Megakaryocytic Differentiation Induced in 416B Myeloid Cells by GATA-2 and GATA-3 Transgenes or 5-Azacytidine Is Tightly Coupled to GATA-1 Expression

By Jane Visvader and Jerry M. Adams

The GATA ‘zinc-finger’ transcription factors are thought to have important roles in the control of hematopoiesis. GATA-1 and GATA-2 are found in the erythroid, mast cell, and megakaryocytic lineages, and GATA-3 in T lymphocytes. GATA-1 is required for erythroid development and has recently been shown by gene transfer to direct megakaryocytic differentiation of the primitive myeloid cell line 416B. Here we show that enforced expression in 416B cells of either the GATA-2 or GATA-3 gene also induces megakaryocytic differentiation, as assessed by cellular morphology, acetylcholinesterase activity, polyloid DNA content, and loss of Mac-1 expression. No erythroid or mast cell differentiation was found. Unexpectedly, the level of endogenous GATA-1 mRNA had increased 20- to 30-fold among the transfectants, whereas that of GATA-2 mRNA was unaltered and endogenous GATA-3 transcripts remained undetectable. This finding suggests that GATA-2 and GATA-3 lie upstream of GATA-1 in a regulatory hierarchy and that, in 416B cells, GATA-1 may mediate the phenotypic changes induced by GATA-2 or GATA-3. Furthermore, 416B cells treated with the DNA demethylating agent 5-azacytidine underwent megakaryocytic differentiation accompanied by a marked increase in the level of GATA-1 mRNA but not that of GATA-2 or GATA-3. These results strongly implicate GATA factors in megakaryocytic differentiation and suggest that, at least for 416B cells, GATA-1 is a dominant regulator of maturation along this lineage.

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mine whether GATA-2 or GATA-3 also had this capacity. Here we report that introduction of a GATA-2 or GATA-3 expression vector into 416B cells also elicited megakaryocytic development. However, the dramatic increase in endogenous GATA-1 expression found in these transfecants suggests that the GATA-1 gene can be activated by either GATA-2 or GATA-3 and that the phenotypic changes may be mediated by GATA-1. By analogy with the myogenic system, the extremely low GATA-1 expression in untransfected 416B cells might reflect methylation of the gene. Therefore, we tested the effect of 5-azaC and found that megakaryocyte production was indeed induced, in concert with markedly elevated expression of GATA-1. Taken to- 

egether, these findings strongly implicate GATA factors in megakaryocytic maturation. Moreover, the tight coupling of GATA-1 expression with megakaryocytic differentiation in 416B cells suggests that it may be a key regulator of that pathway.

MATERIALS AND METHODS

Plasmids. The expression vector pEF-MC1neo has been de- 
scribed previously. A 2.2-kb human GATA-2 cDNA fragment derived from the hGATA-2/pXM expression vector17 and a 2.2-kb human GATA-3 cDNA fragment derived from hGATA-3/ pCDNA1neo3 were inserted into a blunted polylinker 3' of the pEF-IA promoter20 of pEF-MC1neo. Clones in the sense orientation were selected. The vector MC1neoPA pro- 
vided a control.

Cell lines, electroporation conditions, and treatment with 5-aza- 
cytidine. The 416B line13 and transfecants derived from this line were passaged in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal calf serum (FCS). Cell lines other than those generated in this work have been cited previously.18,22

For electroporation, 1 × 10⁶ 416B cells in 0.5-mL HEPESS-buf- 
fered RPMI medium containing 20 μg of SalI-linearized GATA-2 or NdeI-linearized GATA-3 expression vector were left at room temperature for 5 minutes before pulsing at 270 V, 960 μF using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA). After 5 minutes on ice, the cells were diluted into 50 mL of warm DMEM, 10% FCS. Twenty-four hours later, the cells were plated in 24-well dishes at 5 × 10⁵ cells/mL in medium containing 1.5 mg/mL G418 (Gymec- 
inc, Sigma, St Louis, MO). The electroporation frequency was esti- 
mated to be 1 in 2 × 10⁶ cells. Clonal lines were generated by limit- 
ing dilution in 96-well plates.

For treatment with 5-azaC, 416B cells at 5 × 10⁵ or 10⁶ cells/mL in 24-well plates were exposed to 0.5 to 5.0 μmol/L drug (Sigma) for 48 hours. Cells from 3 to 4 wells at the same concentration of 5-azaC were pooled, washed three times and replated in fresh DMEM/10% FCS. Megakaryocytes were observed in all wells after 5 to 6 days. Clonal lines were derived by limiting dilution in 96-well plates.

