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The C-Terminal Zinc Finger of GATA-1 or GATA-2 Is Sufficient To Induce Megakaryocytic Differentiation of an Early Myeloid Cell Line

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The GATA-1 and GATA-2 transcription factors, which each contain two homologous zinc fingers, are important hematopoietic regulators expressed within the erythroid, mast cell, and megakaryocytic lineages. Enforced expression of either factor in the primitive myeloid line 416B induces megakaryocytic differentiation. The features of their structure required for this activity have been explored. The ability of 12 GATA-1 mutants to promote 416B maturation was compared with their DNA-binding activity and transactivation potential. Differentiation did not require any of the seven serine residues that are phosphorylated in vivo, an N-terminal region bearing the major transactivation domain, or a C-terminal segment beyond the fingers. Removal of a consensus nuclear localization signal following the second finger did not block differentiation or nuclear translocation. The N-terminal finger was also dispensable, although its removal attenuated differentiation. In contrast, the C-terminal finger was essential, underscoring its distinct function. Remarkably, only 69 residues spanning the C-terminal finger were required to induce limited megakaryocytic differentiation. Analysis of three GATA-2 mutants led to the same conclusion. Endogenous GATA-1 mRNA was induced by most mutants and may contribute to differentiation. Because the GATA-1 C-terminal finger could bind its target site but not transactivate a minimal reporter, it may direct megakaryocytic maturation by derepressing specific genes and/or by interacting with another protein which provides the transactivation function.

The GATA proteins (designated GATA-1 to GATA-4) constitute a small family of zinc finger transcription factors, each having a distinctive cell type and developmental expression profile (23). The first three members are abundantly expressed within the hematopoietic compartment and may contribute to commitment and/or differentiation of particular cell types. The GATA-1 transcription factor is a central regulator of erythroid gene expression (23), and targeted disruption of the gene in embryonic stem cells has revealed that it is essential for erythroid development (24). GATA-1 is also expressed in mast cells, megakaryocytes, and multipotential myeloid lines (4, 17, 26). GATA-2 is expressed within the same hematopoietic lineages as GATA-1, while GATA-3 is restricted to T lymphocytes (reviewed in reference 23). Outside the hematopoietic compartment, GATA-1 has been found only in the testis (9) and GATA-3 has been found in the developing nervous system (35), whereas both GATA-2 (6) and GATA-4 (1) are expressed in several nonhematopoietic cell types.

The GATA proteins recognize the consensus target sequence (T/A)GATA(A/G) by virtue of zinc fingers in the configuration Cys-X_2-Cys-X_{17}-Cys-X_2-Cys (7, 31). The highly conserved DNA-binding domain comprises two homologous zinc fingers, except in fungal proteins, which possess a single finger resembling the C-terminal finger of the vertebrate polypeptides (12). GATA target sequences (reviewed in reference 23) were first identified in the promoters and enhancers of a number of erythroid-expressed genes. They have subsequently been found in the α- and β-globin locus control regions, the enhancers of T-cell receptor genes, the mast cell carboxypeptidase A gene, and the promoters of the glycoprotein IIb and platelet factor 4 genes, which are expressed in megakaryocytes.

Structure-function analysis of the murine GATA-1 gene has delineated specific domains (Fig. 1, bottom). The two zinc fingers are functionally distinct: the C-terminal finger is absolutely required for recognition of the GATA consensus sequence, while the N-terminal finger confers full specificity and stability of binding (18, 36). Furthermore, cotransfection assays with deletion mutants have defined a potent transcriptional activation domain near the N terminus of GATA-1 (18). Equivalent regions of GATA-3 have the same functions (37). Despite the distinctive expression patterns of GATA-1, GATA-2, and GATA-3, it has proven difficult to distinguish their transcriptional activity on target promoters in cotransfection assays. They do, however, display preferences in recognition of synthetic target sites (11, 19).

A valuable biological assay for GATA-1 activity was provided by the demonstration that its enforced expression in the early myeloid line 416B elicited megakaryocytic differentiation (34). An important experimental advantage of this cell line is that it originally had some megakaryocytic and granulocytic potential (5) but no longer differentiates in response to chemical differentiative agents or to growth factors that normally promote megakaryocytic maturation (34). Megakaryocytic differentiation can also be induced in these cells by a GATA-2 or GATA-3 transgene or by the demethylating agent 5-azacytidine (33). Because a marked increase in endogenous GATA-1 expression accompanied the latter treatments, it remains un-

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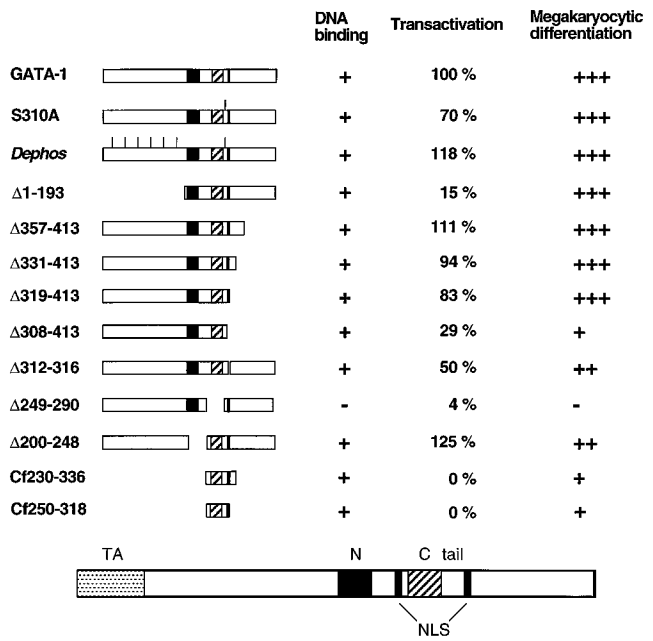

