Dynamic GATA Factor Interplay at a Multicomponent Regulatory Region of the GATA-2 Locus

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Given the simplicity of the DNA sequence that mediates binding of GATA transcription factors, GATA motifs reside throughout chromosomal DNA. However, chromatin immunoprecipitation analysis has revealed that GATA-1 discriminates exquisitely among these sites. GATA-2 selectively occupies the −2.8-kilobase (kb) region of the GATA-2 locus in the active state despite there being numerous GATA motifs throughout the locus. The GATA-1-mediated displacement of GATA-2 is tightly coupled to repression of GATA-2 transcription. We have used high resolution chromatin immunoprecipitation to show that GATA-1 and GATA-2 occupy two additional regions, −3.9 and −1.8 kb of the GATA-2 locus. GATA-1 and GATA-2 had distinct preferences for occupancy at these regions, with GATA-1 and GATA-2 occupancy highest at the −3.9- and −1.8-kb regions, respectively. Activation of an estrogen receptor fusion to GATA-1 (ER-GATA-1) induced similar kinetics of ER-GATA-1 occupancy and GATA-2 displacement at the sites. In the transcriptionally active state, DNase I hypersensitive sites (HSs) were detected at the −3.9- and −1.8-kb regions, with a weak HS at the −2.8-kb region. Whereas ER-GATA-1-instigated repression abolished the −1.8-kb HS, the −3.9-kb HS persisted in the repressed state. Transient transfection analysis provided evidence that the −3.9-kb region functions distinctly from the −2.8- and −1.8-kb regions. We propose that GATA-2 transcription is regulated via the collective actions of complexes assembled at the −2.8- and −1.8-kb regions, which share similar properties, and through a qualitatively distinct activity of the −3.9-kb complex.

The GATA family of transcription factors, GATA-1–GATA-6, controls hematopoiesis and a multitude of other developmental processes (1, 2). Of the GATA factors that regulate erythropoiesis, GATA-2 is more widely expressed than GATA-1, being expressed in hematopoietic stem cells, certain hematopoietic progenitor cells, mast cells, megakaryocytes, endothelial cells, the central nervous system, fetal liver, fetal heart, and the placenta (3–7). GATA-1 expression is restricted to erythroid, megakaryocytic, and mast cells and the testis (8, 9). GATA-2 knockout mice display an embryonic lethal phenotype with a near ablation of blood cell formation, with lethality occurring earlier than in the GATA-1 knockout (3). This analysis and other studies (7, 10) demonstrated that GATA-2 has crucial functions in the early stages of hematopoiesis in which the hematopoietic stem cell gives rise to lineage-committed progenitors.

GATA-2 expression is down-regulated and GATA-1 expression increases as hematopoietic precursor cells differentiate into erythrocyt cells (4, 7, 11, 12). This reciprocal expression pattern has been detected in multiple systems, including mouse embryos and the GATA-1 knockout cells G1E, which mimic the normal proerythroblast (13). Although major questions remain unanswered regarding the exact consequences of GATA-2 down-regulation, this appears to be important for differentiation, as sustained expression of GATA-2 alters hematopoiesis (14–17).

Analysis of the native nucleoprotein structure of the endogenous GATA-2 locus in G1E cells and in hematopoietic precursor cells lacking the GATA factor coregulator Friend of GATA-1 (FOG-1)1 provided molecular insights into the reciprocal expression of GATA-1 and GATA-2 (18, 19). These studies showed that GATA-2 occupies a conserved upstream region of the GATA-2 locus (−2.8 kb) in the active state. GATA-1-mediated repression of GATA-2 transcription was tightly coupled to GATA-1 occupancy and GATA-2 displacement from the −2.8-kb site. Endogenous GATA-1 displaced endogenous GATA-2 upon reintroduction of FOG-1 into FOG-1-null hematopoietic precursors, indicating that the “GATA switch” does not require GATA factor overexpression. FOG-1 co-localized with GATA-1 and GATA-2 at the −2.8-kb region, and FOG-1 was required for the GATA switch. Another component of the chromatin-associated GATA-2-FOG-1 complex is the histone acetyltransferase CBP (18). CBP occupied the −2.8- and −1.8-kb regions in the active state, and occupancy was reduced upon GATA-1 binding, which correlated with reduced di-acetylated histone H3 and monomethylated histone H4 acetylation at the GATA-2 1S promoter and throughout the GATA-2 gene. The level of histone H3 methylated at lysine 4 at the GATA-2 domain was similar in the active and inactive state. These findings support a bimodal repression mechanism in which positive autoregulation conferred by GATA-2 binding to the −2.8-kb region is abrogated by GATA-1-mediated displacement of GATA-2. Subsequent to this GATA switch, complexes assemble that deacetylate the GATA-2 locus, thereby reducing chromatin accessibility and repressing transcription.

