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Ligand-Dependent Repression of the Erythroid Transcription Factor GATA-1 by the Estrogen Receptor

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High-dose estrogen administration induces anemia in mammals. In chickens, estrogens stimulate outgrowth of bone marrow-derived erythroid progenitor cells and delay their maturation. This delay is associated with down-regulation of many erythroid cell-specific genes, including α- and β-globin, band 3, band 4.1, and the erythroid cell-specific histone H5. We show here that estrogens also reduce the number of erythroid progenitor cells in primary human bone marrow cultures. To address potential mechanisms by which estrogens suppress erythropoiesis, we have examined their effects on GATA-1, an erythroid transcription factor that participates in the regulation of the majority of erythroid cell-specific genes and is necessary for full maturation of erythrocytes. We demonstrate that the transcriptional activity of GATA-1 is strongly repressed by the estrogen receptor (ER) in a ligand-dependent manner and that this repression is reversible in the presence of 4-hydroxy-tamoxifen. ER-mediated repression of GATA-1 activity occurs on an artificial promoter containing a single GATA-binding site, as well as in the context of an intact promoter which is normally regulated by GATA-1. GATA-1 and ER bind to each other in vitro in the absence of DNA. In coimmunoprecipitation experiments using transfected COS cells, GATA-1 and ER associate in a ligand-dependent manner. Mapping experiments indicate that GATA-1 and the ER form at least two contacts, which involve the finger region and the N-terminal activation domain of GATA-1. We speculate that estrogens exert effects on erythropoiesis by modulating GATA-1 activity through protein-protein interaction with the ER. Interference with GATA-binding proteins may be one mechanism by which steroid hormones modulate cellular differentiation.

Hematopoiesis is highly regulated in vertebrates and capable of numerous adaptive responses to changing conditions. Among the factors that modulate erythropoiesis are the steroid hormones, including estrogens and glucocorticoids.

High-dose estrogen administration induces anemia in mammals (11, 13, 25, 41). In chickens, estrogens stimulate outgrowth of bone marrow-derived erythroid progenitor cells and delay maturation (32). This delay is associated with reduced expression of many erythroid cell-expressed genes, including α- and β-globins, band 3, band 4.1, and the erythroid cell-specific histone H5 (32). The inhibition of erythroid gene expression seems to be a direct effect of the action of estrogens and not just a consequence of the block in differentiation (32). Remarkably, specific DNA binding of the ER is not required for arrest of differentiation and repression of erythroid genes, consistent with the possibility that the ER exerts these functions through formation of contacts with other proteins (4).

Other steroids, such as glucocorticoids, efficiently block dimethyl sulfoxide-induced differentiation of murine erythroleukemia cells (5, 7, 16, 18, 23, 31). As shown below, estrogens reduce the number of erythroid burst-forming units (BFU-E) in primary human bone marrow cultures.

GATA-binding proteins have emerged as important regulators of developmental functions (26). GATA-1 is a zinc finger-containing transcription factor expressed mainly in erythroid cells and related lineages such as mast cells and megakaryocytes (12, 21, 40). GATA-1 participates in the regulation of the vast majority of erythroid cell-specific genes. Gene targeting experiments have demonstrated that GATA-1 is required for the formation of mature erythroid cells in chimeric mice (28), as well as in embryoid bodies derived from in vitro-differentiated murine embryonic stem cells (36). Progenitor assays using cells derived from embryoid bodies have shown that GATA-1 is required for both maturation as well as for survival of erythroid precursor cells (44).

As GATA-1 is a critical transcriptional regulator in erythroid cells, it stands as a potential target through which hormones and other factors could modulate erythropoiesis. Steroid hormone receptors regulate transcription not only through ligand-mediated binding to the hormone response elements but also indirectly through interaction with other transcription factors, such as AP-1 (10, 15, 19, 33, 46). As no obvious estrogen receptor (ER)-binding sites are evident in the promoters of the genes repressed by estrogens in erythroid cells, we asked whether the ER might exert its function through interference with GATA-1 function.

In this report, we show the following. (i) Estrogens reduce the number of BFU-E in primary human bone marrow cultures. (ii) The ER negatively regulates GATA-1 transcriptional activity in a ligand-dependent manner. This effect can be reversed in the presence of the antiestrogen compound 4-hydroxy-tamoxifen (OHT). (iii) Repression occurs in the context of an intact promoter of a GATA-1 target gene. (iv) GATA-1 and ER physically interact in vitro and in vivo. (v) At least two contacts are formed between GATA-1 and the ER, involving the N terminus and the zinc finger region of GATA-1.

MATERIALS AND METHODS

Human bone marrow cultures. Human bone marrow suspension obtained from healthy adult volunteers was separated over Ficoll-Paque (1.077 g/cm^2; Pharmacia Fine Chemicals) at 400 g for 40 min at 20°C, and the interface mononuclear cells were collected, washed three times, and resuspended in Iscove's modified Dulbecco's medium (Hazelton, Lenexa, Kans.) supplemented with 20% fetal calf serum. After incubation for 1 h, nonadherent cells were enriched for CD34$^+$ progenitor cells by incubation with a biotinylated monoclo-

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nal anti-CD34 antibody and captured on an avidin column (Ceprate LC; CellPro, Bothell, Wash.). After washing with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), cells were released by squeezing the column. Approximately 50% of the $CD34^+$ cells were recovered with purity of 50 to 75%. $CD34^+$ cells were plated at 2,500/ml in quadruplicate in serum-deprived methylcellulose cultures containing 1% deionized BSA, 2×10^{-4} M BSA-absorbed cholesterol, 300 μg of iron-saturated transferrin (Boehringer Mannheim, Indianapolis, Ind.) per ml 10 μg of insulin (Sigma) per ml, 10^{-4} M β-mercaptoethanol, 2 U of erythropoietin (R&D Systems) per ml, 1 nM interleukin 3 (gift of Immunex), and human granulocyte-macrophage colony-stimulating factor (gift of Genetics Institute) as previously described (35). After 14 days, BFU-E were counted.