FIG. 1. Murine GATA-1 mutants and their capacity for DNA binding, transcriptional activation, and induction of megakaryocytic differentiation. Deletion mutants are designated by the amino acids removed, shown as gaps in the schematic representations. The N- and C-terminal fingers are indicated by the solid and hatched boxes, respectively. The small shaded area depicts a consensus NLS, and the short lines indicate the positions of serine-to-alanine substitutions in mutants S310A and *Dephos* GATA-1. Column 1 summarizes gel shift assay results (Fig. 2), and column 2 summarizes transactivation results with an M6α-GH reporter in NIH 3T3 cells, expressed as a percentage of wild-type GATA-1 activity. The ability of each mutant to provoke megakaryocytic differentiation of 416B cells was scored from − to +++, on the basis of cell morphology and intensity of staining for AChE (see text). The bottom panel shows a more detailed schematic representation of GATA-1, indicating the major transactivation (TA) domain, N- and C-terminal fingers, the C-terminal tail, and two putative NLSs.

certain whether the differentiation is mediated solely via GATA-1 or whether other GATA proteins can act directly.

We have exploited the 416B system to delineate the regions of GATA-1 required for its differentiative function. The ability of engineered mutants to direct megakaryocytic differentiation was compared with their DNA-binding activity and transactivation potential in cotransfection assays. The minimal region required to evoke differentiation was found to comprise only 69 amino acids spanning the C-terminal zinc finger. The results suggest that this biological activity requires the DNA-binding function but not the presence of a transactivation domain. This finding is reminiscent of the observation that the DNA-binding domain of MyoD was sufficient to elicit myogenesis in 10T1/2 cells (30). We suggest that the single GATA zinc finger triggers the differentiation program in 416B cells either by altering DNA conformation, competing for a site occupied by another factor, or recruiting a protein with transactivation function.

MATERIALS AND METHODS

Expression vectors. The expression vectors pEF-MClneo, GATA-1/pEF-MClneo, and GATA-2/pEF-MClneo have been described previously (33, 34). GATA-1 mutants previously described include deletion mutants Δ1-193, Δ249-290, and Δ200-248 (18); serine-to-alanine substitution mutants (3); and the GATA-1 C-terminal finger (residues 230 to 336) (19). cDNA fragments corresponding to these mutants were recloned into the blunted *Xba*I site of pEF-MClneo, and clones in the sense orientation were selected.

Mutants Δ357-413 and Δ331-413 were generated by cloning blunted *Xho*I-*Pst*I (1.1 kb) or *Xho*I-*Pvu*II (1 kb) fragments from murine GATA-1 cDNA into the blunted *Xba*I site of pEF-MClneo. Deletion mutant Δ308-413 was synthesized by PCR with primers 5′-CGCTCTAGACTGCATCAACAAGCCCAG (forward) and 5′-CGCTCTAGATCAGCGGTTCCTCGTCTGGAT (reverse),while Δ319-413 was synthesized with the same forward primer and 5′-CGCTCTAGATCAC CCCCGCTTCTTTTTCCCTTT (primer B). Cf250-318 was generated with 5′-CGCTCTAGACCACCATGGTCAGCAAACGGGCAGGCA (forward) and primer B. The PCR products were digested with *Xba*I and cloned into the *Xba*I site of pEF-MClneo. The sequence of the PCR-derived clones was confirmed by dideoxynucleotide sequence analysis (U.S. Biochemicals Corp).

GATA-2 mutant Δ414-474 was created by cloning a blunted *Eco*RI-*Sph*I (1.4 kb) fragment derived from full-length human GATA-2 cDNA into the *Xba*I site (blunted) of pEF-MClneo. GATA-2 mutant NCf269-413 was synthesized by PCR with primers 5′-CGCTCTAGACCACCATGGAGGTGGCCTCCAGCT TCACCCC (forward) and 5′-CGCTCTAGATTAGGATGACTTCTCCTGC ATG (reverse; designated primer C), and Cf335-413 was synthesized with 5′-CGCTCTAGACCACCATGGCCGCCAGAAGAGCCGGC (forward primer) and primer C. These PCR products were restricted with *Xba*I plus *Sph*I, blunted, and cloned into the *Xba*I site (blunted) of pEF-MClneo.

DNA-binding and transactivation assays. To test the DNA-binding activity of the GATA-1 mutants, individual constructs were transfected into COS cells. After 36 h, nuclear extracts were prepared and gel shift assays were performed with a 29-bp fragment derived from the mouse α1-globin gene as a probe as described previously (18).

Transactivation assays were carried out in NIH 3T3 cells, with the reporter plasmid M6α-GH exactly as described in reference 18. This plasmid contains six copies of the GATA site from the mouse α1-globin promoter linked to a minimal promoter driving the growth hormone gene (18).

Cell lines and electroporation conditions. The 416B line and transfectants of it were passaged in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For electroporation, 10^7 416B cells in 0.5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered RPMI medium containing 20 μg of a linearized mutant GATA-1 or GATA-2 expression vector were left at room temperature for 5 min before being pulsed at 270 V (960 μF) with a Bio-Rad Gene Pulser (Bio-Rad, Hercules, Calif.). After 5 min on ice, the cells were diluted into 50 ml of warm Dulbecco's modified Eagle's medium–10% fetal calf serum. After 24 to 36 h at 37°C, the cells were plated in 24-well dishes at 5×10^4 cells per ml in medium containing 1.2 mg of G418 (Geneticin; Sigma, St. Louis, Mo.) per ml. The electroporation frequency was estimated to be at least 1 in 2×10^4 cells. Where indicated, clonal lines were generated by limiting dilution in 96-well plates. In general, several pools of cells derived from individual wells of the 24-well dish were investigated for each construct.

