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Rescue of GATA-1-Deficient Embryonic Stem Cells by Heterologous GATA-Binding Proteins

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Totipotent murine embryonic stem (ES) cells can be differentiated in vitro to form embryoid bodies (EBs) containing hematopoietic cells of multiple lineages, including erythroid cells. In vitro erythroid development parallels that which is observed in vivo. ES cells in which the gene for the erythroid transcription factor GATA-1 has been disrupted fail to produce mature erythroid cells either in vivo or in vitro. With the EB in vitro differentiation assay, constructs expressing heterologous GATA-binding proteins were tested for their abilities to correct the developmental defect of GATA-1-deficient ES cells. The results presented here show that the highly divergent chicken GATA-1 can rescue GATA-1 deficiency to an extent similar to that of murine GATA-1 (mGATA-1), as determined by size and morphology of EBs, presence of red cells, and globin gene expression. Furthermore, GATA-3 and GATA-4, which are normally expressed in different tissues, and a protein consisting of the zinc fingers of GATA-1 fused to the herpes simplex virus VP16 transcription activation domain were able to compensate for the GATA-1 defect. Chimeric molecules in which both zinc fingers of mGATA-1 were replaced with the zinc fingers of human GATA-3 or with the single finger of the fungal GATA factor areA, as well as a construct bearing the zinc finger region alone, displayed rescue activity. These results suggest that neither the transcription activation domains of mGATA-1 nor its zinc fingers impart erythroid cell specificity for its action in vivo. Rather, it appears that specificity is mediated through the *cis*-acting control regions which determine spatial and temporal expression of the GATA-1 gene. Furthermore, our results demonstrate that the zinc finger region may have a biological function in addition to mediating DNA binding.

The regulation of globin and other erythroid cell-specific genes in vertebrates provides an important model system for the study of tissue-restricted and developmentally controlled gene expression. Among the erythroid cell-specific transcription factors identified to date, GATA-1 is of particular interest because it binds to a motif (A/TGATAA/G) which is found in the promoters and enhancers of nearly all erythroid cell-specific genes studied so far, including the globin genes and their respective locus control regions (for a review, see reference 25).

GATA-1 binding to DNA is mediated by two zinc fingers of the configuration Cys-X_2-Cys-X_{17}-Cys-X_2-Cys. Related proteins designated GATA-2, GATA-3, and GATA-4 were identified by virtue of their high degree of homology in the zinc finger region. These GATA factors all bind to consensus GATA sites with high affinity and only slightly different specificities (15, 23). GATA-binding proteins are also found in nonvertebrates, including *Drosophila melanogaster*, *Caenorhabditis elegans*, and fungi. Outside of the finger region, GATA-1 is much less conserved between various species. In particular, while murine and human GATA-1 (mGATA-1 and hGATA-1, respectively) are very highly related, except for a few conservative changes, chicken GATA-1 (cGATA-1) is very divergent from mGATA-1 (9, 33, 35). GATA-2 and GATA-3 also bear little similarity to GATA-1 but are more homologous to each other (25).

An important distinction among the GATA-binding proteins is their patterns of expression. GATA-1 is expressed at a high level in maturing erythroid cells and at a lower level in multipotential progenitor cells and also in two closely related lineages (megakaryocytes and mast cells) (9, 22, 35). Like GATA-1, GATA-2 is expressed in mast cells, megakaryocytes, and erythroid progenitor cells (42). However, during maturation of erythroid cells, GATA-2 expression decreases while that of GATA-1 increases (19). In addition, GATA-2 is expressed in multipotential progenitor cells and in nonhematopoietic cells such as embryonic stem (ES) cells, endothelial cells, and embryonic brain cells (7, 18, 39, 40). GATA-3 is highly expressed in T-lymphoid cells and embryonic brain (11, 12, 16, 40). GATA-4 is expressed in ovaries, intestine, and the developing heart (1).

This difference in the expression patterns has led to the hypothesis that each member of the GATA family might serve a tissue-specific function. This specificity, in turn, might be determined by the unique primary structure of each family member. An alternative hypothesis suggests that it is not the primary structure of the GATA factors but their timing and patterns of expression that determine their tissue-specific functions.

We have used murine ES cells to address the role of GATA-1 in erythroid development, as a loss-of-function mutation at this locus has been created by gene targeting and its consequences have been examined both in vivo in mouse chimeras and in vitro through embryoid body (EB) differentiation. Through these approaches, GATA-1 has been shown to be essential for normal erythroid cell development. In chimeric mice, GATA-1$^-$ ES cells fail to contribute to the formation of mature erythroid cells but give rise to all other tissues, including leukocytes (26), megakaryocytes, and mast cells (25a). In GATA-1$^-$ EBs differentiating in vitro, mature red cells are absent and globin RNAs of either primitive or definitive lineages (ζ and β, respectively) are nearly undetectable (30). As

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developmental deficits of GATA-1$^-$ ES cells were rescued following introduction of an intact GATA-1 gene (30), we are confident that no inadvertent mutations affecting erythroid cell development were introduced at the time of the initial gene targeting. More recently, with a replating assay in which EBs are disaggregated during their maturation to enumerate hematopoietic progenitors, it has been shown that GATA-1$^-$ ES cells fail to produce primitive erythroid progenitor cells but are capable of forming definitive erythroid colonies containing cells blocked at the proerythroblast stage (37). Remarkably, these GATA-1$^-$ proerythroblasts express GATA-2 at a greatly elevated level and express globin mRNAs at levels comparable to those of wild-type proerythroblasts. While wild-type cells progress to mature red cells, GATA-1$^-$ cells die following proerythroblast arrest (37). From these findings, it has been proposed that differentiation to the proerythroblast stage and expression of globins result from partial compensation by GATA-2. The absence of globin RNAs in differentiating EBs, compared with pure proerythroblasts, appears to be due to accelerated death of these arrested erythroid cell precursors within the context of EBs. In fact, as shown below, GATA-1$^-$ EBs fail to enlarge beyond day 5 of EB development when grown under the specified conditions. An analogous situation seems to occur in chimeric mice generated with GATA-1$^-$ ES cells, in which anemia is disproportionate to the degree of chimerism and reflects unexplained detrimental effects of GATA-1$^-$ cells on the survival of host erythroid cells. Taken together, these studies demonstrate the requirement of GATA-1 for both survival and maturation of erythroid cells.