Transient transfections and transactivation assays. NIH 3T3 cells were transfected by the calcium phosphate precipitation method (30). COS cell transfections were performed by the DEAE-dextran method (30). In transactivation experiments, NIH 3T3 cells were initially transfected with various ratios of inducer plasmids (pXM-GATA-1 [20] and pSG5-HEO [14]) and reporter plasmids (M1α-GH [20] and EKLF-GH [9]) to optimize transactivation by GATA-1 and repression by the ER, respectively. We routinely used 1.5 μg of M1α-GH or 1.5 μg of EKLF-GH, 2.5 μg of pXM-GATA-1, and 0.25 μg of pSG-HEO plasmid DNA (Qiagen purified) per 30-mm-diameter tissue culture dish.

When different combinations of plasmids were assayed, the total amount of transfected DNA was always kept constant by using nonexpressing plasmid vectors; 3.5 μg of salmon sperm DNA was added as carrier to all transfections.

Following transfection, cells were grown in phenol red-free Dulbecco's modified Eagle's medium (Gibco/BRL) and charcoal-treated bovine calf serum (Cocalico Biologicals Inc.), essentially free of steroid hormones. β-Estradiol was added to a final concentration of 100 nM. Where indicated, 1 μM OHT (ICI) was added. Growth hormone levels were determined 48 h after transfection, using an Allegro radioimmunoassay kit (Nichols Institute), and the average results of at least three independent experiments are shown.

Plasmids and constructs. The reporter plasmid (M1α-GH) contained one GATA-binding site derived from the mouse α1-globin promoter fused the rabbit β-globin TATA box containing core promoter (20). The plasmids expressing GATA-1 (pXM-GATA-1) and the human ER (pSG5-HEO) were described previously (14, 20, 40).

For in vitro binding studies, cDNA constructs were subcloned into pGEX (Pharmacia) such that the GATA-1 start codon is in frame with glutathione *S*-transferase (GST). Deletion constructs of GATA-1 were generated by PCR using PFU DNA polymerase (Stratagene). For the in vivo binding studies, the GST fusion constructs were introduced into pEBB, in which the EF1α promoter drives expression in mammalian cells (kind gift of Bruce Mayer). Correctness of the constructs was verified by sequencing. Bacterially expressed fusion proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining, while proteins expressed in mammalian cells were analyzed by Western blotting (immunoblotting) using anti-GST antibodies (kind gift of Margaret Chou).

EKLF-GH contained the erythroid Krüppel-like factor (EKLF) promoter region from −77 base to +34 with respect to the transcription start site of the EKLF gene. This region is sufficient for erythroid expression when placed in front of the growth hormone gene (9). It contains two potential GATA-binding sites, one of which (site 2) is important for activity in murine erythroleukemia cells and for activation by GATA-1 in cotransfection experiments using heterologous cells (9).

Gel mobility shift experiments. Nuclear extracts prepared from transfected COS cells were analyzed as described previously (20).

In vitro binding studies. GST fusion proteins were purified from bacteria and coupled to glutathione-agarose beads (Sigma) as described previously (37). Labeled ER protein was generated by in vitro translation using rabbit reticulocyte lysates (Pharmacia) in the presence of [^{35}S]methionine. Equal amounts of coupled GST or GST fusion proteins were incubated with ER protein in 150 mM NaCl–20 mM Tris-HCl (pH 7.5)–0.3% Nonidet P-40–1 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–5 μg of leupeptin per ml–2 μg of aprotinin per ml for 1 h at 4°C and then washed five times. Bound protein was analyzed by PAGE and autoradiography.

In vivo binding studies. pXM-GATA-1 and pSG5-HEO were cotransfected into COS cells. At 48 h after incubation in the presence or absence of 100 nM β-estradiol, cells were harvested in PBS. Nuclei were prepared as described previously (2). To release nuclear proteins without using high salt concentrations, nuclei were vortexed in 150 mM NaCl–50 mM Tris-HCl (pH 7.5)–5 mM EDTA (pH 8.0)–0.1% Nonidet P-40–1 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–5 μg of leupeptin per ml–2 μg of aprotinin per ml in the presence or absence of 100 nM β-estradiol and then centrifuged for 5 min at 14,000 rpm at 4°C. Equal amounts of protein were used for immunoprecipitation using an antiserum directed against a peptide spanning 15 C-terminal amino acids of GATA-1 (kind gift from Len Zon). Immunoglobulins were precipitated by using protein G (Boehringer Mannheim). After five washes in the above-described buffer in the presence or absence of β-estradiol, proteins were analyzed by sodium dodecyl sulfate (SDS)-PAGE and then subjected to Western blotting using a rat monoclonal antibody against the ER (H222; generous gift of Geoffrey Greene) and the enhanced chemiluminescence detection method (Amersham).

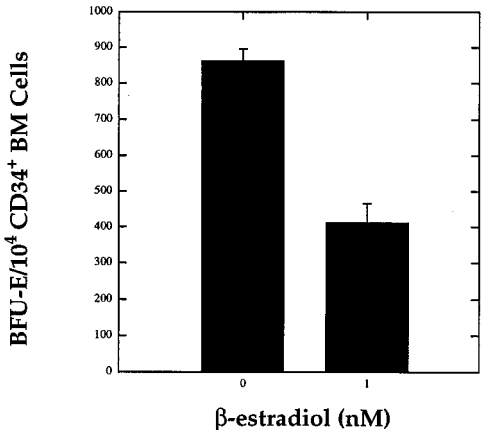

FIG. 1. β-Estradiol reduces the number of BFU-E derived from primary human bone marrow (BM) $CD34^+$ progenitor cells.

For in vivo mapping studies, COS cells cotransfected with GST fusion constructs and pSG5-HEO were lysed by vortexing in 150 mM NaCl–50 mM Tris-HCl (pH 7.5)–5 mM EDTA (pH 8.0)–0.1% Nonidet P-40–1 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–5 μg of leupeptin per ml–2 μg of aprotinin per ml. After centrifugation to remove debris, protein concentrations were determined in the supernatants. Glutathione-agarose beads were added to equal amounts of total protein and incubated for 30 min at 4°C. After five washes, bound protein was analyzed by SDS-PAGE followed by Western blotting using anti-ER or anti-GST antibodies.