RNA analysis. Nucleic acids were isolated and fractionated as described previously (2). The probes used for Northern (RNA) analysis included the following: mouse GATA-1, a 1.8-kb *Xho*I fragment (31); 3′ mouse GATA-1, a 0.35-kb *Pvu*II-*Xho*I fragment from the 3′ untranslated region; and human GATA-2, a 2.2-kb *Eco*RI fragment (6). Mouse cDNA probes were hybridized in buffer containing 50% formamide, whereas human cDNA probes were hybridized under reduced stringency (30% formamide) and were also washed at lower stringency, at 65°C in 2× sodium chloride-sodium citrate (SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate])–0.1% sodium dodecyl sulfate (SDS) instead of 0.2× SSC–0.1% SDS. A rat glyceraldehyde-3-phosphate dehydrogenase cDNA probe was used to monitor RNA loading and transfer efficiency. Probes were labeled with [α-^{32}P]dATP by priming with random hexamers (Bresatec, Adelaide, South Australia, Australia).

Cytochemical and immunofluorescent staining. Cytocentrifuge preparations were stained with modified Wright's stain (Diff-Quik; Lab-Aids [20]) and for acetylcholinesterase (AChE) activity as described previously (10).

For subcellular immunolocalization, stable transfectants of GATA-1 mutants Δ308-413 and Δ312-316 were allowed to adhere to poly-L-lysine-coated chamber slides (8 chamber; Nunc, Inc.). Either glass or plastic chamber slides were used, with (~1 to 2) $\times 10^5$ cells per chamber. Cells were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. After a rinse with PBS, the cells were permeabilized for 10 min with 0.1% Triton X-100 in PBS at room temperature. After two washes with PBS, the cells were incubated for 30 min with 1% bovine serum albumin–0.05% Tween 20–1% fetal calf serum (blocking buffer) in PBS. Monoclonal antibody N-6-1 (9), diluted 1 in 4, was then added to individual chambers and left for 20 min at room temperature. The slides were subsequently washed three times in blocking buffer before a 1-h incubation with goat $F(ab')_2$ anti-rat fluorescein isothiocyanate-conjugated immunoglobulin G (Caltag Laboratories, San Francisco, Calif.). The cells were then washed several times with PBS and placed on a coverslip with 0.23 M 1,4-diazabicyclooctane (DABCO) in 90% glycerol–10% PBS (pH 8.6). Cell staining was visualized and photographed on a Bio-Rad MRC 600 confocal fluorescence microscope.

RESULTS

In vitro DNA binding and transactivation by GATA-1 mutants. Twelve mutants of the murine GATA-1 gene, shown schematically in Fig. 1, were cloned in the expression vector

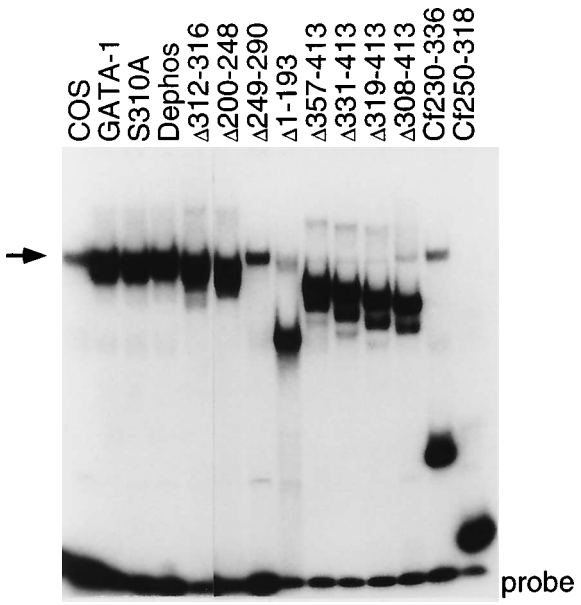

FIG. 2. DNA-binding activity of mouse GATA-1 mutant proteins expressed in COS cells. A 29-bp DNA fragment spanning the TGATAA site from the mouse α-globin promoter was used as a probe in electrophoretic mobility shift assays. The arrowed band present in mock-transfected cells results from an endogenous GATA-binding protein in COS cells.

