Ectopic expression of a conditional GATA-2/estrogen receptor chimera arrests erythroid differentiation in a hormone-dependent manner

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The GATA factors are a family of transcriptional regulatory proteins in eukaryotes that share extensive homology in their DNA-binding domains. One enigmatic aspect of GATA factor expression is that several GATA proteins, which ostensibly share the same DNA-binding site specificity, are coexpressed in erythroid cells. To elucidate the roles of individual GATA factors in erythropoiesis, conditional alleles of GATA-1, GATA-2, and GATA-3 were prepared by fusing each of the factors to the hormone-binding domain of the human estrogen receptor (ER). These GATA/ER chimeric factors were shown to be hormone-inducible *trans*-activating proteins in transient transfection assays. When stably introduced into primary erythroblasts or conditionally transformed erythroid progenitor cells, exogenous GATA-2/ER promoted proliferation and inhibited terminal differentiation in an estrogen-dependent manner. These phenotypic effects are specifically attributable to the action of ectopically expressed GATA-2/ER because erythroblasts expressing exogenous GATA-2 are constitutively arrested in differentiation and because erythroid progenitors expressing either Gal/ER or GATA-3/ER do not display a hormone-responsive block in differentiation. Thus, the GATA-2 transcription factor appears to play a role in regulating the self-renewal capacity of early erythroid progenitor cells.

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The GATA factors are a family of (C₄) zinc finger transcriptional regulatory proteins in eukaryotes that bind to the consensus DNA sequence WGATAR (for review, see Orkin 1990). In vertebrate organisms, each member of this family has a distinct tissue distribution pattern. Thus, GATA-1 is expressed in the erythroid, megakaryocytic, mast cell, and germ cell lineages (Tsai et al. 1989; Martin et al. 1990; Romeo et al. 1990; Ito et al. 1993), whereas GATA-2 is expressed more widely in both hematopoietic and nonhematopoietic lineages (Yamamoto et al. 1990; Zon et al. 1991). The expression of GATA-3 is confined principally to definitive erythroid cells, specific neuronal cells, and T lymphocytes (Yamamoto et al. 1990; Ko et al. 1991; J.M. Kornhauser, M.W. Leonard, M. Yamamoto, J.H. La Vail, K.E. Mayo, and J.D. Engel, in prep.). GATA-1 represents a major transcriptional regulator in the erythroid lineage, as demonstrated by gene inactivation experiments (Pevny et al. 1991).

A curious aspect of GATA factor expression is that in

at least two developmentally distinct lineages (i.e., in both erythroid cells and specific subsets of neurons), multiple members of the GATA gene family are coexpressed (Yamamoto et al. 1990; J.M. Kornhauser, M.W. Leonard, M. Yamamoto, J.H. La Vail, K.E. Mayo, and J.D. Engel, in prep.). In addition, during erythroid cell differentiation the relative abundance of the GATA factors with respect to one another undergoes specific developmentally controlled changes that are highly conserved in several species (M.W. Leonard, K.-C. Lim, and J.D. Engel, in prep.). These data therefore suggested that the regulation of the precise quantitative balance of the GATA factors might play a role in influencing the developmental decisions available to erythroid progenitor cells, for example, to undergo either self-renewal or terminal differentiation. Thus, we hypothesize that the ectopic expression of these regulatory proteins in erythroblasts might describe novel phenotypes that would lend insight into the roles that individual GATA family members might play in erythropoiesis.

To empirically address this question, conditional mutants of the chicken GATA factors were prepared. The

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experimental strategy consisted of fusing each of the GATA cDNA clones (Yamamoto et al. 1990) with the hormone-binding domain of the human estrogen receptor (ER) cDNA, a paradigm that has been shown to render hybrid transcription factors susceptible to estrogen-dependent activity (see Boehmelt et al. 1992, and references therein). We demonstrate that GATA/ER chimeric proteins *trans*-activate reporter genes in a manner characteristic of the GATA factor family but in an estrogen-dependent fashion. In addition, we provide evidence that the *trans*-activation activity displayed by these chimeric proteins *in vivo* is not attributable to blocking of DNA binding by the ER hormone-binding domain but, rather, appears to be attributable to hormone-dependent unmasking of *trans*-activation domain(s) of GATA/ER factors.

To directly assess the roles of the GATA proteins in erythropoiesis, retroviral vectors containing the various GATA and GATA/ER cDNAs were used to stably express these transcription factors in normal or temperature-sensitive (ts)-oncogene-transformed avian erythroid progenitors. GATA-2/ER was found to arrest (or severely retard) erythroid differentiation and to promote proliferation of the immature erythroblasts in a strictly estrogen-regulated fashion. These effects are attributable specifically to the ectopic expression of GATA-2/ER because the same phenotype is constitutively induced by ectopically expressed wild-type GATA-2 protein, whereas no phenotypic alteration is observed in erythroid progenitors expressing Gal/ER or GATA-3/ER proteins. These results strongly implicate the transcription factor GATA-2 as an intimate participant in the maintenance of erythroid progenitors in an immature state.

Results

GATA/ER chimeric proteins function as hormone-dependent trans-activators

Conditional GATA-1, GATA-2, and GATA-3 proteins were prepared by fusing each of the GATA factor cDNAs (Yamamoto et al. 1990) 5' to a cDNA encoding the hormone-binding domain of the human ER (Fig. 1A; Kumar et al. 1986). To address the question of whether or not the *trans*-activation function of the GATA/ER chimeric proteins is modulated by hormone, cotransfection *trans*-activation experiments were performed. For these studies, the parental GATA and GATA/ER cDNAs were inserted into a eukaryotic expression plasmid TFAnco (Federspiel et al. 1989; Yamamoto et al. 1990), and the activator and reporter plasmids were cotransfected into QT6 quail fibroblasts. To monitor GATA activity, the C3 β GH reporter plasmid, which has three copies of the GATA inverted repeat motif of the chicken β -globin enhancer (Emerson et al. 1987; Gallarda et al. 1989) cloned 5' to the rabbit β -globin TATA box, was used. This artificial promoter directs transcription of the human growth hormone (hGH) gene in the C3 β GH reporter plasmid (Yamamoto et al. 1990).

