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The transcription factors c-*myb* and GATA-2 act independently in the regulation of normal hematopoiesis

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ABSTRACT **The transcription factors c-*myb* and GATA-2 are both required for blood cell development *in vivo* and *in vitro*. However, very little is known on their mechanism(s) of action and whether they impact on complementary or overlapping pathways of hematopoietic proliferation and differentiation. We report here that embryonic stem (ES) cells transfected with c-*myb* or GATA-2 cDNAs, individually or in combination, underwent hematopoietic commitment and differentiation in the absence of added hematopoietic growth factors but that stimulation with c-*kit* and *flt*-3 ligands enhanced colony formation only in the c-*myb* transfectants. This enhancement correlated with c-*kit* and *flt*-3 surface receptor up-regulation in c-*myb*-(but not GATA-2-) transfected ES cells. Transfection of ES cells with either a c-*myb* or a GATA-2 antisense construct abrogated erythromyeloid colony-forming ability in methyl cellulose; however, introduction of a full-length GATA-2 or c-*myb* cDNA, respectively, rescued the hematopoiesis-deficient phenotype, although only c-*myb*-rescued ES cells expressed c-*kit* and *flt*-3 surface receptors and formed increased numbers of hematopoietic colonies upon stimulation with the cognate ligands. These results are in agreement with previous studies indicating a fundamental role of c-*myb* and GATA-2 in hematopoiesis. Of greater importance, our studies suggest that GATA-2 and c-*myb* exert their roles in hematopoietic gene regulation through distinct mechanisms of action in nonoverlapping pathways.**

The process of blood cell formation rests on the ability of a small number of hematopoietic stem cells to undergo self renewal or commitment to lineage restricted progenitors. The mechanism(s) underlying these processes remains largely unclear; in contrast, the differentiation of committed progenitors is known to be controlled by lineage-specific transcription factors (e.g., the GATA-1 gene) that regulate the appearance of markers defining the properties of morphologically recognizable blood cells. Knock-out of transcription factor function in embryonal stem (ES) cells has been instrumental in determining the requirement of such genes for *in vitro* hematopoiesis and for blood cell formation *in vivo* (1–7). Those studies have pointed to the remarkable similarity among certain transcription factors with respect to the developmental stage and the phenotype associated with their loss of function (1–3). On the other hand, transcription factors such as GATA-2, c-*myb*, and PU.1 are expressed by multipotent progenitor cells and appear to regulate nonoverlapping stages of hematopoietic development (4–6). These observations prompted us to investigate whether there is a functional redundancy in hematopoiesis-specific transcription factors and whether they impact on different cytokine-restricted circuits of hematopoietic proliferation and differentiation.

We have recently shown that introduction of a c-*myb* full-length cDNA (but not a DNA binding-deficient mutant) in ES cells induces erythromyeloid commitment and accelerates erythromyeloid differentiation (7). This process appears to rest in the ability of c-*myb* to activate the expression of hematopoiesis-specific targets such as c-*kit*, *flt*-3, GATA-1, and CD34, but not GATA-2 (7). The induction of c-*kit* and *flt*-3 expression and the dependence of c-*myb*-transfected ES cells on the expression of these cytokine receptors for their proliferation (7) strongly suggest that the up-regulation of growth factor receptor levels is of fundamental importance for the expansion of progenitor cells. In turn, such a process is probably a requirement for completion of the differentiation program.

Using ES cells constitutively expressing c-*myb* and/or GATA-2, we asked whether these two genes synergize in inducing hematopoietic colony formation. In addition, by expressing c-*myb* or GATA-2 in ES cells in which the expression of either GATA-2 or c-*myb* has been disrupted by transfection with the corresponding antisense constructs, we asked whether we could rescue the GATA-2 or c-*myb* hematopoiesis-deficient phenotype. The results of these investigations suggest functional redundancy and mutual independence of these genes.

MATERIALS AND METHODS

Vector Construction. The pPolyII vector was generated by removing the *neo* gene from the pPol2shortneobpA vector (kind gift of Dr. S. May, The Johns Hopkins University, Baltimore, MD). pPolyII-*myb* was constructed as described previously (7). pXG2 was prepared by blunt-end ligation of *Bgl*II-linearized pXT1 vector (Stratagene, La Jolla, CA) and the Klenow-blunted full-length human GATA-2 cDNA (kind gift of Dr. Stuart H. Orkin) isolated by digestion with *Eco*RI. pV3-G2 was generated by cloning the *Eco*RI fragment including the full-length human GATA-2 cDNA in the *Eco*RI-linearized pPPDV3+ vector. The fragment of the murine c-*myb* cDNA from nucleotides 1231 to 1914 (8) synthesized by RT-PCR using mRNA from mouse erythroleukemia (MEL) cells was cloned into the pCRII vector (InVitrogen, Madison, WI), excised by digestion with *Eco*RI, and cloned in the *Eco*RI site of pPPDV3+ (9). The clone containing the c-*myb* sequence in the sense orientation with respect to the simian virus 40 early promoter was named pV3-Sm, whereas that containing the fragment in the antisense orientation was named pV3-ASm. A ~720-bp fragment of the murine GATA-2 cDNA sequence was amplified by RT-PCR using mRNA from MEL cells and primers as described (10), isolated by digestion with *Eco*RI, and cloned in the *Eco*RI site of pPPDV3+ (9). Clones with the GATA-2 sequence in the sense and the antisense orientations were named pV3-Sg and pV3-ASg, respectively.