1 The abbreviations used are: FOG-1, Friend of GATA-1; CBP, cAMP-response element-binding protein (CREB-binding protein; ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; HS, hypersensitive site; H3-meK4, histone H3 methylated at lysine 4; MEL, mouse erythroleukemia; ER, estrogen receptor; kb, kilobase(s).
Our previous analysis that revealed highly restricted GATA-1 occupancy of the −2.8-kb region of the GATA-2 locus utilized primer sets that amplified ~100-bp DNA fragments residing −1 kb apart (18). Because the input chromatin fragments averaged ~500 bp, we reasoned that this primer set would density provide sufficient coverage of the locus for a first-generation analysis. Herein, we describe a high resolution analysis with an increased primer set density. This analysis confirmed the results of the previous GATA-2 analysis and work with the β-globin locus (20), revealing considerable discrimination among the numerous GATA motifs in the genome. Most importantly, GATA-1 was shown to occupy an additional region of the GATA-2 locus, the −3.9-kb region, which contains two conserved GATA motifs. In the previous analysis (18), GATA-2 occupancy was only analyzed at the −2.8-kb region and at the 1S and 1G promoters; occupancy was detected solely at the −2.8-kb region. The high resolution analysis detected low GATA-2 occupancy at the −3.9-kb region. GATA-2 strongly occupied the −1.8-kb region, in which GATA-1 occupancy was lower than at the −3.9- and −2.8-kb regions. Combined with DNase I HS and functional analyses, these results considerably extend the previous analysis of the native nucleoprotein structure of the endogenous GATA-2 locus. These results are discussed vis à vis a model of GATA-2 transcriptional regulation involving dynamic GATA factor interplay at multiple upstream conserved regions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—G1E cells were maintained in Iscove’s modified Dulbecco’s medium (Invitrogen) containing 2% antibiotic-antimycotic (Invitrogen), 2 units/ml erythropoietin, 120 nM monothioglycerol (Sigma), 0.6% conditioned medium from a Kit ligand-producing Chinese hamster ovary cell line, and 15% fetal bovine serum (Invitrogen). G1E-ER-GATA-1 cells (18, 19, 21), which stably express GATA-1 as a fusion to the human estrogen receptor ligand binding domain (ER-GATA-1), were maintained as were G1E cells with the addition of 1 μg/ml puromycin. Mouse erythroleukemia (MEL) cells were maintained in Dulbecco’s modified Eagle’s medium (Biofluids) containing 5% FBS, 5% calf serum, and 1% antibiotic-antimycotic (all components were from Invitrogen). MEL cells were incubated for 96 h with 1.5% Me2SO for chromatin immunoprecipitation (ChIP) analysis of endogenous GATA-1 occupancy, which was previously demonstrated to be required for transcriptional activation. NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Biofluids) containing 10% calf serum and 1% antibiotic-antimycotic.

**Antibodies**—Rat monoclonal IgGα, anti-GATA-1 (N-6, sc-265) and rabbit anti-GATA-2 polyclonal (H-116, sc-9008) antibodies were obtained from Santa Cruz Biotechnology, Inc. AffiniPure rabbit anti-rat IgG (H + L) (Jackson ImmunoResearch) was used as the secondary antibody for the GATA-1 ChIP analysis. Rabbit anti-CBP antibody was generated against a carboxyl-terminal fragment of purified bacterially expressed murine CBP (22). Preimmune serum served as controls for these antibodies. For Western blot analysis, the rat monoclonal anti-GATA-1 antibody and goat anti-rat horseradish peroxidase (sc-6006, Santa Cruz Biotechnology, Inc.) were used to detect GATA-1. FOG-1 polyclonal antibody was raised in rabbits (23). Two independently generated FOG-1 rabbit polyclonal antibodies were used for the ChIP analysis and gave similar results.2 The antigen used to inoculate rabbits was a glutathione S-transferase fusion to FOG-1 (amino acids 19–248), which was expressed and purified as described previously (23).