Figure 1. Transcriptional activity of the conditional chicken GATA/ER factors in transient transfection. (A) The cDNAs of chicken GATA-1, GATA-2, and GATA-3 and their respective ER counterparts are illustrated schematically. The three additional amino acid residues (PDP) introduced at the GATA-ER junction and the zinc finger DNA-binding domain (solid boxes) are indicated. The parental GATA and the chimeric GATA/ER cDNAs were cloned into the recombinant retroviral vectors pSFCV-LE (Fuerstenberg et al. 1990) and TFAnco (Federspiel et al. 1989). (B) The *trans*-activation activities of wild-type GATA and conditional GATA/ER factors were analyzed using transient cotransfection experiments in QT6 quail fibroblasts. Transfected cells were treated with ethanol (-E2), estrogen (+E2), or estrogen antagonist ICI 164,384 (+ICI). To assay for GATA-dependent transcription, the C3 β GH reporter plasmid (with multiple GATA sites upstream of the hGH) was used. The *trans*-activation activity of each factor was normalized to the background activity of the TFAnco vector alone and plotted against a logarithmic scale.

As anticipated, the presence or absence of estrogen did not influence the ability of the three wild-type GATA factor to *trans*-activate the C3 β GH reporter plasmid (Fig.

1B). The GATA/ER proteins, on the other hand, activated the reporter gene expression only after the addition of estrogen to the medium. GATA-1/ER was the most powerful transcriptional activator in this assay and, surprisingly, stimulated the reporter plasmid five- to eight-fold more strongly than did the parental factor, GATA-1. The level of *trans*-activation by GATA-2/ER was approximately half that of GATA-2 while GATA-3/ER appeared to be the poorest activator in this assay (even though its activity was quite comparable to that of the parental GATA-3 factor). Whereas GATA-2/ER and GATA-3/ER were completely inactive in the presence of the estrogen antagonist ICI 164,384 (ICI; Wakeling and Bowler 1988a,b), some residual *trans*-activation activity of GATA-1/ER remained even in the presence of 10^{-6} M ICI. Similar hormone-dependent *trans*-activation activities of these GATA chimeras were also seen in the *v-erbA*/ts-*v-erbB*-transformed HD3 erythroblast cell line (Beug et al. 1982a,b), showing that the conditional GATA factors can *trans*-activate GATA-dependent reporter gene in avian erythroid cells (data not shown).

DNA-binding activity of the GATA/ER factors is not regulated by estrogen

To determine whether the hormone-dependent *trans*-activation activity of the GATA/ER proteins is the result of the induction of sequence-specific DNA binding or of the activation of the domain(s) required for transcriptional stimulation, we performed electrophoretic gel mobility shift assays (EGMSA) to compare the ability of GATA/ER fusion proteins and wild-type GATA proteins to bind to a GATA consensus probe, derived from the mouse α 1-globin gene promoter (M α P; Tsai et al. 1989), *in vitro*. As shown in Figure 2A, the parental GATA proteins were capable of binding to the M α P probe which was, as expected, unaffected by the presence or absence of estrogen. Surprisingly, the chimeric GATA/ER factors also formed specific DNA complexes irrespective of the presence or absence of hormone (Fig. 2A; data not shown), although they were transcriptionally inactive when estrogen was absent. The molecular complexes formed by each of the GATA and GATA/ER factors were competed by the addition of a 100-fold excess of the same (unlabeled M α P) oligonucleotide but not with a 100-fold excess of mutant (mM α P) oligonucleotide (Fig. 2B), indicating that the binding of these hybrid GATA/ER proteins is sequence specific. All three GATA/ER proteins derived from cytoplasmic fractions showed a high-molecular-mass complex band (compared with wild-type GATA proteins) in EGMSAs (Fig. 2A). As the calculated molecular masses of GATA-1/ER, GATA-2/ER, and GATA-3/ER proteins are 67, 85, and 83 kD, respectively (Yamamoto et al. 1990), the mobility shift bands do not appear to represent monomeric GATA/ER protein-DNA complexes. Hence, the GATA/ER fusion proteins may be complexed with one another or with other cellular factors whose identities are presently unknown.

Ligand-bound GATA/ER proteins localize to the nucleus

Figure 2A shows that specific GATA/ER-DNA complexes were observed only when cytoplasmic extracts of untreated cells were used for EGMSA (lanes 10,12,14), whereas no complex was formed when cytoplasmic extracts of estrogen-treated cells were used. However, when nuclear extracts were used for EGMSA, specific complexes were seen for both untreated and estrogen-treated cells (data not shown). These observations suggested that in the absence of estrogen, the GATA/ER factors might be distributed to both the nucleus and the cytoplasm; the hormone-activated factors, on the other hand, appeared to accumulate predominantly in the nucleus, a notion in keeping with other studies in which the cellular localization of an estrogen-inducible *v-re*/ER fusion protein was examined (Boehmelt et al. 1992). These preliminary conclusions, derived from experiments based on the biochemical fractionation of transfected cells, were tested further by indirect immunofluorescence assays.

QT6 cells transfected with each of the GATA/ER plasmids in the presence or absence of estrogen were reacted with H222 monoclonal antibody that recognized an epitope in the hormone-binding domain of the human ER (Greene et al. 1984). In the absence of estrogen, the cytoplasm and nuclei of transfected QT6 cells displayed relatively uniform fluorescence while estrogen-treated transfected QT6 cells showed only nuclear fluorescence (Fig. 3). In addition, GATA/ER-transfected cells treated with the estrogen antagonist ICI also showed only nuclear fluorescence (data not shown). Thus, only ligand-bound GATA/ER species are selectively partitioned to the nuclear compartment, whereas in the absence of ligand, hybrid GATA/ER factors appear to be freely diffusible and are found in both the cytoplasmic and nuclear compartments.

Hormone-activated GATA-2/ER retards terminal differentiation and enhances self-renewal of transformed erythroblasts

Because the chimeric GATA/ER proteins were shown to be fully functional transcriptional activators *in vivo* when activated by estrogen, we examined the effects of ectopic expression of these factors on erythroid differentiation. Parental and chimeric GATA factors were stably expressed in the *v-erbA*/ts-*v-erbB*-transformed erythroblast cell line HD3 (Beug et al. 1982a,b), which can be induced to differentiate by inactivating the ts-*v-erbB* oncogene product at 42°C and by inhibiting the phosphorylation of the *v-erbA* oncoprotein with the protein kinase inhibitor H7 (Zenke et al. 1988; Glineur et al. 1990).