DNA and RNA analysis. Nucleic acids were isolated and frac- 
tionated as described previously.23 The probes used for Southern or Northern analysis included the following: mouse GATA-1, a 1.8-kb XhoI fragment24; 5 mouse GATA-1, a 0.35-kb PvuII-XhoI fragment from the 3′ untranslated region; human GATA-2, a 2.2-kb Eco RI fragment12; human GATA-3, a 2.2-kb NotI fragment; mouse SCL, a 1.9-kb EcoRI fragment18; a 0.6-kb fragment from the mouse AChE gene26; a mouse platelet factor 4 fragment (nucleotides 1 to 306); and a 0.7-kb XbaI-EcoRI fragment from pEF-BOS28 span- 
ning the human granulocyte-colony stimulating factor (G-CSF) polyadenylation region. Mouse cDNA probes were hybridized in standard buffer containing 50% formamide whereas human cDNA probes were hybridized under reduced stringency (30% formamide) and also washed at lower stringency, at 65°C in 2× sodium chloride/sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) instead of 0.2 × SSC/0.1% SDS. A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA28 probe was used to monitor RNA loading and transfer efficiency. Probes were labeled with α-32P-dATP by priming with random hexamers (Bresatec, Ade- laide, South Australia).

Flow cytometric analysis and measurement of DNA content. The monoclonal antibodies (MoAbs) Ter-11927 and M1/70 (aMac-1)28 were used in flow cytometric analyses as described.29

Ter-119 was directly conjugated to fluorescein isothiocyanate (FITC) while aMac-1 was biotinylated and used in conjunction with phycoerythrin (PE)-streptavidin (Caltag, San Francisco, CA). All staining was performed in the presence of MoAb 2.4G2 (anti- 
FcY receptor) to block nonspecific binding of antibodies.

DNA content28 was estimated as follows: 1 × 10⁶ cells in 1 mL of propidium iodide solution (50 μg/mL) containing 0.1% sodium ci- 
trate, 0.1% Triton X-100 (BDH Chemicals, Victoria), were lysed for 10 minutes at room temperature, then filtered through a 44-μm/L nylon membrane before flow cytometric analysis. Fluorescence above 570 μmol/L was measured and the percent cells with a DNA content greater than 4 N calculated.

Cytotoxic staining. Cytocentrifuge preparations were stained for acetylcholinesterase as described.31 Standard cytochemi-


standard buffer containing 50% formamide whereas human cDNA probes were hybridized under reduced stringency (30% formamide) and also washed at lower stringency, at 65°C in 2× sodium chloride/sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) instead of 0.2 × SSC/0.1% SDS. A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA28 probe was used to monitor RNA loading and transfer efficiency. Probes were labeled with α-32P-dATP by priming with random hexamers (Bresatec, Adela-ide, South Australia).

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trate, 0.1% Triton X-100 (BDH Chemicals, Victoria), were lysed for 10 minutes at room temperature, then filtered through a 44-μm/L nylon membrane before flow cytometric analysis. Fluorescence above 570 μmol/L was measured and the percent cells with a DNA content greater than 4 N calculated.

Cytotoxic staining. Cytocentrifuge preparations were stained for acetylcholinesterase as described.31 Standard cytochemi-
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Gene products in Fig 3A appear to result from alternative expression vector, as indicated by its hybridization to the polyadenylation signal in the introduced GATA-3 cDNA. The 2.2-kb transcript in Fig 3A presumably terminates after the exogenous polyadenylation sequence contributed by the vector probe (3' transgene: Fig 3B). whereas the GATA-2 transgene (2.9-kb transcript in Fig 2A) was generated by transfection of the intact EF-la expression cassette and confirmed that the transgene-derived GATA-2 transcripts could be distinguished from the exogenous polyadenylation region (Fig 2B). Similarly, the level of GATA-3 mRNA expressed from the EF-1α promoter was at least 20-fold higher than that of the endogenous (3.5-kb) transcript in three murine T-cell lines, illustrated by Tikaut in Fig 3A. The two transgene products in Fig 3A appear to result from alternative sites of polyadenylation. The 2.9-kb mRNA reflects use of the exogenous polyadenylation sequence contributed by the expression vector, as indicated by its hybridization to the relevant vector probe (3' transgene; Fig 3B), whereas the 2.2-kb transcript in Fig 3A presumably terminates after the polyadenylation signal in the introduced GATA-3 cDNA.

GATA-2 and GATA-3 provoked megakaryocytic differentiation of 416B cells. After 6 days of selection in G418, large megakaryocytes appeared in every pool of 416B cells electroporated with the GATA-2 expression vector (48 wells) or the GATA-3 vector (24 wells), whereas none appeared with the control vector MC1 neo (72 wells). The pools of transfectants did not contain hemoglobinized (benzidine-) erythroid cells, nor were any mast cells evident on staining with May-Grunwald-Giemsa.