Culture, Differentiation, and Transfection of ES cells. The ES-D3 cell line has been described (11). Undifferentiated ES cells were maintained in gelatin-treated tissue culture dishes in DMEM supplemented with 15% heat-inactivated fetal calf

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Abbreviations: ES, embryonal stem; RT, reverse transcriptase; LIF, leukemia inhibitory factor; Epo, erythropoietin; FITC, fluorescein isothiocyanate.

serum, 2 mM glutamine, 0.1 mM β-mercaptoethanol, and 1500 units of recombinant leukemia inhibitory factor (LIF) (Genetics Institute, Cambridge, MA). Differentiation was carried out in suspension culture in 100-mm bacterial Petri dishes in DMEM supplemented with 15% heat-inactivated fetal calf serum and 2 mM glutamine in the absence of LIF and with or without recombinant murine c-*kit* ligand (25 ng/ml) (Immunex, Seattle, WA), recombinant FLAG human *flt*-3 ligand (50 ng/ml) (Immunex Corp., Seattle), and erythropoietin (Epo) (3 units/ml) (Stem Cell Technology, Vancouver). Parental ES cells were transfected by electroporation with pXT1 or pXG2 and selected with G418 as described (7). Parental, insert-less and c-*myb*-transfected ES cells (7) were also electroporated with pPPDV3+ or pV3G2 and selected 24 h later with 5 μg/ml puromycin. After a 2-week selection in medium containing 3 μg/ml puromycin, expression of the transfected cDNAs was analyzed in several clones first by RT-PCR and then by Western blot analysis.

Western Blot Analysis. c-*myb* and GATA-2 proteins were detected in lysates of parental or transfected ES cells using anti c-*myb* (Upstate Biotechal, Lake Placid, NY) or anti-GATA-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) monoclonal antibodies as described (7). Lysates of MEL cells were used as positive controls.

Assays for Hematopoietic Precursors within Embryoid Bodies. At various times during differentiation, cells within embryoid bodies were dissociated and cultured in 0.9% methyl cellulose; 10^4 ES cells were plated in 35-mm diameter bacterial grade dishes. Colonies (>125 μm diameter) were scored 12 days later. Individual colonies were picked from the same dishes (20 single colonies/dish). RNA was extracted as described (12) and used for single-colony RT-PCR as described (13). This two-step *in vitro* differentiation assay was also performed in the presence of recombinant human Epo (3 units/ml), recombinant murine c-*kit* ligand (25 ng/ml), and recombinant FLAG human *flt*-3 ligand (50 ng/ml).

Expression of Hematopoietic Markers in ES-derived Colonies. RT-PCR analysis was performed for 30 cycles using a pair of synthetic primers corresponding to nucleotides 824–847 (5′ primer), and 1476–1500 (3′ primer) of the published murine myeloperoxidase sequence (14). Oligonucleotide primers for amplification of βH1-globin and a c-*fms* have been utilized as described (7). To exclude amplification from genomic DNA, RT-PCR reactions were also performed in the absence of reverse transcriptase. As a negative control, RT-PCR amplifications were performed in the absence of RNA. Amplified DNA was subjected to electrophoresis, transferred to Zetabind nylon filters (Cuno) and detected by Southern hybridization with a [γ-^{32}P]ATP end-labeled oligoprobe. Endogenous β-actin mRNA levels were also measured to ensure that similar amounts of RNA were utilized for mRNA expression analysis.

Cell Surface Marker Expression Analysis. Exponentially growing cells were harvested and incubated (30 min on ice in PBS containing 0.1% gelatin, 0.01% sodium azide, 5% fetal calf serum) with biotinylated murine c-*kit* or *flt*-3 ligand, irrelevant biotinylated horse IgG as negative control, rabbit antiserum to murine CD34 (kind gift of Dr. Laurence Lasky, Genentech), rabbit anti-mouse IgG (Sigma) as negative control, or monoclonal antibody to major histocompatibility class I (mouse IgG2a; Organon Teknika–Cappel) as positive control. Biotinylation was performed as described (15). Cells were washed and incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated avidin or goat anti-mouse or anti-rabbit Ig F(ab′)$_2$. Cells were washed and analyzed by flow cytometry on a EPICS Profile Analyzer (Coulter). Staining of cells harvested from 12-day methyl cellulose colonies from undifferentiated c-*myb*-transfected ES cells was performed with FITC-conjugated anti-mouse Ly-6G (GR-1), FITC-labeled anti-mouse c-*kit*, FITC-conjugated anti-mouse H-2K^b phycoerythrin-conjugated anti-mouse TER119/erythroid cell, and phycoerythrin-labeled anti-mouse H-2D^b, or irrelevant phycoerythrin- or FITC-labeled mouse IgG as negative control. All of these antibodies were obtained from PharMingen.

