, London, UK

[3]The first two authors contributed equally to the work reported here

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The transcription factor GATA-1 is required for the normal development of erythroid cells. GATA-1 is also expressed in other hemopoietic cells, suggesting that it might be initially activated in a multipotent progenitor. To immortalize GATA-1-expressing progenitors, we generated mice transgenic for a thermosensitive SV40 T gene, driven by the GATA-1 promoter–enhancer. Immortalized marrow cells grow in culture at 32°C but not at 38°C, and are dependent on erythropoietin (Epo) or interleukin 3 (IL-3). Epo dependent cells express hemoglobin, high levels of GATA-1, GATA-2 and NF-E2 p45 mRNAs, and are positive for stem cell antigen 2 (Sca-2) and the early myeloid marker ER-MP12. IL-3 dependent cells can be derived from Epo dependent lines, and are hemoglobin-, Sca-2- and ER-MP12-negative, have low GATA-1 and NF-E2 p45 mRNA levels, and express myeloid markers Mac-1, F4/80 and Gr-1. Brief treatment of Epo dependent cells with myeloid growth factors (plus Epo) leads to the induction of Mac-1, F4/80 and Gr-1, concomitant with the disappearance from most cells of Sca-2, ER-MP12 and GATA-1 driven T antigen nuclear expression. Thus, the immortalized Epo dependent cells have the property of a progenitor capable of differentiation towards either the erythroid or myeloid lineages. These cells initiate transcription of a proportion of GATA-1 RNA molecules at an upstream promoter, previously known to be expressed only in testis cells.

Key words: alternative promoters/erythroid cells/erythropoietin/transcription factor

Introduction

The many cell types which are present in the blood and their precursors in the bone marrow are all derived from a single pluripotent progenitor, the hemopoietic stem cell. Commitment to specific lineages is thought to occur by progressive loss of potential for alternative fates (Suda *et al.*, 1984).

The molecular events underlying lineage choice are not well understood. Cytokines affect the production of blood cells by interacting with membrane receptors which regulate the proliferation of specific precursor cells and by maintaining their viability. In addition it is possible that cytokines may act as inducers of differentiation and lineage choice (Metcalf, 1989). Inductive (Dexter *et al.*, 1990), stochastic (Suda *et al.*, 1984) and hybrid (Fairbairn *et al.*, 1993) models of commitment have been proposed.

Another category of important regulators is represented by transcription factors. The gene encoding the transcription factor GATA-1 appears to play a major role in the development of the erythroid lineage. Chimeric mice resulting from embryonic stem (ES) cells in which GATA-1 had been disrupted by homologous recombination show a complete lack of red cells derived from the mutant ES cells (Pevny *et al.*, 1991). Conversely, forced expression of retrovirally transduced mouse GATA-1 genes in myeloid cells causes the down-regulation of myeloid markers and the appearance of megakaryocytic features (Visvader *et al.*, 1992). Similar results have been preliminarily reported in chicken cells (Graf, 1992). These changes correlate with the expression pattern of GATA-1, whose level was reported to be high in erythroblasts, megakaryocytes and mast cells, and very low or undetectable in mature monocytic or granulocytic cells. In addition, many genes are known to be targets of GATA-1 in erythroblasts or megakaryocytes (reviewed by Orkin, 1992), although other transcription factors, such as GATA-2, may substitute for GATA-1 in GATA-1 'knock-out' cells (Weiss *et al.*, 1994). In particular, regulatory elements of genes encoding globins, enzymes involved in heme synthesis, and membrane proteins including the erythropoietin receptor (EpoR) have GATA-1 binding sites, which appear to be functionally important in transfection experiments (Martin *et al.*, 1989; Mignotte *et al.*, 1989; Nicolis *et al.*, 1989; Chiba *et al.*, 1991; Talbot and Grosveld, 1991; Rahuel *et al.*, 1992; Ellis *et al.*, 1993).

If GATA-1 is involved in lineage choice, as suggested above, it will be important to identify the signals and the transcription factors which regulate it. In established erythroid cell lines, transfected GATA-1 genes are autoregulated by GATA-1 itself (Nicolis *et al.*, 1991; Tsai *et al.*, 1991) which binds to GATA-1 sites in an upstream enhancer which are absolutely necessary for activity (Nicolis *et al.*, 1991). However, GATA-1 is presumably first activated in a morphologically unidentified progenitor cell that is present at very low abundance in bone marrow. It has been hypothesized that this cell might be a multipotent erythro-megakaryocytic-myeloid progenitor (Martin *et al.*, 1990), and that GATA-1 is up-regulated during erythro-megakaryocytic differentiation and down-regulated during myeloid differentiation (Crotta *et al.*, 1990; Migliaccio *et al.*, 1991; Orkin, 1992; Sposi *et al.*,

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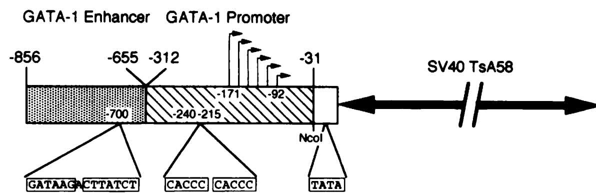


Fig. 1. Structure of the GATA-1 driven tsA58 expression construct. The SV40 large T thermosensitive mutant tsA58 (Jat *et al.*, 1991), truncated at the *Nco*I site within the promoter, is linked to the minimal promoter of the mouse GATA-1 gene, joined to the upstream enhancer (Nicolis *et al.*, 1991). Shown are the TATA-like box of the T gene promoter, the CACC boxes of the GATA-1 gene, and the dimeric GATA-1 sites in the enhancer. Multiple transcription start sites are indicated by arrows. Numbering of nucleotides is according to Nicolis *et al.* (1991).

1992). To identify and immortalize early progenitors expressing GATA-1, we obtained mice transgenic for an SV40 thermosensitive T gene driven by the promoter–enhancer region of GATA-1. The SV40 T gene serves both as an immortalizing agent and as a reporter for GATA-1 promoter activity. We describe the immortalization of a mouse bone marrow cell having properties of a multipotent GATA-1-expressing progenitor.

Results

Temperature and growth factor dependent immortalization of mouse bone marrow progenitors

The product of the SV40 T gene mutant tsA58 reversibly immortalizes cells at 32°C (the permissive temperature), but not at 38°C (the non-permissive temperature) (Jat *et al.*, 1991). We reasoned that, if GATA-1 is involved in lineage choice, the expression of tsA58 under the control of regulatory elements of the GATA-1 gene might lead to immortalization of hemopoietic progenitors at critical branchpoints of their development. We previously showed that the GATA-1 promoter is essentially inactive in transfection experiments, but can be converted into a powerful erythroid specific element by the addition of an upstream enhancer that is itself completely dependent for activity on a strong dimeric GATA-1 binding site (Nicolis *et al.*, 1991). We therefore linked this construct to the tsA58 gene at the upstream *Nco*I site to remove the SV40 21 bp repeat promoter elements and the usptream enhancer, which are both necessary for activity (Gorman *et al.*, 1982) (Figure 1). Using this construct, we generated nine transgenic mice. These mice did not show any obvious hematologic abnormality, as expected due to the lack of activity of tsA58 at mouse body temperature. Two of the founders (carrying five to 10 copies of the transgene per genome) were sacrificed, and their bone marrow was removed and cultured at 32°C in medium supplemented with erythropoietin (Epo) and 10% Wehi cell-conditioned medium as a source of interleukin 3 (IL-3). As shown in Figure 2B, these cells resemble 'myeloid' blasts with large nuclei and little basophylic cytoplasm. These cells (GATA-1 ts Wehi-Epo) are completely dependent on both the addition of growth factors and the permissive temperature. Depriving cells of growth factors leads to death of 55–80% of the cells within 5–6 days, with typical DNA degradation patterns (Rösl, 1992) indicating apoptosis; by 7–10 days the culture is completely extinct. 'Spontaneous' immortalization of mouse cells during *in vitro* culture is known to be a relatively common event. To test this possibility, we took advantage of the thermosensitivity of the SV40 tsA58 product. When the cells are switched to growth at 38°C, they die rapidly (within 24 h). This result rules out the possibility that GATA-1 ts Wehi-Epo cells are immortalized as the consequence of an unknown genetic event that occurred either *in vivo* or during *in vitro* propagation. Furthermore, when bone marrow from non-transgenic mice is grown *in vitro* at 32°C under the same conditions as GATA-1 ts Wehi-Epo cells, no immortalized cells can be recovered. Thus, GATA-1 ts Wehi-Epo cells are immortalized as the consequence of the activity of the SV40 T protein. The immortalization appears to be selective for hemopoietic cells, as stroma-derived adherent cells (i.e. fibroblasts, adipocytes and endothelia) are rapidly lost during the initial phase of the culture, even if care is taken to recover these cells at each passage.