The parental GATA, as well as the GATA/ER, cDNAs were cloned into the recombinant avian retroviral vector pSFCV (Fuerstenberg et al. 1990) containing the neomycin resistance gene as a selectable marker. To generate HD3 clones stably expressing the various GATA and GATA/ER factors, cells were transfected with the re-

Figure 2. Sequence-specific DNA binding of GATA and GATA/ER chimeric factors. (A) Cytoplasmic extracts were prepared from QT6 fibroblasts transiently transfected with various GATA and GATA/ER plasmids [(lanes 2,3) TFNeo vector; (lanes 4,5) GATA-1 cDNA; (lanes 6,7) GATA-2 cDNA; (lanes 8,9) GATA-3 cDNA; (lanes 10,11) GATA-1/ER cDNA; (lanes 12,13) GATA-2/ER cDNA; (lanes 14,15) GATA-3/ER cDNA] in the presence of ethanol (–) or estrogen (+). EGMSAs were performed as described previously (Yamamoto et al. 1990) using 32 P-labeled M α P probe, which contained a GATA consensus site derived from the mouse α 1-globin gene promoter (Tsai et al. 1989). The position of migration of free probe (lane 1; open arrow) and specific protein–DNA complexes (solid arrows) are indicated. (B) Cytoplasmic extracts prepared from QT6 cells transfected with various GATA and GATA/ER cDNAs [(lanes 2–4) TFNeo vector; (lanes 5–7) GATA-1; (lanes 8–10) GATA-2; (lanes 11–13) GATA-3; (lanes 15–17) GATA-1/ER; (lanes 18–20) GATA-2/ER; (lanes 21–23) GATA-3/ER] in the absence of estrogen were used in EGMSAs. In oligonucleotide competition experiments, no competitor oligonucleotide (–), a 100-fold excess of unlabeled M α P oligonucleotide (+), or mutant M α P oligonucleotide (m, which has the core recognition sequence changed from GATA to GGCA) was added to the gel shift reactions. The position of migration of free probe (lanes 1,14; open arrow) and specific protein–DNA complexes (solid arrows) are indicated.

spective retroviral vector DNAs and seeded into methocel medium containing G418. Neomycin-resistant clones were then screened for an estrogen-dependent differentiation phenotype. Because HD3 erythroblasts do not express endogenous ER (Schroeder et al. 1993), any hormone-induced response in GATA/ER-expressing HD3 cells should be strictly the result of the action of the chimeric protein. The effect of hormone addition and depletion on the morphology of erythroblasts was assayed by neutral benzidine staining (Beug et al. 1982b) to detect hemoglobin accumulation and by quantitatively measuring [3 H] thymidine incorporation to assay for withdrawal of differentiated cells from the cell cycle (Kowenz et al. 1987).

Whereas GATA-1/ER- and GATA-3/ER-expressing clones showed no obvious phenotypic changes, GATA-2/ER-expressing HD3 cells displayed dramatic alteration in morphology upon differentiation induction (see below); we therefore focused our attention on these GATA-

2/ER-expressing HD3 clones. Of 70 GATA-2/ER HD3 erythroblast colonies examined, 4 showed a strong hormone-dependent inhibition in terminal differentiation while numerous others displayed a similar, but weaker, response. On the other hand, none of the 10 control clones (pSFCV vector alone) examined showed any hormone-regulated effect on differentiation. Of 94 HD3 clones stably expressing wild-type GATA-2, the majority displayed an inhibition in terminal differentiation, albeit in a hormone-independent manner. Finally, a total of five HD3 clones [two GATA-2/ER clones (C5 and E6), one representative clone expressing parental GATA-2 (C8), and one control clone harboring the pSFCV vector alone (E11; Fuerstenberg et al. 1992)] were subjected to detailed analysis.

To assess the effects of GATA-2 and GATA-2/ER expression on erythroid differentiation, cells were induced to differentiate in the presence of estrogen or the estrogen antagonist ICI. Four to five days later, cells were

Figure 3. Hormone-activated GATA/ER proteins localize to the nucleus. QT6 fibroblasts transfected with GATA-2/ER cDNA were treated with estrogen (+ estrogen) or ethanol (– estrogen). Confluent cells on coverslips were then fixed and incubated with H222 monoclonal antibody (that recognized an epitope in the hormone-binding domain of the human ER), followed by binding with FITC-conjugated secondary antibody. Only transfected cells showed staining. Whereas estrogen-treated cells showed only nuclear fluorescence, control (ethanol-treated) cells were uniformly stained. Similar results were obtained with fibroblasts transfected with GATA-1/ER and GATA-3/ER cDNAs [data not shown].

examined for their stage of maturation by neutral benzidine staining to detect hemoglobin (Beug et al. 1982b). As anticipated, differentiation of the control clone expressing pSFCV vector alone was not affected by the presence of estrogen or ICI (Fig. 4A,B). In the presence of ICI, the GATA-2/ER clones terminally differentiated into erythrocytes and late reticulocytes with kinetics similar to the control clone containing pSFCV vector only. However, when GATA-2/ER was activated by estrogen, terminal differentiation was strongly inhibited: A large proportion of the cells remained arrested in an immature state while the remaining cells matured with delayed kinetics (Fig. 4A,B). Accordingly, the differentiation response of HD3 cells expressing the wild-type GATA-2 mimicked that of estrogen-activated GATA-2/ER but in a hormone-independent fashion (Fig. 4A,B).