**RNA Isolation and Reverse Transcription (RT)-PCR**—RNA was prepared from the same cultures used for ChIP. Total RNA was purified with TRIzol (Invitrogen). cDNA was prepared by annealing RNA (1 μg) with 250 ng of a 5:1 mixture of random and oligo(dT) primers heated at 68 °C for 10 min. This was followed by incubation with 100 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) combined with 10 mM dithiothreitol (Invitrogen), 20 units of RNasin (Promega), and 0.5 mM dNTPs at 42 °C for 1 h. Reactions were diluted to a final volume of 150 μl and heat-inactivated at 85°C for 5 min. Real-time PCR reactions (15 μl) contained 2 μl of cDNA, 7.5 μl of SYBR Green PCR Master Mix (Applied Biosystems), and the appropriate primers. Product accumulation was monitored by SYBR Green fluorescence. Control reactions lacking reverse transcriptase yielded very low or no signals. Relative expression levels were determined from a standard curve of serial dilutions of G1E-ER-GATA-1 cDNA samples. Forward and reverse primers for real-time reverse transcription-PCR (5′-3′): glyceraldehyde-3-phosphate dehydrogenase, TGCCCCCATGTTTGGTAG and TTGGTGCATGGATCTTC; GATA-2-intron2/exon2 (to detect primary transcripts), AGTTGCTCACATTTCTGGTGTTT and TCTTGGGAAACCGGCGAA; GATA-2-exon3/exon4 (to detect mRNA transcripts), GCAGAAGAAGGCTGCG and CCTGTTGACACTCCTCCGC.

**Quantitative ChIP Assay**—Real-time PCR quantitative ChIP analysis was conducted as described (24, 25). For analysis of CBP and FOI-GATA-1 occupancy, cells were cross-linked with 1% formaldehyde. Chromatin fragments averaged ~500 bp for cells cross-linked with 0.4% formaldehyde, and larger fragments were isolated from the 1% formaldehyde cross-linked cells. Before trypsification for 24 h in medium containing 15% charcoal-stripped FBS. Cells were then grown in 7.5% FBS, 7.5% charcoal-stripped FBS for 24 h and treated with 1 μM tamoxifen (Sigma) for the indicated times. In Fig. 1, cells were grown in medium containing 7.5% FBS, 7.5% charcoal-stripped FBS with or without 1 μM tamoxifen for 10 h. In Fig. 3A, cells were grown in medium containing 7.5% FBS, 7.5% charcoal-stripped FBS for various times. Cells were treated for 12 h with 0, 8, 40, and 200 nM tamoxifen. Immunoprecipitated DNA was analyzed by real-time PCR (ABI Prism 7000, PE Applied Biosystems). Primers were designed by PRIMER EXPRESS 1.0 (PE Applied Biosystems) to amplify 50–150-bp amplicons and were based on GenBank™ accession number AB000927 and sequences in Ensembl (www.ensembl.org/Mus_musculus/geneview?gene=ENS- MUSG00000015053). Samples from three or more immunoprecipitations were analyzed. Product was measured by SYBR green fluorescence in 15-μl reactions with 1 μl of sample. The amount of product was determined relative to a standard curve of input chromatin. Dissociation curves and agarose gel electrophoresis showed that PCR reactions yielded homogeneous products.

**Forward and Reverse Primers for Quantitative ChIP Assay**—All primers were used at 100 μM unless otherwise noted (5′-3′): −4.9 F, AAGAAGGCGGCTCATAACCTCTT −4.9 R, TGGTGGCTCATTGGTACTGT; −4.7 F, CAGCCGATGACTTCTATATCGT −4.7 R, TGTTGAATTCTGACGACTATTGG; −4.2 F, GAATTTCTCTGGCGGCTT −4.2 R, GACGGCTTGGTTTGTGTTG (200 nM); −3.9 F, GAGATGATATACTCGGGTGA (500 nM); −3.9 R, AAGGGCTGATTTTTCGAGCC; −3.6 F, AGGACCCATCTTACCA −3.6 R, TTTTTCGCGGGTGTTAAG −3.3 F, CAGGCGGCTAAGGATTT −3.0 F, GGTAATGCTGCTTGGCTTGGT; −3.0 R, TCCGACCCCTTCCAGAG; −2.8 F, GCCCCGTGTAACCCACCTTTG −2.8 R, TTGTGCCGGGGCGGAAATA −2.6 F, CAGTCGAGGTCCTCACTTTCTG −2.6 R, CGGAGGGAGAGAAGGAGGG; −2.2 F, AGGACCCCTGTGCTTGTCTGGT −2.2 R, CTGCCATGAGGATGATATTGG; −1.8 F, CGCCCTCCTCTTTACCA −1.8 R, CAGGCGGGCTCCTTCCATTAA; −1.4 F, CACCCTGCCCAATGTAATAC −1.4 R, TGCAATATGCTGGTCTGACTTA −1.1 F, CTATTAGATACGACTGCTATCACATT −1.1 R, TGGCCATATGCTACCAGATGATGAC; −0.8 F, AGGCCAGCTTGGGACATTAAC (200 nM); −0.8 R, TGCAACCACCATCTTGGTACGA (200 nM); −0.7 F, CATGCAAGGCTTCTCCGAGGAAG −0.7 R, GCCCTGCGCATCTCTAACTTT; −0.6 F, TCTGGTCTCTGGCTTCTTGC −0.7 F, GCCCTGCCATCTTCTGTGTTTG (500 nM); +0.7 R, CTGGCCGCCTGCGCTTCTTATGG (500 nM); 1G F, AGTACCCAGAAGGGCTGACCT −1G R, GCAGACCCCTGCGACCT.