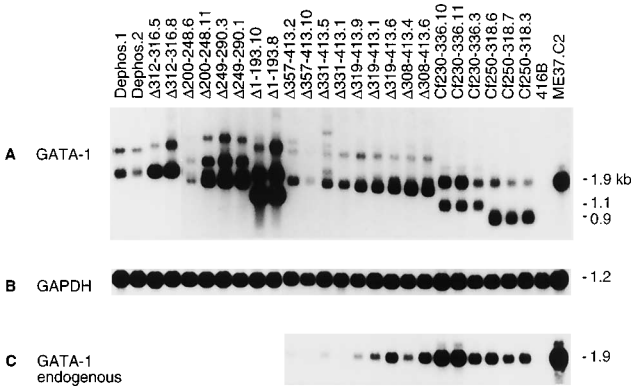

FIG. 3. Northern analysis of 416B lines harboring mutant GATA-1 transgenes. Filters containing poly(A)$^+$ RNA samples (3 μg per lane) were hybridized at high stringency with each probe sequentially. Clonal lines were examined for mutants Δ1-193, Δ357-413, Δ331-413, and Δ319-413, while cell pools containing a small number of individual clones were analyzed for the remainder. In panel A, the larger transcripts observed for Cf230-336 and Cf250-318 transfectants correspond to endogenous GATA-1 mRNA induced in these megakaryocytic lines. The two prominent transcripts detected in mutants *Dephos*, Δ312-316, Δ200-248, Δ249-290, and Δ1-193 result from use of both the GATA-1 polyadenylation signal and that in the vector (33). The uppermost transcripts in other lanes reflect read-through of the G-CSF polyadenylation signal of the vector into the Neo^r gene and/or incomplete splicing of the EF-1α gene intron (see reference 34). In panel C, only transfectants in which endogenous GATA-1 mRNA could be distinguished from transgene-derived transcripts by using the probe from the 3′ untranslated region are shown (the other transfectants contain this sequence in the transgene). Hybridization with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe provided a control for RNA loading. ME37.C2 is a mouse erythroid line.

pEF-MClneo. The mutants, some of which have been described previously (3, 18), include amino-terminal, carboxy-terminal, and internal deletions, as well as two substitution mutants. In one of the last mutants, S310A, alanine replaces the serine residue at position 310, phosphorylation of which increases during murine erythroleukemia cell differentiation. In the other substitution mutant (*Dephos*), all seven serines that are phosphorylated in murine erythroleukemia cells (positions 26, 49, 72, 142, 178, 187, and 310) are changed to alanines (3).

GATA-1 DNA-binding activity can be assessed by an electrophoretic mobility shift assay of nuclear extracts prepared from transfected COS cells (31). The DNA-binding activity of each GATA-1 mutant was determined in this way, and the results are shown in Fig. 2 and summarized in Fig. 1. The faint band observed in extracts from untransfected cells (Fig. 2, lane COS) corresponds to a nonspecific binding activity. Even the small polypeptides encoded by the C-terminal zinc finger constructs Cf230-336 and Cf250-318 bound DNA with relatively high affinity (Fig. 2). The only mutant that did not recognize the GATA target was one missing the C-terminal finger (Δ249-290). Its requirement for DNA binding has been documented (18). A mutant lacking the N-terminal finger (Δ200-248) appeared to associate with DNA efficiently under these conditions, which favor saturation of binding sites, but this polypeptide has been shown to have an increased dissociation rate and therefore to bind less stably (18).

The major transactivation domain of GATA-1 resides near the N terminus (18). In the present study, removal of the N-terminal 193 residues (Δ1-193) suppressed 85% of the transactivating activity in transiently transfected NIH 3T3 fibroblasts (Fig. 1). In agreement with previous work (3), transactivation potential was not significantly altered by mutagenesis of either serine 310 or all seven of the serines phosphorylated in murine erythroleukemia cells, even though two of these serines lie within the major transactivation domain (Fig. 1). However, deletion of the consensus nuclear localization signal (KGKKK) adjacent to the C-terminal finger tail (Δ308-413 and Δ312-316) diminished the activity by two- to threefold (Fig. 1). Because these mutants still translocate to the nucleus (see below), it seems likely that these deletions lower the DNA-binding affinity, which was slightly reduced in independent electrophoretic mobility shift experiments (data not shown). As expected, the C-terminal finger domain (Cf230-336 or Cf250-318) lacked any transactivation capacity (Fig. 1).

Much of GATA-1 is dispensable for induction of megakaryocytic differentiation. A high level of transgene expression can be obtained in 416B cells with the vector pEF-MClneo, in which the promoter from the EF-1α gene drives expression of the gene of interest and a neomycin resistance cassette allows selection (33, 34). G418-resistant transfectants of GATA-1 mutant constructs were expanded from individual wells, or clonal lines were generated by limiting dilution. Southern blots confirmed that the EF-1α expression cassette remained intact (data not shown). Northern blot analysis (Fig. 3A) showed that most transfectants expressed high levels of the mutant GATA-1 transcripts, and the sizes of the fastest-migrating species were those expected for the transgene-derived mRNAs. For example, the level of transcripts from mutant Δ249-290 or Δ1-193 was at least 30-fold higher than that of the endogenous GATA-1 mRNA (1.9 kb species) in 416B cells. The mRNAs from the smallest constructs, Cf230-336 and Cf250-318, were probably at least as abundant, given that they contain only a small portion of the probe sequence.

Because the 416B line comprises a homogeneous population of small blast-like cells, megakaryocytes could readily be distinguished by their large size, multilobed nucleus, and granular cytoplasm (34). Transfectants were monitored cytologically 7 to 10 days after selection in G418 in multiple independent

experiments. In all, 48 to 120 pools of transfectants were analyzed in the case of mutants inducing a high degree of differentiation and 72 to 312 pools were analyzed for those producing only a low degree of differentiation or none. Control cultures electroporated with the control MClneo vector occasionally contained a few wells (~2%) with rare intermediate-size cells, some of which stained for the megakaryocytic enzyme marker AChE. This evidence that rare 416B cells can spontaneously differentiate indicates that enforced expression of GATA-1 activates a latent differentiation potential rather than reprogramming the cells.