These results suggest that GATA-2 blocks erythroid differentiation. This possibility was addressed further by measuring cell proliferation (because partially mature and mature cells withdraw from the cell cycle, whereas immature cells continue to proliferate; Kowenz et al. 1987). Four days after differentiation, the same erythroblast clones were analyzed for their proliferative capacity by [3 H] thymidine incorporation. Figure 4C shows that

Figure 4. GATA-2/ER-expressing HD3 erythroblasts show hormone-dependent inability to terminally differentiate. (A) HD3 erythroblasts stably expressing GATA-2 (clone C8), GATA-2/ER (clone C5), or pSFCV vector only (clone E11) were induced to differentiate in the presence of estrogen or estrogen antagonist ICI. Photographs of cells subjected to cytocentrifugation and staining with neutral benzidine and histological dyes (Beug et al. 1982b) after 4 days of differentiation are shown. (B) Aliquots from the same cultures as in A were evaluated for their stage of maturation after cytocentrifugation and neutral benzidine staining. At least 300 cells were scored, and the results were plotted in histograms. (ery) Erythrocytes; (late r) late reticulocytes; (early r) early reticulocytes; (erbl) erythroblasts. (C) Four days after differentiation induction in the presence of estrogen or ICI, erythroblasts from the same cultures as in A were assayed for [3 H]thymidine incorporation (Kowenz et al. 1987). Incorporated radioactivity was normalized to cell number.

thymidine incorporation of estrogen-treated GATA-2/ER clones was about twofold higher than that of ICI-treated GATA-2/ER cells, whereas no increase in thymidine uptake was seen for the vector control clone cultivated in the presence of either drug. The clone expressing wild-type GATA-2 displayed high levels of thymidine incorporation (about sevenfold over vector alone) under similar growth conditions (Fig. 4C), underscoring a possible increase in the proliferative activity of erythroblasts owing to ectopic GATA-2 expression. Finally, hemoglobin levels were measured as a third parameter for erythroid differentiation. Because all cell samples already contained some mature erythrocytes (Fig. 4A,B), the differences in hemoglobin levels (normalized to viable cell number) were not dramatic. Nevertheless, erythroblast clones expressing estrogen-activated GATA-2/ER exhibited approximately twofold reduction in hemoglobin levels compared with either ICI-treated GATA-2/ER cells or vector control cells (data not shown). Taken together, these data suggest that GATA-2 is able to maintain a considerable fraction of transformed erythroblasts in a self-renewing state, suggesting that a developmentally regulated change in GATA-2 expression is a prerequisite for normal erythroid differentiation.

Exogenous GATA-2 or GATA-2/ER is expressed at comparable levels to endogenous GATA-2 in transformed erythroblasts

To ensure that the phenotypic effects in erythroblasts stably expressing GATA-2 and GATA-2/ER were not attributable to aberrantly high expression levels, we examined the amounts of endogenous versus exogenous GATA-2 (and GATA-2/ER) mRNA in these cells by Northern blot analysis. As a control, HD3 erythroblast clones expressing GATA-3/ER or the human ER (Schroeder et al. 1993), or empty vector were used. A GATA-2-specific probe corresponding to the entire cDNA (probe B in Fig. 5B) detects the cellular GATA-2 mRNA (4.3-kb), as well as both the retroviral genomic (5.35-kb) and subgenomic (4.35-kb) transcripts (with the subgenomic RNA partially superimposing the endogenous GATA-2 mRNA; Fig. 5A). Cryptically spliced retroviral RNAs were also detected, as reported previously (Fuerstenberg et al. 1990). To efficiently discriminate between cellular and retrovirus-derived GATA mRNAs, two other probes, corresponding to the GATA-2 3'-untranslated region (3' UTR) and the ER domain (Fig. 5B, probes C and A, respectively), were employed. The former detects only cellular GATA-2 transcripts while the latter only hybridizes to RNAs encoding either part of or the complete ER. From the data shown in Figure 5A we conclude that the retrovirus-transduced GATA-2 and GATA-2/ER mRNAs are expressed at levels comparable to the endogenous GATA-2 mRNA. Similar expression patterns were obtained for all GATA-2- and GATA-2/ER-expressing HD3 clones analyzed. Additionally, we confirmed that upon induction of differentiation, a con-

stant level of ectopic GATA-2/ER expression is maintained in these cells, whereas the endogenous GATA-2 mRNA gets down-regulated (Fig. 5C).

We were also curious as to the relative levels of chimeric GATA-3/ER expression in GATA-3/ER HD3 erythroblasts, because no obvious phenotypic changes were observed for a series of GATA-3/ER clones analyzed (data not shown). However, GATA-3/ER mRNA was found to be expressed at levels similar to GATA-2/ER mRNA, suggesting that the specific activities observed for GATA-2 (and GATA-2/ER) could not be induced by this closely related member of the GATA factor family.

Finally, a GATA-2-specific anti-peptide antibody was generated and used to examine the level of GATA-2 factor species in a representative erythroblast clone stably expressing GATA-2/ER. As shown in Figure 5D, bands corresponding to the anticipated sizes of GATA-2 and GATA-2/ER proteins were visualized after immunoprecipitation with the anti-GATA-2 serum (lane 2) but not with preimmune serum (lane 1). Both signals are specific, as they could be competed with bacterially expressed GATA-2/glutathione S-transferase (GST) fusion protein (data not shown). Whether the rather weak signal obtained for GATA-2/ER (as compared with endogenous GATA-2) reflects low levels of expression or is attributable to a less avid interaction of the antibody with the chimeric protein as compared with the unmodified GATA-2 is not known at present. Exogenous GATA-2/ER protein was also detected in these erythroblasts by Western blot analysis using the ER-specific monoclonal antibody F3 (kindly provided by P. Chambon and D. Metzger, LGME, Faculte de Medecine, Strasbourg, France; data not shown).

In conclusion, the ectopic expression of the chimeric GATA proteins from the pSFCV vector changes the total concentration of these factors within the cells only modestly. Thus, the physiological consequence of ectopic expression of GATA-2 (and of a hormone-activated GATA-2/ER) is not attributable to dramatically altering the absolute concentration of these factors but is more probably the result of their continued activity.

