GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblats, and erythroblasts

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The transcription factor GATA-1 is expressed in early hematopoietic progenitors and specifically down-regulated in myelomonocytic cells during lineage determination. Our earlier observation that the differentiation of Myb-Ets-transformed chicken hematopoietic progenitors into myeloblasts likewise involves a GATA-1 down-regulation, whereas expression is maintained in erythroid, thrombocytic, and eosinophilic derivatives, prompted us to study the effect of forced GATA-1 expression in Myb-Ets-transformed myeloblasts. We found that the factor rapidly suppresses myelomonocytic markers and induces a reprogramming of myeloblasts into cells resembling either transformed eosinophils or thromboblats. In addition, we observed a correlation between the level of GATA-1 expression and the phenotype of the cell, intermediate levels of the factor being expressed by eosinophils and high levels by thromboblats, suggesting a dosage effect of the factor. GATA-1 can also induce the formation of erythroblasts when expressed in a myelomonocytic cell line transformed with a Myb-Ets mutant containing a lesion in Ets. These cells mature into erythrocytes following temperature-inactivation of the Ets protein. Finally, the factor can reprogram a v-Myc-transformed macrophage cell line into myeloblasts, eosinophils, and erythroblasts, showing that the effects of GATA-1 are not limited to Myb-Ets-transformed myeloblasts. Our results suggest that GATA-1 is a lineage-determining transcription factor in transformed hematopoietic cells, which not only activates lineage-specific genetic programs but also suppresses myelomonocytic differentiation. They also point to a high degree of plasticity of transformed hematopoietic cells.

[**Key Words:** Hematopoietic cell differentiation; lineage commitment; GATA-1; viral oncogenes; transcription factors]

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Hematopoietic differentiation is believed to involve the progressive restriction of differentiation potential of pluripotent progenitors, ending with irreversibly committed cells of various lineages (Metcalf 1989; Fairbairn et al. 1993). Commitment is thought to be mediated by extracellular signals provided by hematopoietic growth factors as well as by cell-autonomous, stochastic events (Suda et al. 1984). Ultimately, it is assumed that a specific combination of transcription factors determines the phenotype of a cell. Although the function of transcriptional regulators in lineage-specific gene expression is well established in committed cells (e.g., Brown and David 1994, and references therein), their role in the lineage determination of multipotent progenitors remains poorly understood.

Among the different transcription factors that are known to control the expression of lineage-specific genes, GATA-1 has emerged as a candidate regulator of hematopoietic cell differentiation. GATA-1 was originally identified as an erythroid-specific factor involved in globin gene regulation (Evans and Felsenfeld 1989; Tsai et al. 1989). It has subsequently been found to be

expressed in megakaryocytes and mast cells (Martin et al. 1990; Romeo et al. 1990) where it regulates lineage-specific genes (Lemarchandel et al. 1993; Zon et al. 1991). Recently, expression of GATA-1 has also been reported for eosinophils, making neutrophil granulocytes and macrophages the only nonlymphoid hematopoietic cell types that do not express the factor (Sposi et al. 1992; Zon et al. 1993). Gene inactivation experiments in mouse embryonic stem cells demonstrated that GATA-1 is essential for terminal erythroid differentiation *in vivo* and *in vitro* (Pevny et al. 1991; Simon et al. 1992; Weiss et al. 1994). The apparent lack of an effect on other hematopoietic lineages could be explained by a functional redundancy. Thus, two related factors, GATA-2 and GATA-3, which share a highly conserved Zn finger-type DNA-binding domain with GATA-1 and recognize the same DNA consensus sequence, exhibit a partially overlapping expression pattern in hematopoietic cells (Yamamoto et al. 1990). GATA-2 is coexpressed with GATA-1 in most hematopoietic cell types, whereas the expression of GATA-3 is more restricted and most abundant in the T-cell lineage (Leonard et al. 1993a,b). Forced

expression of GATA-1, GATA-2, and GATA-3 has shown that each of these factors can induce megakaryocytic differentiation and the loss of Mac-1 antigen expression in a particular mouse myeloid cell line (Visvader et al. 1992; Visvader and Adams 1993). In spite of these similarities GATA family factors seem to be functionally distinct because GATA-2, but not GATA-1 or GATA-3, blocks terminal erythroid differentiation in vitro (Briegleb et al. 1993), and recent gene inactivation experiments indicate that GATA-2 controls the growth of early hematopoietic cells in vivo (Tsai et al. 1994).

In earlier work we developed an avian model system for assessing the influence of various signal transducers and transcription factors on hematopoietic lineage commitment by use of viral oncogenes to transform early hematopoietic progenitors. Two types of Myb-Ets progenitors [also designated as MEPs; Graf et al. 1992] can be obtained after infection of yolk sac cells derived from early chick embryos, depending on whether wild-type or mutated protein is used for transformation. As outlined in Figure 1, one type of progenitor transformed by the Myb-Ets-encoding E26 virus resembles predominantly thromboblats [the avian homologs of megakaryoblasts (Dieterlen-Lièvre 1988)] and can be induced to mature into thrombocytes following inactivation of Myb (Frampton et al. 1995). These progenitors, which differentiate spontaneously at low frequency along the erythroid lineage (Graf et al. 1992), can also be induced to differentiate into eosinophils or myeloblasts (macrophage-granulocyte precursors) after forced expression of Ras or kinase-type oncogenes or after treatment with the phorbol ester TPA (Graf et al. 1992). The other type of Myb-Ets-transformed progenitor, obtained after infection with a mutant containing a temperature-sensitive lesion in Ets, resembles erythroblasts and can be induced to mature into erythrocyte-like cells after shift to the nonpermissive temperature (Golay et al. 1988; Kraut et al. 1994) and into eosinophils and myeloblasts following TPA treatment (Kraut et al. 1994). Similar to what has been reported for the differentiation of normal he-

matopoietic progenitors (Sposi et al. 1994), GATA-1 is highly expressed in the thrombocytic and erythroid progenitors and is completely down-regulated during myelomonocytic differentiation (Graf et al. 1992). Here, we have addressed the role of GATA-1 in lineage determination by forcing its expression in Myb-Ets- and v-Myc-transformed chicken myelomonocytic cell lines. Our results show that ectopic expression of the factor suppresses the myelomonocytic phenotype of transformed myeloblasts and macrophages, converting them into cells resembling either eosinophils, thromboblats, or erythroblats.