**Transient Transfection and Luciferase Assays**—For transfection of GI5 cells, DNA (4 μg) was diluted into 50 μl of Opti-MEM1-reduced serum medium per well. DMRIE-C reagent (4 μl) (Invitrogen) was diluted into 500 μl of Opti-MEM1-reduced serum medium per well. The DNA solution was combined with the DMRIE-C solution and incubated at room temperature for 30 min. GI5 and MEL cells were isolated via centrifugation at 240 × g for 8 min at 4 °C. Cells were diluted to 5 × 10^6 per ml with Opti-MEM1-reduced serum medium. One million cells were added per well and incubated for 4.5 h at 37 °C. Iscove’s modified Dulbecco’s medium supplemented with 15% FBS (for GI5 cells) or Dulbecco’s modified Eagle’s medium supplemented with 7.5% FBS and 7.5% calf serum (for MEL cells) was added (2 ml), and cells were incubated for 48 h at 37 °C. Cells were isolated by centrifugation at 240 × g at 4 °C and washed with phosphate-buffered saline. Lysates were analyzed for luciferase activity and protein concentration, and luciferase activity was normalized by the protein concentration of the lysate.

2 J. A. Grass and E. H. Bresnick, unpublished data.
Multicomponent GATA-2 Regulatory Region

Plasmid Construction—Upstream GATA-2 sequences were cloned from murine 129SV Bacterial Artificial Chromosome DNA isolated by Research Genetics/Invitrogen (Huntsville, AL). The integrity of cloned sequences was confirmed by DNA sequence analysis. The 1S promoter region was amplified with the following primers (5′ to 3′): F, TTTTCCGGGTCGAGCCGGCGCTGAG; R, TTTTCCGGGTCGAGCCGGCGCTGAG. The amplified fragment was digested with KpnI and SacI and ligated into 1SLuc. The (−3.9mt)1S Luc construct was generated by replacing the double-GATA motif in the 1S promoter (Fig. 1A) with an XhoI site (GTCGAC). The (−4.9mt)1S Luc construct was generated by replacing the AGATAAGGCTTATCA to ATAATA (GGATAA) and the CTATCT with a BamHI site (GGCTCGAG). The amplified fragment was digested with KpnI and XhoI and ligated into 1SLuc. The (−2.6kb) locus was amplified with the following primers: F, TTTTCCGGGTCGAGCCGGCGCTGAG; R, TTTTCCGGGTCGAGCCGGCGCTGAG. The amplified fragment was digested with KpnI and XhoI and then ligated into 1SLuc. The (−1.8kb) region was amplified with the following primers: F, TTTTCCGGGTCGAGCCGGCGCTGAG; R, TTTTCCGGGTCGAGCCGGCGCTGAG. The amplified fragment was digested with KpnI and XhoI and then ligated into 1SLuc. The (−1.5kb) region was amplified with the following primers: F, TTTTCCGGGTCGAGCCGGCGCTGAG; R, TTTTCCGGGTCGAGCCGGCGCTGAG. The amplified fragment was digested with KpnI and XhoI and then ligated into 1SLuc. The (−0.8kb) region was amplified with the following primers: F, TTTTCCGGGTCGAGCCGGCGCTGAG; R, TTTTCCGGGTCGAGCCGGCGCTGAG. The amplified fragment was digested with KpnI and XhoI and then ligated into 1SLuc. The (−0.25kb) region was amplified with the following primers: F, TTTTCCGGGTCGAGCCGGCGCTGAG; R, TTTTCCGGGTCGAGCCGGCGCTGAG. The amplified fragment was digested with KpnI and XhoI and then ligated into 1SLuc. The (−0.05kb) region was amplified with the following primers: F, TTTTCCGGGTCGAGCCGGCGCTGAG; R, TTTTCCGGGTCGAGCCGGCGCTGAG. The amplified fragment was digested with KpnI and XhoI and then ligated into 1SLuc. The (−0.005kb) region was amplified with the following primers: F, TTTTCCGGGTCGAGCCGGCGCTGAG; R, TTTTCCGGGTCGAGCCGGCGCTGAG. The amplified fragment was digested with KpnI and XhoI and then ligated into 1SLuc.