In wild-type GATA-1 transfectants of 416B cells, nearly all wells contain 20 to 40% intermediate-size cells, as well as 5 to 10% mature megakaryocytes, and the majority of cells stain intensely for AChE (34). For each mutant, the extent of differentiation was evaluated both in the wells containing primary transfectants and in expanded cell pools. On the basis of cell size, morphology, and intensity of staining for AChE, each was assigned a score up to +++, the extent obtained with wild-type GATA-1. Moderately differentiated cultures (++) contained 7 to 12% megakaryocytic cells of either intermediate or large size, whereas those with limited differentiation (+) comprised 3 to 8% megakaryocytic cells, mainly of intermediate size. In general, the level of staining for AChE paralleled the extent of morphological differentiation: 40 to 85% for highly differentiated cultures, 20 to 54% for moderately differentiated cultures, and 6 to 25% for cultures displaying limited differentiation. With mutants that induced limited differentiation, the proportion of wells displaying megakaryocytes varied between experiments, perhaps reflecting the number of passages of 416B cells before transfection, their degree of recovery from electroporation, or the level of transgene expression attained.

GATA-1 molecules with major structural modifications retained the capacity to induce megakaryocytic differentiation (Fig. 1). With the serine-to-alanine substitution mutants, almost every pool of cells (>95%) contained as many megakaryocytes as those seen with the wild-type GATA-1 transgene. Thus, phosphorylation of GATA-1 may be irrelevant for megakaryocytic differentiation. Although deletion of the N-terminal region (Δ1-193) nearly abolished transactivation activity, it did not impair the ability to induce differentiation, because mature megakaryocytes were numerous in every well. Part of the C-terminal region also proved dispensable. Removal of up to 95 residues from that end (Δ319-413) had little effect. However, differentiation capacity was markedly reduced by deletion of only another 11 residues (in Δ308-413), which removed the nuclear localization signal (NLS) consensus motif that adjoins the tail of the C-terminal finger (see Fig. 1 and Discussion). This mutant gave a lower proportion of wells exhibiting megakaryocytes (average of 46%), and large mature megakaryocytes were rare. In contrast, deletion of the NLS alone (Δ312-316) gave only a slight reduction. Hence, this consensus NLS is dispensable for differentiation.

The two GATA-1 zinc fingers behaved differently. Deletion of the N-terminal finger (Δ200-248) only modestly reduced the capacity to induce megakaryocytic maturation, whereas excision of the C-terminal finger (Δ249-290) abolished this ability. Surprisingly, the short polypeptides spanning the C-terminal finger (Cf230-336 and Cf250-318) could direct megakaryocytic differentiation (Fig. 4C and D). Megakaryocytes were evident in an average of 30% of the wells, whereas vector control transfectants gave an average of only 0.03% positive wells. Moreover, a substantial proportion of the cells (6 to 25%) in C-terminal finger transfectants stained for AChE. The longer C-terminal finger polypeptide produced more differentiated cultures than the shorter one, although both yielded fewer megakaryocytes than wild-type GATA-1. Thus, this single finger suffices to trigger limited maturation.

Nuclear transport does not require the C-terminal NLS. To determine whether removal of the C-terminal NLS (Fig. 1) in mutants Δ308-413 and Δ312-316 affected their subcellular distribution, immunofluorescence staining was carried out in conjunction with confocal laser scanning microscopy. Consistent with their ability to promote differentiation, both proteins were abundantly expressed within the nucleus of the majority of cells, as illustrated for mutant Δ312-316 in Fig. 5. Nuclear staining was intense except for the nucleoli, which were unstained (Fig. 5B). As expected, control 416B cells or transfectants of the C-terminal finger, which lacked the epitope recognized by the anti-mouse GATA-1 antibody (3), did not stain at all (data not shown).

The C-terminal zinc finger of GATA-2 can also induce megakaryocytic differentiation. To check the generality of certain conclusions reached with GATA-1, three GATA-2 mutant constructs were also tested in 416B cells (Fig. 6). The level of their transcripts (Fig. 7) was severalfold higher than that of the endogenous GATA-2 transcripts (3.5 and 2.9 kb) in 416B cells, and the level from the smallest mutants (NCf269-413 and Cf335-413) probably was at least 30-fold higher, given that they contain only a small fraction of the probe sequence. Curiously, certain lines expressing mutant GATA-2 constructs, such as Cf335-413.8 and Cf335-413.15, had much higher levels of endogenous GATA-2 transcripts than other lines. Because this elevated level only appeared in certain lines, its significance is uncertain.

The biological effects of the GATA-2 mutants (Fig. 6) paralleled the findings with GATA-1. Again, both termini were dispensable, and most activity was confined to the DNA-binding domain. Transfectants bearing both fingers (NCf269-413) contained a higher proportion of cells committed to the megakaryocytic pathway (17 to 36%) than those carrying only the C-terminal finger (5 to 8%), but the latter was clearly sufficient for limited differentiation. Removal of the C-terminal 61 residues of GATA-2 (Δ414-474) did not reduce its biological activity (Fig. 6) or impair its DNA-binding activity (data not shown).

Endogenous GATA-1 expression in mutant GATA transfectants. Although GATA-1, GATA-2, and GATA-3 transgenes all elicit megakaryocytic maturation in 416B cells, these genes are not equivalent in their ability to activate expression of the endogenous GATA-1 gene. In every GATA-2 or GATA-3 transfectant, but not in GATA-1 transfectants, endogenous GATA-1 mRNA levels are elevated 20- to 30-fold (33). These data suggest that enforced expression of GATA-2 and GATA-3 ultimately activates transcription of the GATA-1 gene. Megakaryocytic differentiation elicited by most GATA-2 mutants was accompanied by a marked increase in endogenous GATA-1 transcript levels (Fig. 7B). Moreover, the GATA-2 mutant having both zinc fingers (NCf269-413) was more effective than the single finger (Cf335-413) at stimulating endogenous GATA-1 gene expression (Fig. 7B).