RESULTS

c-*myb* and GATA-2 Induce the Development of Hematopoietic Precursors from ES Cells. ES cells transfected with the full-length c-*myb* and/or GATA-2 cDNA expressed the respective protein constitutively (Fig. 1*A*), whereas the endogenous proteins were only detected during ES cell differentiation induced upon removal of LIF (not shown). Two different cell transfectants were used in methyl cellulose colony assays to test whether c-*myb* and/or GATA-2 regulate the development of hematopoietic precursors. Hematopoietic colonies formed from c-*myb*- and/or GATA-2-transfected ES cells (undifferentiated and at various days after LIF removal) plated in the absence of added hematopoietic growth factors (Fig. 1*B*). When cultures were supplemented with c-*kit* and *flt*-3 ligands and Epo (Fig. 1C), colony formation was significantly greater (especially for c-*myb*-transfected ES cells). Such increased colony formation by c-*myb*-transfected ES cells correlated with the ability of a significant proportion of these cells to express c-*kit* and *flt*-3 (Fig. 2). In contrast, GATA-2-transfected ES cells failed to express these surface receptors (Fig. 2). c-*myb*-, but not GATA-2-transfected ES cells, also expressed the CD34 antigen, a marker of hematopoietic stem/progenitor cells (16, 17). Immunophenotypic analysis of hematopoietic colonies grown in methycellulose for 14 days revealed the expression of the myeloid- and the erythroid-specific markers Gr-1 and TER119, respectively, in a high proportion of cells (Fig. 3). Morphological examination of cytospin preparations from pooled colonies revealed erythromyeloid precursor of various stages of differentiation (data not shown). RT-PCR assessment of individual colonies (20 per experiment) for the expression of erythroid (embryonal β-globin)- or myeloid (c-*fms* and myeloperoxidase)-specific markers indicated that 50–60% of the colonies were erythroid and 20–30% of the colonies were myeloid (not shown). In each culture condition, 10–20% of the colonies were negative for each of the markers tested (not shown).

c-*myb* and/or GATA-2 Constitutive Expression Rescues the Hematopoiesis-Deficient Phenotype of ES Cells with Disrupted GATA-2 or c-*myb* Expression. To inactivate c-*myb* or GATA-2 function, ES cells were transfected with a c-*myb* or a GATA-2 cDNA fragment cloned in the antisense orientation in the appropriate vectors (see *Materials and Methods*). Individual clones were first tested for down-regulation of either c-*myb* or GATA-2 protein and then selected for colony formation assays. All of the experiments were conducted using two different clonal populations, but for simplicity, representative experiments with single clones are shown. Following ES cell differentiation induced by LIF removal, Myb or GATA-2 protein was not detected in cells carrying the corresponding antisense construct (Fig. 4*A* lane 3); Myb or GATA-2 protein was readily detected in ES cells transfected with the GATA-2 or the c-*myb* antisense construct, respectively (Fig. 4*A* lane 3). The GATA-2 or the c-*myb* antisense construct also transfected in ES constitutively expressing either the c-*myb* or GATA-2 cDNA, in order to obtain GATA-2$^+$/myb$^-$ and myb$^+$/GATA-2$^-$ ES cells (Fig. 4*A*).

As expected, disruption of either c-*myb* or GATA-2 expression suppressed hematopoietic colony formation (Fig. 4*B*). The c-*myb*-deficient phenotype was rescued by constitutive expression of GATA-2 (Fig. 4*B*); conversely, the GATA-2-deficient phenotype was rescued by constitutive c-*myb* expression (Fig. 4*B*). Colony formation from ES cells constitutively expressing c-*myb* (but not GATA-2) was significantly higher in cultures grown in the presence of c-*kit* and *flt*-3 ligands and Epo