The original GATA-1 ts Wehi-Epo cells can be switched from Wehi conditioned medium plus Epo to recombinant IL-3 plus Epo, or Epo alone. Under these conditions they maintain the same growth characteristics (Table I) indicating that, in the presence of Epo, most or all GATA-1 ts Wehi-Epo cells are independent of IL-3. This allowed us to develop cell lines which grow in the presence of Epo as the only added growth factor. These Epo dependent cells (subsequently named GATA-1 ts Epo, Figure 2C) have been grown continuously for >9 months without any apparent changes, and remain absolutely Epo- and temperature dependent (Table I). On the other hand, GATA-1 ts Wehi-Epo cells can also be switched to growth in IL-3 giving rise to GATA-1 ts IL-3 cells. Switching to IL-3, results in a slightly lower rate of growth (Table I) and lower saturation density (0.9×10^6 versus 1.2×10^6 cells/ml). However, no significant increase in cell mortality is observed upon switching, suggesting that a large proportion of the GATA-1 ts Wehi-Epo cells are responsive to IL-3. Following 3–5 weeks of growth in IL-3, the cells progressively develop a monocytoid-like morphology, with extensive vacuolization (Figure 2D). At this stage their growth rate is slightly increased (Table I). GATA-1 ts IL-3 cells might arise either by a gradual phenotypic transformation of Epo dependent progenitors displaying IL-3 receptors or by selection of a subpopulation of cells responsive exclusively to IL-3, or both. GATA-1 ts Epo cells can also be switched to grow in IL-3 alone (Figure 2 and Table I), and progressively develop antigenic and molecular characteristics (see below) similar to GATA-1 ts IL-3. On the other hand, GATA-1 ts IL-3 cells initially respond poorly to Epo alone (Table I), and only after several weeks of Epo treatment is efficient growth observed. This suggests that among the GATA-1 ts IL-3 cells there exist at a very low frequency Epo responsive cells which can be selected by culture in Epo. These cells, however, only partially reexpress the properties of GATA-1 ts Epo cells (see below).

GATA-1 ts Wehi-Epo and GATA-1 ts Epo cells show antigenic properties of early myeloid progenitors

Analysis by fluorescence activated cell sorting (FACS) shows that both GATA-1 ts Wehi-Epo and Epo cells are

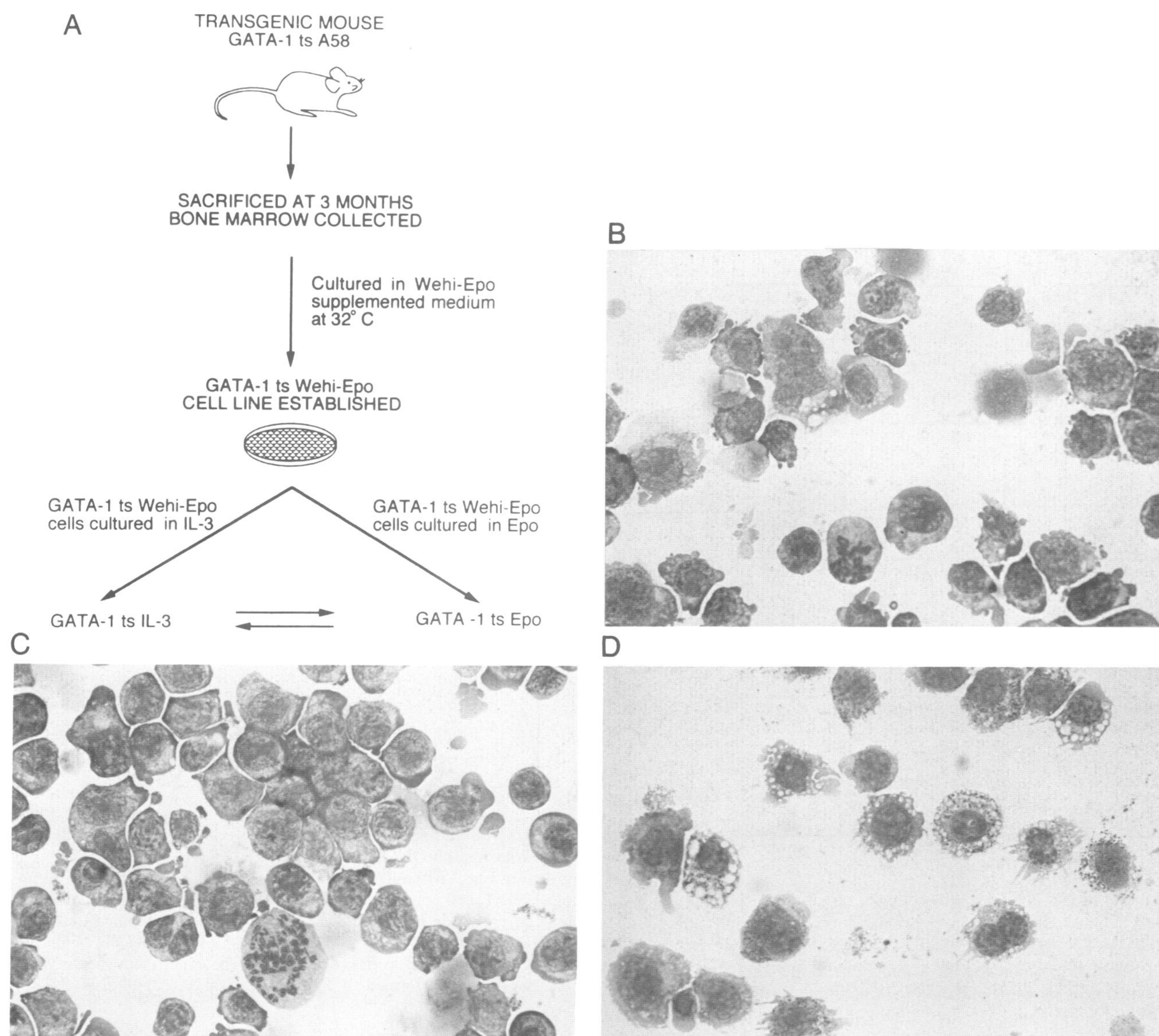


Fig. 2. Morphology of tsA58-immortalized GATA-1-expressing cell lines. (**A**) Outline of the experimental procedure. Note that IL-3 dependent lines with similar characteristics can be derived both from Wehi-Epo and Epo dependent cell lines. Similarly, Epo dependent cells can be derived from Wehi-Epo or IL-3 dependent lines, but in the latter case benzidine-positivity and some antigenic properties are not regained. (**B**) GATA-1 ts Wehi-Epo cells; (**C**) GATA-1 ts Epo cells; (**D**) GATA-1 ts IL-3 cells. Original magnification ×630.

Table I. Growth factor responsiveness and temperature dependence of GATA-1 ts cells

Cells	Doubling times (h) in the presence of growth factors at 32°C				
	Wehi-Epo[a]	Epo	IL-3	No factor	38°C[b]
GATA-1 ts Wehi-Epo	34 ± 6.5	35.5 ± 6.2	40.2 ± 3	−	−
GATA-1 ts Epo	ND	32.3 ± 6.4	38.9 ± 9.4	−	−
GATA-1 ts IL-3	ND	−/+	32.8 ± 8.6	−	−

Results are the averages of at least four experiments. ND: not done. − indicates no growth, followed by extinction of the population. −/+ indicates initial cessation of growth, followed by emergence of responsive cells after long-term (>3 weeks) treatment.
[a]Similar results were obtained in the presence of IL-3 and Epo instead of Wehi conditioned medium and Epo.
[b]Experiments were carried out in the presence of various combinations of Epo, IL-3, GM- and G-CSF and conditioned media.

mostly positive for stem cell antigen 2 (Sca-2), an early hemopoietic progenitor cell marker (Figure 3), although they are negative for stem cell antigen 1 (Sca-1, not shown), a marker of the pluripotent stem cell (Spangrude *et al.*, 1988; Spangrude and Scollay, 1990). In addition, most cells within the GATA-1 ts Wehi-Epo and Epo populations are positive for ER-MP12, an antigen that is present on 6–9% of bone marrow cells, representing early, but not late, myelo-monocytic precursors (Leenen *et al.*, 1990a,b). The panmyeloid CD45 antigen is also present.