Results

Clones derived from GATA-1 transfected Myb-Ets-transformed myeloblasts resemble either eosinophils or thromboblats

To determine whether GATA-1 can alter the phenotype of myelomonocytic cells, we introduced the cDNA into Myb-Ets-transformed myeloblasts. Because of the limited life-span of primary transformed myeloblasts we used a cell line (HD50M) that was derived from the thromblast-MEP cell line HD50 after treatment with the phorbol ester TPA and subsequent recloning. HD50M myeloblasts were electroporated with the retroviral expression vector pNEO-GATA-1 or with a control vector containing only the neomycin resistance gene (pSFCV). The transfected cells were seeded in semisolid medium containing G-418, and resistant colonies were scored and isolated 2–3 weeks after. In three separate electroporation experiments a total of 250 and 400 G-418-resistant colonies were observed with the GATA-1 expression vector and the control vector, respectively. Twenty-six of the clones transfected with pNEO-GATA-1 and 20 with pSFCV were isolated and, following expansion in liquid culture for 2 weeks, analyzed microscopically after cytocentrifugation and May-Grünwald-Giemsa staining. They were also tested for the expression of several lineage-restricted cell-surface antigens by use of monoclonal antibodies specific for myelomonocytic cells (MYL51/2, and cIa, which detects the major histocompatibility (MHC) class II complex), eosinophilic cells (EOS47, which detects melanotransferrin, K. McNagny and T. Graf, unpubl.) and thrombocytic cells (MEP21, and 11C3, which detects the integrin $\alpha_{IIb}\beta_3$).

As summarized in Table 1, all of the 20 clones transfected with the control vector resembled the original HD50M myeloblast line both morphologically and in their expression of MYL51/2 antigen. In contrast, dramatic changes in morphology and the pattern of cell-surface antigen expression were observed in most of the GATA-1-transfected clones analyzed. Five clones were indistinguishable from the parental HD50M myeloblasts, consisting of large cells with a weakly basophilic cytoplasm (Fig. 2A) and expressing MYL51/2 and MHCII (Fig. 2D). Six clones consisted of small cells, many of which exhibited cytoplasmic granules (Fig. 2B). These were MYL51/2⁺, MHCII⁺, EOS47⁺, MEP21⁺, and

Figure 1. Phenotype and differentiation potential of Myb-Ets-transformed hematopoietic cells. The expression of GATA-1 is indicated by cross-hatching of the nuclei (for explanation, see text).

Table 1. Correlation of ectopic GATA-1 expression and phenotypic changes induced in transfected myeloblasts

Phenotype ^a	Number of clones transfected with		GATA-1 expression level in pNEO-GATA-1-transfected clones ^b		
	pSFCV	pNEO-GATA-1	–	{ + / – }	+
Myeloblast	20	5	3	2	0
Eosinophil	0	6	0	0	6
Thromboblast	0	5	0	0	5
Myeloblast/eosinophil	0	9	0	0	9
Myeloblast/eosinophil/thromboblast	0	1	0	0	1

^aThe phenotypes of individual clones are determined with antibodies against lineage-specific cell-surface antigens.

^bGATA-1 expression was analyzed by Western blotting. (–) No detectable expression; { + / – } marginally detectable GATA-1 expression, corresponding to ~1% of the level detected in thromboblast-MEPs; (+) GATA-1 expression corresponding to at least 10% of the level observed in thromboblast-MEPs.

$\alpha_{\text{IIB}}\beta_3^-$ (Fig. 2E). Five clones consisted of small cells, which tended to grow in aggregates, and exhibited a strongly basophilic cytoplasm (Fig. 2C). They were MYL51/2[–], MHCII[–], MEP21⁺, $\alpha_{\text{IIB}}\beta_3^+$ and contained a subpopulation of EOS47⁺ cells during the first weeks of cultivation (Fig. 2F). Nine clones consisted of MYL51/2⁺ and EOS47⁺ subpopulations, and one clone contained subpopulations of MYL51/2⁺, EOS47⁺ and MEP21⁺ cells.

The finding that a high proportion of the pNEO-GATA-1-transfected HD50M clones expressed either EOS47 or MEP21 antigens suggested that they had converted into both eosinophils and thromboblasts. To characterize these cells in more detail we analyzed the expression of an additional set of differentiation markers in three phenotypically altered clones of each type and in three randomly chosen pSFCV transfected clones, as controls. All three EOS47⁺ GATA-1-transfected clones

contained peroxidase-positive granules (>50% of the cells) and expressed low amounts of MEP26 antigen (shared between a subset of eosinophilic cells, thrombocytic, and immature erythroid cells) but not the erythroid/thrombocyte-specific histone H5 (Fig. 3A) or hemoglobin. In comparison, the three MEP21⁺ clones contained a low percentage (<5%) of peroxidase/EOS47⁺ cells, 20%–80% of histone H5-expressing cells (Fig. 3A), and high levels of MEP26 antigen but no hemoglobin. As expected, the clones transfected with the control vector showed no significant differences to the parental myeloblasts in any of the markers tested. As shown by the Northern blot in Figure 3B, all three eosinophil- and thromboblast-like clones down-regulated the expression of the mRNA for the invariant chain of the MHCII complex. In contrast, the eosinophil but not the thromboblast clones retained expression of C/EBP- β and *mim-1*.