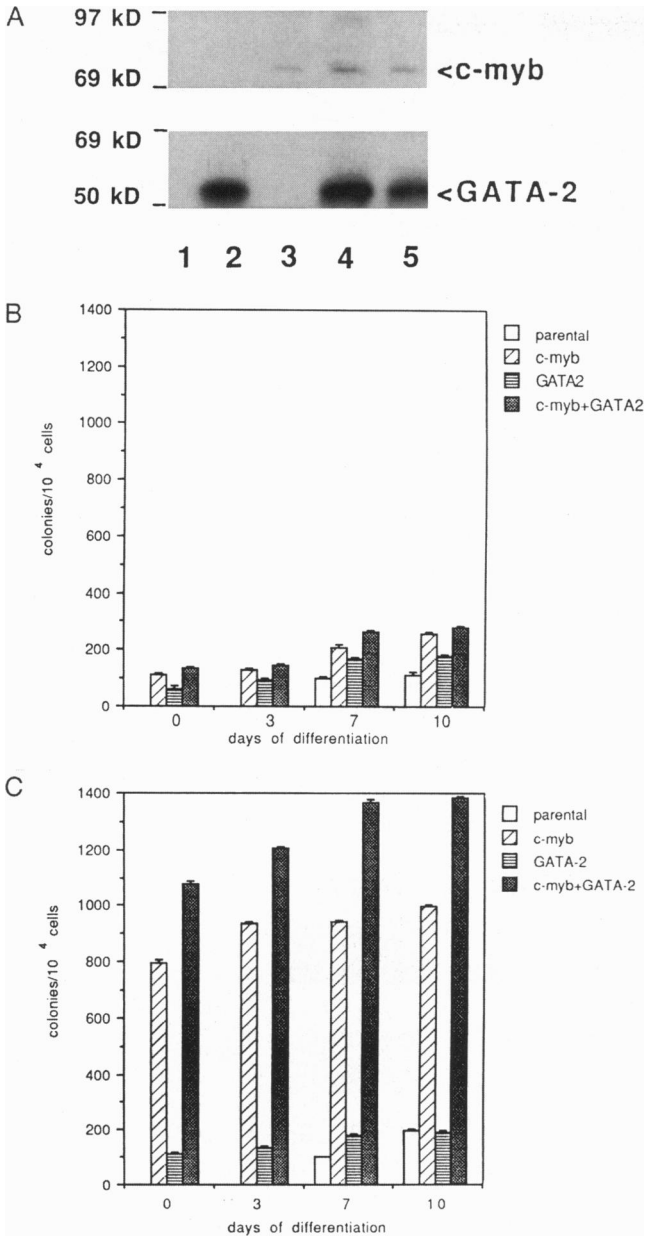

FIG. 1. Development of hematopoietic colonies from ES cells constitutively expressing c-*myb* and/or GATA-2. (*A*) Levels of c-*myb* (top) and GATA-2 (bottom) protein were determined by Western blot analysis in undifferentiated parental (lanes 1), pXG2-transfected (lanes 2), pPolyII-*myb*-transfected (lanes 3), pV3-G2 plus pPolyII-*myb*-transfected (lanes 4) ES cells and MEL cells as positive control (lanes 5). (*B*) Parental, pPolyII-*myb* (c-*myb*)-, pXG2 (GATA-2)-, and pPolyII-*myb* plus pV3-G2 (c-*myb*+GATA-2)-transfected ES cells were tested for colony formation in methyl cellulose. Days of differentiation are indicated. Error bars indicate ± SD of the mean of three independent experiments. (*C*) Parental, pPolyII-*myb* (c-*myb*), pXG2 (GATA-2), and pPolyII-*myb* plus pV3-G2 (c-*myb*+GATA-2) -transfected ES cells were tested for colony formation in methyl cellulose in the presence of *kit* ligand (25 ng/ml), *flt*-3 ligand (50 ng/ml), and Epo (3 units/ml). Days of differentiation are indicated on the bottom. Error bars indicate ± SD of the mean of three independent experiments.

(Fig. 4*C*). Such increased colony formation correlated with the ability of c-*myb* to up-regulate c-*kit* and *flt*-3 surface receptor levels in cells with disruption of GATA-2 expression (Fig. 5). Immunophenotyping of hematopoietic colonies in which the constitutive expression of c-*myb* or GATA-2 rescued the GATA-2-or *myb*-deficient phenotype, respectively, revealed comparable levels of expression of the myeloid (Gr-1) and the