For the study of gene function, ES cells differentiated in vitro provide an experimental system which is superior to the use of available cell lines. For one, development proceeds from a totipotent to a differentiated cell. Second, development occurs in the presence of normal hematopoietic growth factors, rather than chemical inducing agents. Third, development of erythroid precursors within EBs in vitro closely parallels the appearance of primitive and definitive erythroid cells in the murine yolk sac and fetal liver, respectively (14). Hence, in the studies described here we have used GATA-1$^-$ ES cells as recipients to explore several questions, including the following. (i) Are GATA-1 proteins of different species functionally equivalent? (ii) Does erythroid cell specificity reside in the zinc finger region of GATA-1, or can its in vivo function be subserved by the fingers of other GATA factors? (iii) Can GATA-1 functions be replaced by heterologous GATA proteins that are normally expressed in a different tissue pattern? (iv) Is an erythroid cell-specific activation domain critical to the function of GATA-1?

Our results demonstrate that other members of the GATA family, as well as GATA-1 from different species, can compensate for the GATA-1 deficiency in ES cells as judged from the development of EBs, from the presence of mature erythroid cells, and from the expression of the globin genes. Furthermore, we show that the zinc fingers of GATA-1 can be replaced by the zinc fingers of GATA-3 and even by the single zinc finger of the fungal GATA (fGATA) factor areA, without apparent loss of rescue activity. In addition, we found that a chimera of the zinc fingers of GATA-1 when fused to the herpes simplex virus VP16 transactivation domain, or even the zinc fingers alone, can also rescue the GATA-1 defect. These results suggest that the erythroid cell-specific function of GATA-1 may reside not in its primary sequence but rather in its tissue restriction and timing of expression. In addition, these results demonstrate that the conserved zinc finger region confers function beyond DNA-binding activity alone.

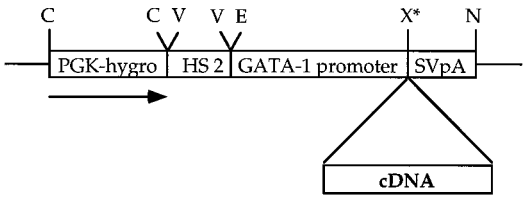

FIG. 1. Design of the rescue vector. A 1.2-kb fragment containing DNase HS2 was placed in front of 2.7 kb of the GATA-1 promoter upstream region. The cDNAs encoding GATA-binding proteins were introduced at the *Xba*I site. PGK-hygro represents the promoter of the phosphoglycerate kinase promoter driving the hygromycin phosphotransferase gene. The polyadenylation site is derived from simian virus 40. The backbone is pBluescript KS+. C, *Cla*I; X, *Xba*I; V, *Eco*RV; E, *Eco*RI; N, *Not*I; *, artificial site.

MATERIALS AND METHODS

Cells and in vitro differentiations. The 129/Sv-derived ES cell line CCE (27) and a GATA-1$^-$ CCE-derived cell line (clone 74 [26]) were grown in the presence of leukemia-inhibitory factor without feeder cells as described elsewhere (14). For in vitro differentiation, cells were trypsinized and resuspended in 0.8% methylcellulose in medium supplemented with 10% fetal calf serum, 2 U of erythropoietin (Amgen) per ml, 500 U of interleukin-1α (IL-1α; Hoffmann-La Roche), IL-3 (conditioned medium from myeloma cells transfected with the IL-3 gene) (13), 5 ng of Kit ligand (Amgen) per ml, and 4.5×10^{-5} M monothioglycerol (38). At day 10, EBs were scored by light microscopy for their morphology, the presence of mature erythroid cells, and expression of ζ-, α-, and β-globin genes. EB size was determined by averaging the diameters of 20 EBs of each clone. Two measurements on each EB were performed. To rule out clonal variation as a cause for differences in erythroid development, several clones of each construct (specified in Results) were examined. Experiments were repeated at least three times to guard against day-to-day variations in plating efficiency and quality of differentiations.

Plasmid constructions. In order to drive expression of transgenes in erythroid cells, the following plasmid was constructed. A fragment containing 2,642 bp of the GATA-1 promoter upstream region extending from an *Eco*RI site to an artificial *Xba*I site at −20 with respect to the first intron (35) was introduced into pBluescript KS+ (Stratagene). To enhance expression in erythroid cells, a 1.2-kb fragment of DNase I-hypersensitive site 2 (HS2) of the human β locus control region (*Asp* 718-*Xho*I [31]) was blunted and inserted upstream of the GATA-1 promoter at an *Eco*RV site (Fig. 1). *Xba*I linkers were attached to the following cDNAs and inserted between the promoter and a simian virus 40-derived polyadenylation sequence: mGATA-1, cGATA-1, hGATA-3, and two chimeric mGATA-1 constructs in which the two zinc fingers (amino acids 202 to 305) were replaced with the corresponding region (residues 260 to 365) of hGATA-3 [mGATA-1(fG3)] or with the single zinc finger (amino acids 501 to 567) of the *Aspergillus nidulans* GATA factor areA [mGATA-1(*fare*A)] (17). The last two constructs were kindly provided by Menie Merika. mGATA-4 cDNA (*Eco*RI fragment) was blunted and inserted into the blunted *Xba*I site of Bluescript. In addition, the mGATA-1 zinc finger region (residues 194 to 318, fGATA-1) alone or fused at the C terminus to the herpes simplex virus VP-16 transactivation region (fGATA-1-VP-16 [29, 34]) was constructed such that the mGATA-1 5′ untranslated region to the natural start codon (an *Nco*I site) was intact. These constructs were placed next to the GATA-1 promoter.