GATA-2/ER inhibits terminal differentiation of primary erythroid progenitor cells in a hormone-regulated manner

Whereas the HD3 cell line has been shown to be representative as a model for erythroid differentiation in many examples (Foley and Engel 1992 and references therein), we also wished to investigate the consequences of expressing the conditional GATA factors in nonestablished primary erythroblasts. Various GATA/ER chimeras were therefore introduced into normal or ts-oncogene-transformed primary chicken erythroid progenitor cells. To control for potential effects on erythroid differentiation attributable to endogenous ER (Schroeder et al. 1993) and to rule out possible side effects contributed by the ER moiety in the chimeric GATA proteins (e.g., sequestration of factors interacting with this domain), a

Figure 5. Expression of GATA and GATA/ER transcription factors in HD3 erythroblast clones. (A) Total RNA (20 μ g per lane) recovered from HD3 erythroblast clones expressing GATA-2/ER, GATA-2, and GATA-3/ER [clones C5, C8, and C3 (lanes 1, 2, and 3, respectively)], pSFCV vector only [clone E11 (lane 4) Fuerstenberg et al. 1992], or the human ER [clone 7 (lane 5) Schroeder et al. 1993] were examined using Northern blot analysis. Blots were hybridized separately to specific probes for the hormone-binding domain of the human ER, GATA-2, GATA-2 3' UTR (specific to the GATA-2 3'-untranslated region; probes A, B, C, respectively, in B), and GATA-3 as described in Materials and methods. (B) Schematic representation of the pSFCV-GATA-2/ER retroviral vector. The expected genomic (5.35-kb) and subgenomic (4.35-kb) retroviral RNAs, and the cellular GATA-2 (4.3-kb) mRNA are shown. Probes A, B and C indicate the ER-, GATA-2-, or GATA-2 3' UTR-specific probes used in A. (C) HD3 erythroblast clones stably expressing GATA-2/ER [clone C5 (lanes 1–5)] or empty pSFCV vector [clone E11 (lanes 6–10)] were allowed to differentiate for various periods of time as indicated. Total RNA was harvested from these cells and subjected to Northern blot analysis (8 μ g of RNA per lane). A full length GATA-2 cDNA was used as a probe (probe B in B). Please note that the 4.35-kb GATA-2/ER-specific subgenomic RNA (lanes 1–5) is not down-regulated upon induction of differentiation owing to continuous expression from the retroviral vector. (D) Immunoprecipitation analysis of a representative GATA-2/ER-expressing HD3 erythroblast clone (E6) employing a GATA-2-specific anti-peptide antibody [α -GATA-2 (lane 2) see Materials and methods]. Preimmune serum was used as a control (lane 1). The positions of the anticipated GATA-2/ER (85-kD) and GATA-2 (56-kD) proteins are indicated.

recombinant Gal/ER fusion construct [containing the DNA-binding domain of the yeast transcription factor GAL4 fused to the hormone-binding domain of the human ER [Metzger et al. 1988; I. Leitner, unpubl.]] was also introduced into erythroblasts as an experimental control.

Normal TGF- α (transforming growth factor- α)-dependent erythroid progenitors [Pain et al. 1991; Schroeder et al. 1993] were infected with recombinant retroviruses expressing various GATA/ER or Gal/ER chimeras. Additionally, ts-v-sea-transformed erythroblasts expressing GATA/ER or Gal/ER were generated. Following selection, G418-resistant erythroblast clones were analyzed for GATA/ER and Gal/ER mRNA expression by Northern blot analysis (data not shown) and then used in subsequent experiments.

Whereas no GATA-1/ER-expressing TGF- α -dependent erythroblast clones could be obtained (for reasons that are not clear at the present time), clones expressing GATA-2/ER, GATA-3/ER, and Gal/ER were isolated.

To induce differentiation, these TGF- α -dependent erythroblasts were cultivated for 2 days in the absence of TGF- α under conditions that support erythroid differentiation. Either estrogen or ICI was added to modulate the activities of GATA/ER and Gal/ER fusion proteins.

Erythroid differentiation was markedly retarded in estrogen-treated, but not ICI-treated, TGF- α -dependent erythroid cells expressing GATA-2/ER, whereas Gal/ER control cells exhibited only minor differences in their differentiation kinetics (possibly owing to endogenous ER activity; Fig. 6A,B). Thus, the ectopic expression of GATA-2/ER in primary erythroblasts resulted in a hormone-dependent suppression of differentiation, as seen in HD3 cells. Several independent observations also indicated that GATA-2/ER (and GATA-2)-expressing TGF- α -dependent erythroblasts grew significantly more robustly than ordinary TGF- α -dependent erythroblasts cultured under the same conditions (data not shown), implicating that GATA-2 may also affect the growth properties of these cells.

Figure 6. GATA-2/ER retards maturation of normal and ts-v-sea-transformed erythroid progenitors in a hormone-regulated fashion. (A) TGF- α -dependent erythroblasts expressing GATA-2/ER (clone L3), GATA-3/ER (clone M13), or Gal/ER (clone N1) were induced to differentiate for 2 days in the presence of estrogen or ICI. Cytospin preparations were stained and photographed as in Fig. 4A. (B) Aliquots of the cells shown in A were evaluated for stages of erythroid differentiation, and the results were plotted in histograms (see Fig. 4B). (C) Ts-v-sea-transformed erythroblasts expressing GATA-2/ER (clone E13) or Gal/ER (clone G1) were induced to differentiate at 42°C for 2 days, subjected to cytocentrifugation and neutral benzidine staining, and evaluated for stages of erythroid maturation as in Fig. 4B. (See Fig. 4 for abbreviations.)

To investigate whether the observed hormone-dependent block in differentiation is specific to GATA-2/ER, erythroblast clones expressing GATA-3/ER were examined. In contrast to GATA-2/ER clones, these cells showed normal erythroid differentiation regardless of the drug used (Fig. 6A; data not shown). Thus, the effects seen for GATA-2/ER could not be induced by another closely related member of the GATA transcription factor family, indicating that the GATA-2-induced differentiation arrest is specific for this factor.

Finally, GATA-2/ER also repressed differentiation of ts-v-sea-transformed erythroblasts in a hormone-responsive fashion (Fig. 6C; data not shown), whereas GATA-3/ER- and Gal/ER-expressing control cells showed only a slight effect (again, probably the result of the action of the endogenous ER). Although the specific clones of GATA-2/ER and Gal/ER shown here appear to differ slightly in the number of mature erythrocytes eventually developed, we have not observed any consistent difference in a large number of analogous clones examined.

In conclusion, these findings support the contention that GATA-2, but not GATA-3, enhances self-renewal in erythroid progenitor cells, thus influencing the developmental decision in these cells to undergo self-renewal

rather than terminal differentiation. These results therefore raise the distinct possibility that GATA-2 might function as a negative regulator of erythroid differentiation.