RESULTS AND DISCUSSION

Distinct Preferences for GATA-1 and GATA-2 Occupancy of Chromosomal Regions within the Endogenous GATA-2 Locus—The GATA-2 1S promoter (Fig. 1A) is active only in certain hematopoietic cell types, whereas the 1G promoter is active in these hematopoietic cells and in diverse cell types (26). To identify further determinants of hematopoietic specificity, we analyzed the sequence conservation of the GATA-2 locus using...
the VISTA program (27). Three regions upstream of the 1S promoter exhibit greater than 75% sequence identity from mouse to man (Fig. 1A). These regions were designated −3.9, −2.8, and −1.8 kb based on their distance upstream of the 1S transcription start site. The −2.8-kb region corresponds to the region occupied by GATA-2 and GATA-1 in the active and inactive states of the locus, respectively (18). Our previous ChIP analysis also detected low level GATA-1 occupancy at the −1.8-kb region; GATA-2 occupancy was not measured at this site nor at the −3.9-kb region (18).

A high resolution quantitative ChIP analysis was conducted to assess GATA-1 occupancy of upstream sequences of the GATA-2 locus in tamoxifen-treated G1E cells that stably express ER-GATA-1 (G1E-ER-GATA-1). GATA-2 occupancy was measured in untreated G1E-ER-GATA-1 cells, which express endogenous GATA-2. PCR amplicons spanned the 11 conserved GATA and WGATAR motifs from −4.7 to +0.7 kb (Fig. 1, A and B) as well as additional sequences containing nonconserved or no GATA motifs. Similar to our previous analysis (18), ER-GATA-1 occupancy was detected at the −2.8-kb amplicon and at the nearby −3.0-kb amplicon. Low level occupancy was detected at the −1.8-kb amplicon (Fig. 1C). High level occupancy was detected at the −3.9-kb amplicon, which contained two conserved WGATAR motifs. Previously, we had assessed occupancy near the −3.9-kb region with a primer set that amplified a sequence −300 bp upstream of the two GATA motifs (at −4.2 kb) (18). Only low enrichments relative to the preimmune control were detected with the −4.2-kb primer set, which highlights the need to design amplicons as close as possible to prospective binding sequences, despite the size of chromatin fragments (−500 bp). Additional weak signals were obtained at certain additional amplicons, but these signals were considerably lower than at the −2.8-kb region (Fig. 1C).

In our previous analysis (18) GATA-2 occupancy was assessed at three regions, the −2.8-kb region and the 1S and 1G promoters. In the active state, GATA-2 occupied the −2.8-kb region but not the 1S and 1G promoters, and ER-GATA-1 rapidly abrogated GATA-2 occupancy. High resolution analysis of GATA-2 occupancy revealed strong enrichments at the −3.0, −2.8, and −1.8-kb amplicons (Fig. 1D). A considerably lower enrichment was detected at the −3.9-kb amplicon, where a high ER-GATA-1 enrichment was detected. No significant enrichments were detected at multiple additional amplicons. Importantly, these results indicate that the patterns of ER-GATA-1 and GATA-2 cross-linking to regions of the GATA-2 locus differ. Furthermore, these results reinforce our previous conclusion (18) that GATA factors discriminate exquisitely among the many GATA motifs of the GATA-2 locus.

The distinct patterns of ER-GATA-1 and GATA-2 cross-linking might result from differential DNA recognition by ER-GATA-1 and GATA-2 or from differential stabilities of the protein-DNA complexes. Although ER-GATA-1 and GATA-1 are seemingly indistinguishable in binding DNA and activating transcription (18, 21, 25, 28, 29), the DNA binding activity of ER-GATA-1 has not been analyzed in detail. Alternatively, the distinct patterns might reflect intrinsic differences in the competence of GATA-1 and GATA-2 to occupy GATA motifs within chromatin. To distinguish between these possibilities, quantitative ChIP analysis was used to measure endogenous GATA-1 occupancy in MEL cells. The greatest enrichments obtained with an anti-GATA-1 antibody, which is absolutely specific for GATA-1 via Western blot analysis of whole cell lysates (Fig. 2A), were detected at the −3.9- and −2.8-kb amplicons (Fig. 2B), similar to the ER-GATA-1-cross-linking pattern in G1E-ER-GATA-1 cells (Fig. 1C). Moreover, analogous to the findings with ER-GATA-1 (Fig. 1C), only a small enrichment was detected at the −1.8-kb amplicon, which was only slightly greater than the preimmune signal. Thus, both endogenous GATA-1 and expressed ER-GATA-1 are preferentially cross-linked to the −3.9- and −2.8-kb amplicons. Endogenous GATA-2 is preferentially cross-linked to the −2.8- and −1.8-kb amplicons. Beyond highlighting the specificity of GATA motif utilization within an endogenous chromatin domain, this analysis revealed an unprecedented difference between GATA-1 and GATA-2 chromatin occupancy patterns in living cells. Such differential occupancy was unexpected, since GATA-1 and GATA-2 bind with similar affinities and specificities to naked DNA in vitro (30, 31) and engage in similar protein-protein interactions (23, 32, 33). Electrophoretic mobility shift analysis revealed that GATA-1 and GATA-2 in MEL and G1E cell nuclear extracts, respectively, formed high affinity complexes with oligonucleotides containing WGATAR motifs of the −1.8- and −2.8-kb regions (data not shown). The electrophoretic mobility shift assay analysis did not provide evidence for distinct DNA binding preferences in vitro.