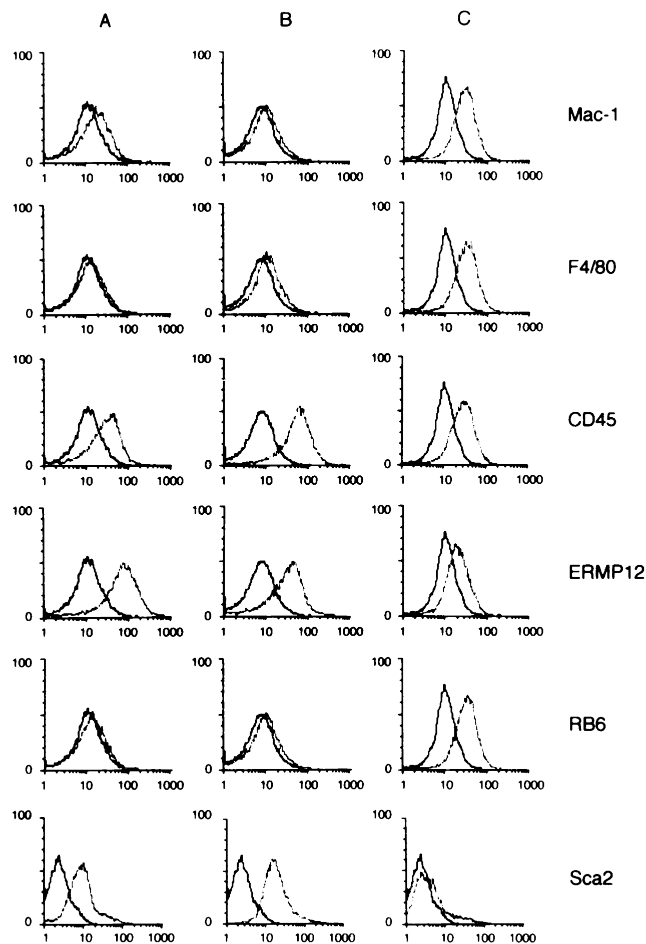


Fig. 3. Cytofluorimetric analyses of cell surface markers on (**A**) GATA-1 ts Wehi-Epo cells, (**B**) GATA-1 ts Epo cells and (**C**) GATA-1 ts IL-3 cells. Frequency versus FITC fluorescence intensity profiles of the markers indicated on the right are given by the thin line. The heavy line indicates non-specific staining due to the second antibody. The experiment shown is one out of six performed with similar results.

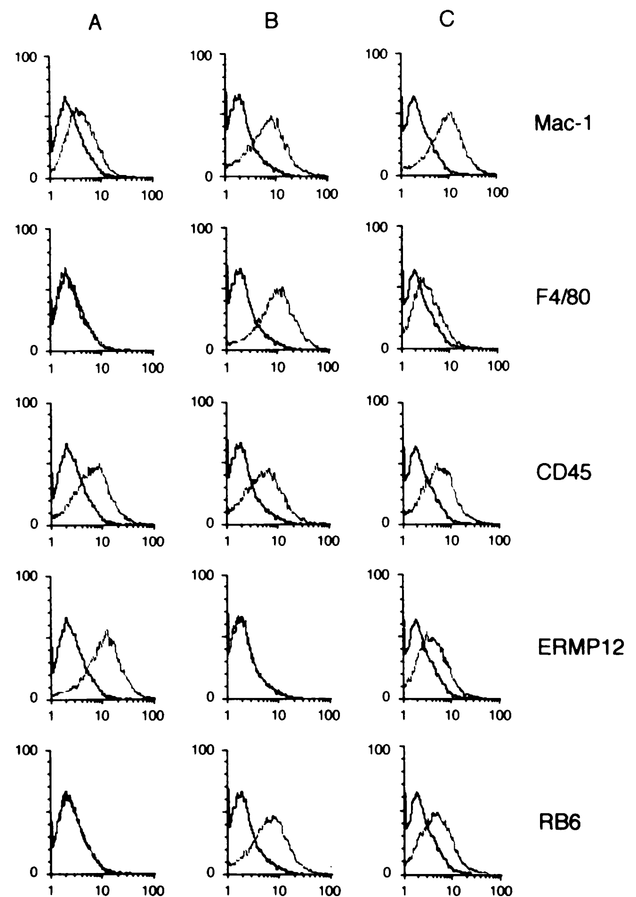


Fig. 4. Cytofluorimetric analyses of the effects of treatments with growth factors on GATA-1 ts Epo cells. (**A**) GATA-1 ts Epo cells grown in Epo, (**B**) GATA-1 ts Epo cells after 2 days of growth in Epo plus 10% L-cell conditioned medium, (**C**) GATA-1 ts Epo cells after 2 days of growth in Epo plus GM-CSF and G-CSF. Combined treatment with Epo, L-cell conditioned medium, GM-CSF and G-CSF yields a profile similar to that in (B) (not shown). The experiment shown is one out of four performed.

More specific lineage markers, such as F4/80 (monocytic lineage) (Austyn and Gordon, 1981; Starkey *et al.*, 1987), Mac-1 (monocytic and polymorphonucleate) (Springer *et al.*, 1979) and Gr-1 (RB-6) (granulocytic) (Holmes *et al.*, 1986) are either absent or expressed at low level (Mac-1) on small numbers of cells. In contrast, most GATA-1 ts IL-3 cells (irrespective of their derivation from Wehi-Epo or Epo dependent cells) clearly express F4/80, Mac-1 and Gr-1, lack Sca-2 and show a low level of ER-MP12. Thus, the antigenic pattern of the GATA-1 ts Wehi-Epo and Epo cells, taken together with their growth factor responsiveness, is consistent with their immature morphology and suggests an identification with a pluripotent erythroid-myeloid progenitor; the antigenic pattern and morphology of the GATA-1 ts IL-3 cells identify them as more advanced progenitors.

GATA-1 ts Epo cells can be induced to express myeloid markers

When GATA-1 ts Epo cells, continuously passaged in Epo for >3 months, are further treated with additional cytokines (in the presence of Epo), antigenic changes follow within 2–3 days. In particular, treatment with L-cell conditioned medium (as a source of M-CSF) leads to the complete disappearance of ER-MP12 (Figure 4) and Sca-2 (not shown), and to the expression on a large proportion of cells of Mac-1, F4/80 and Gr-1 (Figure 4). Treatment with recombinant GM-CSF and G-CSF has a similar effect, except that the disappearance of ER-MP12 is not always as complete, and F4/80 is not induced (Figure 4). Addition of GM-CSF and G-CSF along with L-cell conditioned medium slightly potentiates its effect.

No significant changes are obtained when IL-3 alone is substituted for Epo (not shown). These results indicate that cytokines active on the myeloid lineage can induce ER-MP12$^+$, Sca-2$^+$, Mac-1$^-$, F4/80$^-$ and Gr-1$^-$ to progress to some degree of myeloid differentiation, converting into ER-MP12$^-$, Sca-2$^-$, Mac-1$^+$, F4/80$^+$ and Gr-1$^+$ cells. It should be noticed that, when L-cell conditioned medium and myeloid cytokines are added to the culture in the absence of Epo, the cells stop growing and 20–30% of the cells die within 3 days, whereas in the same period treatment with Epo plus myeloid cytokines allows substantial growth (one or two cell doublings). This observation rules out the possibility that the observed effect of myeloid cytokines is simply due to the overgrowth of an

Epo-unresponsive myeloid progenitor population relative to the Epo responsive population.

Essentially identical results to those with GATA-1 ts Epo cells are obtained using GATA-1 ts Wehi-Epo cells; in contrast, the myeloid phenotype of GATA-1 ts IL-3 cells is not significantly modified by any of these treatments (not shown). As previously indicated (Figure 2, Table I) Epo responsive cells can be selected by long-term treatment of GATA-1 ts IL-3 cells; these cells never fully reexpress both ER-MP12 and Sca-2 (data not shown).

