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Erythroid-Cell-Specific Properties of Transcription Factor GATA-1 Revealed by Phenotypic Rescue of a Gene-Targeted Cell Line

MITCHELL J. WEISS,[1] CHANNING YU,[1] AND STUART H. ORKIN[1,2]*

Division of Hematology-Oncology, Children's Hospital, Dana-Farber Cancer Institute, and Department of Pediatrics, Harvard Medical School,[1] and Howard Hughes Medical Institute,[2] Boston, Massachusetts 02115

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The zinc finger transcription factor GATA-1 is essential for erythropoiesis. In its absence, committed erythroid precursors arrest at the proerythroblast stage of development and undergo apoptosis. To study the function of GATA-1 in an erythroid cell environment, we generated an erythroid cell line from in vitro-differentiated GATA-1$^-$ murine embryonic stem (ES) cells. These cells, termed G1E for GATA-1$^-$ erythroid, proliferate as immature erythroblasts yet complete differentiation upon restoration of GATA-1 function. We used rescue of terminal erythroid maturation in G1E cells as a stringent cellular assay system in which to evaluate the functional relevance of domains of GATA-1 previously characterized in nonhematopoietic cells. At least two major differences were established between domains required in G1E cells and those required in nonhematopoietic cells. First, an obligatory transactivation domain defined in conventional nonhematopoietic cell transfection assays is dispensable for terminal erythroid maturation. Second, the amino (N) zinc finger, which is nonessential for binding to the vast majority of GATA DNA motifs, is strictly required for GATA-1-mediated erythroid differentiation. Our data lead us to propose a model in which a nuclear cofactor(s) interacting with the N-finger facilitates transcriptional action by GATA-1 in erythroid cells. More generally, our experimental approach highlights critical differences in the action of cell-specific transcription proteins in different cellular environments and the power of cell lines derived from genetically modified ES cells to elucidate gene function.

Hematopoietic development is regulated in large part by nuclear proteins that activate (or repress) sets of genes characteristic of individual lineages. Studies of globin gene expression have formed a conceptual framework for investigating the transcriptional control of erythropoiesis. Among erythroid transcription factors, GATA-1 is of particular interest. GATA-1 was identified as a protein that binds to a consensus GATA motif initially appreciated in globin gene promoters and enhancers and was thereafter found in the *cis*-regulatory elements of virtually all erythroid-cell-expressed genes (30, 52). Forced expression of GATA-1 in selected hematopoietic cell lines alters their phenotype. For example, GATA-1 expression induces megakaryocytic differentiation in the myeloid cell line 416B (48–50) and reprograms transformed avian myelomonocytic cell lines to three lineages—erythroid, eosinophilic, and megakaryocytic—apparently in a concentration-dependent manner (19). In parallel with lineage programming, myeloid markers of host cells are down-regulated, suggesting that reciprocal transcriptional effects may underlie commitment to a unique pathway. How GATA-1 functions to regulate transcription in different cellular contexts is unknown.

GATA-1 is the founding member of a small family of transcription factors that recognize the consensus motif (A/T)GATA(A/G) through a highly conserved zinc finger domain. Six different GATA members (designated GATA-1 thorough GATA-6) have been identified in vertebrates (20, 57). Proteins with related GATA fingers are also present in yeasts, fungi, *Drosophila melanogaster*, and *Caenorhabditis elegans* (1, 5, 12, 18, 42). In hematopoietic tissues, GATA-1, GATA-2, and GATA-3 exhibit unique but overlapping patterns of expression (52). GATA-1 is expressed in erythroid, eosinophilic, mast, and megakaryocytic lineages and in multipotential progenitors. GATA-2 is present in early hematopoietic progenitors, mast cells, and megakaryocytes, whereas GATA-3 is expressed primarily in T lymphocytes. Each family member is also expressed in selected nonhematopoietic tissues.

GATA-1 is the predominant family member expressed in maturing erythroid cells (21). Like other transcription factors, GATA-1 appears to be a modular protein comprised of separable DNA-binding and transcriptional activation domains (25, 55, 58). The DNA-binding region consists of two highly conserved zinc fingers: the carboxyl (C) finger is necessary and sufficient for binding, while the amino (N) finger stabilizes this interaction, most clearly at a small subset of GATA motifs (46). Excluding the finger domains, murine (or human), chicken, and frog GATA-1 polypeptides exhibit little amino acid similarity (9, 60), an unusual finding for transcription factors believed to function in similar pathways in different species. Potential erythroid target genes for GATA-1 include globins, erythroid transcription factors (including SCL/tal-1, erythroid Krüppel-like factor [EKLF] and GATA-1 itself), the Duffy antigen/chemokine receptor (45), and the erythropoietin (Epo) receptor (EpoR) (reference 52 and references therein).

Gene-targeting experiments in mouse embryonic stem (ES) cells reveal that GATA-1, the product of an X chromosome locus, is essential for normal erythropoiesis (35, 40). Erythroid cell precursors lacking GATA-1 arrest at the proerythroblast stage of development and undergo apoptosis (34, 51, 54). Surprisingly, GATA-1$^-$ proerythroblasts express all GATA target genes examined. Transcription of these genes is believed to occur through the action of GATA-2, whose expression is

* Corresponding author. Mailing address: Howard Hughes Medical Institute, Division of Hematology, Children's Hospital, 300 Longwood Ave., Harvard Medical School, Boston, MA 02115. Phone: (617) 355-7910. Fax: (617) 355-7262. E-mail: orkin@rascal.med.harvard.edu.

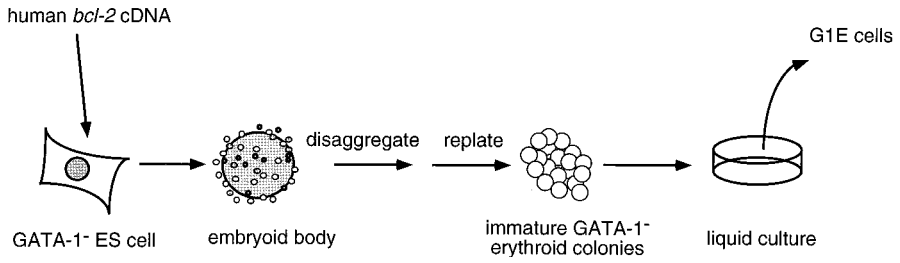

FIG. 1. Isolation of G1E cells, a murine GATA-1$^-$ erythroid cell line. Human *bcl-2* cDNA in an erythroid expression vector was introduced into GATA-1$^-$ ES cells. Stable lines containing the transgene were used to derive pure erythroid cell colonies in methylcellulose cultures by a two-step in vitro differentiation method. Individual erythroid cell colonies were isolated and expanded in liquid medium.

elevated approximately 50-fold compared to wild-type proerythroblasts.