**Dynamic Changes in the Nucleoprotein Architecture of the Endogenous GATA-2 Locus—ER-GATA-1- and GATA-1-mediated displacement of GATA-2 from the −2.8-kb region in G1E-ER-GATA-1 (18) and FOG-1-rescued FOG-1-null cells (19), respectively, correlated with transcriptional repression. Because the high resolution analysis revealed additional regions of GATA factor occupancy (Fig. 1, C and D), we asked whether**
ER-GATA-1 occupies these regions with similar kinetics upon tamoxifen treatment of G1E-ER-GATA-1 cells. ER-GATA-1 might occupy the regions simultaneously or sequentially. ER-GATA-1 occupancy of the −3.9- and −2.8-kb regions was nearly maximal by 30 min of tamoxifen treatment and was maintained similarly during the 10-h treatment (Fig. 3A). Similar to Fig. 1C, ER-GATA-1 occupancy was low at the −1.8-kb region. Moreover, ER-GATA-1 activity was titrated by culturing G1E-ER-GATA-1 cells with increasing concentrations of tamoxifen for 12 h, and quantitative ChIP analysis was used to measure ER-GATA-1 occupancy and endogenous GATA-2 occupancy at the −3.9-, −2.8-, and −1.8-kb regions (mean ± S.E.; three independent experiments). ○, preimmune; ●, anti-GATA-1 (top graphs) or anti-GATA-2 (bottom graphs).

Because ER-GATA-1 and GATA-1 displace GATA-2 from the −2.8-kb region, we tested whether ER-GATA-1 displaces GATA-2 from the −1.8- and −3.9-kb regions with similar kinetics. Repression might only require ER-GATA-1-mediated displacement of GATA-2 from a single region. In this scenario GATA-2 would remain associated with the other region(s) until GATA-2 transcription is repressed and GATA-2 protein levels decline below a critical level. However, tamoxifen-mediated activation of ER-GATA-1 resulted in nearly identical kinetics for loss of GATA-2 occupancy from the −2.8- and −1.8-kb regions (Fig. 3). Similar to Fig. 1D, GATA-2 occupancy at the −3.9-kb region was low. Thus, ER-GATA-1 occupies the −3.9- and −2.8-kb regions with indistinguishable kinetics, as measured by ChIP, resulting in the rapid displacement of GATA-2 from multiple regions of the locus. The rapid displacement of GATA-2 from all regions supports a model in which GATA-2 occupancy of multiple regions is required for establishment and/or maintenance of GATA-2 transcription.

ChIP analysis provides a snapshot of specific protein components bound at a given chromosomal region. The ChIP results of Figs. 1–3 show that dynamic GATA factor interplay occurs at multiple regions of the GATA-2 locus. Given the high conservation of these regions and correlation between occupancy and transcriptional activity, the GATA factor interplay is likely to control GATA-2 transcription. In the active state of the GATA-2 locus, the −3.9-, −2.8-, and −1.8-kb regions reside within a zone of enriched histone H3 and H4 acetylation as well as H3-methyllysine 18. Histone acetylation counteracts higher order chromatin folding and increases factor access to nucleosomes as H3-methyllysine 18. Histone acetylation co-localizes with acetylated histones as it is enriched in the chromatin of active genes and that are poised to be activated. Thus, it seems reasonable to assume that upstream sequences encompassing the −3.9-, −2.8-, and −1.8-kb regions are accessible to trans-acting factors in the active state. Because repression correlates with reduced domain-wide acetylation, this might broadly decrease chromatin accessibility. However, GATA-1 remains associated with the −3.9- and −2.8-kb regions in the inactive state, suggesting that either local niches of accessibility remain or that GATA-1 has the unique ability to stably associate with inaccessible chromatin. Despite expectations that the access of
factors to heterochromatin should be greatly impaired, occupancy by heterochromatin protein-1 (43, 44), heat shock transcription factor, TATA-binding protein, and RNA polymerase II (45) have been demonstrated.