A clonal population of GATA-1 ts Epo cells can initiate myeloid differentiation upon treatment with myeloid cytokines

The GATA-1 ts Epo cells described in the previous paragraph may include a heterogeneous mixture of early hemopoietic progenitors. To minimize this heterogeneity, we obtained by limiting dilution at 0.5 cells/well clonal populations of GATA-1 ts Epo cells. One of the clones is Sca-2$^+$, ER-MP12$^+$, Mac-1$^-$ or Mac-1^{low}, F4/80$^-$ and Gr-1$^-$. When treated with L-cell conditioned medium and/or GM- and G-CSF (in the presence of Epo) these cells convert to Sca-2$^-$ or Sca-2^{low} (not shown), ER-MP12$^-$, Mac-1$^+$, F4/80$^+$ (Figure 5A). Induction of Mac-1 and F4/80 is obtained preferentially with L-cell conditioned medium, whereas Gr-1 induction is better obtained with GM-CSF and G-CSF (Figure 5B).

These data confirm that even a clonal Epo dependent population can switch on myeloid markers upon appropriate treatments.

The induction of myeloid differentiation correlates with extinction of GATA-1 promoter driven T antigen expression

The expression of the SV40 T gene product is expected to 'freeze' the cells in their actual state of differentiation. The question arises how the GATA-1−T gene behaves following cytokine-induced differentiation. Indirect immunofluorescence with a monoclonal antibody specific for the SV40 T antigen shows uniform positive fluorescence in ~95% of the GATA-1 ts Epo (Figure 6A) and the GATA-1 ts Wehi-Epo (not shown) cells. Following cytokine treatments, T antigen fluorescence is drastically reduced in a large proportion of GATA-1 ts Epo cells. In particular, after 3 days' culture in medium supplemented with Epo, G-CSF and GM-CSF, only 10% of the cells fluoresced brightly similar to GATA-1 ts Epo. Fifty percent fluoresced dully and 40% were completely negative (Figure 6C). When cells were cultured under the same conditions, with the further addition of L-cell conditioned medium, no brightly fluorescent cells were obvious but ~70% of the cells were weakly fluorescent and the remaining 30% were negative (not shown). In addition, cells maintained in IL-3 on a long-term basis, GATA-1 ts IL-3, show a uniformly low but significant level of T antigen staining (Figure 6B).

Taken together, these observations suggest that cytokine-induced myeloid differentiation leads to repression of the GATA-1−T gene construct. Presently, we cannot distinguish between two mechanisms. Cytokine treatments might primarily inhibit GATA-1-driven T gene expression, relieving the differentiation block. Alternatively, cytokines might force the cells into some degree of differentiation, secondarily causing decreased expression of the GATA-1−T gene. The two mechanisms are not mutually exclusive, and might reinforce each other.

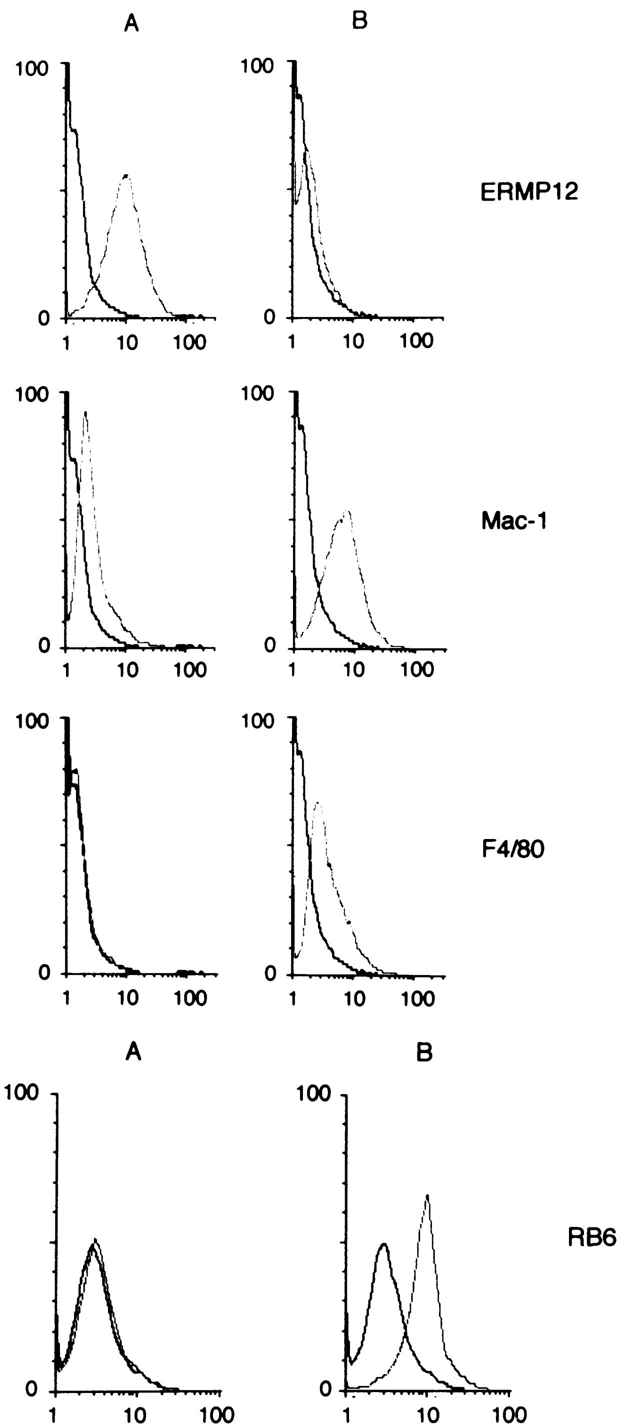


Fig. 5. Cytofluorimetric analyses of the effects of treatments with growth factors on cloned GATA-1 ts Epo cells. (**A**) Effect of L-cell conditioned medium. (A) Control cells grown in Epo. (B) Cells grown in conditioned medium plus Epo. (**B**) Effect of GM- and G-CSF. (A) Control cells grown in Epo. (B) Cells grown in GM and G-CSF plus Epo.

Northern analysis of erythroid and progenitor cell markers

As shown in Figure 7, GATA-1 ts Wehi-Epo and Epo cells express high levels of endogenous GATA-1 mRNA

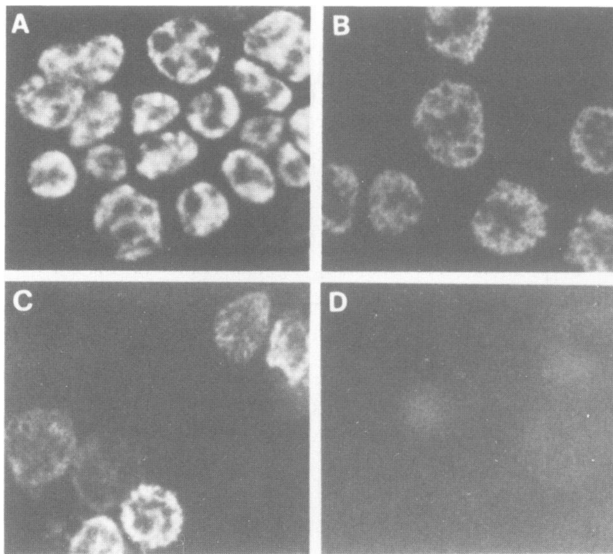
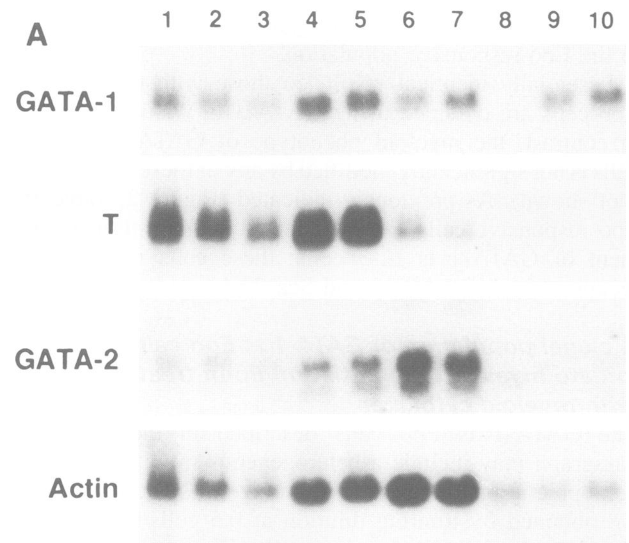


Fig. 6. Analysis by immunofluorescence of SV40 T antigen expression in cell lines. (A) GATA-1 ts Epo cells showing >95% positivity. Similar results were obtained with GATA-1 ts Wehi-Epo cells. (B) GATA-1 ts IL-3 cells. Most cells are weakly positive (C) GATA-1 ts Epo cells treated with L-cell conditioned medium, GM-CSF, G-CSF and Epo (see Figure 4B). Most cells are negative or very weakly positive; a few cells are still bright; (D) control Friend's cells stained with both first and second antibody. Similar results are obtained with GATA-1 ts Epo cells stained with the second (labeled) antibody only.