The marker expression pattern of the altered clones in

Figure 2. Morphology and cell-surface antigen expression of HD50M myeloblasts transfected with GATA-1. May-Grünwald-Giemsa staining (A–C) and FACScan profiles (D–F) of three morphologically distinct G-418-resistant GATA-1-transfected HD50M clones. The cell-surface antigens tested are indicated.

Figure 3. Marker expression by eosinophil- and thromboplast-like HD50M-GATA-1 clones. (A) Histone H5 immunofluorescence of myeloblast-, eosinophil-, and thromboplast-like clones. Note the nuclear fluorescence in the cells of the thromboplast-like clone. (B) Northern blot with 10 μ g of total RNA hybridized sequentially with the indicated probes. (Lane 1) Parental HD50M myeloblast cell line; (lane 2) eosinophil cell line derived from HD50 thromboplasts by TPA treatment; (lane 3) original HD50 thromboplast cell line; (lanes 4–6), myeloblast-like HD50M clones transfected with the empty vector; (lanes 7–9) eosinophil-like HD50M-GATA-1 clones; (lanes 10–11) thromboplast-like HD50M-GATA-1 clones. (C) Summary of the lineage-specific markers expressed in GATA-1-transfected myeloblast clones as compared with Myb-Ets-transformed myeloblast, eosinophil, and thromboplast cell lines. The data were obtained from three or more GATA-1-transfected HD50M clones of each type. To simplify the representation, the percentage of marker-positive cells and relative expression levels are indicated by the filling of the boxes (1/4 = weak; 1/2 = moderate; 3/4 = high; 1 = maximal).

comparison to HD50 thromboplasts, HD50-derived eosinophils, and HD50-derived myeloblasts is summarized in Figure 3C. It illustrates that the HD50 myeloblast clones reprogrammed by exogenous GATA-1 expression are essentially indistinguishable from the original HD50 thromboplasts or HD50-derived eosinophils. It is not yet clear whether GATA-1-induced thromboplasts are multipotent, as is the case for primary Myb-Ets-transformed cells. By use of an inducible GATA-1 construct it should be possible to obtain the corresponding cell types and to determine whether in the absence of the inducer these thromboplasts can be triggered to differentiate along the myelomonocytic and eosinophilic lineages. Preliminary experiments with HD50 clones transfected with a constitutively expressed GATA-1 showed that phorbol-ester treatment fails to induce their differentiation along the myelomonocytic lineage; instead, they begin to express hemoglobin and high levels of eosinophilic markers.

The induced phenotype correlates with the GATA-1 expression level

To determine whether there is a correlation between the phenotype of a particular GATA-1-transfected clone and its GATA-1 expression level, cell extracts were prepared and subjected to Western blotting. As summarized in Table 1, all of the 21 GATA-1-transfected clones that exhibited an altered phenotype expressed significant levels of GATA-1 protein. In contrast, of the five clones that remained unaltered, three were completely negative, whereas two expressed marginally detectable levels of GATA-1 protein. In addition, we observed a slight but significantly higher GATA-1 expression level in thromboplasts as compared with eosinophils. These results are compatible with the notion that a threshold amount of GATA-1 expression is both necessary and sufficient to cause a phenotypic change. Therefore, the GATA-1 expression levels of three randomly chosen eosinophil and thromboplast clones were each examined quantitatively. As can be seen from the Western blot in Figure 4A, all six clones expressed high levels of GATA-1 protein, comparable to or higher than that of the original HD50 cell line. In addition, the thromboplast clones expressed more GATA-1 than those resembling eosinophils. To distinguish between exogenous and endogenous GATA-1 expression, RNA from each clone was isolated and analyzed by Northern blotting. As shown in Figure 4B, all six HD50M-GATA-1 clones expressed the 4.2-kb full-length viral and the 2.5-kb spliced GATA-1 mRNA and, in addition, the 1-kb endogenous GATA-1 mRNA. Quantification of the GATA-1 bands relative to those of the β -actin control by use of a PhosphorImager (Fig. 4C) showed that all thromboplast HD50M-GATA-1 clones expressed two to four times as much GATA-1 mRNA as the eosinophil clones. Interestingly, a similar ratio was also seen between the GATA-1 mRNA levels of the original HD50 and the HD50 eosinophil cell line. However, the total expression level was found to be 4–10 times as

Figure 4. Expression of GATA-1 in HD50M-derived eosinophils and thromboblats. (A) Western blot with extracts of 10^6 cells, probed with anti GATA-1 antibody and visualized by ECL. (B) Northern blot with $10\mu\text{g}$ of total RNA, probed sequentially with GATA-1 and β -actin probes. The three bands obtained with GATA-1 are the 4.2-kb genomic viral RNA, the 2.5-kb spliced viral RNA encoding exogenous GATA-1, and the 1.0-kb mRNA encoding endogenous GATA-1. (C) Quantification of the GATA-1 mRNA expression shown on the autoradiograph in B by use of a PhosphorImager. The values are normalized to the β -actin mRNA expression. The expression level in the HD50 thromboblats cell line was arbitrarily set as 1.

high in the transfected clones as in the corresponding types of cell lines that had not been transfected.