The morphologic changes induced by enforced GATA-2 or GATA-3 expression in clonal derivatives are depicted in Fig 4, where the top panel shows cell suspensions and the lower panel shows stained cytocentrifuge preparations. The control 416neo line, which was indistinguishable from the parental line, consisted of small blast cells with a large nuclear to cytoplasmic ratio. In contrast, GATA-2 and GATA-3—expressing transfectant lines comprised not only 416B-like blast cells and intermediate-sized cells, but also a distinctive subpopulation (approximately 4% to 8%) of mature megakaryocytes, characterized by their large size, multi-lobed nuclei, and granular cytoplasm (Fig 4).

An important megakaryocytic marker is acetylcholinesterase (AChE) activity. Cytochemical staining for AChE activity showed very high levels within the cytoplasm of the mature megakaryocytes and intermediate levels within the smaller cells (data not shown). In three independent GATA-2—expressing lines, from 50% to 90% of cells exhibited moderate or intense staining and another 7% to 25% had a lower level. Similarly, in two GATA-3 transfectants, 79% to 85% of cells exhibited at least moderate staining and 11% to 18% had a lower level, whereas neither the parental line nor the control 416neo cells exhibited any detectable AChE activity. Thus, a large proportion of the cells expressing either GATA-2 or GATA-3 appeared to be committed to the megakaryocytic lineage. Consistent with these cytochemical data, Northern blot analysis (Figs 2D, E; Figs 3D, E) showed that the level of mRNAs for both the AChE gene and another megakaryocyte-specific gene, platelet factor 4 (PF4), was 3- to 10-fold higher in lines showing megakaryocytic differentiation than in the control lines.
eloid lineage marker Mac-1 was substantially downregulated in the lines expressing the GATA-2 or GATA-3 transgene (Fig 5B). Whereas only approximately 3% of 416B cells lacked this surface marker, 29% of cells in the megakaryocytic line GATA-2.C6 and 89% in the GATA-3.F7 line were negative. Similarly, in three other 416GATA lines (GATA-2.12, GATA-3.C5, GATA-3.A3), from 14% to 89% of cells lacked Mac-1. The bimodal distribution, particularly evi-

Mature megakaryocytes are polyploid and both the GATA-2 and GATA-3 transfectants contained a subpopulation of such cells. Flow cytometric analysis of DNA content showed that 3.7% of cells in the GATA-2 transfectant C6 (Fig 5A) and 1.9% in the I2 line (not shown) contained nuclei with greater than 4 N DNA content. Similarly, for the GATA-3 transfectants F7 (Fig 5A) and C5 (not shown), 5.6% and 1.9% of cells were polyploid whereas only 0.2% of cells in the control 416neo contained nuclei with greater than 4 N DNA content.

As noted previously for GATA-1 transfectants, the my-
Fig 4. Morphology of 416B transfectants. The upper panel depicts cell suspensions of (A) 416B cells electroporated with the neo<sup>+</sup> gene alone (416neo), (B) a GATA-2 transfectant, and (C) a GATA-3-expressing clonal line. Original magnification × 250. The lower panel shows cytocentrifuge preparations stained with modified Wright’s (Diff-Quick) of (A) 416neo control cells, (B) and (C) GATA-2 and GATA-3 expressing lines, respectively. Original magnification × 500.

Fig 5. Analysis of DNA content and cell-surface marker expression in 416neo cells and GATA-gene transfectants. (A) Relative DNA content of 416B cells transfected with MC1-neoPA (neo), a GATA-2 transfectant, and a GATA-3 transfectant. DNA content was estimated by flow cytometry of propidium-iodide-stained DNA complexes. The relative positions of 2N and 4N are shown. Both axes represent linear scales. (B) Flow cytometric analysis of Mac-1 and Ter-119 surface antigens on control 416neo cells (neo), the GATA-2 expressing clone GATA-2.C6 and the GATA-3 transfectant GATA-3.F7. Unstained cells gave fluorescent intensities within the lower left quadrant.

GATA factors in megakaryocytic development

GATA-I mRNA is markedly elevated in GATA-2 and GATA-3 transfectants. In the parental 416B cells, GATA-2 transcripts (2.9 and 3.5 kb) were moderately abundant, but the level of GATA-1 mRNA (1.9 kb) was very low (Fig 1) and no GATA-3 mRNA (3.5 kb) was detectable (not shown). Among megakaryocytic lines generated by any of the three GATA transgenes, the amount of endogenous GATA-2 transcripts remained unaltered and no endoge-
A.  
Probe  
3' GATA-1  

B.  