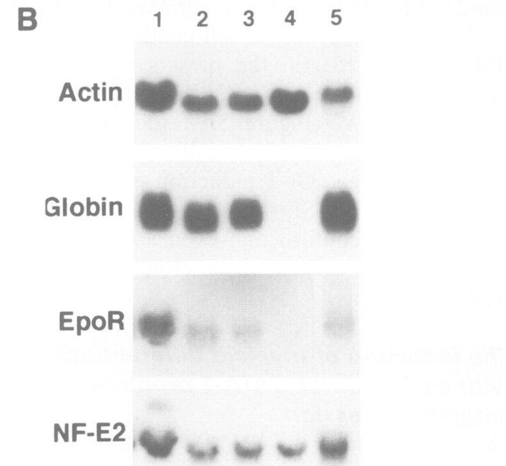


Fig. 7. Northern analysis of cell lines harboring the tsA58 transgene (A). The same filter was sequentially hybridized with probes representing mouse GATA-1, SV40 T gene, mouse actin and GATA-2. Lanes 1–3: GATA-1 ts Wehi-Epo cells, 10, 5 and 2.5 μg of total RNA. Lanes 4–5: GATA-1 ts Epo cells, two different clones, 10 μg of RNA. Lane 6: GATA-1 ts IL-3 cells, 20 μg of RNA. Lane 7: a myeloid subclone (obtained by limiting dilution) of GATA-1 ts Wehi-Epo cells, 20 μg of RNA. Note that lanes 6 and 7 were overloaded to allow visualization of weak T and GATA-1 bands. Lane 8: NIH 3T3 cells, 2.5 μg of RNA. Lane 9: mouse erythroleukemia (MEL) cells 2.5 μg. Lane 10: 32D Epo cells, 2.5 μg. (B) The filter was sequentially hybridized with probes representing mouse actin, β-globin, Epo R and NF-E2 p45. Lanes 1 and 2: GATA-1 ts Wehi Epo cells, 10 and 5 μg of total RNA. Lane 3: GATA-1 ts Epo cells, 5 μg of RNA. Lane 4: GATA-1 ts IL-3 cells, 10 μg of RNA. Lane 5: 32D Epo cells (Migliaccio et al., 1991), 5 μg of RNA. In (B), the exposure time was 16 h for hybridization to Epo R; note that the extremely weak EpoR band in lane 4 is clearly visible in the original and is clearly detected after 5 days exposure.

as well as of GATA-1-driven T gene mRNA; in addition, both cell types show high expression of NF-E2 p45 subunit mRNA (Andrews *et al.*, 1993), encoding a transcription factor important for the locus control region dependent activity of the globin gene clusters. Target genes of GATA-1 and/or NF-E2, such as β-globin (Figure 7B), α-globin (not shown) and EpoR (Figure 7B; compare with Friend's cells and another Epo dependent line), are highly expressed; the presence of globin mRNA correlates with the observation of 7-8% and 10-15% benzidine positivity, indicating hemoglobinization, in these cells. In contrast to GATA-1 ts Wehi-Epo and Epo cells, GATA-1 ts IL-3 cells have much lower, though still significant, levels of NF-E2 p45, GATA-1 and GATA-1 driven T mRNAs (Figure 7). The latter observation is consistent with results obtained by immunofluorescence (Figure 6A and B). However, in these cells, globin and EpoR mRNA levels are very low, detectable only after 8- to 20-fold overexposure, and benzidine staining is negative. A discrepancy between the presence of significant levels of GATA-1 and the absence or low expression of globin and EpoR has been reported previously in myeloid cells (Crotta *et al.*, 1990; Migliaccio *et al.*, 1991). Taken together with their inability to respond to Epo (Table I), these data suggest that some EpoR and globin synthesis may be confined to a very small subset of the population in GATA-1 ts IL-3 cells.

Another member of the GATA family, GATA-2, is known to be expressed in early progenitors, possibly in the same cells which express GATA-1, but its activity greatly declines relative to that of GATA-1 during terminal differentiation (Leonard *et al.*, 1993). GATA-2 mRNA is expressed in all three GATA-1 ts cell types (Figure 7A; compare with Friend's cells and an Epo responsive cell line).

An upstream GATA-1 promoter is active in GATA-1 ts Wehi-Epo and Epo cells

GATA-1 transcription in mouse erythroid cells starts at many sites within a G-rich, 90 nt long region, ~600 nt downstream of the GATA-1 dependent enhancer (Nicolis *et al.*, 1991, see Figure 1). The latter is probably implicated in maintaining GATA-1 transcription but is unlikely to be involved in the initial activation. It has been speculated (Ito *et al.*, 1993) that an additional promoter, located ~8 kb

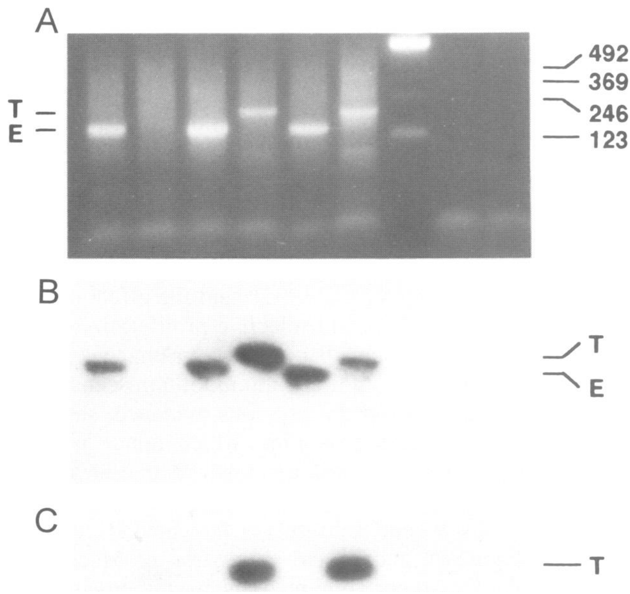


Fig. 8. RT-PCR analysis of the transcription start sites of the GATA-1 gene in GATA-1 ts Wehi-Epo and GATA-1 ts Epo cells. Following reverse transcription using a primer mapping to the second exon (common to the testis and erythroid transcripts), the transcripts corresponding to the specific testis and erythroid exons were differentially amplified. Aliquots from the amplification reaction (35 cycles) were loaded onto two parallel gels. After ethidium bromide staining (**A**) the two gels were blotted onto membranes and respectively hybridized to a cloned probe containing the first testis exon fused to the second (common) exon (**B**) or to an oligonucleotide specific to the first testis exon (**C**). Only one of the parallel ethidium bromide stained gels is shown. Erythroid- and testis-specific amplifications of each RNA sample are on alternate lanes. Lanes 1 and 2: RT-PCR from a myeloid subclone of 32D cells (Greenberger *et al.*, 1983). Lanes 3 and 4: GATA-1 ts Epo. Lanes 5 and 6: GATA-1 ts Wehi-Epo. Lane 7: size marker. Lanes 8 and 9: amplification carried out in the absence of added cDNA. Same result (not shown) when reverse transcription was omitted from the initial reverse transcription reaction. T and E indicate the testis and erythroid bands, respectively. Molecular weight markers are shown on the right.

upstream, and found to be active exclusively in testis cells, might be involved in the initial activation of GATA-1 in a rare progenitor cell. Taking advantage of the availability of early multipotent GATA-1 expressing cells, we looked for transcription of the testis promoter. RNA from GATA-1 ts Wehi-Epo and Epo cells was reverse transcribed using a primer mapping in the second exon, and amplified with *Taq* polymerase using a common antisense primer located in the second erythroid exon and specific sense 'testis' or erythroid first exon primers, respectively. Both the erythroid and 'testis' transcripts are detected in GATA-1 ts Wehi-Epo and Epo cells (Figure 8A). The identity of the amplified 'testis' band was confirmed both by hybridization to an internal testis-specific oligonucleotide (Figure 8C) and by sequencing (not shown). Blotted RT-PCR at low cycle numbers (25-30) indicates that the testis transcript is ~2–3% of the erythroid transcript (not shown).