Activation of GATA-1 rapidly suppresses myelomonocytic markers in myeloblasts transfected by an inducible GATA-1 construct

The time span between electroporation and the first analysis of GATA-1 transfected clones (3–4 weeks) leaves open the possibility that GATA-1 reprograms only a small percentage of cells that subsequently grow out. To address this question we fused GATA-1 with the hormone-binding domain of the estrogen receptor, thus creating an estrogen-inducible construct designated GATA-1-ER. This approach was justified by the finding that estrogen has no effects on the growth, viability, or differentiation of thromboblats or myeloblasts transformed by the E26 virus (H. Kulesa, unpubl.) and that no estrogen receptor can be detected in these cell types (Schroeder et al. 1993). As found with a GATA-1-responsive reporter in fibroblasts, the GATA-1-ER protein is inactive in the absence of hormone but can be activated to approximately one-third of the activity of wild-type GATA-1 after estradiol addition (see Materials and methods). After electroporation of HD50M myeloblasts

with a retroviral expression vector for GATA-1-ER (pNEO-GER) and selection for G-418-resistant colonies in Methocel, eight clones were isolated and found to express the expected 70-kD GATA-1-ER protein (data not shown). These clones resembled myeloblasts morphologically, although three clones showed a slight reduction in the expression of MYL51/2 antigen. Two clones with high MYL51/2 expression were treated with β -estradiol and found to respond similarly. As illustrated for one of these clones in Figure 5 (A, B), treatment with 10^{-6} M β -estradiol led to a two- to threefold decrease in the expression level of MYL51/2 and MHCII antigen

Figure 5. Changes in cell-surface antigen expression of HD50M-GATA-1-ER myeloblasts treated with estradiol. HD50M-GATA-1-ER clone 1 cells were treated with 10^{-6} M β -estradiol and cell-surface antigen expression monitored by immunofluorescence and flow cytometry at different days thereafter. (A) FACSscan profiles showing the expression of MYL51/2, MHCII, and EOS47 antigens. Note the decrease in fluorescence intensity of both MYL51/2 and MHCII as early as 1 day after estradiol addition. (B) Kinetics of cell-surface antigen expression recorded as the proportion of marker-positive cells at different days after estradiol treatment (part of the data correspond to those shown in A).

within 1 day and a complete suppression after 4 days. Conversely, 8% of the cells expressed EOS47 antigen after 1 day and 22% of the cells after 4 days. No MEP21 antigen-expressing cells could be detected during this period. Prolonged treatment with 10^{-6} M β -estradiol led to substantial cell death, which was followed by the outgrowth of an EOS47⁺ and eventually MEP21⁺ population (data not shown).

Forced GATA-1 expression reprograms Myb-Ets^{ts}-transformed myeloblasts into erythroblasts

Although GATA-1 is known to play an important role in erythroid differentiation, none of the GATA-1-transfected myeloblast-derived clones examined had an erythroid phenotype. This is perhaps not surprising in view of the fact that the original HD50 thromboplast cell line, in contrast to primary Myb-Ets-transformed cells, shows no spontaneous erythroid differentiation. However, as mentioned in the introductory section, progenitors transformed by a temperature-sensitive mutant of E26 virus with a lesion in Ets (called *ts1.1*) predominantly resemble erythroblasts and can be induced to mature into erythrocyte-like cells following shift to 42°C (Golay et al. 1988; Kraut et al. 1994; Frampton et al. 1995).

We therefore asked whether ectopic expression of GATA-1 would convert myelomonocytic cells transformed by the *ts1.1* mutant into erythroblasts and, if so, whether these could be induced to mature following temperature shift. For this purpose we generated a *ts1.1*-transformed myelomonocytic cell line (designated HD13) that expresses MYL51/2 and MHCII antigens but no erythroid or thrombocytic markers. After electroporation of the HD13 cell line with pNEO-GATA-1 and G-418-selection in Methocel, nine colonies were isolated and expanded for another 4 weeks at 35°C (in these and the following experiments with HD13 and its transfected derivatives 5% anemic serum was added to the growth medium as a source of erythropoietin). When shifted to 42°C for 3 days, seven of the nine GATA-1 clones showed an increase in hemoglobin-positive cells (0.5–20%, average 8%). Three of these clones were also positive for eosinophilic peroxidase. In a parallel experiment, none of 10 clones electroporated with pSFCV showed any up-regulation of these markers. An aliquot of the GATA-1-transfected clone 2 kept at 35°C was re-cloned in Methocel at 35°C and subclones 17 and 18 were analyzed further. Both were found to resemble Myb-Ets-transformed erythroblasts, being positive for MEP26 and JS4, weakly positive for hemoglobin and MEP21 and negative for $\alpha_{Hb}\beta_3$, peroxidase, EOS47, and MYL51/2. When shifted to 42°C for 4 days, an ~10-fold increase in hemoglobin expression was observed, as judged by benzidine staining (Fig. 6A, B) and by Northern blotting of α - and β -globin RNAs (Fig. 6C). At the same time, the cells completely down-regulated their MEP21 and MEP26 antigens and further up-regulated their JS4 antigen. One of the other GATA-1-transfected HD13 clones was predominantly eosinophilic with a low per-

Figure 6. Induced erythroid differentiation of GATA-1-transfected myelomonocytic cells transformed by the *ts1.1* mutant of E26. (A) Benzidine staining of HD13-GATA-1 clone 2/17 cells grown either at 35°C or shifted for 4 days to 42°C. (B) Kinetics of hemoglobin induction of HD13-GATA-1 clone 2/17 cells as determined by benzidine staining following temperature shift to 42°C. (C) Northern blot with 10 μ g of total RNA from untransfected HD13 cells or HD13-GATA-1 clone 2/17 cells. The cells were either grown at 35°C or shifted for 4 days to 42°C prior to RNA isolation. The filter was sequentially hybridized to the indicated probes.

centage of the cells expressing hemoglobin following shift to 42°C (T. Graf, unpubl.).