RESULTS

Estrogens reduce the number of human bone marrow-derived BFU-E. To define more precisely the effects of estrogens in primary erythroid cells of mammalian origin, human bone marrow $CD34^+$ cells were cultured in semisolid medium supplemented with 2 U of erythropoietin per ml, 1 nM granulocyte-macrophage colony-stimulating factor, and 1 nM interleukin 3 in the presence or absence of 1 nM β-estradiol. After 14 days in culture, the number of BFU-E was scored. Addition of estrogens reduced the number of BFU-E by 50% (Fig. 1). The difference between the means of the cultures with and without β-estradiol was significant ($P = 0.0002$, paired Student's t test). In contrast to the findings for chicken cells (32), no outgrowth of immature cells was observed.

Estrogens inhibit GATA-1 activity. The strict requirement of GATA-1 for erythropoiesis raised the possibility that estrogens exert their effects by interfering with GATA-1 function.

As a first step to identifying a possible interaction between the ER and GATA-1, we examined whether the presence of ligand-bound ER affects transactivation by GATA-1 in cotransfection experiments. NIH 3T3 cells were transfected with plasmids expressing cDNAs encoding murine GATA-1 (pXM-GATA-1 [20]) and the human ER (14) and a reporter containing the human growth hormone gene driven by a minimal rabbit β-globin promoter to which the mouse α-globin GATA-binding site was juxtaposed (M1a-GH [20]). Initial titration experiments were designed to determine the appropriate input of transfected DNA for each construct in order to optimize transactivation by GATA-1 and the effects of the cotransfected ER (data not shown). Transactivation by GATA-1, which averaged 35- to 40-fold, was reduced 10-fold in the presence of ligand-bound ER, while basal-level transcription, i.e., transcription in the absence of GATA-1, was minimally affected (Fig. 2a). No repression was observed in the absence of ER or β-estradiol. Inhibition of GATA-1 transactivation was not due to nonspecific toxicity of estrogens, as it was abrogated by

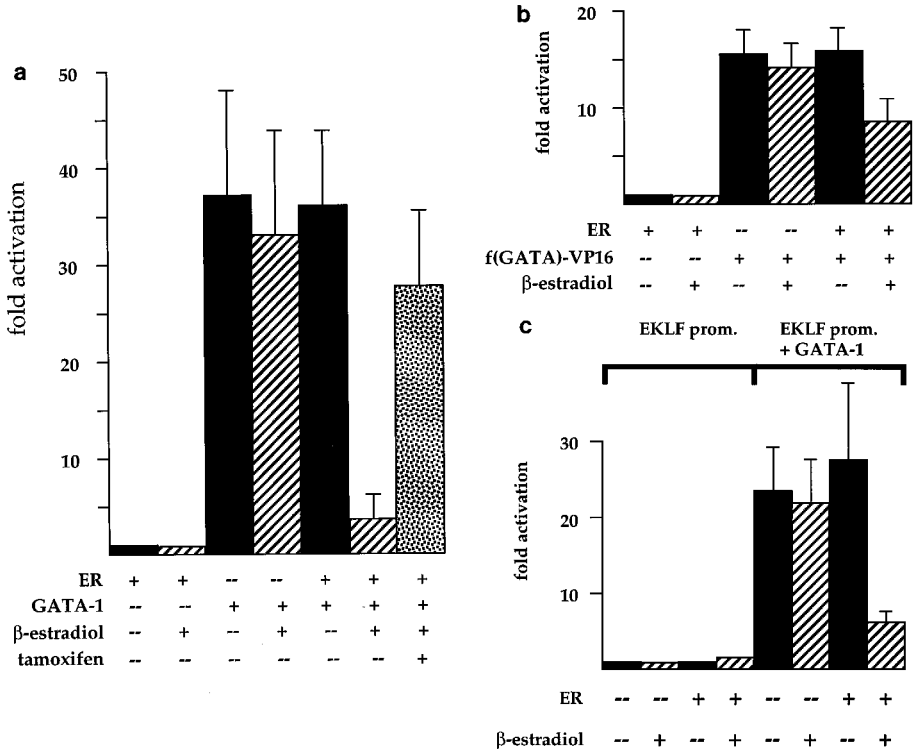

FIG. 2. Inhibition of GATA-1 activity by the ligand-bound ER is about 10-fold and reversible in the presence of OHT (a), whereas f(GATA)-VP16 is inhibited only 1.6-fold (b). The ER represses GATA-1 activity in the context of the intact EKLF promoter (prom.) (c). Note that basal-level activities are not affected by the presence or absence of estrogens.

addition of the antiestrogen compound OHT (Fig. 2a). As a further control, an artificial GATA-binding protein consisting of the GATA-1 zinc finger region fused to the herpes simplex virus VP16 activation domain [f(GATA)-VP16] was tested. While this construct activated transcription about 16-fold, inhibition by estrogens was minimal (less than 2-fold) (Fig. 2b). The activities of other transcription factors such as Sp1, GAL4-VP16 (29), and GAL4 fused to the p65 subunit of NFκB (gift of Dimitri Thanos) were not affected by the ligand-bound ER (data not shown), indicating that inhibition of GATA-1 is not due to a nonspecific squelching effect by the ER. Transactivation by chicken GATA-1 was also repressed by ligand-bound ER (data not shown).

Inhibition of GATA-1 activity was not due to reduced expression of GATA-1 in response to estrogen, as shown below by anti-GATA-1 Western blotting using extracts from transfected cells.

ER-mediated repression of a GATA-1 target gene promoter. Steroid hormone receptors act in a promoter context-dependent manner (10, 24, 27, 34). As the experiments described above were performed with an artificial reporter plasmid containing a single GATA-binding site, we tested if the ER might interfere with GATA-1 function in the context of the intact GATA-1-regulated promoter of the gene encoding the erythroid transcription factor gene EKLF (9). Expression of the ER repressed GATA-1 transactivation of the EKLF promoter fivefold (Fig. 2c). The activity of the EKLF promoter in the absence of GATA-1 was not inhibited. Thus, inhibition is mediated by GATA-1 and not by other factors binding to the promoter.