As a selectable marker, a cassette containing a hygromycin phosphotransferase gene driven by the phosphoglycerate kinase promoter (kindly provided by R. Mortenson) was placed upstream of HS2 by use of *Cla*I linkers. All constructs were verified by restriction analysis and sequencing.

Transfections. A quantity (2×10^7) of GATA-1$^-$ cells (clone 74 [26]) grown in gelatinized plates in the absence of feeder cells were electroporated with 20 μg of DNA in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (Bio-Rad electroporator; 280 V and 960 μF). Stable transfectants were selected in 0.3 mg of hygromycin B (Calbiochem) per ml. Southern blot analysis was performed to determine the presence of intact transgenes and to confirm that all clones had a mutant endogenous GATA-1 gene.

RNase protection assays. Total cellular RNA was isolated from embryoid bodies between day 10 and day 12 of differentiation by the acid phenol method as described previously (30). Antisense riboprobes for α-, β-, and ζ-globin and for γ-actin were generated as described elsewhere (2).

RESULTS

Design of a rescue vector. GATA-1$^-$ ES cells fail to generate mature erythroid cells upon in vitro differentiation of EBs (30). The capacities of a series of constructs to restore erythroid development in EBs of stably transfected GATA-1$^-$ ES cells

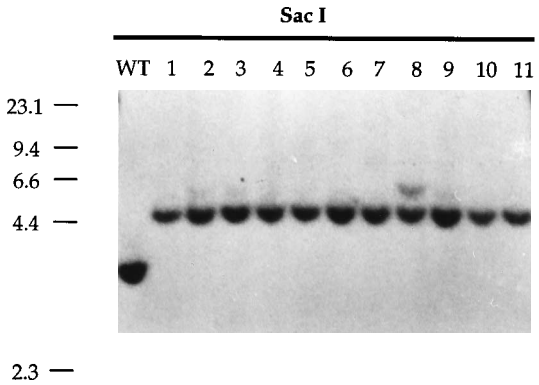

FIG. 2. Southern blot analysis of cell lines 1 to 11 transfected with an mGATA-1 cDNA expressing vector. WT, wild-type ES cells. DNA was digested with *Sac*I. An *Eco*RI-*Dra*I fragment of the first intron of the GATA-1 gene served as a probe. The sizes of the bands in wild-type and mutant DNAs are 3.8 and 4.9 kb, respectively.

were assayed. We reasoned that rescue would best be achieved through expression of transgenes in a manner approximating that of the normal, endogenous GATA-1 gene. In an effort to accomplish this, we constructed a vector in which the GATA-1 promoter is enhanced by addition of a β-globin locus control region element (HS2) (Fig. 1). Addition of HS2 to reporter constructs introduced into transgenic mice and into erythroleukemia cells provides for erythroid cell-specific expression, largely free of chromosomal position effects (5, 28). cDNAs were cloned into the vector downstream of the GATA-1 promoter, and stable cell transfectants were isolated by selection with hygromycin B after electroporation into GATA-1$^-$ ES cells.

Southern blotting was performed to identify clones containing intact transgenes (data not shown) and to verify that upon cell passage the mutant endogenous GATA-1 gene had not reverted to the wild type through deletion or rearrangement. A representative Southern blot, shown in Fig. 2, in which *Sac*I-digested DNA of 11 cell lines was probed with a labelled fragment of the GATA-1 gene, demonstrates that the GATA-1 locus is disrupted in all lines.

Rescue was assayed through the in vitro EB differentiation method in the presence of serum and growth factors (see Materials and Methods). For each experiment, clones to be tested and control cells (wild-type and GATA-1$^-$ ES) were seeded at three different densities to allow for variation in plating efficiencies. After 10 days in culture, EBs were examined for size and morphology, the presence of visible mature erythroid cells, and globin RNA transcripts by RNase protection assay. GATA-1$^-$ EBs, which served as a negative control, routinely showed poor growth beyond day 5 of development (Fig. 3 and 4) with apparent cell death in the periphery of the EBs. Red cells as well as globin transcripts were undetectable (see reference 30, and see below).

Rescue by cGATA-1. Outside the finger region, cGATA-1 and mGATA-1 are highly divergent, a finding not typical of other transcription factors thought to serve an important developmental function. We have asked, therefore, whether cGATA-1 can substitute for mGATA-1 in erythroid cell development. ES cell clones containing an intact cGATA-1 cDNA in the rescue vector developed into robust EBs in which, occasionally, islands of mature red cells were observed (Fig. 3). As shown in Fig. 4, EBs expressing cGATA-1 grew to the same extent as wild-type EBs. Rhythmically contracting EBs ("beaters") were sometimes seen, indicating full developmental potential (6, 24). As judged from morphology and the number of visible red cell islands, these EBs were similar to those harboring mGATA-1 cDNA in the rescue vector as a positive control (Fig. 3).