C.  

D.  

Fig 6. Northern analysis of 416B cells transfected with GATA genes or treated with 5-azaC. Filters containing poly(A)+ RNA samples (3 μg) were hybridized sequentially with mouse GATA-1 (high stringency) and human GATA-2 probes (moderate stringency). The 3' GATA-1 probe used in (A) corresponds to a 3' untranslated sequence excluded from the expression vector construct and is therefore specific for endogenous GATA-1 mRNA. The control GAPDH hybridizations for the GATA-2 and GATA-3 clones are displayed in Figs 2 and 3, respectively, whereas that for these GATA-1 expressing lines has been shown previously. In (D), four clonal megakaryocytic lines isolated after treatment of 416B cells with 5-azaC are shown. Equivalent amounts of intact RNA were loaded on the basis of ethidium bromide staining of the gel.

DISCUSSION

Although the immature myeloid line 416B originally could differentiate in vivo along the megakaryocytic and granulocytic lineages,4 no differentiation now occurs spontaneously, nor can any be induced by interleukin-6 (IL-6) or erythropoietin (Epo), growth factors that normally promote megakaryocytic maturation. We recently showed that introduction of a GATA-1 expression vector into this line provoked megakaryocytic differentiation16 and report here that enforced expression of the GATA-2 or GATA-3 gene induced the same phenotypic change (Fig 4). Like GATA-1 transfectants, the lines bearing a GATA-2 or GATA-3 transgene comprised a heterogeneous population that included undifferentiated 416B-like cells in addition to cells at
Megakaryocyte

416B early myeloid cell

GATA-2

GATA-1

GATA-3

Fig 7. A model depicting the relationship of the GATA genes to megakaryocytic differentiation in the early myeloid line 416B. Expression of the endogenous GATA-1 gene is likely to mediate the megakaryocytic differentiation in the GATA-2 and GATA-3 expressing lines, although this pathway may also be activated directly by GATA-2.

Various stages of megakaryocytic maturation. A subpopulation of 4% to 8% mature megakaryocytes was evident morphologically and by polyploid DNA content (Fig 5A). The majority of cells in the cultures were apparently committed to the megakaryocytic lineage, as 50% to 90% exhibited strong AChE staining. As well as AChE, the megakaryocytic lines contained increased levels of mRNA for the megakaryocytic pathway.

Although GATA-2 is also normally expressed in erythroid and mast cells (Fig 1), cytochemical staining of the transfectant lines provided no evidence that enforced expression of this gene (or GATA-3) prompted differentiation to mature cell types of either of these lineages. These results parallel those obtained with GATA-1 transfectants and suggest either that these pathways have been impeded in 416B cells or that neither GATA-1 nor GATA-2 is sufficient to induce erythroid or mast cell differentiation in a progenitor cell.

Although enforced expression of the SCL (or TAL) gene, which has a hematopoietic expression pattern remarkably similar to that of GATA-1 and GATA-2, has been shown to provoke maturation in an erythroid line it did not provoke megakaryocytic (or erythroid) maturation of 416B cells. Nevertheless, SCL may well cooperate with other factors in regulating megakaryocytic maturation, because SCL mRNA levels increased several fold in all the GATA-2 and GATA-3 transfectants (Figs 2C and 3C) as well as in those expressing the GATA-1 transgene. Whereas this increase might be an indirect consequence of megakaryocytic maturation, one (or more) of the GATA factors may instead act directly on the SCL promoter, which contains a GATA binding site that can mediate transcriptional activation by GATA-1. Members of a gene family often have overlapping functions, presumably to protect critical cellular pathways. Despite the distinctive expression patterns of the GATA factors, transient transfection assays and DNA-binding experiments have not delineated specific roles for these factors. Disruption of the GATA-1 gene has established that this factor is essential for erythroid development, but functional redundancy could hold for the megakaryocytic and mast cell lineages as development along these pathways may still be operative in GATA-1 null embryos.