The ER binds to GATA-1 in vitro. To test if the inhibition of GATA-1 was caused by a direct physical interaction with the ER, bacterially expressed purified GATA-1 fused to GST (GST–GATA-1) or controls (GST alone or GST fused to the p45 subunit of the erythroid transcription factor NF-E2 [GST–NF-E2] [1]) were coupled to glutathione-coated agarose beads. Beads loaded with equivalent amounts of protein were incubated with in vitro-translated [^{35}S]methionine-labeled ER, washed extensively, and analyzed by SDS-PAGE followed by autoradiography. Figure 3a demonstrates that the ER binds to GST–GATA-1 but not to GST. Only trace amounts of ER protein bound to GST–NF-E2. We further show that the interaction was unaffected by the presence of excess amounts of oligonucleotides containing GATA-binding sites (Fig. 3a), indicating that binding occurs through protein-protein interactions and not through contaminating DNA.

To assess whether the ER–GATA-1 complex is dependent on the presence of ligand, 0.1 μM β-estradiol was included in the binding reaction mixtures and all subsequent washes. Figure 3b shows that in vitro binding was ligand independent, suggesting that the ligand dependence of GATA-1 inhibition seen in intact cells is not a result of β-estradiol modulating the affinity of the ER to GATA-1.

The stability of the GATA-1–ER complex was tested by increasing the concentrations of NaCl in the washes following the binding reactions. Remarkably, a significant proportion of ER protein remained bound to GATA-1 even after a 1.2 M NaCl wash (Fig. 3c).

The ER binds to GATA-1 in vivo. COS cells were cotransfected with plasmids expressing GATA-1 and the ER and incubated in the presence or absence of β-estradiol for 48 h. Nuclear extracts were immunoprecipitated with an anti-GATA-1 antibody or preimmune serum. Precipitates were washed, fractionated by SDS-PAGE, and Western blotted with

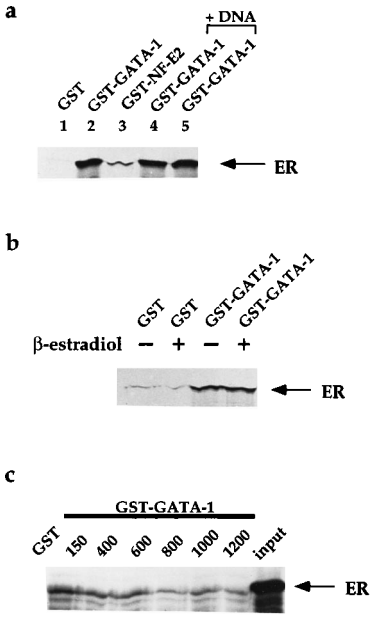

FIG. 3. GATA-1 and the ER interact in vitro. ER binds to GST–GATA-1 but little, if at all, to GST–NF-E2 or GST alone (a). In vitro binding is not ligand dependent (b). The GATA-1–ER complex is stable at high salt concentrations (c). Numbers indicate millimolar NaCl concentrations. Equal amounts of GST or GST fusion proteins were used in all reactions.

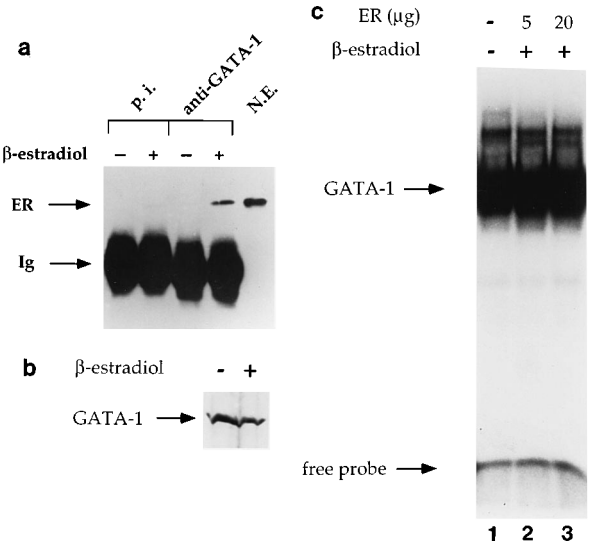

FIG. 4. GATA-1 and the ER form a ligand-dependent complex in vivo. (a) Equal amounts of nuclear extracts of transfected COS cells were immunoprecipitated with preimmune serum (p.i.) or with an anti-GATA-1 antibody. Precipitates were analyzed by Western blotting using an anti-ER antibody. Unprecipitated nuclear extracts (N.E.) were run in parallel as a control. Comparable amounts of immune and preimmune immunoglobulins (Ig) were used. (b) The presence of ligand-bound ER does not affect the expression of transfected GATA-1. Unprecipitated nuclear extracts were run in parallel and probed with an anti-GATA-1 antibody. (c) GATA-1 binding to DNA is unaffected in the presence of ligand-bound ER. Gel mobility shift analysis was performed with nuclear extracts from COS cells transfected with plasmid expressing GATA-1 alone (lane 1) or in combination with 5 and 20 μg of plasmid expressing ER (lanes 2 and 3).

a monoclonal antibody against the ER (h222; kind gift of Geoffrey Greene). The results show that the ER specifically coprecipitates with GATA-1 in a ligand-dependent manner (Fig. 4a). A control anti-GATA-1 Western blot analysis using unprecipitated nuclear extracts demonstrates the presence of equal amounts of transfected GATA-1 in the presence and absence of estradiol (Fig. 4b). The GATA-1–ER interaction is ligand independent in in vitro binding assays, which suggests that subcellular distribution and/or binding of the ER to other proteins such as the heat shock proteins may affect its availability to interact with GATA-1.

As the zinc finger region of GATA-1 participates in the association with the ER (see below), one possible mechanism by which ER represses the transcriptional activity of GATA-1 is through inhibition of GATA-1 binding to DNA. Gel shift experiments, however, indicate that GATA-1 DNA-binding activity was the same in nuclear extracts from COS cells transfected with plasmids expressing GATA-1 alone or in combination with plasmids expressing ER (Fig. 4c).