To assess whether the chromatin accessibility of sequences upstream of the GATA-2 locus is dynamically controlled by GATA factors, DNase I hypersensitive site mapping was conducted in untreated and tamoxifen-treated G1E-ER-GATA-1 cells. GATA-2 transcription was active and repressed in untreated and tamoxifen-treated cells, respectively, based upon measurements of mRNA and primary transcripts (Fig. 4A). Previously, DNase I HSs were detected at \(-2.7\) and \(-0.4\) kb upstream of the 1S exon of the GATA-2 locus in the P815 mouse mastocytoma cancer cell line that expresses GATA-2 (6). Three HSs were detected in untreated G1E-ER-GATA-1 cells at \(-3.7\), \(-2.7\), and \(-1.8\) kb, corresponding closely to the \(-3.9\), \(-2.8\), and \(-1.8\) kb regions. The \(-3.7\)- and \(-1.8\)-kb HSs yielded the strongest signals (Fig. 4B). Extensive efforts to increase the signal of the \(-2.7\)-kb HS with different DNase I concentrations and digestion times were unsuccessful (data not shown). Upon treatment of G1E-ER-GATA-1 cells with tamoxifen for 23 h under conditions in which GATA-2 transcription is fully repressed, the \(-2.7\)- and \(-1.8\)-kb HSs were lost. However, the \(-3.7\)-kb HS remained and was undiminished in intensity. Additional DNase I cleavage sites were detected in the repressed locus with a periodicity of 223 to 344 bp. The HSs were cell-type-specific, as they were not detected in murine 3T3 fibroblast cells (Fig. 4C).

The presence of the \(-3.7\)-kb HS in the active and inactive states suggests that the chromatin structure within this region is highly accessible under both conditions. In contrast, the loss of the \(-2.7\)- and \(-1.8\)-kb HSs suggests that the ER-GATA-1-mediated reconfiguration of nucleoprotein complexes decreases chromatin accessibility at these regions. Intriguingly, ER-GATA-1 occupancy is high at the \(-2.8\)-kb region, in which hypersensitivity is lost, providing further evidence that ER-GATA-1 is competent to stably associate with inaccessible chromatin. Alternatively, other parameters might preclude the detection of hypersensitivity at this region upon ER-GATA-1 binding.

Because FOG-1 co-localizes with both GATA-2 and GATA-1 at certain chromatin sites (19), including the \(-2.8\)-kb region, we asked whether FOG-1 occupies the \(-3.9\) and \(-1.8\)-kb regions in the transcriptionally active and inactive states. FOG-1 occupancy was detected at the \(-3.9\)- and \(-2.8\)-kb regions, with a weaker signal at the \(-1.8\)-kb region and no significant signal at the 1S promoter (Fig. 5A). Under conditions in which ER-GATA-1 abrogated the \(-1.8\)-kb HS and the \(-3.7\)-kb HS persisted, FOG-1 occupancy was unchanged at these regions and at the \(-2.8\)-kb region. The dynamic change in HS formation does not correlate with altered FOG-1 occupancy, and therefore, FOG-1 occupancy is insufficient to maintain hypersensitivity. In contrast, our previous analysis of CBP occupancy (18) revealed reduced CBP occupancy at the \(-2.8\)- and \(-1.8\)-kb regions upon activation of ER-GATA-1 and repression of the GATA-2 locus. CBP occupancy correlates with the presence of the \(-2.7\)- and \(-1.8\)-kb HSs, and CBP occupancy at the \(-3.9\)-kb region was unaffected by ER-GATA-1 activation (Fig. 5B), reinforcing the correlation between hypersensitivity and CBP occupancy.

**Distinct Functional Properties of the Conserved GATA Binding Regions of the GATA-2 Locus**—The transcription of a subset of mammalian genes is controlled by multiple upstream HSs rather than a single primary control element. Multiple HSs can function cooperatively (additively or synergistically) or can be redundant. This issue has been addressed with the \(\beta\)-globin locus, in which erythroid-specific transcription is controlled by
gative for maintenance of DNase I hypersensitivity. A, quantitative ChIP analysis of FOG-1 occupancy at the −3.9-, −2.8-, and −1.8-kb and 1S promoter sites of the GATA-2 locus (mean ± S.E., at least three independent experiments). The dashed line indicates the average background value obtained from analysis of the preimmune immunoprecipitations. B, quantitative ChIP analysis of CBP occupancy at the −3.9-kb region of the GATA-2 locus (mean ± S.E., at least three independent experiments).