ζ- and β-globin mRNAs serve as markers for predominantly primitive and definitive erythropoiesis, respectively. EBs derived from six independent clones containing cGATA-1 expressed high levels of both ζ- and β-globin transcripts, comparable to EBs of clones rescued with mGATA-1 cDNA (Fig. 5). α-Globin RNA, expressed in both primitive and definitive erythroid cell lineages in mice, was also present at an increased level (Fig. 6). In contrast, as observed previously, GATA-1$^-$ EBs did not contain detectable globin mRNAs (Fig. 5a and 6). Because of the smaller size of GATA-1$^-$ EBs, total extractable mRNA was low and autoradiographs therefore required longer exposures. γ-Actin RNase protection provided an internal standard for RNA recovery from EBs. While some variation was observed among the different cGATA-1 clones, all showed significant rescue with regard to both morphology and globin mRNA levels. For comparison, additional rescued clones are shown in Fig. 5b. Parental GATA-1$^-$ cells and three clones that underwent hygromycin selection but did not harbor an intact transgene served as negative controls. None generated large, red EBs or displayed detectable globin mRNAs (Fig. 4 and 5a).

EBs derived from wild-type ES cells consistently revealed larger and more readily visible red cell islands than EBs carrying cGATA-1 or mGATA-1 cDNA constructs (Fig. 3), and globin mRNA levels were correspondingly higher (Fig. 5). We speculate that this difference reflects incomplete rescue due to suboptimal expression of transgenes in the rescue vector (see Discussion).

mGATA-1 zinc finger replacements. The zinc finger DNA-binding region is highly conserved among members of the GATA family of transcription factors. Yet, subtle differences among these proteins with respect to their DNA-binding specificities have been uncovered by PCR-mediated site selection (15, 23). We have asked, therefore, whether differences in binding specificity conferred by the zinc fingers might underlie erythroid specificity of GATA-1 action in vivo. Chimeric molecules in which zinc fingers of mGATA-1 were replaced with the zinc fingers of human GATA-3 [mGATA-1(fG3)] or with the single finger of the *A. nidulans* GATA factor areA [mGATA-1(f*are*A)] were generated. ES cells harboring mGATA-1(fG3) or mGATA-1(f*are*A) generated robust EBs (Fig. 3 and 4). Eight mGATA-1(fG3) clones and four mGATA-1(f*are*A) clones were assayed by RNase protection. ζ- and β-globin mRNA levels were comparable to those observed in mGATA-1-expressing EBs (Fig. 5). In addition, significant amounts of α-globin mRNA were produced in the mGATA-1(fG3) clones (Fig. 6) and in the mGATA-1(f*are*A) clones (data not shown). This suggests that the high degree of sequence conservation in the zinc fingers is manifest in their function and that subtle specificity differences noted in vitro may not exert a predominant effect in vivo. Rescue by the mGATA-1(f*are*A) construct is noteworthy, in that it suggests that a two-finger DNA-binding domain structure may not be required for action in erythroid cells. The areA zinc finger is more homologous to the C-terminal than to the N-terminal zinc finger of mGATA-1 and recognizes the same consensus binding site (23). Previous studies have shown that deletion of the N-terminal finger of mGATA-1 or cGATA-1 leads to subtle changes in DNA binding in vitro and transactivation function (21, 42).

Rescue by heterologous and artificial GATA factors. The finding that highly divergent cGATA-1 rescued the mGATA-1$^-$