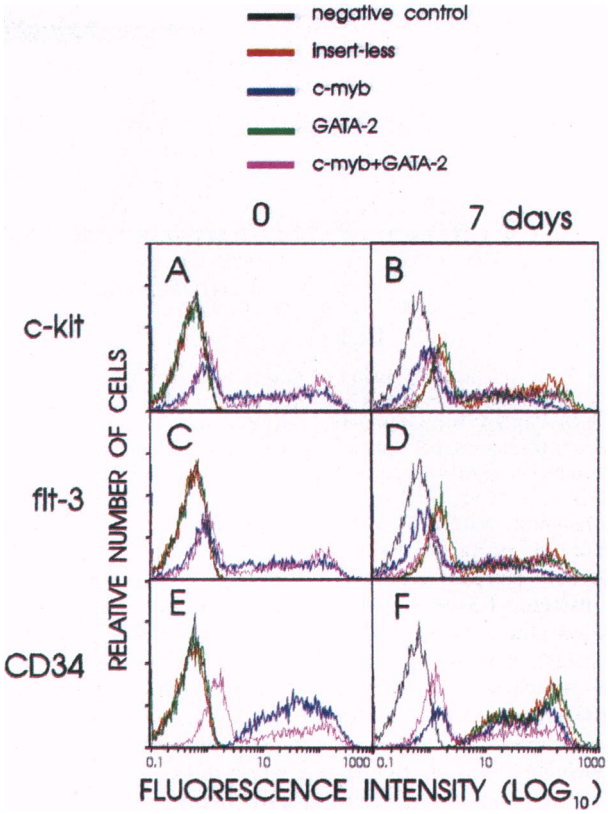

FIG. 2. Surface expression of c-*kit*, *flt*-3, and CD34 on ES cells constitutively expressing c-*myb* and/or GATA-2. Expression of c-*kit*, *flt*-3, and CD34 on pPolyII (insertless), pPolyII-*myb* (c-*myb*), pXG2 (GATA-2), and pPolyII-*myb* plus pV3-G2 (c-*myb* + GATA-2)-transfected ES cells, as determined by flow cytometry analysis, on day 0 (*A*, *C*, and *E*) and day 7 (*B*, *D*, and *F*). Data are representative of two separate experiments with superimposable results.

erythroid (TER119) markers tested (Fig. 6). Such levels were similar to those found in parental cells plated 7 days after LIF removal and grown under identical culture conditions. In agreement with the flow cytometry analysis, RT-PCR phenotyping of individual colonies assessed for β-globin, c-fms, and myeloperoxidase expression revealed that 50–60% of the colonies were erythroid and 20–30% of myeloid origin (not shown). A small fraction (10–20%) of colonies was negative for the expression of each marker tested.

DISCUSSION

The goal of the present study was to assess whether two transcription factors, c-*myb* and GATA-2, with a defined requirement in normal hematopoiesis act independently or regulate the same pathway(s) involved in the activity of hematopoiesis-specific cytokines. This is becoming a fundamental question as a wealth of *in vivo* and *in vitro* studies suggest that there are striking similarities in the phenotype of hematopoietic cells with altered (by disruption or overexpression) transcription factor function (18). Using a two-step assay of ES cells hematopoietic differentiation, we found that constitutive expression of either c-*myb* or GATA-2 induces a cohort of ES cells to undergo hematopoietic commitment and differentiation at earlier times than parental cells (Fig. 1). Interestingly, hematopoietic colony formation from c-*myb*-transfected ES cells was stimulated in cultures grown in the presence of c-*kit* and *flt*-3 ligands, whereas these cytokines had no apparent effect on GATA-2-transfected cells (Fig. 1). The co-expression of c-*myb* and GATA-2 stimulate ES colony-forming ability above the levels of cultures constitutively

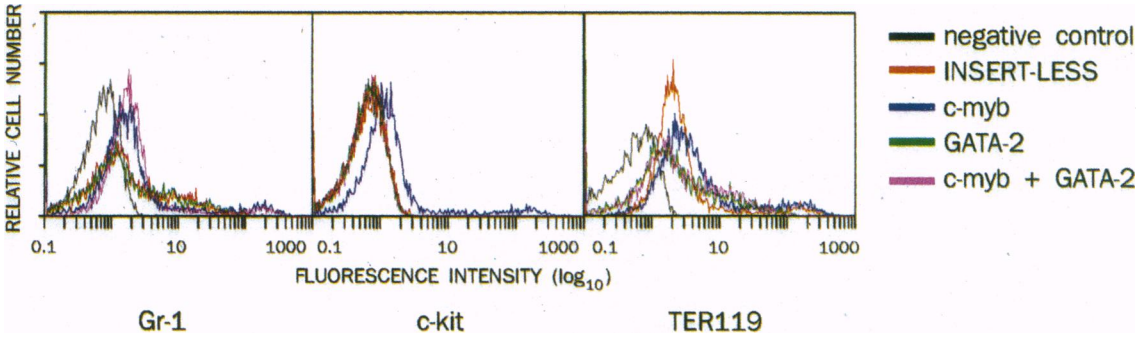

FIG. 3. Surface expression of GR-1, c-*kit*, and TER119 markers in colonies derived from ES cells constitutively expressing c-*myb* and/or GATA-2. Expression of GR-1, c-*kit*, and TER119 on pPolyll (insertless), pPolyll-*myb* (c-*myb*)-, pXG2 (GATA-2)-, and pPolyll-*myb* plus pV3-G2 (c-*myb*+GATA-2)-transfected ES cells, as determined by flow cytometry. Three days (c-*myb*, GATA-2, and c-*myb* + GATA-2) or 7 days (insertless) after LIF removal, ES cells were plated in methyl cellulose in the presence of *kit* ligand, *flt*-3 ligand and Epo and harvested 14 days later. Flow cytometry analysis was performed twice with superimposable results.