Discussion

Previous models addressing the initial activation of GATA-1 suggested two alternative hypotheses. According to these hypotheses, GATA-1 is either activated independently in early committed precursors belonging to each of the lineages in which it is known to be expressed (i.e. erythroid, megakaryocytic, basophilic and possibly myelo-monocytic) or it is activated in an earlier progenitor common to several lineages (Crotta *et al.*, 1990; Martin *et al.*, 1990; Romeo *et al.*, 1990; Orkin, 1992; Sposi *et al.*, 1992). The available evidence favors the latter model; indeed, a number of multipotent hematopoietic cell lines do express very low levels of GATA-1 (Crotta *et al.*, 1990; Heberlein *et al.*, 1991; Orkin, 1992). However, this evidence is indirect, as the analysis was not carried out at the single cell level; the low GATA-1 expression observed might simply reflect the presence within the multipotent cell population of a small number of cells already committed to terminal differentiation. A similar problem arises with FACS purified cells, representing a mixture of multipotent and lineage committed progenitors, which express low levels of GATA-1 after 2–3 days of *in vitro* growth and differentiation (Sposi *et al.*, 1992).

In this work we approached these problems by the use of an immortalizing gene driven by GATA-1 regulatory elements. We asked several questions. (i) Can hemopoietic progenitors be immortalized by such a construct? (ii) If so, are these progenitors multipotent or committed to specific lineages? (iii) How does the level of expression of the construct relate to the type of progenitor immortalized?

We immortalized two different types of growth-factor dependent cells. These cells appear to be progenitors 'frozen' at early stages, and able to initiate, but not to complete, the process of differentiation. GATA-1 ts IL-3 cells respond to, and are dependent on, IL-3 exclusively. Most cells express myeloid antigens Mac-1, F4/80 and Gr-1 (Figure 3), but are negative for Sca-2 and the very early myelo-monocytic ER-MP12 antigen. As expected, the cells do not significantly express globin and EpoR RNAs (Figure 7B). Intriguingly, however, these cells express significant levels (Figure 7B) of mRNA encoding the p45 subunit of transcription factor NF-E2 (Andrews *et al.*, 1993), a molecule that was previously reported in erythroid and megakaryocytic cells and in multipotent cell lines (Romeo *et al.*, 1990; Andrews *et al.*, 1993). In addition, GATA-2 mRNA, which is expressed in early myeloid and erythroid progenitor populations, but greatly declines during terminal differentiation (Leonard *et al.*, 1993; Mouthon *et al.*, 1993), is also present at relatively high levels (Figure 7A). Thus, we suggest that GATA-1 ts IL-3 cells might be at a stage immediately downstream of a bipotential myeloid-erythroid progenitor.

GATA-1 ts Epo cells actively grow in response to either Epo, as the sole added growth factor, or IL-3. When Epo is withdrawn from GATA-1 ts Epo cells cultured in the presence of Epo only, the cells stop growing and the culture becomes extinct within a few days. These data indicate that most GATA-1 ts Epo cells have an erythroid characteristic, Epo dependence, and are ultimately derived from an erythroid (Epo dependent) progenitor. Additionally, these cells show a high level of NF-E2 p45 and globin RNAs (Figure 7) and a significant proportion are benzidine positive, i.e. hemoglobinized. In spite of these erythroid properties, the large majority of these cells are positive for the early myeloid antigen ER-MP12 (Figure 3) and, upon stimulation with myeloid cytokines, most

cells progress to express the myeloid antigens F4/80, Mac-I and Gr-1, while switching off ER-MP12 (Figure 4). This is true for both the total (Figure 4) and the clonal (Figure 5) GATA-1 ts Epo cells. Finally, GATA-1 ts Epo cells are positive (Figure 7A) for the multipotent progenitor marker GATA-2. Thus, we suggest that most GATA-1 ts Epo cells may be immortalized at a level corresponding to a myeloid-erythroid progenitor.

Current models suggest that the ability to proliferate and differentiate in response to Epo is the property of a cell already committed (BFU-E or CFU-E) to the erythroid lineage (Metcalf, 1989; Youssoufian *et al.*, 1993). However, from our results it is clear that many of the GATA-1 ts Epo cells are not irreversibly committed and can still be diverted towards myeloid differentiation. Interestingly, several human cell lines, (discussed in Hermine *et al.*, 1992) arising from leukemic patients, are able to express some myeloid and erythroid markers concomitantly, although only one shows long-term Epo responsivness. Whether these properties arise as the consequence of the malignant process is not known; however, these leukemic cells might represent the 'frozen' progeny of a normal human progenitor (Papayannopoulou *et al.*, 1988) similar to that described in this work. In addition, 'early' erythroid chicken cells obtained by E26 leukemia virus transformation have been shown to extinguish erythroid markers and to differentiate into myeloid cells following treatment with tumor promoters or superinfection with retroviruses containing kinase-type or *ras* oncogenes (Graf *et al.*, 1992). However, while the chicken leukemic 'erythroblasts' are triggered into myeloid differentiation by non-physiological stimuli, in our system murine erythroid cells can be switched towards a myeloid phenotype simply by treating them with cytokines. These observations in three different experimental models suggest that early 'erythroid' cells capable of differentiation into myeloid cells do exist in man, chicken and mouse.

Does the progenitor immortalized in our experiments represent the first cell to express GATA-1? As the promoter driving tsA58 expression is strongly dependent on GATA-1 itself, it is expected that as soon as a cell expresses a sufficient level of GATA-1 to activate the construct, this cell will become immortalized. Sufficient levels of tsA58 are apparently attained even in myeloid GATA-1 ts IL-3 cells expressing very low GATA-1 levels (Figures 6 and 7), possibly due to the relatively high copy number of the transgene. This suggests that at least some cells in our population may be close to the first cell expressing GATA-1. It is of interest that at least some GATA-1 transcripts arise from an upstream promoter, previously shown to be active only in testis cells. It has been hypothesized that this promoter may be the first to be expressed in early hemopoietic cells (Ito *et al.*, 1993); work in progress (with A.R.Migliaccio *et al.*, New York) indicates that these transcripts are already expressed and more abundant in highly purified early progenitors from normal mouse bone marrow. The fact that our cells, contrary to other erythroid lines such as Friend cells (Ito *et al.*, 1993) express the 'testis' transcript, is further in keeping with their immature phenotype.

An important aspect of GATA-1 biology is the observation (Crotta *et al.*, 1990; Migliaccio *et al.*, 1991) that GATA-1 is expressed at relatively high levels in some early myeloid lines, and at much lower or undetectable levels in more mature myeloid cells. These data indirectly suggested that GATA-1 is selectively down-modulated during myeloid differentiation. In addition, mixed progenitors (BFU-E, CFU-GM, CFU-mix) grown *in vitro* in the absence of Epo, transiently express GATA-1 before differentiating into myeloid cells (Sposi *et al.*, 1992; Leonard *et al.*, 1993). However, in these experiments it is not possible to assess which cells express GATA-1 (discussed in Leonard *et al.*, 1993), and the effect of Epo deprivation on apoptosis and selective proliferation could not be taken into account. By monitoring changes in the level of T antigen by indirect immunofluorescence, we now confirm and extend the previous evidence, showing at the single cell level that cytokines which induce myeloid differentiation lead to a striking down-regulation of the GATA-1 promoter construct. The inverse correlation between GATA-1 gene activity (as indicated by experiments in Figures 6 and 7) and expression of Mac-1 and other myeloid markers (Figure 4) is in agreement with the recent observation (Visvader *et al.*, 1991) that, in a myeloid cell line, forced expression of GATA-1 results in a substantial loss of Mac-1 expression. These results suggest that GATA-1 might, directly or indirectly, inhibit the expression of some myeloid markers.