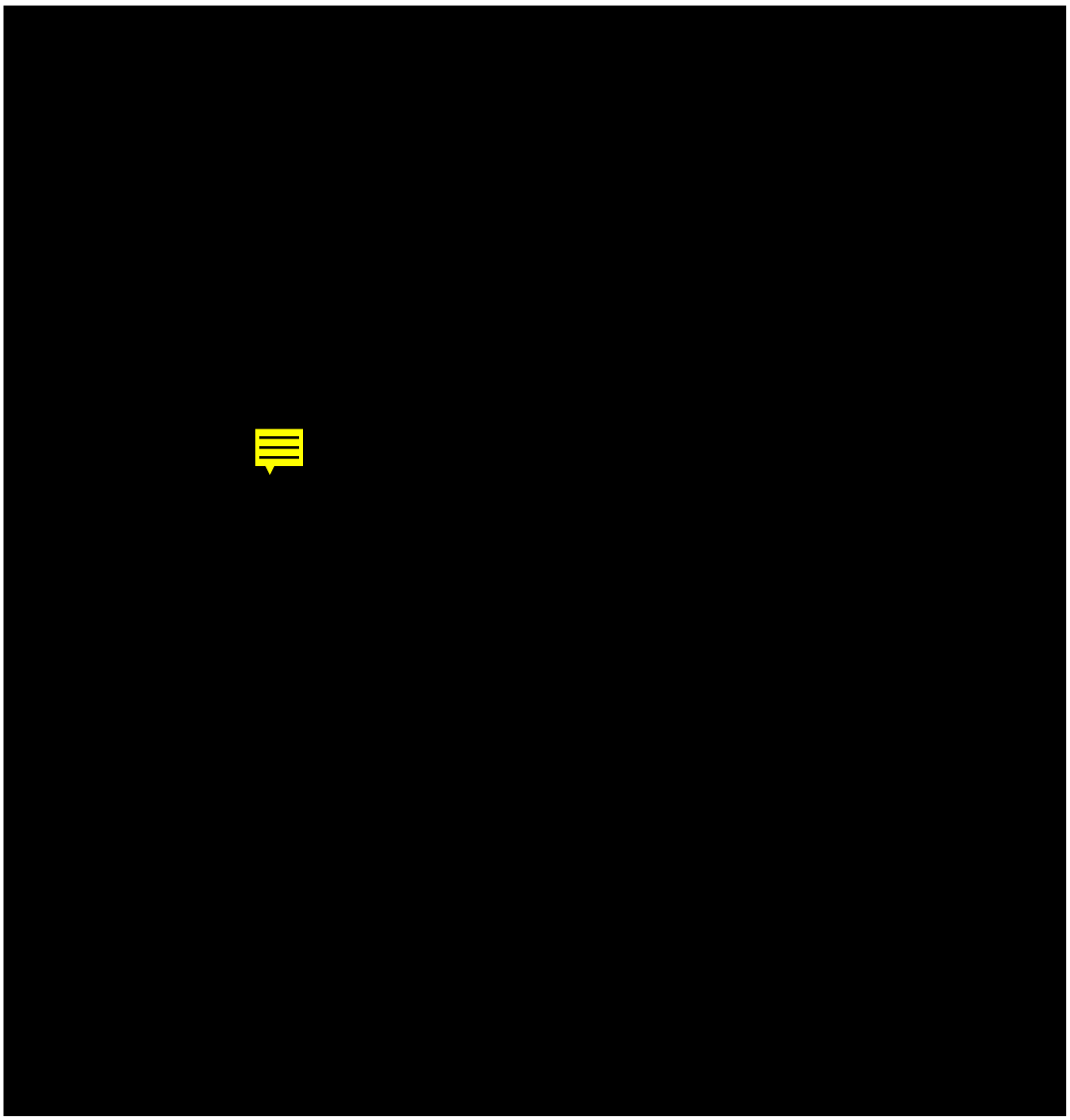

FIG. 3. Embryoid bodies from GATA-1-deficient ES cells (a), from wild-type ES cells (b), and from GATA-1-deficient ES cells expressing the cDNA of mGATA-1 (c), cGATA-1 (d), mGATA-1(*fare*A) (e), and GATA-VP16 (f). Under the conditions used, GATA-1$^-$ EBs were smaller and more irregularly shaped than wild-type EBs. Note the red cells at the periphery of the wild-type EB shown. Redness was also observed in EBs expressing cGATA-1 and GATA-VP16. Magnification, ×40 in all panels.

phenotype led us to consider whether more distantly related GATA-binding proteins with different tissue distributions, such as GATA-3 and GATA-4, might also compensate for the mGATA-1 deficiency. ES cells transfected with hGATA-3- or mGATA-4-expressing plasmids developed into large, healthy EBs, similar to those obtained with mGATA-1 or cGATA-1 transgenes (Fig. 3). Beaters were found frequently. While red cell islands were not visible by light microscopy, ζ- and β-globin mRNA levels in eight GATA-3 clones and in six GATA-4 clones were similar to those in mGATA-1 transfectants (Fig. 5). α-Globin levels were also high (Fig. 6). These data indicate substantial rescue of the mGATA-1 defect.

To confirm and extend these findings, a construct designed to express a novel GATA-binding protein (designated fGATA-1-VP16) was generated. In it, the herpes simplex virus VP16 transactivation domain was fused to the zinc finger region of mGATA-1. In cotransfection experiments with NIH 3T3 cells, we demonstrated that transactivation of a reporter by fGATA-1-VP16 approximates that of normal mGATA-1 (data not shown). ES cells expressing fGATA-1-VP16 developed into normal-appearing EBs (Fig. 3 and 4). Many rhythmically beating EBs were seen. Remarkably, in one of the clones rescue was sufficiently complete that a few EBs displayed some visible red cells (Fig. 3). Three independent clones expressed ζ- and β-globin mRNAs. Two are shown in Fig. 7a. Taken together, these results show that functionally GATA-1 can be substantially replaced by unrelated GATA-binding proteins.

The GATA-1 zinc fingers alone display rescue potential. In standard cotransfection experiments in heterologous cells, the zinc fingers of GATA-1 bind DNA but fail to activate transcription (21). However, in light of the above results it seemed possible that in an erythroid environment the DNA-binding domain confers activity beyond DNA recognition, as it is the only region common to all constructs tested. A precedent for such a scenario is provided by the myogenic transcription factor MyoD. Its basic helix-loop-helix domain, which is required

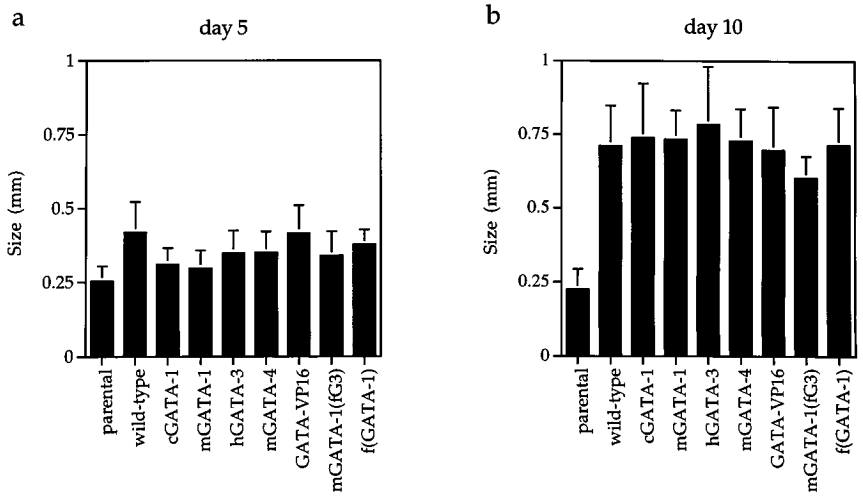

FIG. 4. Average diameters of EBs at day 5 (a) and day 10 (b) of in vitro differentiation. While GATA-1$^-$ EBs stopped enlarging at day 5, rescued EBs more than doubled their diameter by day 10. Error bars represent standard deviations.