expressing individual transcriptional factors, but the effects were not synergistic. As observed previously (7), c-*kit* and *flt*-3 surface receptor expression was up-regulated in c-*myb*-transfected ES cells, and this correlated with c-*kit* and *flt*-3 ligand stimulation of colony formation (Figs. 1 and 2). In contrast, constitutive expression of GATA-2 had no effect on ES cell expression of these cytokine receptors (Fig. 2). These differences in the colony-forming ability of c-*myb*- and GATA-2-transfected ES cells suggest that the primary mechanism of c-*myb* activity is via the up-regulation of two cytokine receptors expressed and required by early hematopoietic cells (19, 20). In contrast, the lack of effects of constitutive GATA-2 expression on c-*kit* and *flt*-3 levels suggests that GATA-2 either acts on a c-*kit*/*flt*-3-independent pathway or, less likely, at a postreceptor level along the c-*kit*/*flt*-3 signal transduction-regulated pathways. The lack of effects of GATA-2 overexpression on c-*kit* levels is consistent with the detection of c-*kit* expression in homozygous deficient GATA-2 ES cells (4). Despite the differential ability of c-*myb* and GATA-2 to up-regulate, upon LIF removal, markers of early stages of hematopoiesis (Fig. 2), ES cells that constitutively expressed c-*myb* and/or GATA-2 formed normal-appearing hematopoietic colonies expressing late markers of erythroid and myeloid differentiation (Fig. 3 and data not shown).