Elucidating the functions of GATA-1 in erythroid cell development has been challenging, in part due to the lack of suitable cellular systems. An ideal experimental system would take advantage of genetically modified, committed erythroid cells for functional complementation. While introduction of GATA expression constructs into GATA-1$^-$ ES cells partially restores viability and globin mRNA accumulation in embryoid bodies generated by in vitro differentiation, full analysis of GATA-1 function by this approach is compromised by suboptimal transgene expression and, most notably, the failure to rescue erythroid maturation to a late stage (2). Here, we describe structure-function studies in a novel GATA-1$^-$ erythroid cell line, termed G1E, that we have isolated from gene-targeted ES cells. G1E cells closely resemble primary GATA-1$^-$ erythroblasts and, importantly, undergo terminal erythroid maturation upon restoration of GATA-1 activity. Therefore, these cells constitute the first experimental system for stringent assay of GATA-1 function in an erythroid cellular context. Using these cells, we have identified critical differences between the function of GATA-1 assessed in erythroid and nonhematopoietic cells, an observation that raises caution regarding extrapolating biological conclusions from experiments performed in heterologous cell contexts. More generally, our findings illustrate the value and promise of studying gene function in lineage-committed cell lines generated from ES cells with targeted mutations.

MATERIALS AND METHODS

Plasmids. To express bcl-2 in erythroid cells, we modified the vector HS II β (1.9), a plasmid containing the human β-globin gene linked to a 1.9-kb fragment from DNase-hyper sensitive site II of the β-globin locus control region (36). The β-globin initiation codon was removed by *Nco*I digestion followed by mung bean nuclease treatment and insertion of a *Not*I linker. Human *bcl-2* cDNA was inserted in the sense orientation into the newly created *Not*I site. A hygromycin resistance cassette driven by the human *EF1*α promoter was cloned into a unique *Sal*I site of the plasmid.

The GATA-1 mutant cDNAs Δ63 (ID5) and Δ200–248 (XN4) were isolated as *Nco*I-*Not*I fragments from previously described plasmids (25) and subcloned into the retroviral vector MFG (8), which was modified to contain a 3′ *Not*I cloning site. Chimeric GATA finger constructs mGATA-1(fareA), mGATA-1(Nf/areA), and mGATA-1(fhGATA-3) (see Fig. 5A) were generated by PCR with previously described plasmids as templates (26). All PCRs were performed with *Pfu* DNA polymerase (Stratagene). PCR-derived clones were checked by restriction enzyme mapping, and at least two independent clones were tested in all functional studies.

Cell lines. The human *bcl-2* erythroid expression vector was stably introduced into GATA-1$^-$ ES cells (clone 74 [35]) by electroporation followed by selection with hygromycin. Clones containing the *bcl-2* transgene were isolated, and erythroid cell colonies were generated by a two-step in vitro differentiation assay (14). Briefly, ES cells were induced to form embryoid bodies, aggregates of differentiated cells that include erythroid cell precursors. Embryoid bodies cultured for 10 days were disaggregated by incubation in 0.5% trypsin at 37°C for 5 min followed by passage through a 20-gauge needle. The resultant cell suspension was plated at a density of 10^5 cells/ml in methylcellulose cultures containing Epo at 2 U/ml and kit-ligand (KL) at 50 ng/ml. These conditions favor the development of definitive (adult-type) erythroid cell colonies (14). After 4 to 5 days, individual colonies were transferred to liquid cultures containing Iscove's modified Dulbecco's medium with 15% fetal calf serum, 4.5×10^{-5} M monothioglycerol, 2 U of Epo per ml, and 50 ng of KL per ml. Cells from one group of colonies continued to proliferate in liquid culture and gave rise to the G1E cell line.

Retroviral infections. A transient-transfection system was used to create retroviruses harboring GATA-1 cDNA and various derivatives (32, 33). G1E cells were infected by cocultivation with virus-producing BOSC 23 lines for 36 h and transferred to fresh growth medium for 2 to 3 days prior to analysis.

Immunofluorescence. Cells were cytocentrifuged onto glass slides, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 3 min. After being blocked in 5% goat serum, the cells were incubated with a mixture of polyclonal rat anti-mGATA-1 (1:300; a gift from D. Engel) and polyclonal rabbit anti-murine band 3 (1:500; gift from R. Kopito) sera for 1 h. Bound antibodies were detected with fluorescein isothiocyanate-labeled goat anti-rat and rhodamine-labeled goat anti-rabbit immunoglobulin Gs (Jackson ImmunoResearch, West Grove, Pa.).

Gel shift assay. To test the DNA-binding activity of GATA-1 mutants, nuclear extracts were prepared from G1E cells 48 h after retroviral infection as described previously (25). Gel shift experiments were performed, with a 29-bp oligonucleotide derived from the mouse α_1-globin gene as a probe, as described previously (25).

Western blotting. Nuclear extracts were prepared from murine erythroleukemia (MEL) cells and G1E cells infected with various retroviral constructs. Protein extracts were fractionated on sodium dodecyl sulfate–12% polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. GATA proteins were detected with either rat anti-mGATA-1 polyclonal serum (1:500 dilution) or rat anti-mGATA-1 monoclonal antibody N6 at 20 ng/ml (Santa Cruz Biotechnology, Santa Cruz, Calif.). Bound antibody was detected with peroxidase-labeled secondary antibody followed by chemiluminescence with the ECL kit (Amersham) as specified by the manufacturer.

RNA analysis. Total cellular RNA isolated with the RNeasy Total RNA kit (Qiagen, Chatsworth, Calif.) was fractionated on a 1.3% agarose–formaldehyde gel, transferred to a nitrocellulose membrane (Hybond C+; Amersham), and hybridized to radiolabeled mouse α-globin and β-actin probes as specified by the manufacturer. Northern blots were washed at 65°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate.

RESULTS

Isolation of G1E cells, a GATA-1$^-$ erythroid cell line. To assess the role of GATA-1 in erythroid maturation, we originally sought to determine whether developmental arrest of GATA-1$^-$ proerythroblasts (51) and apoptosis (54) could be uncoupled. Our strategy was to forestall the death of GATA-1$^-$ proerythroblasts by expressing human *bcl-2* (17) (Fig. 1). We reasoned that if apoptosis were blocked by bcl-2, developmentally arrested GATA-1$^-$ cells might continue to proliferate. GATA-1$^-$ ES cells stably transfected with human *bcl-2* in an erythroid expression vector were subjected to in vitro differentiation under conditions designed to generate predominantly definitive (adult-type) erythroid cell colonies (see Materials and Methods). One clone of ES cells gave rise to normal numbers of erythroid cell colonies that exhibited prolonged survival. From these colonies, we expanded a hematopoietic cell line, termed G1E (for GATA-1$^-$ erythroid). This

TABLE 1. Characteristics of G1E cells

Parameter	Characteristic in:	
	G1E cells	Primary GATA-1$^-$ erythroblasts
Morphology	Proerythroblast	Proerythroblast
Karyotype	40XY (normal male)	40XY (normal male)
Growth factor requirements	Epo and KL	Epo and KL
Growth in liquid or methylcellulose cultures	Divide continuously	Undergo spontaneous apoptosis
Expression of erythroid markers (globins, heme biosynthetic enzymes, band 3, EKLF)	Yes	Yes
Expression of macrophage (Mac-1), granulocyte (Gr-1), T-cell (CD2, CD3, CD5), B-cell (B220), megakaryocyte (4A5) surface markers	No	ND[a]
Expression of GATA-1 (Western blot, gel shift, immunofluorescence)	No	No
Expression of GATA-2	Yes (mRNA elevated >20-fold relative to MEL cells)	Yes (mRNA elevated ~50-fold relative to wild-type erythroblasts)
Spontaneous erythroid maturation	No	No
GATA-1-dependent terminal erythroid maturation	Yes	Yes

[a] ND, not done.

line has been maintained in continuous culture for more than 1 year.