Forced GATA-1 expression reprograms v-myc-transformed macrophages into various new cell types

The experiments described so far were all performed in the background of Myb-Ets-transformed myeloblasts, raising the question of whether GATA-1 can only reprogram myelomonocytic cells containing the fusion protein. We therefore determined whether GATA-1 is also capable of reprogramming the v-myc-transformed macrophage cell line HD11. As illustrated in Figure 7, of eight stable HD11 clones obtained after transfection with pNEO-GATA-1, three clones (4, 7, and 8) had a dramatically altered morphology, containing cells that were significantly smaller, rounder, and less adherent than the parental HD11 cells. In addition, clone 4 cells contained peroxidase-positive granules characteristic of eosinophils. As can be seen in Figure 8A, the same three clones that exhibited an altered morphology expressed

Figure 7. Morphology of GATA-1-transfected HD11 cells. May-Grünwald-Giemsa staining of a HD11 macrophage control clone and three phenotypically altered HD11 clones transfected with pNEO-GATA-1 prepared by cytoentrifugation. (Top right) Clone 4 shows cells stained for eosinophilic peroxidase.

high levels of exogenous GATA-1. All three altered clones specifically lost expression of the *c-fms* gene (the CSF-1 receptor), a marker for mature myelomonocytic cells and instead expressed the c-Myb protein (Fig. 8A,B), suggesting that they had acquired an immature phenotype.

A more detailed analysis of the three altered GATA-1-transfected HD11 clones confirmed that clone 4 has a predominantly eosinophilic phenotype (Fig. 9). In contrast, clone 7 consisted of immature erythroid cells that could be induced to express hemoglobin after treatment with DMSO (data not shown). Interestingly, clone 8 had neither acquired eosinophilic, erythroid, or thrombocytic markers nor activated expression of the endogenous GATA-1 gene and instead resembled immature myelomonocytic cells. It is not clear why this clone expresses such low amounts of GATA-1 protein despite its high levels of exogenous GATA-1 mRNA (Fig. 8A,B). The GATA-1 protein levels and the phenotypes of these three clones are in agreement with the correlation noted in the Myb-Ets-transformed cells, with myeloblasts expressing the lowest and erythroblasts the highest levels of GATA-1.

Discussion

Our results have shown that exogenous GATA-1 expres-

sion induces the suppression of myelomonocytic markers in the Myb-Ets-transformed myeloblast cell line HD50M, converting them into cells that closely resemble either Myb-Ets-transformed eosinophils or thrombocytes (Fig. 10A). Similarly, expression of GATA-1 in the Myb-Ets^{ts}-transformed myeloblast cell line HD13 converts them into erythroblasts, which, after Ets inactivation by temperature shift, mature into erythrocyte-like cells (Fig. 10B). The capacity of GATA-1 to reprogram myelomonocytic cells does not depend on the presence of either the Myb or the Ets oncoprotein because the Myc-transformed macrophage cell line HD11 can also be converted into cells resembling either immature eosinophils, erythroblasts, or myeloblasts (Fig. 10C). In these properties GATA-1 therefore resembles the MyoD family of transcription factors, which have been shown to induce muscle differentiation in a variety of cell lines (Davis et al. 1987). However, in contrast to the myogenic factors, GATA-1 can apparently promote the commitment of a cell into several different phenotypes.

Induction or selection?

A critical question in the experiments described is whether the altered phenotypes observed after GATA-1 transfection of myeloid cells are attributable to an in-

Figure 8. Expression of GATA-1, *c-fms*, and c-Myb in HD11 cells transfected with pNEO-GATA-1. (A) Northern blot with 10 μ g of total RNA, probed sequentially with GATA-1, *c-fms*, and β -actin probes. The 4.2-kb genomic viral RNA, the 2.5-kb spliced viral RNA (exogenous GATA-1 mRNA), and the 1.0-kb endogenous GATA-1 mRNA are indicated. (B) Western blot with lysates of 10^6 cells, probed sequentially with GATA-1- and c-Myb-specific antibodies and visualized by ECL.

Figure 9. Summary of lineage-specific marker expression in GATA-1-transfected HD11 clones. (For explanation, see legend to Fig. 3C.)

structive role of the factor or whether they originate through the selection of spontaneously differentiated cells, endorsed with a growth advantage by GATA-1 overexpression. It seems unlikely that pre-existing (but undetectable) subpopulations of eosinophils, thrombocytes, or erythroblasts in the myelomonocytic cell lines are the targets for the observed GATA-1 effect for several reasons: First, it would be surprising if all three myelomonocytic cell lines studied exhibited spontaneous differentiation. Second, many of the clones expressing ectopic GATA-1 were found to consist of mixed populations. Third, the numbers of stably transfected clones obtained with the GATA-1 vector were not significantly smaller than with the control vector. However, selection might also be involved if it is assumed that the myelomonocytic cell lines spontaneously differentiate into one or more of the above-mentioned lineages and that the differentiated cells do not persist in culture. In this case, exogenous GATA-1 would again not directly affect the fate of the myeloid cells but would cause a stabilization and the eventual outgrowth of spontaneously differentiated cells. This seems also unlikely because a rapid down-regulation of myelomonocytic markers was observed following activation of GATA-1 in myeloblasts-transfected by GATA-ER, paralleled by an up-regulation of an eosinophil marker in part of the population. We therefore think that the induction hypothesis is essentially correct, with GATA-1 first turning off myelomonocytic genes while turning on eosinophil-specific genes, and activating thrombocyte lineage genes, at a later stage.