Discussion

The paradigm of generating hormone-inducible transcription factors by fusing them to the hormone-binding domain of the human ER has been applied with success previously (e.g., *myc*, *myb*, *fos*, and *rel*; see Boehmelt et al. 1992 and references therein). In this paper we show that a hormone-inducible GATA-2/ER fusion protein specifically arrests or retards erythroid differentiation in an estrogen-dependent fashion. The *in vivo* activity of the GATA-2/ER chimera mirrors its ability to *trans*-activate a GATA-responsive reporter gene in a hormone-dependent manner.

How is the activity of GATA/ER chimeras modulated by estrogen?

The analysis of various functional properties of the GATA/ER factors (subcellular localization, sequence-

specific DNA binding, and activation of transcription) provided initial clues as to how they might act on a molecular basis. In their hormone-activated state, the GATA/ER proteins are selectively partitioned to the nucleus (probably the result of the estrogen-inducible nuclear localization signal in the ER moiety; Ylikomi et al. 1992), whereas they are distributed throughout the cell in the absence of ligand. In addition, the anti-estrogen ICI induced nuclear localization of the GATA/ER factors (data not shown), as also observed with the chimeric *v-rel*/ER fusion protein (Boehmelt et al. 1992). However, because ICI failed to fully activate GATA-dependent transcription in initial cotransfection *trans*-activation assays, properties of the GATA/ER proteins other than their nuclear translocation must be regulated by hormone. Using fractionated extracts from transfected fibroblasts, we show that unliganded GATA/ER factors also bind efficiently to the GATA DNA motif in EGMSAs and that this binding is sequence specific. These data indicate that the DNA-binding ability of these chimeric factors is not subjected to regulation by hormone and, therefore, cannot account for the estrogen-dependent transcriptional activities of the GATA/ER proteins. In contrast, the *v-rel*/ER fusion protein is estrogen dependent for DNA binding (Boehmelt et al. 1992).

The appearance of a GATA-binding site-specific high-molecular-mass mobility band in EGMSA (which migrates far more slowly than the parental GATA protein–DNA complex) suggests that the chimeric GATA/ER factors could be complexed either with other cellular factors or as homodimers via the dimerization interface contained in the ER moiety of the fusion protein (Kumar and Chambon 1988; Fawell et al. 1990). Although hormone-dependent dimerization may occur, this does not seem to be crucial for sequence-specific DNA binding, as the GATA/ER proteins do bind DNA in the absence of hormone. It is therefore tempting to speculate that binding of estrogen to the GATA/ER proteins might induce a change in protein folding, leading to the unmasking of the *trans*-activation domain(s), which thereby triggers the transcriptional activities of the GATA/ER factors in a hormone-dependent manner.

The GATA/ER chimeric factors harbor at least two transcriptional activation domains: one or more GATA-specific domain(s) and the ligand-dependent transcriptional activator function TAF-2 contained in the ER moiety (Gronemeyer 1991; Danielian et al. 1992 and references therein). In these proteins the juxtaposition of the *trans*-activation domains of the ER and the GATA factors might lead to greater transcriptional stimulation as compared with parental factors. However, of the three chimeras, only GATA-1/ER stimulated reporter gene expression to a level greater than that of the parental GATA-1 factor. The differences in the biological response of the GATA-1/ER, GATA-2/ER, and GATA-3/ER fusion proteins to estrogen (and the estrogen antagonist ICI; see below) could reflect fundamental differences in the organization of the various functional domains of these different GATA transcription factors.

It has been shown previously that ICI behaves as a

pure estrogen antagonist both for the bona fide ER and for (e.g.) the *v-rel*/ER chimeric protein (Boehmelt et al. 1992 and references therein). Although GATA-1/ER can *trans*-activate GATA site-dependent reporter gene expression in the presence of ICI, albeit weakly (7-fold vs. 274-fold in the presence of estrogen), GATA-2/ER and GATA-3/ER are transcriptionally inactive in the presence of this drug. The identical response of these two factors is not surprising because chicken GATA-2 and GATA-3 are much more similar to one another in primary amino acid sequence than either is to GATA-1 (Yamamoto et al. 1990), which is also true for the mammalian and amphibian GATA factor families (Zon et al. 1991).

What is the functional role of transcription factor GATA-2 in erythropoiesis?

Although an important role of GATA-1 in erythropoiesis has been clearly demonstrated by gene inactivation experiments (Pevny et al. 1991), such studies provide only inferential evidence for how GATA expression might control erythroid differentiation. In particular, why are all three GATA factors (which apparently share similar DNA-binding characteristics) simultaneously expressed within the same erythroid progenitor during its maturation (Yamamoto et al. 1990)? Two general observations suggest that this transcription factor family plays an important, conserved role in erythropoiesis. First, the expression pattern of all three GATA factors is conserved in avian, human, and murine erythroid cells. Second, the changes in the relative abundance of all three GATA factors during erythroid differentiation are very similar across species (Yamamoto et al. 1990; M.W. Leonard, K.-C. Lim, and J.D. Engel, in prep.). This suggests that the precise quantitative balance of these factors might determine the developmental options available to erythroid progenitor cells, that is, to undergo either self-renewal or terminal differentiation. It was therefore of interest to perturb the relative abundance of the endogenous GATA factors by introducing conditional GATA/ER chimeras into ts-oncogene-transformed erythroblasts in which self-renewal and terminal differentiation can be modulated at will (Beug et al. 1992).

In such ts-oncogene-transformed erythroblasts, similar to the situation in ovo, the expression pattern of GATA-1, GATA-2, and GATA-3 undergoes distinct, developmentally regulated changes: GATA-1 expression is high throughout and increases only modestly late during erythroid differentiation; GATA-2 is down-regulated shortly after the induction of differentiation; GATA-3 abundance is markedly induced during the same period (when these factors are quantified on a “per cell” basis; Yamamoto et al. 1990). These experiments point to the importance of high-level GATA-1 expression throughout erythroid differentiation and suggest that GATA-3 up-regulation during late maturation may be important for globin gene switching (M.W. Leonard, K.-C. Lim and J.D. Engel, in prep.).