GATA-1 forms at least two contacts with the ER. To delineate the regions of GATA-1 required for ER binding, GATA-1 derivatives with various deletions were tested in in vivo binding studies.

Initial mapping experiments made use of the in vitro binding assay described above. Throughout the course of these experiments, we noticed that in vitro binding of the ER to some mutant GATA-1 proteins was subject to variation between experiments (data not shown). Therefore, cDNAs encoding wild-type GATA-1 and various mutant GATA-1 proteins fused to GST were introduced into a mammalian expression vector (pEBB; kind gift of Bruce Mayer). The resulting constructs were cotransfected into COS cells together with plasmids expressing the ER. Whole cell lysates were incubated with glutathione-agarose beads, washed, and subjected to PAGE followed by immunoblot analysis using anti-ER antibodies. Figure 5a demonstrates that the ER bound to intact GST–GATA-1 but not to GST alone, consistent with the results obtained in the coimmunoprecipitation experiments. In addition, ER bound to GATA-1 lacking the zinc finger region and to the zinc finger region alone (Fig. 5a). Both the N terminus (amino acids 1 to 63) and the C terminus (amino acids 308 to 413) of GATA-1 contain transactivation domains (20). Constructs bearing deletions of these portions of GATA-1 also bound ER protein (Fig. 5a). The Western blot shown in Fig. 5a was stripped of the anti-ER antibodies and reprobed with antibodies directed against GST. Figure 5b shows that comparable amounts of GST fusion proteins were precipitated from all of the cell lysates. As an additional control, unprecipitated whole cell lysates were analyzed in parallel Western blot experiments using ER antibodies. Figure 5c demonstrates that all samples contained comparable amounts of ER protein. Taken together, these results suggest that GATA-1 forms at least two contacts with the ER.

An N-terminal activation domain of GATA-1 is inhibited by ER. The importance of the N- and C-terminal activation domains of GATA-1 for ER-mediated repression was tested in transient transfection experiments in 3T3 cells. While the transcriptional activity of GATA-1 bearing the C-terminal deletion (DX) was less than that of wild type, as previously reported, it could still be repressed to almost basal level by ligand-bound ER (Fig. 6a). Deletion of the N-terminal activation domain (ID5) resulted in a pronounced reduction of transactivation. In contrast to DX, however, inhibition by the ER of the residual activity was disproportionatly reduced (Fig. 6a). These results suggest that the N-terminal but not the C-terminal activation domain is inhibited by the ER. To confirm this in a gain-of-function experiment, the N-terminal 63 amino acids of GATA-1 were fused to the GATA-VP16 construct [1-63f(GATA)-VP16].

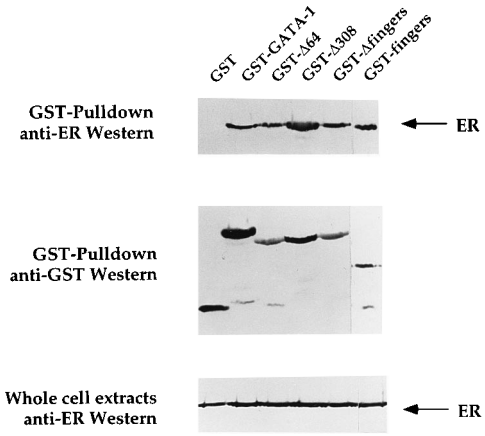

FIG. 5. The ER forms at least two contacts with GATA-1 in vivo. The indicated constructs were transfected into COS cells together with the ER. Following cell lysis, GST fusion proteins were isolated by using glutathione-agarose beads and analyzed by Western blotting using anti-ER antibodies (upper panel). The following constructs were fused to GST: full-length GATA-1, GATA-1 with a deletion of the N-terminal activation domain down to amino acid 64 (Δ64) or with a deletion of the C-terminal activation domain up to amino acid 308 (Δ 308), GATA-1 without the zinc finger region (Δfingers), or the zinc fingers alone. To determine the amounts of GST fusion proteins in each binding reaction, the filter was stripped and reprobed with anti-GST antibodies (middle panel). As further control, equal amounts of cell lysates were directly analyzed by Western blotting using anti-ER antibodies. The lower panel shows the presence of equal amounts of transfected ER in all samples.

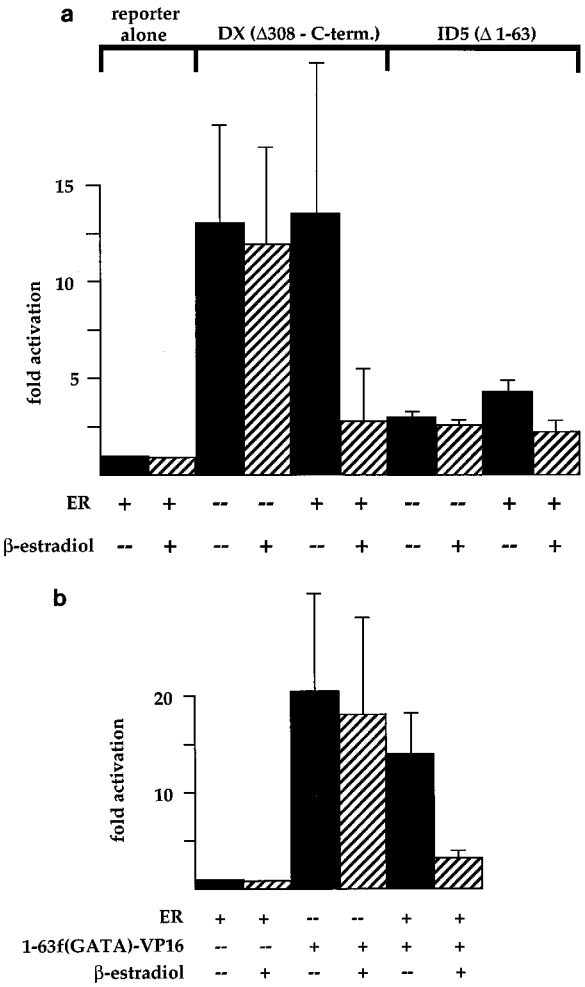

FIG. 6. The N terminus of GATA-1 is required for ER-mediated repression. (a) A GATA-1 construct bearing a deletion of the C-terminal (C-term.) activation domain comprising amino acids 308 to 413 (DX) activates transcription although to a lesser extent than the wild type but is still repressible by the ER. In contrast, a deletion removing the N-terminal activation domain (amino acids 1 to 63; ID5) reduces transactivation and also extent of repression by the ER. (b) Addition of the N-terminal activation domain to f(GATA)-VP16 [1-63f(GATA)-VP16] increases the extent of repression (4.5-fold) by ligand-bound ER.