Whereas the endogenous GATA-1 gene was not expressed in wild-type GATA-1 transfectants (33), it was expressed in lines bearing certain GATA-1 mutants. A probe specific for the endogenous gene revealed a 10- to 30-fold increase in its mRNA levels over that in 416B cells with mutants Δ308-413, Δ319-413, Cf230-336, and Cf250-318 (Fig. 3C). Surprisingly, however, mutants containing more of the C-terminal region (Δ331-413 and Δ357-413) exhibited no increase or a minimal one. In addition, no significant increase was evident for mutant Δ1-193, which contains an intact C terminus (data not shown). Because several mutants (e.g., S310A, *Dephos*, and Δ312-316)

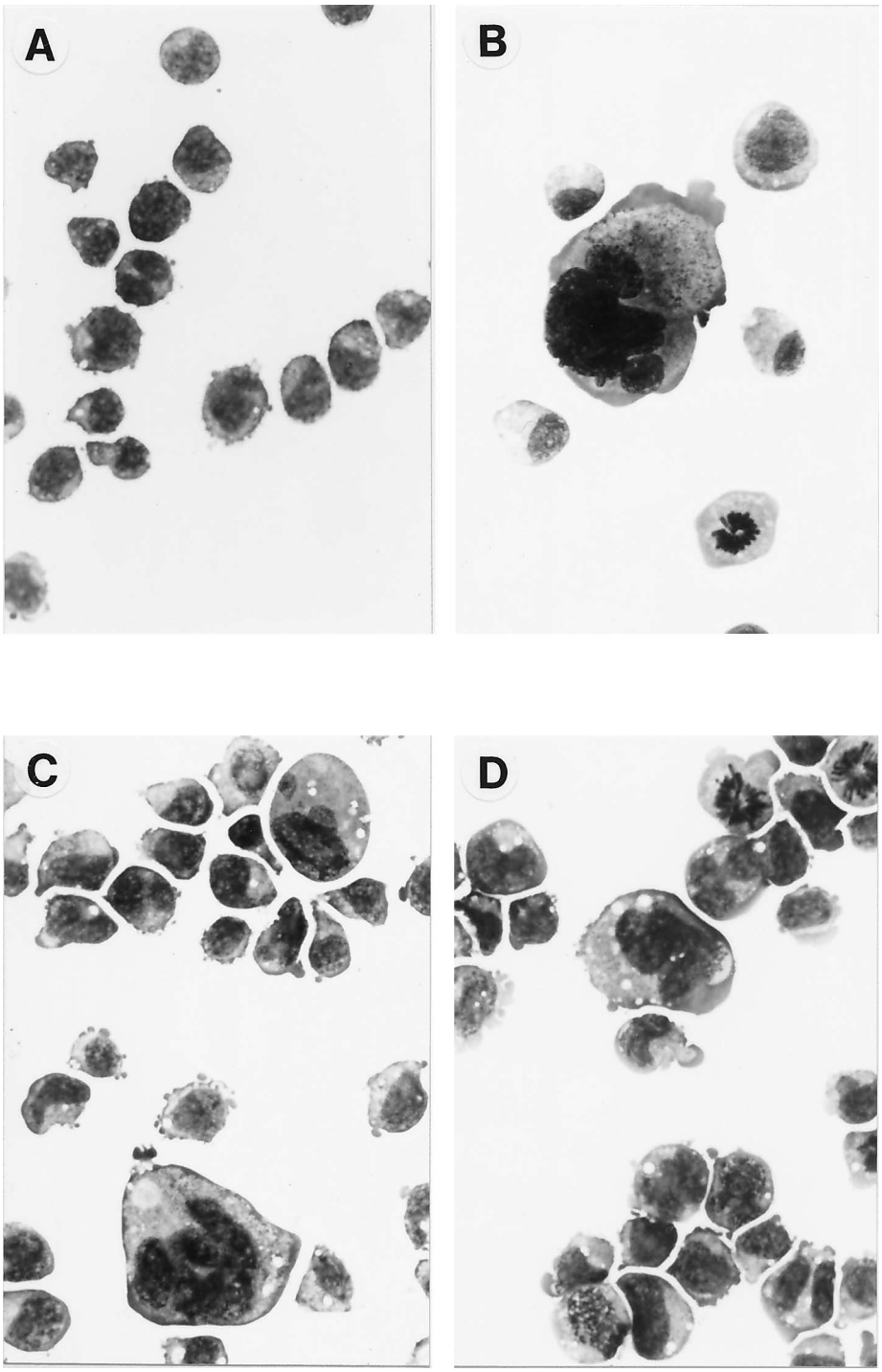

FIG. 4. Morphology of 416B transfectants. Cytocentrifuge preparations stained with modified Wright's stain (Diff-Quik) of 416B cells transfected with the Neo^r gene alone (416neo) (A), the wild-type GATA-1 gene (B), and GATA-1 mutants Cf230-336 (C) and Cf250-318 (D) are shown. Original magnification, ×500.

contained the 3′ untranslated region of the GATA-1 gene, electrophoresis was extended to help resolve the endogenous 1.9-kb GATA-1 transcript from the 1.8-kb transgene-derived mRNA. No increase in the endogenous GATA-1 mRNA level was apparent (data not shown). These findings suggest that a C-terminal domain of GATA-1 can block its ability to stimulate transcription of the GATA-1 gene.