Fig. 5. FOG-1 occupancy is insufficient for maintenance of DNase I hypersensitivity. A, quantitative ChIP analysis of FOG-1 occupancy at the −3.9-, −2.8-, and −1.8-kb and 1S promoter sites of the GATA-2 locus (mean ± S.E., at least three independent experiments). The dashed line indicates the average background value obtained from analysis of the preimmune immunoprecipitations. B, quantitative ChIP analysis of CBP occupancy at the −3.9-kb region of the GATA-2 locus (mean ± S.E., at least three independent experiments).

Fig. 6. Cell type-specific activity of the GATA-2 hypersensitive sites in transient transfection assays. G1E and MEL cells were transiently transfected with (−3.9)1SLuc, (−2.8)1SLuc, (−1.8)1SLuc or 1SLuc reporter plasmids in which the GATA motifs within the −3.9-, −2.8-, and −1.8-kb regions were intact or the (−3.9mt)1SLuc, (−2.8mt)1SLuc, or (−1.8mt)1SLuc plasmids, in which the GATA motifs were mutated. The plots depict luciferase activity of the cell lysates normalized by the protein concentration of the lysates (mean ± S.E., four and five independent experiments for G1E and MEL transfections, respectively). In each independent experiment transfections were performed in triplicate. RLU, relative luciferase units.

Multicomponent GATA-2 Regulatory Region

A multi-HS locus control region. Deletion of individual HSs of the endogenous β-globin locus control region yielded incremental decreases in β-globin expression, consistent with a mechanism involving additivity (46, 47). In contrast, deletion of HSs from the intact β-globin locus within a yeast artificial chromosome (48, 49) and transfection experiments (50, 51) reveal synergism between the HSs.

Individual complexes assembled on HSs can function distinctly if the complexes are targeted by distinct cellular signals, they recruit unique ensembles of coregulators, or they uniquely engage in interactions that control the subnuclear localization of the chromosomal region. Alternatively, distinct complexes that engage in similar protein-protein interactions and recruit the same coregulators would be expected to function redundantly, assuming that the local concentrations of coregulators are not limiting.

As a first-generation analysis to analyze the functions of the multiple upstream HSs at the GATA-2 locus, DNA fragments spanning the HSs were subcloned into a luciferase reporter vector and analyzed by transient transfections. Transient transfection analysis does not always recapitulate endogenous transcriptional mechanisms but provides an assessment of the enhancer and silencer potentials of prospective regulatory elements. If the individual HSs of the GATA-2 locus function redundantly, it would not be surprising if they conferred identical activities in the transfection assay. In contrast, if the HSs have intrinsic mechanistic differences, their activities would likely differ. These assumptions are reinforced by results from analysis of the β-globin locus. HS2 has stronger erythroid-specific enhancer activity than the other HSs of the locus control region. However, HS3 is uniquely able to confer position-independent expression in single-copy transgenic mice.

Previously, we showed that the −2.8-kb region of the GATA-2 locus had weak cell type-specific enhancer activity, increasing SV40 promoter activity by 2-fold in G1E cells but not in 3T3 cells (18). Constructs containing the −1.8-, −2.8-, and −3.9-kb sequences linked to the GATA-2 1S promoter were transiently transfected into G1E and MEL cells, which express endogenous GATA-2 and GATA-1, respectively. In G1E cells, the −1.8- and −2.8-kb fragments increased promoter activity by 3–4-fold, and the −3.9-kb fragment had no significant activity (Fig. 6). In MEL cells, the −1.8- and −2.8-kb fragments had no significant activity, whereas the −3.9-kb fragment increased promoter activity by 7-fold. The −1.8- and −2.8-kb fragments conferred identical activities in the two cell lines, either weakly enhancing promoter activity (in G1E cells) or not affecting promoter activity (in MEL cells). The −3.9-kb fragment conferred an activity distinct from that of the −1.8- and −2.8-kb fragments in both cell lines. Mutation of a conserved double GATA motif within the −3.9-kb region abolished the
Because GATA-2 is required for differentiation of hematopoietic stem cells into diverse lineages of blood cells (1), mechanisms that regulate GATA-2 levels are likely to be central determinants of hematopoiesis. Our analysis has implicated a multicomponent upstream regulatory region in the regulation of GATA-2 transcription in G1E cells. It will be particularly important to assess the function of this region via targeted deletions of the individual HSs and combinations thereof in embryonic stem cells followed by functional analyses in vivo and in vitro. Such analyses should prove whether indeed the two classes of regulatory elements function distinctly in vivo and will yield powerful tools for dissecting the underlying mechanisms.

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Multicomponent GATA-2 Regulatory Region
Dynamic GATA Factor Interplay at a Multicomponent Regulatory Region of the GATA-2 Locus

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