GATA-1 as a suppressor and activator of lineage-specific genes

The possibility that suppression of myelomonocytic markers represents a distinct function of the factor is suggested by the finding that GATA-1 induced an immature, myeloblast-like phenotype in HD11 clone 8. Most likely, GATA-1 targets a regulator of myelomonocytic genes, either by repressing an activator or by activating a

repressor. This effect need not take place at the level of protein-DNA interactions but might involve protein-

Figure 10. Summary of the reprogramming effects of GATA-1 in myelomonocytic cell lines. Each section (A–C) depicts the results obtained with a different myelomonocytic cell line, transformed by the oncogene indicated in italics. Shading of nuclei indicates expression of GATA-1. (For explanation, see text.)

protein interactions, as has been reported for the interaction of Jun with MyoD. Here, expression of excess Jun inactivates MyoD and promotes cell growth, whereas excess MyoD inactivates Jun and tips the balance toward growth arrest and differentiation (Bengal et al. 1992). Similar results were reported for the interaction of Jun with the glucocorticoid receptor (for review, see Ponta et al. 1992). Combining the potential for positive and negative regulation in a single transcription factor provides a simple means to achieve mutually exclusive growth and developmental decisions. In the case of GATA-1, it ensures that erythroid, thrombocytic, and perhaps eosinophilic cells do not inappropriately activate genes characteristic of myelomonocytic cells.

Are the induced phenotypes dependent on the dosage of GATA-1?

The observed correlation between the expression level of GATA-1 and the induced phenotype, as analyzed in a limited number of clones, suggests that the dosage of GATA-1 plays an important role in lineage determination. Thus, low concentrations of the factor appear either to exhibit no effect on the phenotype or induce an immature phenotype in macrophages. In contrast, intermediate levels correlate with the formation of eosinophils and high levels with that of thromboblats and probably erythroblasts. In addition, activation of endogenous GATA-1 is observed only in the latter two cell types. It will now be interesting to understand how the fine tuning of GATA-1 expression is maintained in Myb-Ets-transformed eosinophils and thromboblats, particularly in view of the known capacity of GATA-1 to regulate its own expression (Hannon et al. 1991; Tsai et al. 1991). Our data also suggest that conversion of myeloblats into either eosinophils or thromboblats requires more than fourfold as high levels of expression of GATA-1 at the RNA level as are found in the corresponding Myb-Ets-transformed cell types. High concentrations of GATA-1 might be needed in myelomonocytic cells to compensate for the absence of factors that cooperate with GATA-1 in other cell types. Alternatively, high expression levels might be required to overcome regulatory mechanisms responsible for turning off GATA-1 expression during myelomonocytic differentiation.

Activation of high and low affinity target genes by GATA-1 in combination with other transcription factors could be responsible for its apparent dosage effect, in a somewhat analogous manner to the concentration-dependent activation of different sets of target genes by the *Drosophila dorsal* gene (for review, see Hill and Treisman 1995). A candidate transcription factor that might act in combination with GATA-1 is C/EPB- β . C/EPB- β is expressed in transformed myeloblats (Ness et al. 1993; Burk et al. 1993) and eosinophils (this study) but not in thromboblats or erythroblats. The observation that activation of a conditional C/EPB- β construct in Myb-Ets-transformed thromboblats induces their differentiation into eosinophils (C. Müller, E. Kowenz-Leutz, S. Grieser-Ade, T. Graf, and A. Leutz, in prep.),

together with the results described here, points toward an important role for the combination of GATA-1 and C/EBP- β in the activation of the eosinophilic differentiation program. The dosage effect of GATA-1 observed in our experiments suggests that lower concentrations of GATA-1 are sufficient to activate eosinophilic genes in combination with C/EBP- β than are required to activate thrombocytic or erythroid genes, possibly in conjunction with other as yet unidentified factors. The presence of C/EBP- β in myeloblats might also explain their rapid reprogramming by GATA-1 into eosinophils, whereas a reprogramming into thromboblats probably requires the activation of additional factors that are not present in myeloblats.

Is GATA-1 unique in its capacity to reprogram myelomonocytic cells?

The results of Visvader and co-workers (Visvader et al. 1992; Visvader and Adams 1993) suggested that the capacity of GATA-1 to induce megakaryocytic maturation is restricted to a cell line that exhibits both myelomonocytic and megakaryocytic properties. However, as shown here, GATA-1 can induce the reprogramming of three independent myelomonocytic cell lines into eosinophils and erythroblats as well as into thrombocytic cells. Preliminary results indicate that GATA-1 might not be unique in its capacity to reprogram myelomonocytic cells because GATA-2 and GATA-3 are also capable of converting Myb-Ets-transformed myeloblats into eosinophils, although no thromboblats could be obtained (data not shown). These observations might reflect functional redundancies as well as differences between GATA family factors, as discussed above. Another transcription factor that is predominantly expressed in erythroid and thrombocytic cells, the helix-loop-helix-type factor SCL, is unable to induce a reprogramming of myelomonocytic cells into the thrombocytic or erythroid lineages, although it can suppress myelomonocytic maturation and promote erythroid differentiation (Aplan et al. 1992; Visvader et al. 1992; Tanigawa et al. 1993). The capacity of SCL to exhibit a subset of the functions ascribed to GATA-1 is consistent with the proposal that the SCL gene is one of the targets of GATA-1 (Aplan et al. 1992).

Our finding that GATA-1 transfection of the HD11 macrophage cell line induces the formation of various immature cell types that express c-Myb suggests a role for c-Myb in retrodifferentiation. This notion is in line with the observation that c-Myb can inhibit the chemically induced maturation of mouse myeloid and erythroid leukemia cell lines (Clarke et al. 1988). Furthermore, we have shown that v-Myb blocks the differentiation of thrombocytic cells (Frampton et al. 1995) and that it induces a retrodifferentiation of macrophage-like cells into myeloblats (Beug et al. 1987; Ness et al. 1987).