As described in Table 1, G1E cells retain features of primary definitive-type erythroblasts derived from GATA-1$^-$ ES cells. The cells are euploid and exhibit blast-like morphology but do not undergo spontaneous terminal differentiation. Growth requires two hematopoietic cytokines, Epo and KL (stem cell factor). GATA-2 mRNA is expressed at a high level (relative to the expression in wild-type proerythroblasts or erythroleukemia cells). Adult-type (definitive) globins α and β are expressed at low levels, whereas embryonic (primitive) globins ε and ζ are undetectable (data not shown). As expected, G1E cells contain no GATA-1 as assessed by gel shift assay, Western blotting, or immunofluorescence (see below). When cultured in Epo and KL, G1E cells do not express cell surface markers for nonerythroid cell lineages, including T cells (CD2, CD3, and CD5), B cells (B220), macrophages (Mac-1), neutrophils (Gr-1), and megakaryocytes (4A5 [3]) (data not shown).

In contrast to primary GATA-1$^-$ erythroblasts, G1E cells fail to undergo spontaneous apoptosis; instead, they divide continuously in liquid culture. However, after several months in culture, no human *bcl-2* mRNA was detected by Northern blot analysis or reverse transcription-PCR (data not shown). Although the role of bcl-2 in generating the G1E cell line is uncertain, our results demonstrate that arrest of development and apoptosis can be uncoupled in GATA-1$^-$ erythroid precursors. Except for their viability, G1E cells are remarkably similar to primary GATA-1$^-$ proerythroblasts.

GATA-1 overcomes the block to erythroid maturation in G1E cells. The immature phenotype of G1E cells might reflect either the specific absence of GATA-1 or unknown genetic (or epigenetic) events taking place during their isolation and subsequent in vitro culture. To distinguish between these possibilities, we infected G1E cells with retrovirus harboring wild-type mouse GATA-1 (mGATA-1) cDNA and thereafter plated the population in methylcellulose medium. Mock-infected G1E cells give rise to white colonies containing only immature erythroblasts (Fig. 2A, left panels). In contrast, G1E cells exposed to GATA-1 retrovirus generate a subpopulation of smaller, hemoglobinized colonies that contain mature erythroid cells, mainly late pronormoblasts and occasional anucleate erythrocytes (Fig. 2A, right panels). Therefore, G1E cells are unique in that they exhibit GATA-1-dependent terminal maturation.

GATA-1-expressing G1E cells maintained in liquid culture also undergo terminal erythroid maturation as evidenced by the appearance of benzidine-positive cells (Fig. 2B), progression to late normoblast stages (see Fig. 3A), and induction of the membrane cytoskeletal protein band 3 (erythrocyte anion exchanger [see Fig. 3A]), a marker for mid- to late pronormoblasts (56). We saw no morphologic evidence for mast cell or megakaryocytic differentiation in liquid or semisolid cultures of GATA-1-expressing G1E cells, despite the capacity of GATA-1 to induce commitment to these lineages in certain transformed hematopoietic cell lines (19, 49). The more limited developmental potential of G1E cells could reflect their immortalization at a committed stage. Alternatively, the culture conditions used here may favor erythroid maturation.

Importantly, we estimate that the GATA-1 protein level in rescued G1E cells is similar to that in MEL cells when normalized for the fraction of the G1E cell population transduced by retrovirus (Fig. 2C). This indicates that a physiologic, rather than supranormal, level of GATA-1 is provided to these cells by the retroviral vector we have used. This is an important control in assessing the biological relevance of the findings presented below.

Use of G1E cells for structure-function analysis of GATA-1. Based on the above properties of G1E cells, we proceeded to test the capacity of various derivatives of GATA-1 to induce maturation of G1E cells. cDNAs were introduced into G1E cells by retroviral transfer, and infected cells were analyzed for protein expression and erythroid maturation. GATA-1 expression was evaluated by Western blotting, immunofluorescence, and gel shift assay. Erythroid maturation was assessed by benzidine staining, immunofluorescence for induction of band 3,