The SV40 tsA58 gene has been previously utilized by others conditionally to immortalize selected cell populations from transgenic mice (Jat *et al.*, 1991; Whitehead *et al.*, 1993). However, its use in conjunction with regulatory regions of a gene encoding a key tissue-specific transcription factor is novel, and might be of general significance for other developmental systems. Within the hemopoietic system, the regulation and the role of the 'testis' promoter may now be studied both by transfection of our cell lines with β-galactosidase constructs or in transgenic mice using further T constructs. Moreover, work is in progress to select additional lines responsive to other growth factors, such as stem cell factor. Finally, the reversibility of the SV40 tsA58 induced immortalization indicates that conditions might be developed for achieving differentiation of these cells either *in vitro* or upon reinjection into mice.

Materials and methods

DNA constructs

The GATA-1 driven tsA58 construct (Figure 1) was obtained by linking the GATA-1 promoter–enhancer described by Nicolis *et al.* (1991) to the mutant SV40 large T tsA58 (Jat *et al.*, 1991). In order to remove the upstream enhancer–promoter elements of the SV40 gene, the following strategy was adopted. First, the GATA-1 promoter–enhancer construct (with enhancer in the sense orientation) (Nicolis *et al.*, 1991) was amplified by PCR using the following primers: sense: a 5′ tail containing an *Eco*RI site followed by nucleotides −856 to −837 of the GATA-1 construct; antisense: a tail containing (5′ to 3′) *Eco*RI and *Nco*I sites, followed by nucleotides −31 to −52 of the GATA-1 construct.

The amplified DNA was cloned into the pUC18 *Eco*RI site upstream of the *Kpn*I–*Bam*HI tsA58 fragment provided by P.Jat. The DNA comprised between the newly introduced and the SV40 *Nco*I sites was deleted by *Nco*I digestion, thereby removing the *Kpn*I–*Nco*I region including the SV40 enhancer and all the promoter functional elements (Gorman *et al.*, 1982). Following religation and plasmid amplification, *Eco*RI and *Bam*HI digestion was used to recover the desired fragment.

Transgenic mice

DNA fragments were purified from vector sequences by agarose gel electrophoresis and injected into the pronuclei of fertilized eggs from a

cross of BDF1 animals (C57 Bl/6×DBA/2). Transgenic animals were identified by Southern blotting analysis of tail DNA. Copy number was estimated by comparison with different loadings of plasmid DNA.

Bone marrow cultures

Marrow cells were obtained from 2–3 month old transgenic mice, by flushing from the femura with PBS. Cells were dissociated by repeat pipetting and plated at 32°C in RPMI-1640 (Bio-Whittaker; Walkersville, MD) supplemented with 10% fetal calf serum (Seromed, Berlin), 10% Wehi-conditioned medium (Bazill *et al.*, 1983), human recombinant Epo (CILAG, AG, Schaffausen, Switzerland) (0.2 U/ml) L-glutamine (2 mM), 50 μM 2-mercaptoethanol (Sigma, St Louis, USA) and antibiotics.

Initially, adherent cells were recovered at each passage after gentle treatments with trypsin, and replated together with the cells which grow in suspension; however, adherent cells were lost after three or four passages (2–3 weeks). Cells were subsequently maintained in flasks at concentrations of $2.5–7.5\times10^5$/ml by bi- or tri-weekly passages. Epo and IL-3 dependent cells were obtained by switching the original Wehi-Epo dependent cells to media containing Epo (0.5 U/ml) or mouse recombinant IL-3 (Bio Source Int.) (5 ng/ml) instead of Wehi-Epo. IL-3 and Epo dependent cell lines were similarly derived also from Epo and IL-3 dependent cells, respectively.

For determination of doubling times cells were plated at 2.5×10^5/ml in the presence of the appropriate growth factor at 32°C. The number of viable cells was estimated daily Trypan blue. When cells reached densities of $>6\times10^5$/ml they were replated at 2.5×10^5/ml. The cultures were maintained for at least 2 weeks.

For short-term treatments with myeloid growth factors, GATA-1 ts Wehi-Epo and Epo cells were repeatedly washed with PBS and cultured for 48–72 h in media supplemented with 0.5 U/ml Epo plus 10% L-cell conditioned medium (Waheed and Shadduck, 1979) and/or recombinant mouse GM-CSF (Pharmingen, San Diego, CA) (0.5 ng/ml) and human G-CSF (Amgen) (50 ng/ml).

Cloning of GATA-1 ts Epo cells was carried out by limiting dilutions in 96-well plates in 0.5 U/ml Epo at 10, 5 and 0.5 cells/well. Only clones growing in wells with 0.5 cells/well were picked and expanded.

Cell surface markers and flow cytometry

Expression of cell surface markers was evaluated by indirect immunofluorescence. Cells (5×10^5) were incubated with the specific monoclonal antibody at 4°C for 30 min. Antibodies included anti-Sca-2 (kindly provided by I.Weissman, Stanford University, CA), ER-MP12 (BMA Biomedicals), anti-Mac-1 (clone MI/70) (Serotec), anti-Gr-1 (clone RB6-8C5) (kindly provided by R.M.Steinman, the Rockefeller University, New York), anti-F4-80 (kindly provided by S.Gordon, Oxford, UK) and anti-CD-45 (Boehringer Mannheim, Mannheim, Germany). Cells were washed three times in PBS supplemented with 1% bovine serum albumin and incubated with fluorescein isothiocyanate-labeled goat $F(ab')_2$ anti-Rat IgG (H+L) (Southern Biotechnology Inc., Birmingham, AL). Unrelated isotypic antibodies were used for negative control staining. Stained cells were analyzed with a FACScan (Becton Dickinson) using the LYSIS II program.

Detection of SV40 nuclear T antigen in cells

For localization of large T antigen, cells were cytospun onto glass slides. After a brief wash in PBS, cells were fixed in 3.7% paraformaldehyde in PBS for 30 min at room temperature, rinsed in PBS and then permeabilized with 0.4% Triton X-100 (Sigma) for 30 min. Cells were incubated with a specific anti-SV40 large T antigen mAb (pAb 419) (Oncogene Science Inc. New York) for 30 min followed by a FITC-labeled goat anti-mouse IgG (H+L). Cells were mounted in Fluoromount-G (Southern Biotechnology Inc., Birmingham, AL) and examined with a Zeiss fluorescence microscope equipped with oil immersion objectives.

Benzidine assay

Benzidine-HCl (Cooper *et al.*, 1974) was used to detect the pseudo-peroxidase activity of hemoglobin in cell suspensions or methylcellulose colonies.

RNA analysis

Total cell RNA was prepared according to Chomczynski and Sacchi (1987). RNA samples were fractionated in 1% agarose containing 20 mM MOPS, 5 mM EDTA, 5 mM sodium acetate, pH 7.0 and 0.66 M formaldehyde as previously described (Crotta *et al.*, 1990), transferred to Hybond N membranes (Amersham) and hybridized according to standard protocols (Sambrook *et al.*, 1989). NF-E2 (Andrews *et al.*, 1993) and GATA-2 (a 700 bp 5′ *Eco*RI fragment) cDNAs were kindly provided by N.Andrews and S.Orkin; for other probes, see Crotta *et al.* (1990) and Migliaccio *et al.* (1991).

RT-PCR of GATA-1 transcripts

One microgram of RNA, heated for 5 min at 90°C, was incubated for 1 h at 37°C in 20 μl of a reaction mixture containing 1 mM deoxynucleotides, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM DTT, 10 mM Tris–HCl pH 9, 0.1% Triton X-100, 20 U of RNAsin (Promega), 200 U of Moloney leukemia virus reverse transcriptase and 100 pmol of a primer complementary to nucleotides 143–164 of the GATA-1 cDNA sequence (Tsai *et al.*, 1989). Following reverse transcription, 10 μl of the mixture were directly diluted in a 50 μl mixture containing the same salts (except DTT) as the previous reaction, and amplified with 2.5 U of *Taq* DNA polymerase (Promega) with 100 pmol of a 'common' antisense primer complementary to nucleotides 118–139 (Ito *et al.*, 1993) of GATA-1 cDNA and 100 pmol of either a 'testis' (nucleotides 1–20; Ito *et al.*, 1993) or erythroid (nucleotides 1–22; Tsai *et al.*, 1989) specific first exon sense primer.