for dimerization and DNA binding, retains the capacity to induce myogenic differentiation in fibroblasts (32). To test the rescue activity of a transactivation domain-deleted version of GATA-1, a construct that encodes amino acids 194 to 318 containing both zinc finger motifs was made. EBs expressing this construct developed normally with respect to size (Fig. 4) and general morphology (data not shown). ζ- and β-globin levels were comparable to those of ES cell lines expressing other rescue constructs (Fig. 7a). To account for some variation between cell lines and between experiments, eight lines

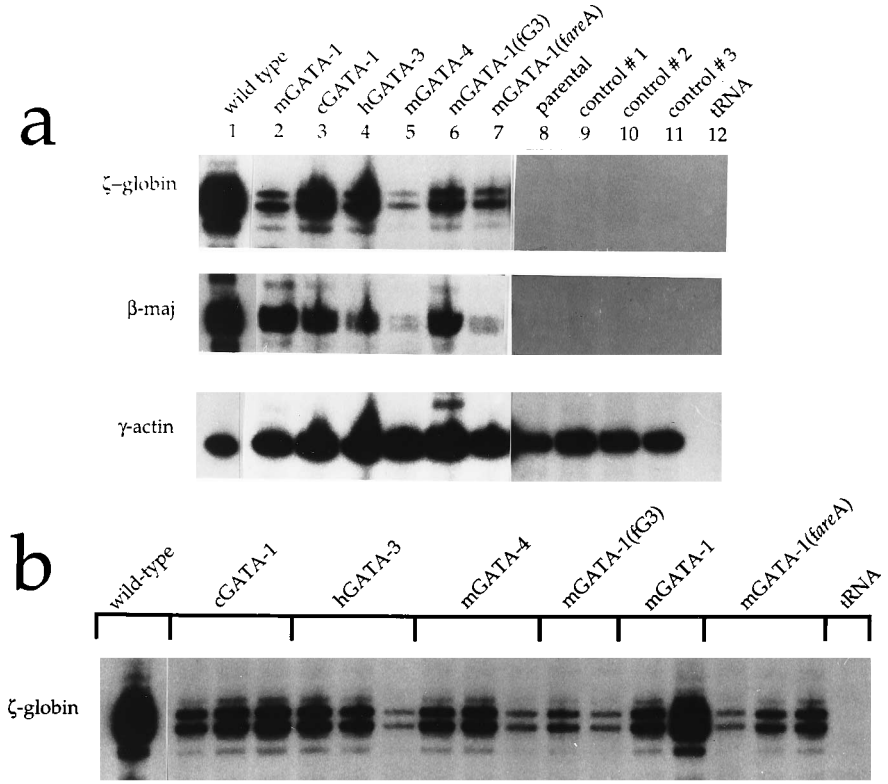

FIG. 5. (a) RNase protection assays for ζ-globin and β-major globin mRNAs of wild-type EBs and EBs carrying the indicated plasmids. As a control for loading, γ-actin was assayed in parallel. Globin transcripts were not detectable in the control EBs derived from the parental mGATA-1-deficient ES cells or in EBs lacking intact transgenes (controls 1, 2, and 3). In lanes 1 to 7, exposure times for ζ-globin, β-major globin, and γ-actin were overnight, 3 days, and 5 h, respectively. Control EBs were smaller, and levels of mRNA were lower, requiring longer exposure times. In lanes 8 to 12, autoradiograms were exposed 3 days for ζ-globin and β-major globin and overnight for γ-actin. tRNA served as a negative control. (b) Overnight exposure of an RNase protection assay for ζ-globin of multiple clones carrying the indicated constructs. Expression levels varied somewhat between clones but were always high in comparison with those of control EBs.

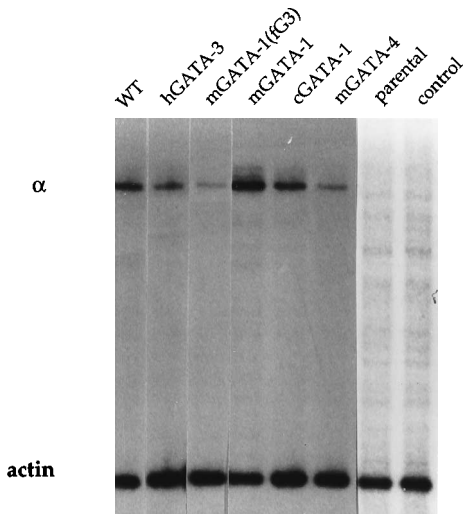

FIG. 6. RNase protection assay for α-globin mRNA of EBs derived from wild-type ES cells (WT) or from GATA-1-deficient ES cells carrying the indicated constructs. α-Globin transcripts were easily detectable in all samples carrying an intact transgene but were absent in parental ES cell-derived EBs or control EBs. Probes for ζ-globin and γ-actin were added to the same reaction mixture. The autoradiogram shown was exposed overnight. Control EBs were smaller and contained less mRNA. In order to compare relative signal intensities, the lanes showing parental and control EBs were exposed for 3 days such that the actin bands were comparable for all lanes.

expressing the zinc fingers alone are shown in Fig. 7b. These results suggest that the finger region itself retains rescue activity when placed into a natural context.