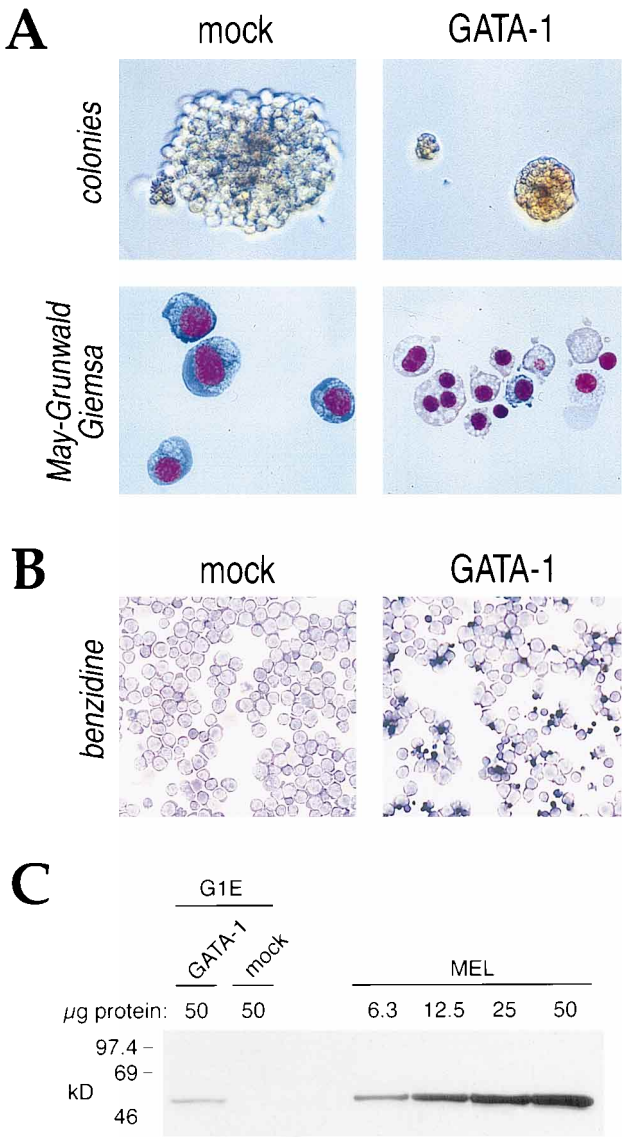

FIG. 2. GATA-1 induces erythroid maturation of G1E cells. Murine GATA-1 cDNA was introduced into G1E cells by retroviral transfer. Infected cells were analyzed for erythroid maturation and GATA-1 expression as described below. (A) Methylcellulose colony assays. Immediately after retroviral infection, cells were seeded into methylcellulose suspension cultures and incubated for 4 days. Mock-infected cells generate large white colonies (upper panel, left) that contain immature erythroblasts (lower panel, left). In contrast, GATA-1-expressing G1E cells form smaller, hemoglobinized colonies (upper panel, right) that contain mainly late pronormoblasts and some anucleate cells (lower panel, right). Original magnifications, ×200 (upper panels) and ×1,000 (lower panels). (B) Benzidine staining. Cytocentrifuge preparations of cultures maintained in liquid medium were analyzed 3 days after infection. G1E cells infected with the GATA-1 expression vector give rise to a population of darkly staining benzidine-positive cells (right). No benzidine-positive cells were observed in mock-infected G1E cells (left). Original magnification, ×400. (C) Western blot analysis with a monoclonal antibody directed against mGATA-1. Nuclear extracts were prepared from G1E cells 2 days after retroviral infection and compared to similar extracts from MEL cells. The amount of total protein loaded per lane is shown. The GATA-1 protein level in rescued G1E cells approximates that in MEL cells when normalized for the fraction of the G1E cell population transduced by retrovirus (~5 to 10% [data not shown]). Extracts from uninduced MEL cells were used in this experiment. The abundances of GATA-1 protein (data not shown) and mRNA (47) do not change appreciably during chemical-induced MEL cell differentiation.

Northern blotting for induction of globin mRNA, and histologic testing. Our findings are summarized below.

An "obligatory" transactivation domain is dispensable for terminal erythroid maturation. Sequences within the 70 N-terminal amino acids of mGATA-1 are strictly required for transactivation in nonhematopoietic cells (such as COS, NIH 3T3, HeLa, and quail fibroblasts) and act as an independent activator domain when fused to a heterologous DNA-binding domain (25). To test if transactivation provided by GATA-1 correlates with its capacity to drive erythroid differentiation, we introduced a derivative of GATA-1 lacking this region (GATA-1 Δ63) into G1E cells. Remarkably, the Δ63 protein induces erythroid maturation to the same extent as does the full-length GATA-1 by all criteria examined, including induction of benzidine-positive cells, morphology characteristic of terminal erythroid differentiation, induction of band 3 (Fig. 3A), and up-regulation of α-globin (Fig. 3B) and β-globin (data not shown) mRNAs. The levels of wild-type GATA-1 and Δ63 proteins expressed in G1E cells are comparable (Fig. 3C and D) and similar to that present normally in MEL cells (Fig. 2C and 3C). Therefore, the capacity of Δ63 to promote terminal erythroid maturation is not due to overexpression of a partially crippled transcription factor but is an intrinsic property of the protein. We conclude that the N-terminal domain of GATA-1, which has been defined as an "obligatory" transactivation domain in nonhematopoietic cells, is dispensable for terminal erythropoiesis.

In an effort to define the minimal region of GATA-1 sufficient for terminal erythroid maturation, we removed more extensive portions of the protein surrounding the two-finger DNA-binding domain. Successive N-terminal deletions beyond amino acid 63 and, to a lesser extent, deletions from the C terminus progressively impaired both protein accumulation and erythroid differentiation (data not shown). The diminished expression of these truncated variants in G1E cells obscures identification of protein domains that participate in terminal erythroid maturation; the small extent of G1E cell rescue might relate to the removal of transactivation functions but might be equally likely to relate to a dependence of induced maturation on the concentration of GATA-1 protein.

Analysis of the DNA-binding domain of mGATA-1. We sought to answer two specific questions regarding the DNA-binding domain of GATA-1. First, are both zinc fingers necessary for erythroid differentiation? Second, can zinc fingers from related proteins substitute for homologous regions of GATA-1? Accordingly, a series of deletion and substitution derivatives of GATA-1 were introduced into G1E cells.

Both fingers of GATA-1 are required for erythroid maturation. We first tested whether DNA binding by GATA-1 is necessary for erythroid maturation. Although the non-DNA-binding mutant C261P (which disrupts the structure of the critical C finger [25]) accumulates to a high level in the nucleus, it fails to induce maturation of G1E cells (Fig. 4). Hence, as anticipated, loss of the DNA-binding capacity of GATA-1 leads to a protein that is biologically inactive for erythroid differentiation.

The role of the N finger in mediating functions of GATA-1 has been less clear. Although the N finger stabilizes GATA-1 binding to a small subset of GATA motifs, transactivation properties of N-finger-deleted versions of GATA-1 are only modestly affected (25, 58). To test N-finger function in the context of erythroid maturation, a derivative lacking the N finger (GATA-1Δ200–248) was examined. Despite its accumulation to a level comparable to wild-type GATA-1 in G1E cells (Fig. 4; also see Fig. 5), the N-finger-deleted protein also fails to induce benzidine positivity (Fig. 4), cell surface band 3 protein,