Possible implications for normal hematopoiesis

The results presented raise the possibility that GATA-1 not only regulates the expression of late erythroid and

megakaryocytic genes but that it can also act as a lineage-determining factor in uncommitted progenitors. In addition, the induction of eosinophilic differentiation shows for the first time the functional importance of GATA-1 in this lineage, which was proposed earlier on the basis of the expression of GATA-1 in human eosinophils (Zon et al. 1993). There are several reasons to think that the observed effects of GATA-1 do not represent cell-line artifacts. First, all three myelomonocytic cell lines tested could be reprogrammed by forced GATA-1 expression. Second, the resulting phenotypes are remarkably similar to those of primary transformed cells and no cells of mixed lineage phenotype were ever observed. Third, infection of blastoderm cultures with a viral construct encoding the Myb-Ets fusion protein together with GATA-1 induces the formation of a reduced number of myeloblast colonies and increased numbers of eosinophil and thromboplast colonies relative to the proportions of colony types observed with the original Myb-Ets virus (J. Frampton, unpubl.).

The observed plasticity of differentiation of transformed myelomonocytic cells supports and extends our earlier findings demonstrating that macrophages can be induced to redifferentiate by activation of the v-Myb protein (Beug et al. 1987). This plasticity is also in line with conclusions drawn from somatic cell fusion and nuclear transplantation experiments that demonstrated, for example, that frog erythrocyte nuclei have the potential to be reprogrammed to form tadpoles (DiBerardino et al. 1986). Such experiments have led to the concept that the phenotype of a cell is continuously and actively controlled by specific gene products (for review, see Blau et al. 1985; Blau 1992; Baron 1993). Together with the present observations, these experiments raise the intriguing possibility that the onset of expression of lineage-specific markers in committed progenitors does not preclude cells from differentiating along other lineages. It will now be important to determine whether GATA-1 can act as a lineage-determining factor in normal hematopoiesis and whether it can also reprogram committed myelomonocytic cells in the absence of a transforming oncogene.

Materials and methods

Retroviral constructs

The retroviral vector pSFCV-LE (abbreviated here as pSFCV) and its derivative pNEO-GATA-1 have been described by Furstenberg et al. (1990) and Briegel et al. (1993). The GATA-1-ER construct was generated by introduction of a *Bgl*III site at position 953–958 of the chicken GATA-1 cDNA and subsequent ligation to the *Bam*HI–*Eco*RI fragment of HE63 (Kumar et al. 1987), coding for the estradiol-binding domain (amino acids 251–595) of the human estrogen receptor (ER). An *Xba*I fragment containing the GATA-1-ER-coding sequence was then cloned into pSFCV-LE to generate pNEO-GER.

Activity of the GATA-1-ER fusion protein

To determine the activity and the hormone dependence of the GATA-1-ER fusion protein, we performed *trans*-activation as-

says on the GATA-1-responsive glycophorin A promoter in quail QT6 fibroblasts. QT6 (3×10^5) cells were seeded on a 3.5-cm dish in 5 ml of medium with or without 10^{-8} M β -estradiol and transfected the following day by CaPO_4 -precipitation (Graham and van der Eb 1973) with the following DNAs: 500 ng of an expression vector for GATA-1, GATA-1-ER, or the empty vector; 250 ng of a reporter plasmid containing the luciferase gene under the control of the glycophorin A promoter; and 200 ng of a β -gal expression vector as a control for the transfection efficiency. Cell extracts were prepared after 48 hr as described earlier (Ness et al. 1989). Luciferase and β -galactosidase activity were determined by standard procedures (Herbomel et al. 1984; de Wet et al. 1987). The GATA-1-ER protein caused a 6.8-fold increase in the luciferase activity over the basal level in the presence of β -estradiol, showing that the ER fusion protein indeed acts as an hormone-dependent transcriptional activator. The maximum activity of the GATA-1-ER protein, however, corresponded to only 30% of the activity of the wild-type protein.

Cell lines and culture conditions

The HD50 cell line was established after infection of 2-day-old chicken embryos (blastoderm stage) by use of a virus carrying variant 4 of the Gag-Myb-Ets fusion protein described by Metz and Graf (1991). The HD50M myeloblast cell line was derived from the HD50 line after treatment with 100 nM TPA by the procedure described in Graf et al. (1992) and was clone-purified in 0.5% methylcellulose-containing medium (Methocel). The HD50 eosinophil cell line was derived from the HD50 line after treatment with 20 nM TPA and 0.1 $\mu\text{g}/\text{ml}$ of ionomycin and subsequent recloning (T. Graf, unpubl.). The v-myc transformed chicken macrophage cell line HD11 was established from chicken bone marrow cells by Beug et al. (1979). The HD13 cell line was derived after infection of bone marrow cultures from a 1-day-old chicken with ts1.1 E26 virus (Golay et al. 1988). A transformed myelomonocytic colony was isolated and established in permanent culture in the presence of cMGF at 39°C. The cell line was subsequently recloned in Methocel.

Cells were grown in DMEM supplemented with 10% fetal calf serum, 2.5% chicken serum, 0.15% NaHCO_3 , 56 $\mu\text{g}/\text{ml}$ of conalbumin, 80 mM 2-mercaptoethanol, 0.9 $\mu\text{g}/\text{ml}$ of insulin, and standard complement of antibiotics at 39°C. HD13 derivatives were grown at 35°C and shifted to 42°C for the inactivation of the Ets protein. Except for the HD50 cell line, conditioned supernatant of concanavalin A-stimulated spleen cells was added to the medium as a source of chicken myelomonocytic growth factor (cMGF; Leutz et al. 1984). HD13 cells used in the experiments described, and their transfected derivatives received an additional 5% anemic chicken serum.