As shown in Fig. 6b, 1-63f(GATA)-VP16 was inhibited 4.5-fold by ligand-bound ER, demonstrating that the N terminus of GATA-1 participates in the ER-mediated inhibition (see also Fig. 2b). The effects of ER binding to the GATA-1 finger region alone or to fingerless GATA-1 cannot be tested in these cotransfection experiments since these constructs do not activate transcription. We conclude that the formation of two contacts, one provided by the zinc finger region and one provided by the N-terminal activation domain, may therefore be necessary for repression to occur.

DISCUSSION

Homeostasis during erythropoiesis is maintained by growth factors and hormones that modulate proliferation and differentiation. The erythroid transcription factor GATA-1 plays an essential role during erythroid development, and GATA-binding sites are present in the regulatory elements of almost all erythroid cell-specific genes. Previous studies showed that estrogens block the differentiation of primary chicken bone marrow erythroid progenitor cells while promoting their proliferation (32). Loss of differentiation correlated with the repression of many GATA-1-regulated genes (32). Repression of these genes was a direct effect of estrogen action and not merely a consequence of a general block in differentiation, as repression could still occur after maturation was complete (32). Mutational analysis of the ER revealed that specific DNA binding is not required for these effects (4). Therefore, regulation of GATA-1 activity through protein-protein interaction presents an attractive mechanism by which steroid hormones, such as estrogens, could exert broad effects on erythroid differentiation. This report demonstrates that the ER represses GATA-1 activity in a ligand-dependent manner. Furthermore, evidence is presented that GATA-1 and the ER form a complex in vitro and in vivo.

The results presented here show that estrogens reduce the number of mature erythroid colonies derived from primary human $CD34^+$ bone marrow cultures. In contrast to the finding in chickens, no increase in proliferation of immature cells was observed. Consistent with the observation in human cells is the preliminary finding that murine embryonic stem cell-derived progenitor cells give rise to fewer erythroid colonies when seeded in the presence of estrogens (data not shown). Murine erythroid cell lines that overexpress the ER undergo apoptotic cell death following treatment with estradiol (5). As GATA-1-deficient erythroid progenitor cells also undergo apoptotic cell death (45), this finding suggests a link between ER action and loss of GATA-1 function.

In support of such a link, we demonstrate that the ER can strongly repress GATA-1 activity in a ligand-dependent manner. The antiestrogen compound OHT reversed estrogen action, demonstrating that the observed effects are not due to nonspecific toxicity of steroids. OHT binds to the transactivation function 2 (TAF-2) of the ER but, in contrast to the physiological ligand, does not cause its activation (43). The other activation function of the ER (TAF-1), which does not

bind estrogens or OHT, activates transcription in a cell-type- and promoter context-dependent manner (38). Therefore, it has been proposed that OHT acts as an estrogen agonist whenever it increases ER binding to a TAF-1-responsive promoter, while it acts as an estrogen antagonist whenever transcription is solely dependent on TAF-2 (3). OHT reverses the effects of the ER on GATA-1 activity, which suggests that the TAF-2 domain is involved in GATA-1 repression in the context of the M1α reporter contruct.

Further control experiments using other transcription factors such as GATA-VP16 as well as non-GATA-binding proteins including Sp1, GAL4-VP16, and GAL4 fused to the p65 subunit of NFκB (not shown) rule out the possibility that repression is due to an unspecific squelching mechanism.

Steroid hormone receptors can positively or negatively regulate transcription depending on the context of the hormone response elements or on the presence of other transcription factors, such as AP-1, that allow for protein-protein interactions (for a review, see reference 39). Therefore, it was of importance to test whether ER-mediated repression of GATA-1 activity could be observed in the context of an intact GATA-1-responsive erythroid cell-specific promoter. We have shown previously that 111 bp (−77 to +34) of the EKLF gene promoter are sufficient to direct erythroid expression of a linked reporter gene (9). Activity of this promoter in the presence of GATA-1 and ligand-bound ER was significantly reduced, while the rate of transcription in the absence of GATA-1 was not affected. Thus, GATA-1 is a target for the ER in the context of an intact promoter. Repression is also specific for GATA-1 and does not involve other factors bound to the EKLF promoter.

To elucidate the mechanism by which the ER interferes with GATA-1 activity, in vitro and in vivo binding studies were performed. The ER coimmunoprecipitates with GATA-1 from nuclear extracts in a ligand-dependent manner, and purified GST–GATA-1 associates with in vitro translated ER. Hence, the two molecules might make direct contact. GST alone, or an irrelevant protein fused to GST (GST–NF-E2), bound little or no ER protein. The complex is remarkably stable, as some ER was bound to GATA-1 after washing in 1.2 M NaCl. Furthermore, the ER–GATA-1 interaction was not mediated by contaminating DNA, since addition of excess amounts of oligonucleotides containing GATA-binding sites had no effect.

Of particular interest, the ER–GATA-1 association in vitro was ligand independent, which suggests that ligand is required to make the ER available for interaction within the intact cell. This might reflect hormone-induced changes in subcellular localization and/or binding to other proteins, such as Hsp90.

We next performed structure-function analysis in order to delineate the site(s) in GATA-1 required for ER binding. Various GATA-1 constructs were assayed for their responses to ligand-bound ER in transient cotransfection experiments and for their abilities to bind to the ER in vivo. The results show that the ER forms at least two contacts with GATA-1, one involving the GATA-1 zinc finger region and the other involving the N-terminal activation domain. While deletion of the N-terminal 63 amino acids of GATA-1 resulted in reduced repression by the ER, addition of this region to GATA-VP16 conferred ER-mediated repression. In contrast, f(GATA)-VP16 alone showed little or no response to the ER. This finding suggests that a functional interaction requires at least two contacts.