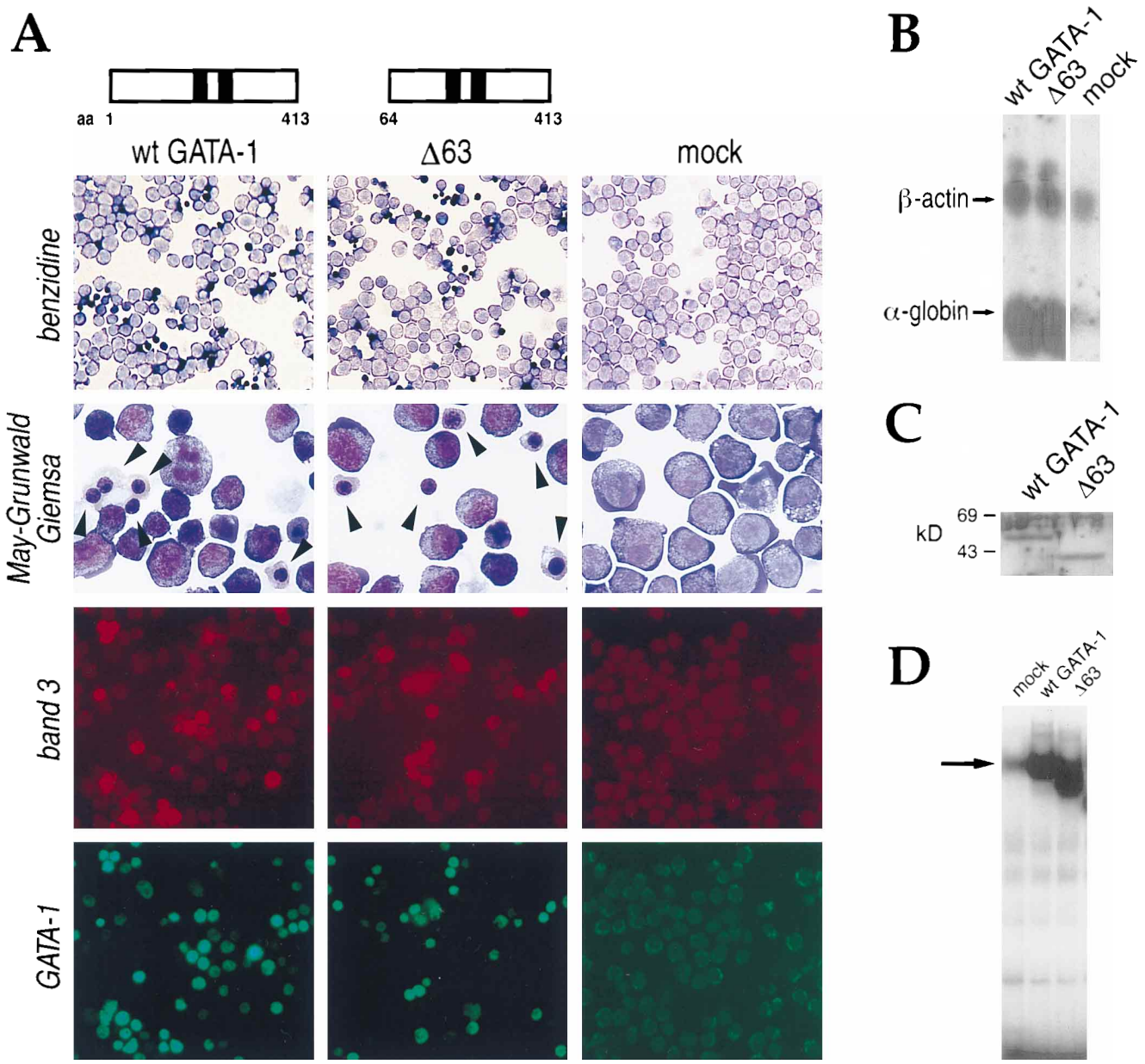

FIG. 3. The N-terminal obligatory activation domain of GATA-1 is dispensable for terminal erythroid maturation. (A) GATA-1Δ63 cDNA, which lacks the N-terminal domain, was introduced into G1E cells and erythroid maturation was determined as shown. The Δ63 protein and wild-type GATA-1 are equivalent with respect to generation of benzidine-positive, morphologically differentiated erythroid cells (arrowheads) and induction of cell surface band 3, a marker for mid- to late pronormoblasts. GATA-1 expression and band 3 expression were generally concordant by coimmunofluorescence, although a minority of cells apparently expressed only one of these proteins. Presumably, cells that stain only for band 3 express GATA-1 below the detectable level, since mock-infected populations express no band 3. GATA-1 expression in the absence of band 3 could reflect cells at an early stage of maturation, or a subpopulation of cells that have lost the capacity to mature. Original magnifications: benzidine staining and immunofluorescence, ×400; May-Grunwald Giemsa, ×1,000. (B) Induction of α-globin mRNA by full-length GATA-1 and the Δ63 mutant. Northern blot analysis with total RNA (10 μg/lane) from G1E cells infected with the indicated constructs is illustrated. A β-actin probe was used to standardize for the amount of total RNA in each lane. (C) Western blot analysis with a polyclonal antiserum directed against mGATA-1, demonstrating that the Δ63 and wild-type mGATA-1 proteins accumulate to similar levels in rescued G1E cells. (D) Gel shift assay of wild-type and GATA-1Δ63 expressed in G1E cells. Nuclear extracts of G1E cells infected with the indicated constructs were tested for DNA-binding activity with a radiolabeled 29-bp oligonucleotide spanning the TGATAA site from the mouse α-globin promoter (24). The arrow indicates the complex containing endogenous GATA-2, which is present in all samples and supershifts with anti-mGATA-2 antiserum (data not shown).

or globin mRNAs (α and β, [data not shown]). We conclude, therefore, that both zinc fingers of GATA-1 are required for terminal erythroid maturation (as summarized in Fig. 5).

Zinc fingers from related proteins are able to function within the mGATA-1 backbone. Although the zinc finger regions are highly conserved among GATA family members, random-site-selection assays have revealed subtle differences in DNA-binding specificities (15, 26, 55). To investigate their biological significance, we asked if erythroid maturation requires fingers of vertebrate GATA-1 proteins in particular or whether homologous fingers of nonerythroid GATA proteins might suffice. We examined GATA proteins in which the two zinc fingers of mGATA-1 were replaced by the zinc fingers of human GATA-3 or the single finger of the fungal protein *areA*