GATA-1 has been shown to be essential for the gener-

ation of the erythroid lineage (Pevny et al. 1991), whereas GATA-3 is principally expressed in neuronal and T-lymphocytic cells (Yamamoto et al. 1990). However, the functional role of GATA-2, which has the widest tissue expression profile of the GATA family, remains enigmatic. Here, we present significant evidence arguing for a functional role for GATA-2 in erythropoiesis. Ectopic expression of a conditional GATA-2/ER allele in various transformed or normal erythroid precursors inhibits terminal erythroid differentiation and concurrently promotes self-renewal of these cells in a hormone-dependent fashion. No comparable activity was observed for the structurally related GATA-3/ER or unrelated Gal/ER transcriptional regulatory proteins. These observations suggest that the GATA-2 transcription factor specifically regulates the maintenance of erythroid progenitors in an immature state and that changes in the relative abundance of GATA-2 might be a key event required for induction of terminal differentiation. Direct tests of this hypothesis will require erythroid cell lines in which the GATA-2 gene has been inactivated by targeted gene disruption or, for example, the expression of dominant-negative GATA-2 versions.

Given the fact that the GATA factors are highly homologous in their DNA-binding domains but very different in their overall primary structure (Yamamoto et al. 1990), it is possible that GATA-1 and GATA-2 might bind to the very same target sequences (or a common subset of those) but engender different activities in erythroid cells. Thus, GATA-1 binding could promote differentiation, whereas GATA-2 might interfere with the very same process. Alternatively, subtle differences in DNA-binding sequence specificity might cause the GATA factors to bind to different transcriptional regulatory sequences whose distinct target genes then elicit separate effects on the physiological program of these cells, that is, promoting either self-renewal or terminal differentiation. A more refined analysis of the GATA factor DNA-binding characteristics indicates that while the various members of this transcription factor family bind to consensus (WGATAR) sites with similar affinities, chicken GATA-2 and GATA-3 are also capable of binding to an alternative consensus sequence with high affinity (L.J. Ko and J.D. Engel, in prep.). Thus, erythroblast clones expressing the various conditional GATA/ER factors may be useful in identifying common and/or specific downstream target genes differentially regulated by each member of the GATA factor family during erythropoiesis.

Materials and methods

Construction of GATA/ER plasmids

PCR technology was used to remove the termination codon from each of the GATA cDNAs (Yamamoto et al. 1990) and to introduce a *Bam*HI linker at the 3' ends of the various GATA-coding regions (adding the 3 amino acid residues Pro-Asp-Pro). These full-length GATA factor cDNAs were joined at their 3' *Bam*HI restriction sites in-frame to a 0.95-kb *Bam*HI-*Eco*RI cDNA fragment encoding the hormone-binding domain of the

human ER (amino acids 282–595; Kumar et al. 1986). The resulting chimeric cDNAs (1.9-kb GATA-1/ER, 2.35-kb GATA-2/ER, and 2.45-kb GATA-3/ER) were then subcloned into the eukaryotic expression vector TFAneo (Federspiel et al. 1989; with the Rous sarcoma virus long terminal repeat (RSV LTR) directing the synthesis of the hybrid GATA/ER mRNAs) for use in transient transfection assays. For stable expression in erythroblasts, various GATA and GATA/ER cDNAs were subcloned into the retroviral vector pSFCV-LE (Fuerstenberg et al. 1990) with the avian erythroblastosis virus (AEV) LTR directing the transcription of these cDNAs.

Cotransfection trans-activation assay

QT6 quail fibroblast cells (5×10^6) (Moscovici et al. 1977) were seeded into a 100-mm petri dish and fed with Dulbecco's modified Eagle medium (DMEM) containing 2% tryptose phosphate broth, 5% fetal calf serum (FCS) and 2% chicken serum. The following day, cells were transfected with 10 μ g of DNA (3 μ g of the various activator plasmids and 7 μ g of C3 β GH reporter plasmid) as described previously (Yamamoto et al. 1990). Sixteen hours post-transfection, the cells were rinsed with phosphate-buffered saline (PBS) and fed with fresh DMEM containing 10% dextran-coated charcoal-treated FCS (to deplete the serum of residual steroid hormone; Schroeder et al. 1993). β -Estradiol (10^{-6} M) or an estrogen antagonist [ICI 164,384 at 10^{-6} M, (Wakeling and Bowler 1988a,b) gift from A. Wakeling, ICI, Cheshire, England] in ethanol was added to one set of plates. To a second set of control plates, an identical volume of ethanol alone was added. Fresh medium containing the same additives was added after 24 hr. Supernatants were assayed the next day by radioimmunoassay for secreted hGH using the Allegro hGH kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). *trans*-activation activity was calculated as described (Yamamoto et al. 1990).

EGMSA

QT6 cells transfected with 10 μ g of activator plasmid were harvested at 60 hr post-transfection for preparation of cytoplasmic cell lysates using the method described (Tsai et al. 1989). EGMSA was performed as described (Yamamoto et al. 1990) using a 32 P-radiolabeled oligonucleotide that contained a single GATA factor-binding site derived from the mouse α 1-globin gene promoter (M α P; Tsai et al. 1989). To examine the sequence specificity of DNA binding, a 100-fold excess of either cold M α P oligonucleotide or unlabeled mM α P oligonucleotide (in which the core recognition sequence is changed from GATA to GGCA) was added to the gel shift reactions. The reactions were incubated for 15 min at 4°C. Protein–DNA complexes were then fractionated by electrophoresis at 150 V for 2 hr at room temperature in a 5% nondenaturing polyacrylamide gel, which was prepared and run in 1 $\times$ TBE buffer (Yamamoto et al. 1990).

Indirect immunofluorescence assay

Twenty-four hours after transfection, QT6 cells were reseeded onto coverslips in the presence of estrogen or ethanol. Confluent cells on the coverslips were rinsed with PBS, briefly fixed in 1% paraformaldehyde (in PBS), and permeabilized with cold acetone. H222 rat monoclonal antibody, specific for the hormone-binding domain of the human ER [(Greene et al. 1984) gift from G. Greene, University of Chicago, IL, USA], was added. After 1 hr of incubation at room temperature, the coverslips were rinsed with PBS, and FITC-conjugated goat-anti-rat antibody (Zymed, San Francisco, CA) was used as a secondary antibody.

The stained cells were then photographed using a Zeiss Axio-phot fluorescent microscope.

Cells and tissue culture

HD3 erythroblasts, transformed with *v-erbA/ts-v-erbB* oncogenes (Beug et al. 1982a,b), were transfected with either the pSFCV vector DNA or various pSFCV-GATA and pSFCV-GATA/ER DNAs and cultivated as described previously (Disela et al. 1991). Transfected HD3 cells ($2-5 \times 10^4$) were then seeded into CFU-E methocel containing 2 mg/ml of G418; and 7–10 days later, G418-resistant colonies were isolated and expanded in CFU-E medium (Radke et al. 1982).