DISCUSSION

Heterologous GATA-binding proteins were assayed for their ability to correct the developmental defect of GATA-1$^-$ cells in the context of in vitro EB differentiation. Expression of cGATA-1, which is highly divergent from mammalian GATA-1, compensated for the defect of GATA-1$^-$ cells with an efficiency similar to that of mGATA-1, as judged from overall growth of EBs, presence of red cells, and expression of globin mRNAs. Moreover, heterologous GATA-binding proteins (GATA-3 and GATA-4) which are normally expressed in different tissues, as well as a GATA chimera with a viral transactivation domain (GATA-VP16), also compensated for the GATA-1 deficiency. Chimeric GATA factors with zinc finger replacements [mGATA-1(fG3) and mGATA-1(f*are*A)] were nearly as effective as mGATA-1 in their abilities to correct the GATA-1 defect. Although subtle differences in DNA-binding specificities between the chimeric GATA factors and normal mGATA-1 exist, transcriptional activity in transient transfection assays in heterologous cells remains unchanged (23). Taken together, these results indicate that the GATA-1 zinc fingers do not impart erythroid specificity in vivo. The activation domains of GATA-1 have originally been defined in heterologous cells, and the question of what role these domains play in erythroid development remains. The lack of conservation of these regions between cGATA-1 and mGATA-1 suggests that they may not be so critical for erythroid cell-specific transcription as conventionally thought.

The finding that mGATA-1 zinc fingers alone displayed rescue activity was unanticipated, since they fail to activate transcription in heterologous cells. Nonetheless, using an assay in which the primitive myeloid cell line 416B is induced to undergo megakaryocytic differentiation by forced expression of GATA-binding proteins, Visvader and coworkers have observed that the C-terminal zinc finger of GATA-1 is sufficient to induce differentiation (reference 35a; see also below). Hence, in two different biological assay systems, the DNA-binding region of GATA-1 appears sufficient for promoting expression of markers of cellular differentiation. This phenomenon is formally analogous to the activity of the basic helix-loop-helix region of MyoD in myogenic conversion of fibroblasts, albeit at a lower efficiency than that of intact MyoD (32).

None of the constructs tested, including the positive control plasmid carrying the mGATA-1 cDNA, rescue to the extent previously seen with the introduction of the intact GATA-1 gene (30). Large conspicuous red cell islands at the borders of the EBs were seen only in wild-type EBs. Consistent with this, globin transcripts were more abundant. We believe this is most likely due to suboptimal expression of the transgenes achieved with the current rescue vector. While mGATA-1 is expressed at very low but detectable levels in whole wild-type EBs (30), transgene expression in rescued EBs was not detectable by RNase protection experiments (data not shown). In an effort to optimize transgene expression, we have tested different vectors

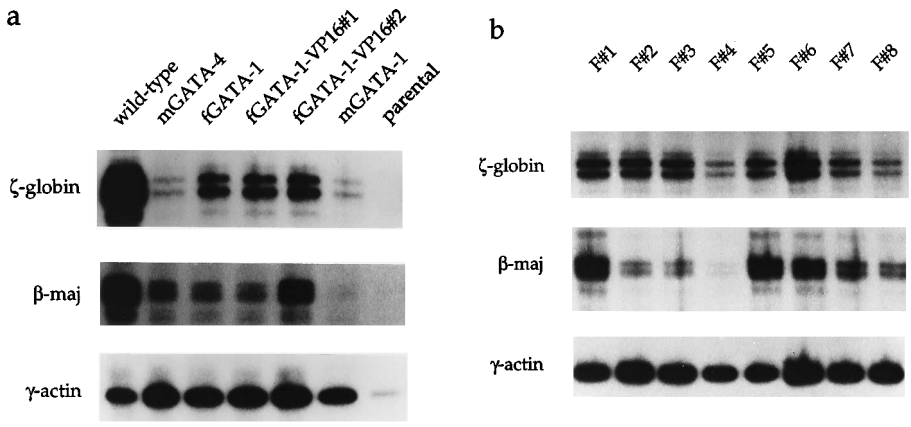

FIG. 7. (a) RNase protection assays for ζ-globin and β-major globin mRNAs of EBs derived from two clones containing an fGATA-1-VP16 construct and one construct expressing only the GATA-1 finger region (fGATA-1). For comparison, a GATA-1- and GATA-4-expressing clone was included in this experiment. Exposure times for ζ-globin and β-major globin were 3 days, and that for γ-actin was overnight. As in previous figures, the actin signal of the parental GATA-1-deficient EBs is lower because of their much reduced size. (b) Eight independent clones expressing fGATA-1. Exposures are the same as in panel a.

containing larger portions of the GATA-1 gene. A vector containing a largely intact GATA-1 gene from kb −2.7 to +10.1, in which the test cDNA was introduced at the mGATA-1 start codon, did not improve rescue efficiency or its extent (data not shown). Attempts to rescue the GATA-1$^-$ phenotype in chimeric mice made with GATA-1$^-$ cells containing cDNA constructs have not been successful to date. To achieve substantial chimerism in peripheral blood, nearly complete compensation for the GATA-1 defect is probably required. Therefore, more faithful transgene expression will be necessary to evaluate subtle differences in the abilities of GATA proteins to facilitate full erythroid maturation. Thus, future improvements of this experimental design may require inclusion of additional genomic regions to provide normal timing and level of GATA-1 expression in developing cells.

While all cDNAs tested here were comparable in rescue, there remains the possibility that subtle, and yet important, differences between GATA-binding proteins would be revealed if expression more closely approximating that of the normal GATA-1 gene were achieved. Nonetheless, the substantial rescue already observed, evidenced by improved EB development and expression of globin mRNAs in total EBs, allows several notable conclusions to be drawn. The emerging view that proteins of the GATA family are often interchangeable in their functions is supported by other studies. (i) cGATA-1 activates transcription in transient transfection assays in human (42) and murine (our unpublished observation) cells, and hGATA-1 functions when expressed in chicken fibroblasts (42). (ii) cGATA-1 and mGATA-1 are equally effective in the stage- and tissue-specific activation of chromatin-assembled β-globin genes in in vitro transcription assays (3). (iii) Remarkably, GATA-1, GATA-2, and GATA-3 induce megakaryocytic differentiation upon forced expression in a primitive myeloid cell line, 416B (36). Notwithstanding, evidence supporting nonoverlapping activities has been reported. For example, forced GATA-2 expression maintains chicken erythroid cell precursors in a proliferative state, whereas expression of GATA-1 and GATA-3 does not (4).