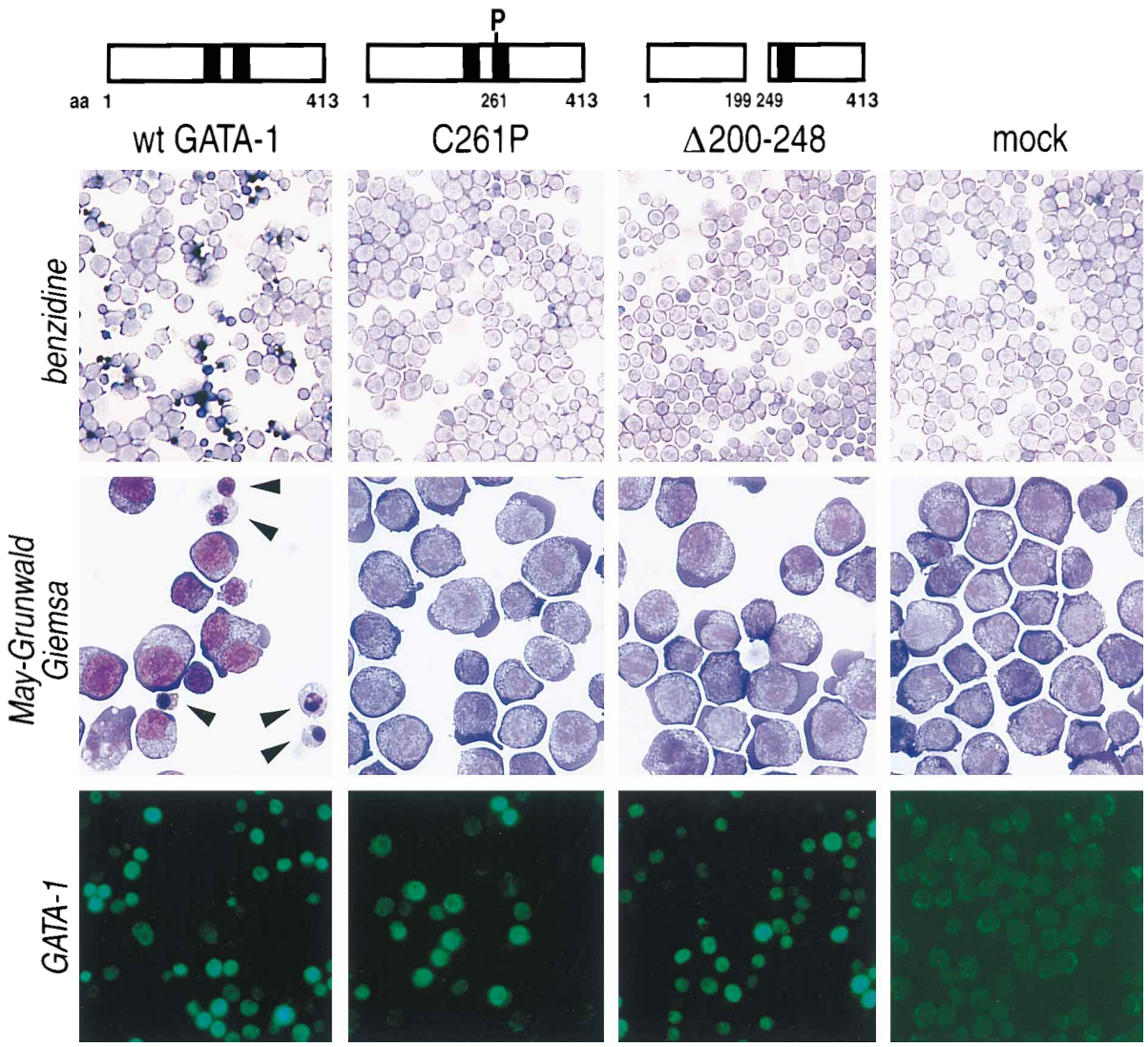

FIG. 4. Both zinc fingers of mGATA-1 are required for erythroid maturation. GATA-1 derivatives were introduced into G1E cells. Erythroid maturation was assessed by benzidine staining and morphology. Mature erythroid cells (normoblasts) are marked by arrowheads. Protein expression was determined by immunofluorescence. Disruption of the C finger (C261P; Cys → Pro, which prevents DNA binding) or removal of the N finger (Δ200–248) abrogates the ability of GATA-1 to induce G1E cell maturation.

(Fig. 5). Among vertebrate GATA proteins, the finger of *areA* most closely resembles the C finger of GATA-1 in structure and DNA-binding specificity (26). All finger substitution constructs localized to the nucleus (data not shown) and accumulated to high levels in G1E cells as assessed by gel shift and Western blot assays (Fig. 5B and C).

mGATA-1(fareA), which contains a single *Aspergillus* finger embedded in the body of mGATA-1, fails to induce erythroid maturation. Thus, despite its presence within a functional GATA protein in fungi, the single *areA* finger is inactive in G1E cells, a finding consistent with our observation that a two-finger arrangement is essential for function in erythroid development. However, a chimeric protein in which the *areA* finger replaces only the carboxyl finger of GATA-1 [mGATA-1(Nf/areA)] allows for substantial erythroid maturation. Likewise, substitution of both GATA-1 fingers with the homologous DNA-binding region of human GATA-3 leads to a functional chimeric protein [mGATA-1(fhGATA-3)]. Therefore, despite subtle differences in DNA-binding specificity defined in vitro, zinc fingers of GATA family members retain common functions. In this regard, GATA-1 differs from MyoD, whose DNA-binding domain contains a "recognition code" for muscle-specific gene activation that is not present in homologous basic regions of nonmuscle basic helix-loop-helix (bHLH) proteins (6). While erythroid maturation capabilities are encoded in the DNA-binding domains of nonerythroid GATA proteins, the reduced rescue efficiencies of the mGATA-1(fareA) and mGATA-1(fhGATA-3) derivatives may reflect functional differences arising from subtle alterations in DNA-binding specificity or protein conformation.

DISCUSSION

Establishing cellular models for analysis of gene knockouts. Dissection of gene function requires suitable genetic systems. The creation of mutant strains of mice from gene-targeted ES cells has provided a tool of extraordinary power. Often, however, detailed analysis of mice or cells isolated directly from