DISCUSSION

As the GATA-1 and GATA-2 factors are normally coexpressed in megakaryocytes (6, 17, 26), their specific roles in this lineage and their potential for interaction and cross-regulation remain to be defined. We have previously shown that enforced expression of either gene (or GATA-3) in the primitive my-

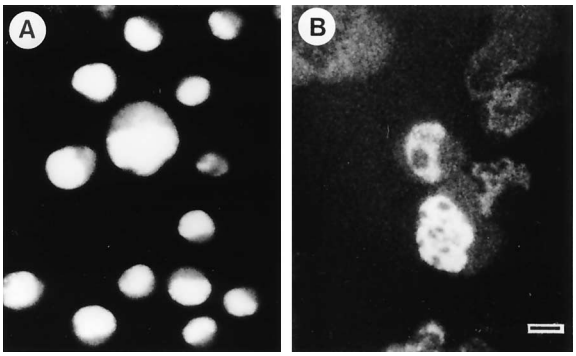

FIG. 5. Immunolocalization of a mouse GATA-1 deletion mutant lacking a consensus NLS. Both panels show indirect immunofluorescence staining of 416B cells transfected with Δ312-316. Because 416B-like cells have a high nucleus/cytoplasm ratio, the small rim of cytoplasm around the intensely stained nucleus is not always apparent, but it is particularly evident for the intermediate-size megakaryocyte in panel A. The unstained nucleoli are visible in panel B. The bar in panel B represents 10 micrometers. The magnification in panel A is half that in panel B. Control 416B cells exhibited no detectable staining with anti-mouse GATA-1 antibody N-6-1 (not shown).

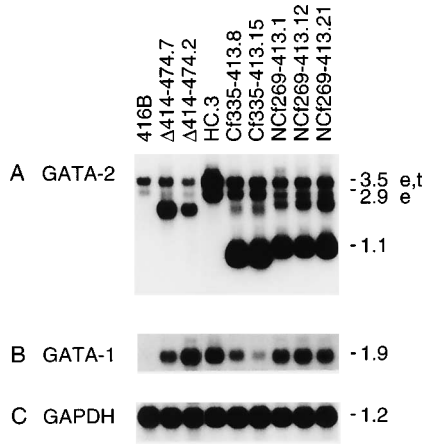

FIG. 7. Northern analysis of 416B lines harboring mutant GATA-2 transgenes. Filters containing poly(A)$^+$ RNA samples (3 μg per lane) were hybridized with the human GATA-2 probe at moderate stringency and with mouse GATA-1 or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes at high stringency. In panel A, e and t denote the endogenous and transgene-derived transcripts, respectively. Unspliced transcripts derived from the Δ414-474 transgene comigrated with the larger endogenous transcript (3.5 kb) but could be distinguished by hybridization with a 3′ fragment from the pEF-MClneo vector that provides the polyadenylation region (data not shown). HC.3 is a murine mast cell line.

eloid cell line 416B triggered megakaryocytic differentiation (33, 34). Since introduction of the GATA-2 or GATA-3 transgene or treatment with 5-azacytidine induced abundant GATA-1 mRNA, GATA-1 might be the critical regulator. However, as ES cells lacking GATA-1 can generate megakaryocytes (23a), GATA-2 may be the normal mediator of megakaryocytic maturation, or both genes may contribute.

This report addresses the features of GATA-1 and GATA-2 structure required to induce megakaryocytic differentiation of 416B cells (33, 34). Mutagenesis of the GATA-1 gene revealed that regions N terminal and C terminal to the zinc finger DNA-binding domain were dispensable for induction of megakaryocytic differentiation (Fig. 1). Similarly, removal of the last 61 residues from GATA-2 (Δ414-474) or 60 residues from GATA-3 (our unpublished results) showed that their C-terminal regions were not required. Although the GATA-1 major transactivation domain lies near the N terminus (18), excision of the first 193 amino acids had no effect on the differentiation capacity (Fig. 1). Thus, most of the activity appeared to reside in the central DNA-binding moiety.

Remarkably, 69 amino acids of GATA-1 (Cf250-318) encompassing the C-terminal zinc finger were capable of inducing megakaryocytic differentiation, albeit less efficiently than wild-type GATA-1. The three-dimensional structure of the homologous chicken GATA-1 domain complexed to its cognate DNA site has recently been elucidated (22). In that structure, the DNA-binding domain comprises a Cys-X_2-Cys-X_{17}-Cys-X_2-Cys core, which coordinates a zinc atom and interacts with the major groove of the DNA, followed by an extended C-terminal tail of eight amino acids that wraps around the minor groove. The smallest biologically active GATA-1 mutant (Cf250-318) contains both the core and tail, as well as a consensus NLS that directly abuts the tail. This mutant could bind DNA in vitro but could not transactivate a reporter plasmid in NIH 3T3 fibroblasts. Thus, megakaryocytic differentiation appears to correlate closely with the DNA-binding activity of GATA-1. A slightly longer overlapping polypeptide (Cf230-336) yielded cultures containing more mature megakaryocytes than those observed with Cf250-318, perhaps because of enhanced DNA-binding affinity or the presence of a weak transactivation domain between residues 319 and 336. The homologous C-terminal finger of GATA-2 (Cf335-413) showed equivalent activity, as might be expected in view of its 77% identity with that of GATA-1.

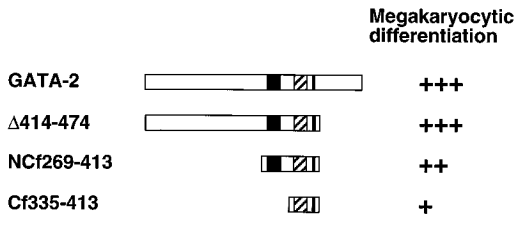

FIG. 6. Ability of human GATA-2 mutants to induce megakaryocytic differentiation. The two deletion mutants are designated by the amino acids removed. The N- and C-terminal fingers are indicated by the solid and hatched boxes, respectively. The thin solid bars mark a nuclear localization signal also present in GATA-1. The differentiative capacity of each mutant in 416B cells was evaluated on the basis of cell morphology and AChE staining intensity and was assigned a score up to +++.