While heterologous GATA-binding proteins could rescue the GATA-1 defect to a significant extent under the conditions tested, it is possible that fine-tuning of GATA-1 activity in response to signals that modulate erythroid cell maturation, such as changing concentrations of growth factors, is mediated through portions unique to GATA-1.

The concept that different members of a transcription factor family with unrelated activation domains may be interchangeable in vivo is consistent with observations made with other systems. In *D. melanogaster*, phenotypes resulting from mutation in certain transcription factor genes may be corrected by genes encoding heterologous proteins. *Drosophila* embryos harboring a *bicoid* (*bcd*) mutation are rescued by *bcd* DNA-binding/activation domain fusion proteins (8). *Paired*, *gooseberry*, and *gooseberry neuro*, three proteins which recognize the same DNA sequences but differ in their spatial and temporal expression patterns, are interchangeable in vivo (20). Taken together, these results suggest that pattern and timing of expression of selected nuclear regulators, rather than a conserved primary sequence, are major determinants of tissue specification.

If cell-type specificity of transcription factor action were mediated through a particular domain, a tissue-specific cofactor(s) would be required to provide a transcriptional signal to the basal transcriptional machinery. Since most enhancer-binding proteins are thought to act through the same basal transcription machinery, they must contain regions with limited tissue specificity that allow them to interact with a smaller group of general transcription factors or TATA-binding protein-associated factors. Since GATA-1 does not as yet appear to have an erythroid cell-specific domain, it might interact directly with a general transcription factor. In support of this, cGATA-1 has been shown to interact with a factor contained in a fraction that stabilized DNA binding of human TFIID (10).

We postulate that the signals which regulate the GATA-1 gene are crucial for determining the erythroid cell phenotype, rather than the properties of GATA-1 itself. Accordingly, attention must now be directed to determining the *cis* elements that regulate the erythroid cell-specific activation and expression of GATA-1.

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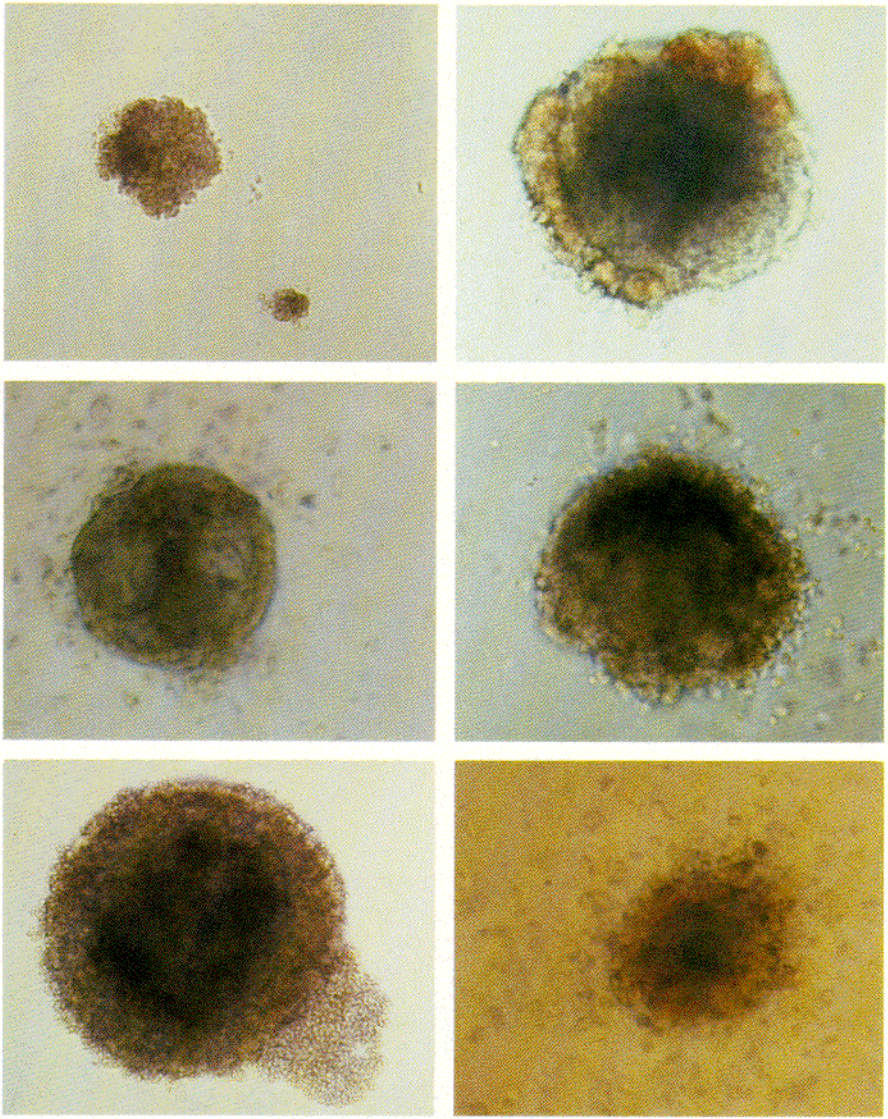
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