One possible mechanism by which the ER could repress GATA-1 transactivation is through inhibition of GATA-1 binding to DNA. Gel mobility shift experiments, however, indicated that the GATA-1–DNA complex is not affected in the presence of the ER. Higher-migrating complexes consisting of GATA-1 and ER were not detected, suggesting that such a species might be unstable under gel shift conditions.

Another possibility is that formation of a complex between GATA-1 and the ER results in mutual masking of the transactivation domains via protein-protein interaction. Such a mechanism has been proposed for the glucocorticoid receptor-mediated inhibition of AP-1 activity, as in vivo footprinting studies demonstrated that inducible AP-1 binding to DNA was not affected by the presence of ligand-bound glucocorticoid receptor (17). One way by which a transcriptionally inactive complex could be generated is through binding of the ER zinc fingers to the GATA-1 activation domain, and vice versa. We are currently testing this model.

From the transient transfection experiments, it seems that formation of two contacts between GATA-1 and ER is required for repression, since a transcriptionally active construct bearing only the finger region [f(GATA-1)-VP16] is not ER sensitive. In addition, two constructs containing different portions of the N-terminal activation domain of GATA-1 (amino acids 2 to 66 and 2 to 193, respectively [20]) fused to a heterologous DNA-binding domain (GAL4) are also ER insensitive (data not shown).

In the experiments described here, the transcriptional activity of GATA-1 has been assayed in cotransfection experiments in heterologous cells. This activity resides in at least two domains outside the zinc finger region (21). However, two recent studies indicate that the zinc finger region of GATA-1 possesses biological activity in addition to mediating DNA binding when assayed in hematopoietic cells. Specifically, the defect in erythroid development caused by GATA-1 deficiency in embryonic stem cells was partially restored by a construct bearing only the zinc finger region (6). Moreover, forced expression of this domain induced megakaryocytic differentiation in the myeloid cell line 416B (42). Independent studies examining protein-protein interactions demonstrate that GATA-1 can form higher-order complexes on DNA with itself and with other transcription factors, such as Sp1 and EKLF (8, 22). These interactions are mediated by the GATA-1 zinc finger region and do not interfere with DNA binding (8, 22). Inhibition of any of the functions conferred by the zinc fingers could play a role during ER-mediated repression of GATA-1 function in erythroid cells. This cannot be easily tested in transfection experiments, as the GATA-1 zinc finger region alone does not activate transcription in heterologous cells.

Recent studies indicate that the ligand-activated ER also interferes with GATA-2 function in cotransfection experiments (5). It is likely that the ER interacts with the zinc finger region of GATA-2, as this region is highly conserved among the GATA-binding proteins (26). In addition, another contact might be formed with one of its activation domains, since transcriptional repression appears to require additional sites of interaction.

In summary, we have shown that GATA-1 and the ER form a functional ligand-dependent complex resulting in the repression of GATA-1 activity. We speculate that this mechanism may account for some of the biological effects of estrogens on erythroid development. GATA-binding proteins are highly conserved in their DNA-binding domains. Therefore, it is likely that different steroid hormone receptors also exert various effects on cellular functions in other cell types by modulating the activity of other GATA factors dependent on their respective expression patterns.