The reactions were heated at 95°C for 3 min, followed by 25–35 cycles of DNA reannealing (66°C, 1 min), elongation (72°C, 90 s) and denaturation (94°C, 75 s). Aliquots of the amplification reaction were fractionated in 3% Metaphor agarose (FMC Bioproducts, Rockland) in Tris–borate buffer at 50 V for 3–5 h, stained with ethidium bromide and transferred to Hybond N^+ membranes (Amersham). Hybridization was carried out according to standard protocols (Sambrook *et al.*, 1989) in 3×SSC at 60°C when using a 'testis' specific oligonucleotide probe (nucleotides 23–44; Ito *et al.*, 1993) and at 65°C when using a probe recognizing both the 'testis' first exon and the 'common' GATA-1 second exon. This probe is a cloned fragment corresponding to the 184 bp band originally described by Ito *et al.* (1993) which we obtained by amplification from mouse bone marrow cDNA. Washing of the membranes was at 65°C at a final stringency of 0.5×SSC (for testis oligonucleotide) and 0.1×SSC (for the common probe).

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References

Andrews,N.C., Erdjument-Bromage,H., Davidson,M.B., Tempst,P. and Orkin,S.H. (1993) *Nature*, **362**, 722–728.

Austyn,J.M. and Gordon,S. (1981) *Eur. J. Immunol.*, **11**, 805–815.

Bazill,G.W., Haynes,M., Garland,J. and Dexter,T.M. (1983) *Biochem. J.*, **210**, 747–759.

Chiba,T., Ikawa,Y. and Todokoro,K. (1991) *Nucleic Acids Res.*, **19**, 3843–3848.

Chomcynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156.

Cooper,M.C., Levy,J., Cantor,L.N., Marks,P.A. and Rifkind,R.A. (1974) *Proc. Natl Acad. Sci. USA*, **71**, 1677–1680.

Crotta,S., Nicolis,S., Ronchi,A., Ottolenghi,S., Ruzzi,L., Shimada,Y., Migliaccio,A.R. and Migliaccio,G. (1990) *Nucleic Acids Res.*, **18**, 6863–6869.

Dexter,T.M., Heyworth,C.M., Spooncer,E. and Ponting,I.L.O. (1990) *Phil. Trans. Roy. Soc. (Lond.) B*, **327**, 85–98.

Ellis,J., Talbot,D., Dillon,N. and Grosveld,F. (1993) *EMBO J.*, **12**, 127–134.

Fairbairn,L.J., Cowling,G.J., Reipert,B.M. and Dexter,T.M. (1993) *Cell*, **74**, 823–832.

Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.

Graf,T. (1992) *EMBL Research Report*, pp. 46–48.

Graf,T., McNagny,K., Brady,G. and Frampton,J. (1992) *Cell*, **70**, 201–213.

Greenberger,J.S., Sakakeeny,M.A., Humphries,R.K., Eaves,C.J. and Eckner,R.J. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 2931–2935.

Heberlein,C., Fischer,K.D., Stoffel,M., Nowock,J., Ford,A., Tessmer,U. and Stocking,C. (1992) *Mol. Cell. Biol.*, **12**, 1815–1826.

Hermine,O. *et al.* (1992) *Blood*, **80**, 3060–3069.
Holmes,K.L., Langdon,W.Y., Fredrickson,T.N., Coffman,R.L., Hoffman,P.M., Hartley,J.W. and Morse,H.C.,III (1986) *J. Immunol.*, **137**, 679–688.
Ito,E., Toki,T., Ishihara,H., Ohtani,H., Gu,L., Yokoyama,M., Engel,J.D. and Yamamoto,M. (1993) *Nature*, **362**, 466–468.
Jat,P.S., Noble,M.D., Ataliotis,P., Tanaka,Y., Yannoutsos,N., Larsen,L. and Kioussis,D. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5096–5100.
Leenen,P.J.M., Melis,M., Slieker,W.A.T. and Van Ewijk,W. (1990a) *Eur. J. Immunol.*, **20**, 15–25.
Leenen,P.J.M., Melis,M., Slieker,W.A.T. and Van Ewijk,W. (1990b) *Eur. J. Immunol.*, **20**, 27–34.
Leonard,M., Brice,M., Engel,D.J. and Papayannopoulou,T. (1993) *Blood*, **82**, 1071–1079.
Martin,D.I.K., Tsai,S.F. and Orkin,S.H. (1989) *Nature*, **338**, 435–438.
Martin,D.I.K., Zon,L.I., Mutter,G. and Orkin,S.H. (1990) *Nature*, **344**, 444–447.
Metcalf,D. (1989) *Nature*, **339**, 27–30.
Migliaccio,A.R., Migliaccio,G., D'Andrea,A., Baiocchi,M., Crotta,S., Nicolis,S., Ottolenghi,S. and Adamson,J.W. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 11086–11090.
Mignotte,V., Eleouet,J.F., Raich,N. and Romeo,P.H. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 6548–6552.
Mouthon,M.A., Bernard,O., Mitajavila,M.T., Romeo,P.H., Vainchenker, W. and Mathieu-Mahul,D. (1993) *Blood*, **81**, 647–654.
Nicolis,S., Ronchi,A., Malgaretti,N., Mantovani,R., Giglioni,B. and Ottolenghi,S. (1989) *Nucleic Acids Res.*, **17**, 5509–5516.
Nicolis,S., Bertini, C.,Ronchi, A., Crotta,S., Lanfranco,L., Moroni,E., Giglioni,B. and Ottolenghi,S. (1991) *Nucleic Acids Res.*, **19**, 5285–5291.
Orkin,S.H. (1992) *Blood*, **80**, 575–581.
Papayannopoulou,T., Nakamoto,B., Kurachi,S., Tweeddale,M. and Messner,H. (1988) *Blood*, **72**, 1029–1038.
Pevny,L., Simon,M.C., Robertson,E., Klein,W.H., Tsai,S.F., D'Agati,V., Orkin,S.H. and Costantini,F. (1991) *Nature*, **349**, 257–260.
Rahuel,C., Vinit,M.A., Lemarchandel,V., Cartron,J.P. and Roméo,P.H. (1992) *EMBO J.*, **11**, 4095–4102.
Romeo,P.H., Prandini,M.E., Joulin,V., Mignotte,V., Prenant,M., Vainchenker,W., Marguerie,G. and Uzan,G. (1990) *Nature*, **344**, 447–449.
Rösl,F. (1992) *Nucleic Acids Res.*, **20**, 5243.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual.* 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Spangrude,G.J. and Scollay,R. (1990) *J. Immunol.*, **145**, 3661–3670.
Spangrude,G.J., Heimfeld,S. and Weissman,I.L. (1988) *Science*, **241**, 58–62.
Sposi,N.M. *et al.* (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6353–6357.
Springer,T., Galfré,G., Secher,D.S. and Milstein,C. (1979) *Eur. J. Immunol.*, **9**, 301–306.
Starkey,P.M., Turley,L. and Gordon,S. (1987) *Immunology*, **60**, 117–122.
Suda,T., Suda,J. and Ogawa,M. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 2520–2524.
Talbot,D. and Grosveld,F. (1991) *EMBO J.*, **10**, 1391–1398.
Tsai,S.F., Martin,D.I.K., Zon,L.I., D'Andrea,A.D., Wong,G.G. and Orkin,S.A. (1989) *Nature*, **339**, 446–451.
Tsai,S.F., Strauss,E. and Orkin,S.H. (1991) *Genes Dev.*, **5**, 919–931.
Visvader,J.E., Elefanty,A.G., Strasser,A. and Adams,J.M. (1992) *EMBO J.*, **11**, 4557–4564.
Waheed,A. and Shadduck,R.K. (1979) *J. Lab. Clin. Med.*, **94**, 180–194.
Weiss,M.J., Keller,G. and Orkin,S.H. (1994) *Genes Dev.*, **8**, 1184–1197.
Whitehead,R.H., VanEeden,P.E., Noble,M.D., Ataliotis,P. and Jat,P.S. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 587–591.
Youssoufian,H., Longmore,G., Neumann,D., Yoshimura,A. and Lodish,H.F. (1993) *Blood*, **81**, 2223–2236.

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