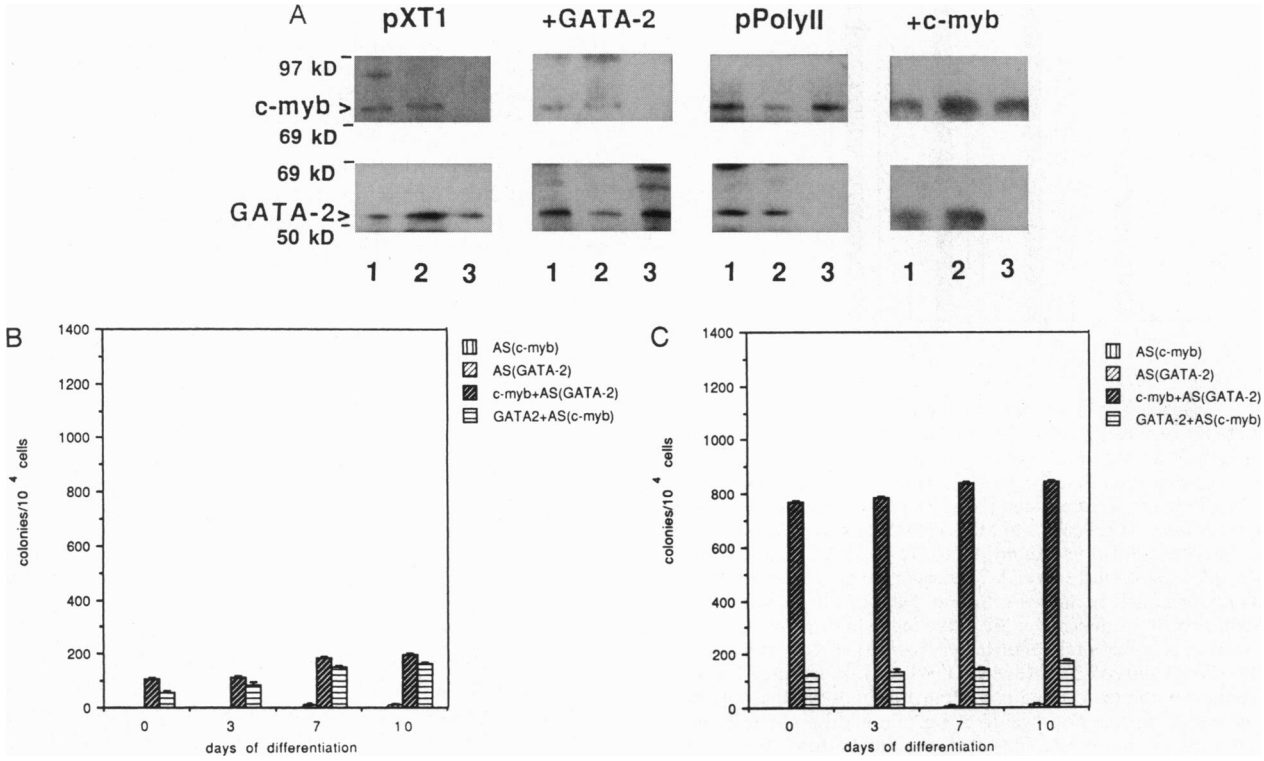

FIG. 4. Development of hematopoietic colonies from ES cells constitutively expressing either a c-*myb* or a GATA-2 antisense construct. (*A*) Levels of c-*myb* (top) and GATA-2 (bottom) protein were determined by Western blot analysis after 7 days of differentiation in pXT1 or pXG2 (+GATA-2) transfected ES cells after cotransfection with pPPDV3+ (lanes 1), pV3-Sm (lanes 2) or pV3-ASm (lanes 3); pPolyll or pPolyll-*myb* (+c-*myb*) transfected ES cells after cotransfection with pPPDV3+ (lanes 1), pV3-Sg (lanes 2) or pV3-ASg (lanes 3). (*B*) Development of hematopoietic colonies in methyl cellulose from pV3-ASm [AS(c-*myb*)], pV3-ASg [AS(GATA-2)], pPolyll-*myb* plus pV3-ASg [c-*myb*+AS(GATA-2)], and pXG GATA-2 plus pV3ASm [GATA-2 +AS(c-*myb*)] transfected ES cells. (*C*) Development of hematopoietic colonies in methyl cellulose in the presence of *kit* ligand, *flt*-3 ligand, and Epo from pV3-ASm [AS(c-*myb*)], pV3-ASg [AS(GATA-2)], pPolyll-*myb* plus pV3-ASg [c-*myb*+AS(GATA-2)], and pXG GATA-2 plus pV3ASm [GATA-2 +AS(c-*myb*)]-transfected ES cells. Days of differentiation are indicated on the bottom. Error bars indicate ± SD of the mean of three independent experiments.

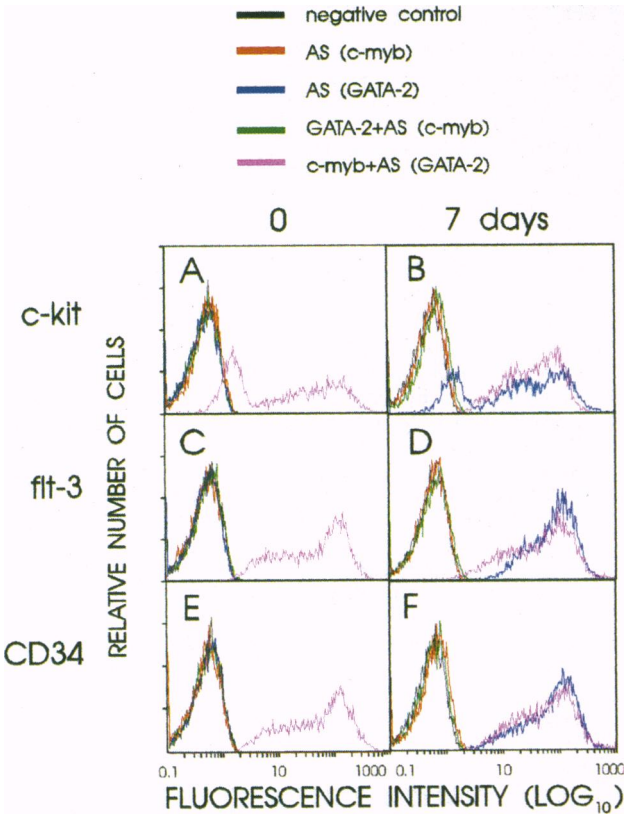

FIG. 5. Surface expression of c-*kit*, *flt*-3, and CD34 in GATA-2- or c-*myb*-deficient ES cells rescued by constitutive expression of c-*myb* or GATA-2. Expression of c-*kit*, *flt*-3 and CD34 on pPolyll (insertless)-, pV3-ASm [AS(c-*myb*)], pV3-ASg [AS(GATA-2)]-, pXG2 plus pV3-ASm [GATA-2+AS(c-*myb*)]-, and pPolyll-*myb* plus pV3-ASm [c-*myb*+AS(GATA-2)] transfected ES cells, as determined by flow cytometry analysis on day 0 (*A*, *C*, and *E*) and day 7 (*B*, *D*, and *F*). The analysis was performed twice with superimposable results.

Down-regulation of either GATA-2 or c-*myb* expression, obtained by transfection of ES cells with antisense constructs, was associated with suppression of colony formation (Fig. 4). In agreement with these observations, treatment of ES cultures with antisense oligodeoxynucleotides targeting either GATA-2 or c-*myb* mRNA was also associated with inhibition of colony formation (not shown). Interestingly, down-regulation of c-*myb* expression did not allow the up-regulation of c-*kit* and *flt*-3 normally occurring during differentiation of ES cells (7); in contrast, the absence of GATA-2 expression did not interfere with the up-regulation of these receptors during ES cell differentiation, and yet colony formation was suppressed.

We finally asked whether constitutive expression of either c-*myb* or GATA-2 could rescue the hematopoiesis-deficient phenotype of ES cells in which either GATA-2 or c-*myb* expression was down-regulated by transfection with antisense constructs. c-*myb* and GATA-2 were able to rescue the hematopoiesis-deficient phenotype of ES cells with disrupted expression of either GATA or c-*myb* protein (Fig. 4). The phenotype of rescued ES cells was remarkably similar, as indicated by flow cytometry analysis of late maturation antigens (Fig. 6) and marker gene expression in individual colonies (data not shown). However, c-*kit* and *flt*-3 expression was not up-regulated in ES cells with disrupted c-*myb* expression, suggesting that GATA-2 acts on a c-*kit*/*flt*-3-independent signal transduction pathway or activates such pathways at a post-receptor level.