Both zinc fingers of GATA-1 were required for efficient induction of mature megakaryocytic lines. Removal of the C-terminal finger (Δ249-290) abolished differentiation, consistent with evidence that both the mouse and chicken GATA-1 proteins require this finger (plus the adjoining tail) for DNA binding (7, 18). However deletion of the N-terminal finger (Δ200-248), which contributes primarily to specificity and stability of binding, only moderately impaired differentiation ability. The lower proportion of mature megakaryocytes obtained with these mutants probably reflects the reduced stability of DNA binding observed in vitro. Similar findings were obtained with GATA-2 mutants (Fig. 6), in which the construct containing both fingers (NCf269-413) produced more differentiated cultures than the C-terminal finger alone (Cf335-413). The two-finger polypeptide was not as effective as wild-type GATA-2 in promoting megakaryocytic differentiation. Hence, the production of highly differentiated lines appears to require more than the DNA-binding function.

Many transcription factors contain more than one NLS (29). Within GATA-1, two short stretches of basic amino acids sim-

ilar to the consensus NLS have been conserved across species (Fig. 1). One (RPKKR) resides in the interfinger region, and the other (KGKKK) follows the tail of the C-terminal finger. Thus, each finger may be followed by an NLS. Because deletion of the second NLS in mutants Δ312-316 and Δ308-413 did not block nuclear transport (Fig. 5), it seems plausible that the interfinger consensus NLS can independently direct nuclear translocation. Although deletion of the equivalent interfinger NLS in GATA-3 did not preclude nuclear accumulation, a region encompassing the N-terminal finger and interfinger motif was important for directing nuclear localization (37). Thus, it seems likely that nuclear localization of GATA proteins is specified by multiple independent signals.

It has been difficult to ascribe distinct transcriptional roles to the different GATA factors on the basis of DNA-binding experiments or transient transfection assays. However, we have reported that GATA-2 and GATA-3 can be distinguished from GATA-1 by their ability to stimulate endogenous GATA-1 gene expression in 416B-derived cells (33). The dramatic rise in GATA-1 mRNA in every megakaryocytic 416B line expressing a GATA-2 or GATA-3 transgene suggested that GATA-2 and GATA-3 might directly stimulate transcription of the endogenous GATA-1 gene via the double-GATA motif in its promoter (32). Although a wild-type GATA-1 transgene did not positively regulate expression of the endogenous GATA-1 gene in 416B-derived megakaryocytes, mutant GATA-1 constructs lacking most of the C terminus (Δ308-413, Δ319-413, Cf230-336, and Cf250-318) unexpectedly did provoke a marked increase in endogenous GATA-1 expression. The relative roles of these mutant polypeptides versus that of the induced endogenous GATA-1 protein in implementing the megakaryocytic program are not known. Mutants containing more of the C-terminal region (Δ331-413 and Δ357-413) or all of it (Δ1-193 and the two serine-to-alanine substitution mutants) did not significantly increase the level of endogenous GATA-1 transcripts. These findings raise the possibility that the C-terminal region of GATA-1 contains an inhibitory domain that interferes with activation of the endogenous gene, at least in megakaryocytic lines.

There are striking similarities between the action of GATA factors in 416B myeloid cells and that of MyoD family members in 10T1/2 embryonic fibroblasts. Myogenesis can be triggered in 10T1/2 cells by enforced expression of MyoD and its relatives or by treatment with 5-azacytidine (15). Furthermore, only 68 amino acids spanning the MyoD DNA-binding domain, a basic/helix-loop-helix motif, were required to elicit myogenesis in stable transfectants (30). The activity of MyoD in differentiation requires its dimerization with the ubiquitously expressed E12/E47 proteins (13). In transfectants harboring only the basic/helix-loop-helix domain of MyoD, it is likely that the transactivation domain provided by its E12/E47 partner allows myoblast formation.

Consistent with the findings reported here, the DNA-binding domain of GATA-1 has also been found to function in an entirely different biological assay; this domain alone partially rescues the block in erythroid development of GATA-1$^-$ ES cells (1a). The mechanism by which a single GATA zinc finger can mediate biological activity remains to be established. One possibility is that the differentiative function is conveyed directly by the DNA-binding activity. For example, binding of the single C-terminal finger may displace repressors bound to critical genes required for differentiation. Pertinently, activity of the glycophorin B promoter in erythroid cells requires displacement of a repressor by GATA-1 (25). DNA binding may also alter the conformation of DNA. GATA-1, like many other transcription factors, can bend DNA (22, 27). DNA bending can strongly influence promoter activity (21), probably by altering contact between other components of the transcription apparatus. Thus, DNA binding by the GATA factors may itself be sufficient to alter the genetic program of the cell.

An alternative hypothesis is that the single GATA finger can recruit another polypeptide with a transcriptional activation domain. There is increasing evidence that the zinc finger motifs within transcription factors can act as protein-protein interfaces, as well as a specific DNA recognition surface. For example, the N-terminal finger of the glucocorticoid receptor mediates homodimerization (16) and the transcription factors YY1 and Sp1 appear to associate via their zinc finger domains (14, 28). Furthermore, the zinc finger region of the E1A transactivation domain can bind to the TATA box binding protein (8). Interestingly, the GATA-1 C-terminal finger region has been found to associate with Sp1 (18a). Thus, the GATA C-terminal finger may directly interact with another protein in 416B cells, perhaps even with the endogenous GATA-2 or induced GATA-1 protein, in order to mediate megakaryocytic differentiation.

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