Transfection protocols

The HD50M and HD13 cell lines were transfected by electroporation. The cells were washed twice in PBS and resuspended at 2×10^6 cells/ml. Linearized plasmid (10 μg), dissolved in 10 μl water, was added to 75 μl of cells, which was then transferred to a 2-mm electroporation cuvette. Electroporation was performed at 160 V/250 μF with a Bio-Rad Gene Pulser. After electroporation, the cells were resuspended in 1–2 ml of medium and seeded 1 day later in Methocel, with the addition of 1.6 mg/ml of G-418. Two to three weeks later G-418-resistant colonies were transferred into liquid medium containing 0.8 mg/ml of G-418. The HD11 cell line was transfected by use of CaPO_4 precipitation (Graham and van der Eb 1973). Cells (10^6) were

Table 2. Monoclonal antibodies recognizing cell-surface antigens of chicken hematopoietic cells

Antibody	Antigen	Specificity ^a	Reference
MYL 51/2	170-kD antigen	myelomonocytic cells	Kornfeld et al. (1983)
cIa	MHC class II	myelomonocytic cells	Ewert et al. (1984)
EOS 47	melanotransferrin	eosinophils	McNagny et al. (1992); Graf et al. (1992)
MEP 21	150-kD antigen	thromboblats and thrombocytes	McNagny et al. (1992); Graf et al. (1992)
11C3	$\alpha_{IIb}\beta_3$ integrin	thromboblats and thrombocytes	Lacoste-Eleau et al. (1994)
MEP 26	47- to 60-kD antigen	erythroblats, thromboblats, thrombocytes, and eosinophils	McNagny et al. (1992)
JS4	unknown	late erythroblats and erythrocytes	Schmidt et al. (1986)

^aCell types tested included Myb-Ets-transformed thromboblats, myeloblats, macrophages, erythroblats, and eosinophils; ErbA- and ErbB-transformed erythroblats; and Myc-transformed macrophages as well as chick bone marrow cells of the myelomonocytic, eosinophilic, and thrombocytic lineages.

seeded on a 10-cm tissue culture dish in 10 ml of medium and grown overnight. CaPO₄ precipitates of 10 μ g of pSFCV-LE or pNEO-GATA-1 linearized with *NotI* were added to the cells for 4 hr. The cells were washed once with medium and grown in fresh medium overnight. Thereafter, they were treated as described for the electroporated cells.

Antibodies

The rabbit polyclonal anti-GATA-1 antibody H62, a gift of G. Goodwin, was raised against the peptide NGQNRPLIRPKKR comprising amino acids 141–153 of chicken GATA-1. The source of the mouse monoclonal antibodies against cell-surface antigens and their specificity is given in Table 2. The 11C3 mouse monoclonal antibody against chicken GP_{IIb/IIIa} ($\alpha_{IIb}\beta_3$) was provided by C. Corbel (Institut d'Embryologie, Nogent-sur-Marne, France). Antisera against histone H5 and Myb have been described earlier (Beug et al. 1979; Ness et al. 1987).

Immunofluorescence

Indirect immunofluorescence and flow cytometric analyses (by use of a Becton Dickinson FACScan) were performed as described earlier (Graf et al. 1992).

Cytochemical assays

For assessment of their morphology, cells were spread onto slides by cytocentrifugation, dried, fixed with methanol, and stained with May-Grünwald-Giemsa (Diff-Quik, Harleco). Eosinophilic peroxidase was detected in cell suspensions as described previously (Kaplow 1965; Graf et al. 1992). Peroxidase-positive cells, containing brown-to-black staining granules, were scored after 15–30 min of incubation, counting 300 cells. Alternatively, cells were stained by adding a drop of peroxidase reagent diluted 1:4 to freshly prepared cytopins for 15 min, washed in PBS, dried, fixed with methanol, and counterstained with May-Grünwald-Giemsa. Hemoglobin expression was detected by acid benzidine staining in cell suspensions as described by Orkin et al. (1975). The percentage of benzidine-positive cells was determined by counting of 300 cells.

Western blotting

The cells were lysed in 0.1 % Triton X-100 [0.1 % Triton X-100, 25 mM Tris/Cl at pH 7.6, 1 mM EDTA at pH 8, 200 mM NaCl, 50 mM NaF, 0.1 mg/ml of leupeptin, 1 mM PMSF, 1% Trasylol (Bayer)], the extracts fractionated on a SDS-polyacrylamide gel

and blotted onto a nylon filter (Immobilon-P, Millipore). Immunodetection was performed by enhanced chemiluminescence (ECL, Amersham) as recommended by the manufacturer.

Northern blotting

Total RNA was prepared by acid/guanidinium/phenol/chloroform extraction (Chomczynski and Sacchi 1987), separated on a 1.2% agarose-formaldehyde gel, and blotted onto a nylon filter (Hybond, Amersham) as described by Sambrook et al. (1989). DNA probes were labeled with [α -³²P]dATP by random hexamer priming (Feinberg and Vogelstein 1983). The following cDNA fragments were used as probes: *Clal* fragment of pNEO-GATA-1 (Briegleb et al. 1993); *EcoRI* fragment of C/EBP- β (Katz et al. 1993); *EcoRI* fragment of *mim-1* (Ness et al. 1989); *EcoRI* fragment of MHCII- γ chain (S.A. Ness and T. Graf unpubl.); *EcoRI* fragment of *c-fms* (Fuhrmann 1989). The α - and β -globin probes were described earlier (Kraut et al. 1994). All probes were hybridized under stringent conditions by use of 0.1 $\times$ SSC, 0.5% SDS, at 68°C for 1 hr for the final wash. They were removed by incubation of the filters for 30 min in 1% SDS at 100°C or in 80% formamide, 0.1 $\times$ SSC, and 1% SDS at 80°C.

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GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblats, and erythroblasts.

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