Although all three GATA genes could induce megakaryocytic differentiation in 416B cells, GATA-1 appeared to be the most important for implementing the megakaryocytic program. Because GATA-1 mRNA levels were elevated 20- to 30-fold in all the lines expressing the GATA-2 or GATA-3 transgene (Figs 6B and C), we suggest that enforced expression of GATA-2 or GATA-3 stimulated GATA-1 expression, which then directed megakaryocytic differentiation (Fig 7). GATA-3 is unlikely to play a role in normal megakaryocytic differentiation as it appears to be largely restricted to T-lymphoid cells. However, because GATA-2 is expressed within the megakaryocytic lineage as well as in multipotential myeloid lines, it is also plausible that GATA-2 elicited megakaryocytic maturation directly in the GATA-2 transfectants (Fig 7). If normal megakaryocytic differentiation can be directed by either GATA-2 or GATA-1, that pathway would be maintained in GATA-1 deficient embryos. Thus, our findings emphasizing the importance of GATA-1 in megakaryocytic differentiation are not in conflict with the phenotype elicited by GATA-1 gene disruption.

Our findings (summarized in Table 1) suggest that the GATA-2 and GATA-3 genes may lie upstream of GATA-1 in a regulatory hierarchy. Consistent with this view, in human hematopoiesis expression of GATA-2 and perhaps even GATA-3 appears to precede that of GATA-1. Each factor may act via the double GATA motif present in the upstream region of the GATA-1 promoter and known to be required for its full activity. However, other factors must participate in GATA-1 gene regulation, because the gene is expressed very poorly in certain hematopoietic cell lines containing abundant GATA-2 mRNA (Fig 1). Interestingly, in 416B cells the GATA-1 transgene did not provoke

| Table 1. Megakaryocytic Differentiation in 416B Cells |
|----------------|----------------|----------------|----------------|
| Agent          | Expression of Endogenous Gene* | Megakaryocytic Differentiation |
| None           | ±               | +               | −               | yes |
| GATA-1 transgene | ±               | −               | +               | ++  |
| GATA-2 transgene | ±               | −               | +               | ++  |
| GATA-3 transgene | ±               | +               | −               | +   |
| SCL transgene  | ±               | +               | −               | +   |
| 5-azaC         | ±               | +               | −               | ND  |

* ± refers to low levels of transcripts, detectable only on longer autoradiographic exposure.

Abbreviation: ND, not done.
expression of its endogenous counterpart (Fig 6A). Evidence from cotransfection experiments has indicated that GATA-1 can drive its own transcription, via the GATA sites in its promoter, but it has not been established whether other GATA factors also regulate this promoter. Our data favors control of GATA-1 gene expression by GATA-2 and GATA-3, but do not exclude the proposed autoregulation of GATA-1 expression during erythroid maturation.

Further support for a dominant role of GATA-1 in the differentiation of 416B cells arose from the experiments with 5-azaC. A substantial proportion (21% to 50%) of the treated cells became committed to megakaryocytic differentiation. By morphologic appearance and AChE staining, representative clonal lines were indistinguishable from those generated by GATA-factor transgenes. Moreover, their megakaryocytic differentiation was accompanied by a dramatic increase in the level of GATA-1 mRNA but not, significantly, that of GATA-2 or GATA-3 (Fig 6D and Table 1). This increase may reflect direct activation of GATA-1 expression by demethylation. The tight coupling in 416B cells of GATA-1 expression with the megakaryocytic differentiation elicited by all three transgenes as well as by the demethylating agent (Table 1) leads us to suggest that GATA-1 is a primary effector of megakaryocytic maturation.

Our results with the 416B cell line are reminiscent of those with the 10T1/2 myogenic progenitor line. The frequency (21% to 50%) with which megakaryocytes arose from 416B cells treated with 5-azaC is comparable with that of myogenic conversion (25% to 50%) of 10T1/2 cells exposed to this agent. The high frequency observed with the 10T1/2 cell line suggested that a single gene might suffice to elicit myogenesis in these cells, as subsequently proved to be the case. The establishment of permanent cell lines and their prolonged culture presumably select against expression of endogenous genes that promote differentiation, such as MyoD in 10T1/2 cells or GATA-1 in 416B cells, and de novo methylation of such genes may contribute to the selection process.

As GATA-1 and GATA-2 are coexpressed in these hematopoietic lineages, it is presumed that each of these factors contributes primarily to the control of maturation along these lineages rather than commitment to any one of them. A crucial role for GATA-1 in erythroid maturation was shown by gene disruption, and a role in megakaryocytic maturation can be inferred from our observations. However, GATA-1 cannot be the sole determinant of differentiation in any of these lineages because erythroid markers known to be regulated by GATA-1 are not present in megakaryocytes or mast cells, despite their abundant GATA-1 expression. Lineage commitment in a presumptive progenitor cell for the erythroid, megakaryocytic, and mast cell lineages is likely to require interplay between the relevant GATA factors and other cell-restricted transcription factors.

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Megakaryocytic differentiation induced in 416B myeloid cells by GATA-2 and GATA-3 transgenes or 5-azacytidine is tightly coupled to GATA-1 expression

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