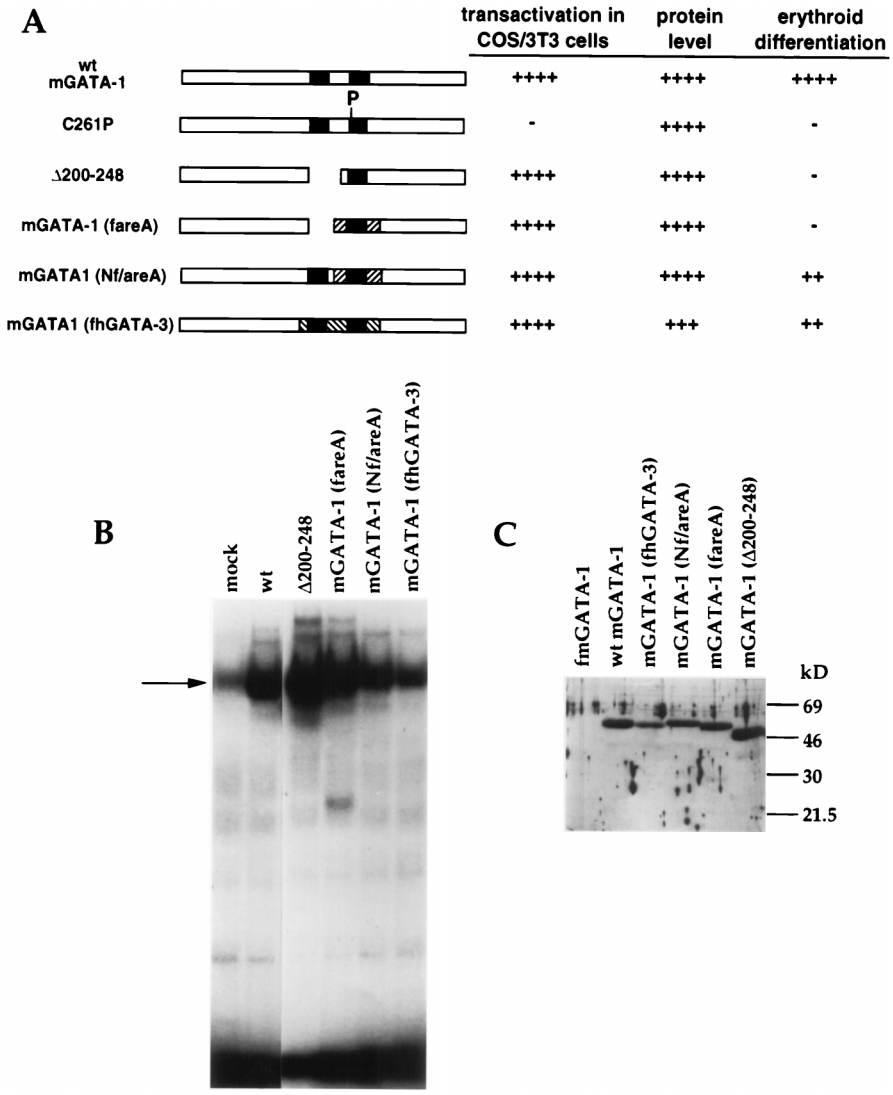

FIG. 5. Structure-function mapping of the DNA-binding region of GATA-1 in G1E cells: effects of zinc finger deletions and substitutions. (A) Summary of the mutant proteins and their activities. Mutants are represented schematically at the left. Shaded regions represent the two zinc finger DNA-binding domains. fareA refers to the single zinc finger present in the *areA* protein of *Aspergillus nidulans*. fhGATA-3 refers to the DNA-binding domain of human GATA-3. Transactivation in heterologous cells (either NIH 3T3 or COS), determined by cotransfection assays with promoter-reporter constructs, is described elsewhere (24, 27, 50). Protein expression refers to accumulation of the respective proteins in G1E cells, as estimated by immunofluorescence (data not shown), gel shift (B), and Western blot (C) assays, which were in general agreement. Induction of erythroid differentiation is graded from − to ++++ according to the percentage of benzidine-positive cells relative to those obtained with rescue by wild-type (wt) GATA-1: −, 0%; ++, 26 to 50%; +++, 51 to 75%; ++++, 76 to 100%. (B) Gel shift assay of zinc finger deletion and substitution mutants. Nuclear extracts of G1E cells infected with the indicated constructs were analyzed for GATA-binding activity as described for Fig. 3D. The arrow identifies the complex containing endogenous GATA-2, which supershifts with anti-GATA-2 antiserum (data not shown). (C) Western blot of mutant proteins expressed in G1E cells. Nuclear extracts of G1E cells infected with the indicated constructs were analyzed with a polyclonal antiserum prepared against mGATA-1. fmGATA-1 designates a minimal finger construct consisting of amino acids 194 to 318 of mGATA-1. This construct expressed no detectable protein in G1E cells.

them is not feasible. Cell lines from mice with targeted mutations complement studies in whole animals. For example, embryonic fibroblasts from p53 gene-targeted mice have been useful in examining cellular responses to DNA damage (7, 22, 38). An Abelson virus-transformed pre-B-cell line from Oct-$2^{-/-}$ fetal liver allowed the identification of a potential Oct-2 target gene (16). As described here, we have taken a novel approach to exploring how a particular transcription factor, GATA-1, acts to promote erythroid cell maturation. We have generated a lineage-committed cell line, G1E, from gene-targeted ES cells differentiated in vitro, which very closely resembles the developmentally arrested GATA-1^{-} proerythroblasts from which it was derived. G1E cells are unique in that they retain the capacity to complete terminal erythroid maturation upon expression of a single nuclear regulator, GATA-1. Besides providing an invaluable tool for dissecting regions of the GATA-1 protein critical for maturation, these cells should permit the study of genes activated (or repressed) by GATA-1 and the mechanisms by which cell cycle arrest is programmed in terminal differentiation of erythroid cells.

Given the growing number of genes shown to be essential for various aspects of hematopoiesis (39), the study of cell lines derived from gene-targeted ES cells offers great promise. Our data underscore the utility of such a cell line, but we caution

that the use of *bcl-2* cannot be considered as a general approach for its derivation. What role, if any, the *bcl-2* transgene played in the generation of the G1E cell line is uncertain. We do not consider this an obstacle to further use of this strategy, as several other approaches are available to immortalize committed ES cell-derived lineages. For instance, hematopoietic cell lines may be isolated from in vitro-differentiated ES cells following infection with retrovirus encoding the murine *Hox 11* gene (13a). Moreover, erythroid-like cell lines have also been obtained from embryoid bodies after infection with myeloproliferative leukemia virus (41) or retrovirus expressing the *myc* and *raf* oncogenes (7a, 28). Although in vitro differentiation of murine ES cells has been used extensively to study hematopoietic lineages, production of muscle, vascular, and neuron-like cells has also been described (reviewed in reference 53). In principle, therefore, immortalized lines representing any of these cell types could be derived from gene-targeted ES cells and used for developmental studies.

Erythroid features of GATA-1 function. Our studies are important in revealing aspects of GATA-1 function specific to its action in an erythroid cell environment. Most of what has been previously deduced regarding domains of the GATA-1 protein rests on conventional promoter/reporter experiments performed in transfected nonhematopoietic cells. While this approach has proved useful in the analysis of many transcription factors, it does not ensure an accurate view of how a particular protein acts within a specific cell context. Our results with G1E cells caution against extrapolating the results of rather artificial reporter assays to biological function in vivo.

In our previous efforts to develop a rescue assay involving GATA-1$^-$ ES cells, we were able to demonstrate partial restoration of globin RNA levels and cell viability but did not observe morphological evidence of terminal maturation, even with the introduction of wild-type GATA-1 cDNA (2). This is reminiscent of the incomplete rescue of G1E cells afforded by some poorly accumulating GATA-1 truncation mutants which induce modest globin RNA expression but no other hallmarks of rescue such as benzidine positivity or morphologic changes (data not shown). Given the ability of large genomic fragments from the GATA-1 locus to rescue GATA-1$^-$ ES cells completely in the embryoid body differentiation assay (25a), it is likely that previous difficulties in rescuing ES cells with various constructs reflected inadequate transgene expression. A requirement for high-level GATA-1 expression for full erythroid differentiation is consistent with our recent observation that erythroblasts expressing GATA-1 at a reduced level due to a targeted mutation in the locus exhibit marked retardation in erythroid maturation both in vitro and in vivo (25a).

Complementation of the maturation block in G1E cells defines two clear-cut instances in which the requirement for domains of GATA-1 differs between erythroid and nonhematopoietic environments. These relate to the roles of the obligatory N-terminal transactivation domain and the N zinc finger.

The dispensability of the obligatory N-terminal acidic transactivation region of mGATA-1 is particularly remarkable. This region confers activation upon transfer to a heterologous DNA-binding domain, and its removal leads to the loss of all transcriptional activation by mGATA-1 in nonhematopoietic cell reporter assays (25). Despite this, the GATA-1Δ63 protein is fully competent to induce α-globin and β-globin RNAs and band 3 protein expression, generate benzidine-positive cells, and promote the morphologic changes of terminal maturation. In considering the validity of these observations, it is important to note that the activity of GATA-1Δ63 cannot be attributed to overexpression of a partially crippled protein in rescued cells (Fig. 3).