To generate virus-producing cells, chick embryo fibroblasts (CEFs) were transfected with 10 μ g of each of the GATA-retroviral vector DNAs together with 1 μ g of RCAN helper virus DNA (Hughes et al. 1987) using the calcium phosphate/DNA coprecipitation method (Graham and Van der Eb 1973). After G418 selection, these virus-releasing CEFs were treated with mitomycin C (1 hr, 5 μ g/ml) and then used to infect normal erythroid progenitors, which were obtained by cultivating bone marrow cells in the presence of 5 ng/ml of TGF- α and 10^{-6} M β -estradiol (Pain et al. 1991; Schroeder et al. 1993). Alternatively, these TGF- α -dependent erythroblasts were infected simultaneously with GATA (or GATA/ER)-expressing retroviruses and the *ts-v-sea* virus (Fuerstenberg et al. 1992) and seeded into CFU-E methocel containing 2 mg/ml of G418. Neomycin-resistant clones were isolated and expanded in CFU-E medium. To control for effects on erythroid differentiation owing to the endogenous ER in normal erythroblasts (Schroeder et al. 1993), a retroviral vector pCRNCM expressing Gal/ER (Metzger et al. 1988; I. Leitner, unpubl.) was included as an experimental control. Both *ts-v-sea*-transformed and normal TGF- α -dependent erythroblast clones stably expressing recombinant GATA, GATA/ER, or Gal/ER factors were then screened in the presence of estrogen or ICI in proliferation and differentiation assays.

Proliferation and differentiation assays

To induce erythroid differentiation, TGF- α -dependent erythroblast clones were incubated at 37°C for 2 days in the absence of TGF- α while the *ts-v-sea* cells were shifted to 42°C (to inactivate the *ts-v-sea* oncogene) for 2 days in differentiation medium (Glineur et al. 1990; Zenke et al. 1990) supplemented with 2% anemic chicken serum and 10 ng/ml of insulin. HD3 erythroblast clones were induced to differentiate for 4–5 days at 42°C (to inactivate *ts-v-erbB*) in pH 8.0 medium containing 20–30 mM H7 [to inactivate *v-erbA*, which blocks temperature-induced differentiation (Zenke et al. 1988, 1990; Glineur et al. 1990)]. Inducible GATA/ER activity was modulated by the addition of either 10^{-6} M β -estradiol or 10^{-6} M ICI 164,384. Erythroid differentiation was assessed qualitatively by examining cytopins stained with neutral benzidine (Beug et al. 1982b) and quantitatively by measuring [3 H] thymidine incorporation as well as hemoglobin accumulation in a photometric assay (Kowenz et al. 1987).

RNA analysis

RNA was isolated from individual erythroblast clones by standard procedures (Chomczynski and Sacchi 1987). Total RNA (8–20 μ g) was analyzed by electrophoresis and blotting to Gene-Screen nylon membranes (Zenke et al. 1988), followed by hybridization to random primer-labeled probes (Feinberg and Vogelstein 1984) corresponding to the human ER hormone-

binding domain (Kumar et al. 1986; nucleotides 1204–2148), to the full-length cDNAs of chicken GATA-2 or GATA-3 (Yamamoto et al. 1990) or to the 3' UTR of GATA-2 (nucleotides 1990–2791). The blots were washed at moderate stringency (Church and Gilbert 1984) and exposed to film.

Preparation of GATA-2-specific anti-peptide antibody

A 19-mer peptide corresponding to a specific epitope of the chicken GATA-2 protein (Yamamoto et al. 1990; amino acids 177–192) plus a carboxy-terminally attached 3-amino-acid spacer—NSTSAASPSSAGARQ-GGC—was synthesized on an Applied Biosystems 431A peptide synthesizer. Subsequently, the reduced form of the peptide was coupled via its carboxy-terminal cysteine residue to soybean trypsin inhibitor (STI; Sigma) carrier protein that had been activated with succinimidyl-pyridinyl-dithio-propionate (SPDP; Pharmacia). The conjugate was dialyzed extensively against PBS and used directly for immunization. For the first immunization, rabbits were injected subcutaneously with an emulsion of 150 μ g of conjugate in PBS and complete Freund's adjuvant (1 : 1). The subsequent booster injections were performed with conjugate (200–500 μ g) emulsified in incomplete Freund's adjuvant. Sera were taken 10–14 days after each injection and tested for immunoreactivity against bacterially expressed GATA-2/GST fusion protein by Western blot analysis. After the third boost, high titer serum was obtained and the IgG fraction was purified by CM Affi-Gel blue chromatography (Bio-Rad). Eluates were concentrated by centrifugation on Centricon 10 columns, dialyzed extensively against PBS, and used in immunoprecipitation studies.

Immunoprecipitation analysis

HD3 erythroblast clones expressing GATA-2/ER chimeric protein were labeled *in vivo* for 2 hr with 250 μ Ci of [35 S]methionine [1000 Ci/mmol; Amersham (Zenke et al. 1988)]. Estrogen was added to a final concentration of 10^{-6} M, 30 min prior to harvesting to achieve the active conformation of the GATA-2/ER fusion protein. After labeling, the cells were washed with ice-cold PBS and lysed for 15 min on ice in 500 μ l of lysis buffer (PBS containing 1% Triton-X 100, 0.1% SDS, 50 mM NaF, 175 μ g/ml of PMSF, and 28 μ g/ml of aprotinin). The lysates were precleared by incubation with 100- μ l aliquots of a 10% suspension of *Staphylococcus aureus* protein A for 1 hr at 4°C and subsequent centrifugation for 30 min in an Eppendorf centrifuge. Aliquots of extracts in the lysis buffer were incubated for 1 hr at 4°C either with 5 μ l of preimmune serum or 5 μ l of Affi-gel-purified anti-GATA-2 antibody; 50 μ l of the 10% *S. aureus* protein suspension was added and, following 30 min of incubation, washed four times in ice-cold lysis buffer. The immunoprecipitates were separated on a 8% SDS-PAGE gel (Laemmli 1970) and detected by fluorography.

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