A major mechanism of c-*myb* regulation of hematopoietic development appears to rest in its ability to activate cytokine receptor (i.e., c-*kit* and *flt*-3) expression; the rescue of the GATA-2-deficient phenotype implies that, in these cultures, the c-*kit* and *flt*-3 mediated post-receptor pathway is intact. The ability of GATA-2 to rescue, at least in part, the hematopoietic phenotype of c-*myb*-deficient ES cells is compatible with a post-receptor mode of action. However, a negative effect of GATA-2 down-regulation on c-*kit* and *flt*-3 post-receptor signaling seems unlikely, since c-*myb* constitutive expression would not rescue the GATA-2 hematopoiesis-deficient phenotype.

Together, our data suggest that c-*myb* and GATA-2 regulate distinct pathways of gene expression required for hematopoietic proliferation and differentiation, although we cannot exclude the possibility that these transcription factors impact on specialized properties of hematopoietic cells.

The regulation of ES cells-derived hematopoiesis by c-*myb* and GATA-2 via distinct nonoverlapping pathways as reflected in the ability of GATA-2 or c-*myb* to rescue the c-*myb* or GATA-2 hematopoiesis-deficient phenotype, is in apparent contrast with the inhibition of hematopoiesis by either c-*myb* or GATA-2 antisense constructs. Perhaps, only the enforced expression of transcription factors can substitute for compensatory changes in endogenous gene expression that might be associated with the knock-out of a given transcription factor. In this regard, endogenous GATA-2 expression is up-regulated in GATA-1 knock-out cells, and yet erythropoiesis is impaired (10).

However, GATA-1-deficient ES cells can be rescued by overexpression of members of the GATA family such as GATA-3 and GATA-4 (21), which are not normally expressed in erythroid precursors. In this regard, our studies have a

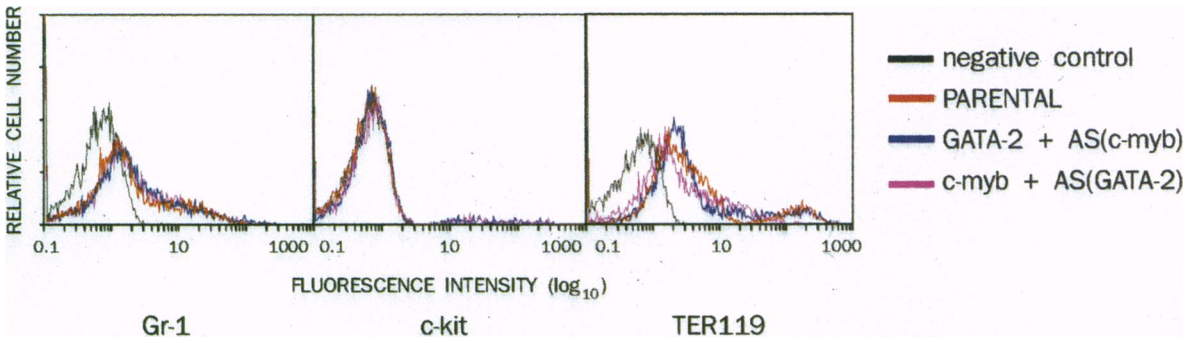

FIG. 6. Surface expression of GR-1, c-*kit*, and TER119/erythroid marker in colonies derived from GATA-2-or c-*myb*-deficient ES cells rescued by constitutive expression of c-*myb* or GATA-2. Expression of GR-1 and the erythroid marker recognized by monoclonal antibody TER119 was determined by flow cytometry analysis of parental ES cells and ES cells transfected with pPolyll (insertless), or -pV3-ASm [AS(c-*myb*)]-pV3-ASg [AS(GATA-2)], pXG2 plus pV3-ASm [GATA-2+(c-*myb*)], or pPolyll-*myb* plus pV3-ASm [c-*myb*+AS(GATA-2)]. Cells were harvested from colonies 14 days after plating in c-*myb*- or GATA-2-transfected ES cells in methyl cellulose at day 3 of differentiation in the presence of *kit* ligand, *flt*-3 ligand, and Epo. Insertless-transfected ES cells were plated 7 days after LIF removal. The analysis was performed twice with identical results.

broader significance since members of the GATA family act as transcription factors recognizing identical targets, whereas the sequence specificity of c-*myb* and GATA-2 is clearly different. The studies reported here may serve as a model for similar investigations to dissect overlapping, complementary, or even antagonistic functions of other transcription factors involved in the regulation of hematopoiesis.

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