A precedent for the dispensability of a major transactivation domain for in vivo function has been reported. In early studies of the action of the myogenic factor MyoD, it was noted that expression of 68 amino acids, which was subsequently shown to contain only the bHLH DNA-binding domain, was sufficient to promote myogenesis in cultured cells (44). It is uncertain in retrospect to what extent overexpression of a partially active MyoD could account for this result. Nonetheless, the more recent demonstration that the bHLH region of myogenic factors can tether myocyte enhancer factor 2 to promoters may provide an explanation for the dispensability of the activation domains of MyoD itself (29).

Similarly, GATA-1 need not contribute a transactivation domain per se; it may acquire transactivation properties through protein interactions. For example, the DNA-binding region of GATA-1 mediates homotypic interactions of GATA factors, as well as interactions with Krüppel-related proteins such as Sp1 and EKLF (4, 27, 59). Murine GATA-1 has been reported to interact with another essential erythroid nuclear protein, rbtn-2 (lmo2) (31), and cGATA-1 may interact with a nuclear fraction that stabilizes the binding of TFIID to DNA (11). We speculate below that GATA-1 may associate with additional proteins through the N finger. Our observation that transactivation functions ascribed to GATA-1 may differ in erythroid and nonhematopoietic cells is compatible with experiments demonstrating markedly reduced activation of GATA-reporter constructs in primary chicken erythrocytes as compared with fibroblasts (10).

The second important finding to emerge from our studies with G1E cells relates to the function of the N finger of GATA-1. The N finger of GATA-1 is dispensable for binding to the vast majority of GATA motifs and for transcription assays in heterologous cells (25). In contrast, the N finger is stringently required for erythroid maturation of G1E cells. Although not absolutely required for DNA binding, the N finger stabilizes binding, specifically at rare sites containing two GATA elements arranged as direct or inverted repeats (25, 46, 55, 58). Therefore, the N finger may refine protein-DNA interactions at a subset of cognate sites within developmentally important genes. Functional bipartite GATA sites are present in the gene promoters of chicken α^D-globin, human ζ-globin, human Aγ-globin, and GATA-1 (chicken, mouse, and human) (reference 46 and references therein). The double-GATA motif in the GATA-1 gene promoter contributes to full transcriptional activity in erythroid cells and may represent a biologically relevant target for discrimination by the N finger (46). However, the role of the N finger in mediating interactions with these sites within the GATA-1 promoter is not relevant to our results in the G1E cell rescue assay, since GATA-1 cDNA is expressed from a retroviral promoter and therefore is independent of the action of the GATA-1 protein at the endogenous promoter.

Our observations reveal a central and complex role for the two-finger DNA-binding domain of GATA-1 for biological function in vivo. While the two-finger arrangement is essential for erythroid maturation to occur, fingers from a nonerythroid protein, such as GATA-3, substitute quite well (Fig. 5). Thus, the highly conserved GATA-1 finger domain does not appear to confer intrinsic erythroid-cell-specific action. In this aspect, GATA-factor DNA-binding domains differ from the bHLH domain of the myogenic factors, which appears to participate in restricting activity in a cell-type-specific manner (6).

It is important to recognize that our conclusions regarding the dispensability of the N-terminal activation domain and the

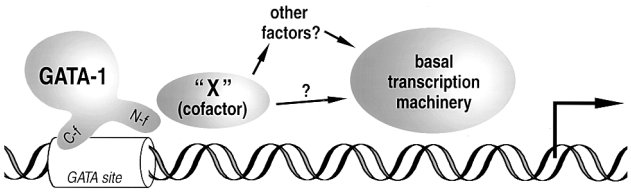

FIG. 6. Model for GATA-1 function in erythroid cells.

requirement for the N-terminal zinc finger are applicable only to the function of GATA-1 in terminal erythroid maturation. Rescue of erythroid maturation in G1E cells does not relate to what role, if any, these domains might play in other hematopoietic lineages (e.g., mast, eosinophil, and megakaryocytic) or an unrelated tissue (e.g., Sertoli cells of the testis). For example, while the N finger of GATA-1 is strictly required for G1E cell maturation, derivatives lacking this region retain some capacity to reprogram myeloid 416B cells to megakaryocytes (50). A role for the N finger in megakaryocyte differentiation remains possible, however, because inclusion of this domain enhances the biological potency of GATA-1 in 416B cells (50). Additional, direct experiments are necessary to address the in vivo functions of GATA-1 in nonerythroid cells.

New view of GATA-1 function in erythroid cells. The dispensability of the "obligatory" N-terminal activation domain for terminal erythroid maturation casts doubt on the conventional view that GATA-1 acts directly as a potent transcriptional activator in erythroid cells. When this is taken together with the stringent requirement for the N finger, we are led to consider an alternate model by which GATA-1 functions. Specifically, we propose that GATA-1 requires an erythroid cofactor(s) to regulate the transcription of its critical target genes during erythroid maturation (Fig. 6). This cofactor (X) is envisioned to interact specifically with the N finger. Erythroid gene expression would then be achieved in a combinatorial fashion whereby GATA-1 binds to its cognate sites in promoters and enhancers and is coupled functionally to transcription complexes by a cofactor. The primary roles of GATA-1 might then be to mark sites in erythroid chromatin and provide a docking site for cofactors. The hypothetical GATA-1 cofactor might contribute transactivation domains to a complex and thereby act in a manner similar to that proposed for the B-cell-specific coactivator OCA-B (OBF-1, Bob1), which interacts with the transcription factors Oct-1 and Oct-2 to drive immunoglobulin gene expression (13, 37, 43). A major difference, however, is that an Oct-1 activation domain is viewed as critical to the function of the Oct-1–OCA-B complex (23) whereas an activation domain from GATA-1 should not be required. Alternatively, cofactor X might merely couple DNA-bound GATA-1 to other proteins within a complex. Finally, we do not exclude the possibility that the cofactor might also recognize DNA and serve to bridge GATA sites to neighboring or distant *cis*-elements or refine the DNA sequence requirements for transcriptional activation through GATA motifs. Erythroid-cell-restricted expression of this cofactor could impose specificity on GATA-1 action and account for the inability of related GATA proteins to activate erythroid genes in other cell types despite marked functional similarities in their DNA-binding regions (Fig. 5). The multifunctional character of the GATA-1 DNA-binding domain central to this model is consistent with prior work demonstrating interactions with other zinc finger proteins of the Krüppel family (27). However, the interacting proteins identified thus far do not appear to exhibit the requisite specificity imposed by our model. Further characterization of the action of GATA-1 in erythroid cells necessitates identification of candidates for the proposed cofactor.

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