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REFERENCES

1. **Andrews, N., H. Erdjument-Bromage, M. Davidson, P. Tempst, and S. H. Orkin.** 1993. Erythroid transcription factor NF-E2 is a hematopoietic-specific basic-leucine zipper protein. Nature (London) **362:**722–728.
2. **Andrews, N., and D. Faller.** 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. **87:**2499.
3. **Berry, M., D. Metzger, and P. Chambon.** 1990. Role of the two activating domains of the estrogen receptor in the cell-type and promoter context dependent agonistic activity of the anti-estrogen 4-hydroxytamoxifen. EMBO J. **9:**2811–2818.
4. **Beug, H., E. W. Müllner, and M. J. Hayman.** 1994. Insights into erythroid differentiation obtained from studies on avian erythroblastosis virus. Curr. Opin. Cell Biol. **6:**816–824.
5. **Blobel, G. A., and S. H. Orkin.** Unpublished data.
6. **Blobel, G. A., M. C. Simon, and S. H. Orkin.** 1995. Rescue of GATA-1-deficient embryonic stem cells by heterologous GATA-binding proteins. Mol. Cell. Biol. **15:**626–633.
7. **Chang, T.-J., B. M. Scher, S. Waxman, and W. Scher.** 1993. Inhibition of mouse GATA-1 function by the glucocorticoid receptor: possible mechanism of steroid inhibition of erythroleukemia cell differentiation. Mol. Endocrinol. **7:**528–542.
8. **Crossley, M., M. Merika, and S. H. Orkin.** 1995. Self-association of the erythroid transcription factor GATA-1 mediated by its zinc finger domains. Mol. Cell. Biol. **15:**2448–2456.
9. **Crossley, M., A. Tsang, J. J. Bieker, and S. H. Orkin.** 1994. Regulation of the erythroid Krüppel-like factor (EKLF) gene promoter by the erythroid transcription factor GATA-1. J. Biol. Chem. **269:**15440–15444.
10. **Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. R. Yamamoto.** 1990. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science **249:**1266–1271.
11. **Dukes, P. P., and E. Goldwasser.** 1961. Inhibition of erythropoiesis by estrogens. Endocrinology **69:**21–29.
12. **Evans, T., and G. Felsenfeld.** 1989. The erythroid-specific transcription factor Eryf1: a new finger protein. Cell **58:**877–885.
13. **Fried, W., T. Tichler, I. Dennenberg, J. Barone, and F. Wang.** 1974. Effects of estrogens on hematopoietic stem cells and on hematopoiesis of mice. J. Lab. Clin. Med. **83:**807–815.
14. **Green, S., P. Walter, V. Kumar, A. Krust, J.-M. Bornert, P. Argos, and P. Chambon.** 1986. Human oestrogen receptor cDNA: sequence, expression and homology to v-*erb*-A. Nature (London) **320:**134–139.
15. **Jonat, C., H. J. Rahmsdorf, K.-K. Park, A. C. B. Cato, S. Gebel, H. Ponta, and P. Herrlich.** 1990. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell **62:**1189–1204.
16. **Kaneda, T., T. Murate, M. Sheffery, K. Brown, R. A. Rifkind, and P. A. Marks.** 1985. Gene expression during terminal differentiation: dexamethasone suppression of inducer mediated α_1 and β-maj-globin expression. Proc. Natl. Acad. Sci. USA **74:**5020–5024.
17. **König, H., H. Ponta, H. J. Rahmsdorf, and P. Herrlich.** 1992. Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation in vivo. EMBO J. **11:**2241–2246.
18. **Lo, S.-C., R. Aft, J. Ross, and G. C. Mueller.** 1978. Control of globin gene expression by steroid hormones in differentiating Friend leukemia cells. Cell **15:**447–453.
19. **Lucibello, F. C., E. P. Slater, K. U. Jooss, M. Beato, and R. Müller.** 1990. Mutual transrepression of Fos and the glucocorticoid receptor: involvement of a functional domain in Fos which is absent in FosB. EMBO J. **9:**2827–2834.
20. **Martin, D. I. K., and S. H. Orkin.** 1990. Transcriptional activation and DNA binding by the erythroid factor GF-1/NF-E1/Eryf1. Genes Dev. **4:**1886–1898.
21. **Martin, D. I. K., L. I. Zon, G. Mutter, and S. H. Orkin.** 1990. Expression of an erythroid transcription factor in megakaryocytic and mast cell lineages. Nature (London) **344:**444–447.
22. **Merika, M., and S. H. Orkin.** 1995. Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Krüppel family proteins Sp1 and EKLF. Mol. Cell. Biol. **15:**2437–2447.
23. **Mierendorf, R. C., and G. C. Mueller.** 1979. Possible role of glucocorticoid receptors in globin gene expression in differentiating Friend cells. Mol. Cell. Endocrinol. **13:**301–316.
24. **Miner, J. N., and K. R. Yamamoto.** 1992. The basic region of AP-1 specifies glucocorticoid receptor activity at a composite response element. Genes Dev. **6:**2491–2501.
25. **Mirand, E. A., and A. Gordon.** 1966. Mechanism of estrogen action in erythropoiesis. Endocrinology **78:**325–332.
26. **Orkin, S. H.** 1992. GATA-binding transcription factors in hematopoietic cells. Blood **80:**575–581.
27. **Pearce, D., and K. R. Yamamoto.** 1993. Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response elements. Science **259:**1161–1165.
28. **Pevny, L., M. C. Simon, E. Robertson, W. H. Klein, S.-F. Tsai, V. D'Agati, S. H. Orkin, and F. Costantini.** 1991. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature (London) **349:**257–260.
29. **Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne.** 1988. GAL-4-VP16 is an unusually potent transcriptional activator. Nature (London) **335:**563–564.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Scher, W., D. Tsuei, S. Sassa, P. Price, N. Gabelman, and C. Friend.** 1978. Inhibition of dimethyl sulfoxide-stimulated Friend cell erythrodifferentiation by hydrocortisone and other steroids. Proc. Natl. Acad. Sci. USA **75:**3851–3855.
32. **Schroeder, C., L. Gibson, C. Nordström, and H. Beug.** 1993. The estrogen receptor cooperates with the TGFα receptor (c-*erb*B) in regulation of chicken erythroid progenitor self renewal. EMBO J. **12:**951–960.
33. **Schüle, R., P. Rangarajan, S. Kliewer, L. J. Ransone, J. Bolado, N. Yang, I. M. Verma, and R. M. Evans.** 1990. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell **62:**1217–1226.
34. **Shemshedini, L., R. Knauthe, P. Sassone-Corsi, A. Pornon, and H. Gronemeyer.** 1991. Cell-specific inhibitory and stimulatory effects of Fos and Jun on transcription activation by nuclear receptors. EMBO J. **10:**3839–3849.
35. **Sieff, C. A., S. C. Ekern, D. G. Nathan, and J. W. Anderson.** 1989. Combinations of recombinant colony stimulating factors are required for optimal hematopoietic differentiation in serum-deprived culture. Blood **73:**688–693.
36. **Simon, M. C., L. Pevny, M. V. Wiles, G. Keller, F. Costantini, and S. H. Orkin.** 1992. Rescue of erythroid development in gene targeted GATA-1$^-$ mouse embryonic stem cells. Nat. Genet. **1:**92–98.
37. **Smith, D. B., and K. S. Johnson.** 1988. Single step purification of polypeptides expressed in E. coli as fusions with GST. Gene **67:**31–40.
38. **Tora, L., J. White, C. Brou, D. Tasset, N. Webster, E. Scheer, and P. Chambon.** 1989. The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell **59:**477–487.
39. **Tsai, M.-J., and B. W. O'Malley.** 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. **63:**451–486.
40. **Tsai, S.-F., D. I. K. Martin, L. I. Zon, A. D. D'Andrea, G. G. Wong, and S. H. Orkin.** 1989. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature (London) **339:**446–451.
41. **Tyslowitz, R., and E. Dingemanse.** 1941. Effect of large doses of estrogens on the blood picture of dogs. Endocrinology **29:**817–827.
42. **Visvader, J. E., M. Crossley, J. Hill, and S. H. Orkin.** 1995. The C-terminal zinc finger of GATA-1 or GATA-2 is sufficient to induce differentiation of an early myeloid cell line. Mol. Cell. Biol. **15:**634–641.
43. **Webster, N., S. Green, J. R. Jin, and P. Chambon.** 1988. The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. Cell **54:**199–207.
44. **Weiss, M. J., G. Keller, and S. H. Orkin.** 1994. Novel insights into erythroid development revealed through in vitro differentiation of GATA-1$^-$ embryonic stem cells. Genes Dev. **8:**1184–1197.
45. **Weiss, M. J., and S. Orkin.** Unpublished data.
46. **Yang-Yen, H.-F., J.-C. Chambard, Y.-L. Sun, T. Smeal, T. J. Schmidt, J. Drouin, and M. Karin.** 1990. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell **62:**1205–1215.