GATA-1-mediated Transcriptional Repression Yields Persistent Transcription Factor IIB-Chromatin Complexes*

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The hematopoietic GATA factors GATA-1 and GATA-2, which have distinct and overlapping roles to regulate blood cell development, are reciprocally expressed during erythropoiesis. GATA-1 directly represses Gata2 transcription, and reduced GATA-2 synthesis promotes red blood cell development. Gata2 repression involves “GATA switches” in which GATA-1 displaces GATA-2 from Gata2 regulatory regions. We show that extragenic GATA switch sites occupied by GATA-2 associate with as much RNA polymerase II (Pol II) and basal transcription factors as present at the active Gata2 promoters. Pol II bound to GATA switch sites in the active locus was phosphorylated on serine 5 of the carboxyl-terminal domain, indicative of elongation competence. GATA-1-mediated displacement of GATA-2 from GATA switch sites reduced Pol II recruitment to all sites except the far upstream −77-kb region. Surprisingly, TFIIIB occupancy persisted at most sites upon repression. These results indicate that GATA-2-bound extragenic regulatory elements recruit Pol II, GATA-1 binding expels Pol II, and despite the persistent TFIIIB-chromatin complexes, Pol II recruitment is blocked.

All blood cell types are derived from a common precursor, the hematopoietic stem cell. The process of hematopoiesis requires highly regulated transcriptional mechanisms, as relatively small changes in transcription factor levels can dictate distinct cell fates (1, 2). The requirement for stringent transcriptional regulation of the hematopoietic transcription factor Gata2 appears to be critical, as deregulated GATA-2 expression either blocks or promotes hematopoiesis (3–9).

During early hematopoiesis, GATA-2 is expressed in multipotent hematopoietic precursors that maintain proliferation and/or survival (10, 11). Gata2 null mice are embryonic lethal at embryonic days 10–11 due to anemia (10). GATA-2 is also expressed outside the hematopoietic system in the developing central nervous system, fetal liver, fetal heart, mast cells, megakaryocytes, and endothelial cells and is involved in pituitary and urogenital development (10–15).

GATA-1 is less broadly expressed, being restricted to the erythroid, megakaryocytic, and eosinophil lineages, as well as the Sertoli cells in testis (16–20). GATA-1 is essential for erythropoiesis, megakaryocyte maturation, and eosinophil production (17, 18, 21, 22).

GATA-2 levels decline as GATA-1 levels rise during erythropoiesis (7, 21, 23, 24). GATA-1 represses Gata2 transcription by displacing GATA-2 from sites at −77, −3.9, −2.8, −1.8, and +9.5 kb relative to the hematopoietic-specific 1S Gata2 promoter (25–27). GATA-1-mediated displacement of GATA-2 or a “GATA switch” is tightly coupled to broad histone deacetylation of the locus, transcriptional repression from the two Gata2 promoters (1S and 1G), and loss of GATA-2 protein (25). DNase I hypersensitive sites (HSs)3 have been mapped to the −77, −3.9, −2.8, and −1.8-kb regions of the Gata2 locus. The GATA switch abrogates the strong −1.8-kb HS and reduces the intensity of the weak −2.8 HS (26). The GATA factor coregulator FOG-1 (28) is required for the GATA switch (29) and Gata2 repression (29, 30), but not for Gata2 transcription (29). FOG-1 interacts directly with the nucleosome remodeling and deacetylase chromatin remodeling complex, which is an important mediator of repression (31).

It is instructive to compare the structure/function of GATA factor complexes at the GATA-1-activated β-globin locus (32, 33) and the GATA-1-repressed Gata2 locus. Pol II occupancy at the far upstream β-globin locus control region (LCR) is restricted to the four HSs (33). Intergenic transcripts were detected in the vicinity of the HS-bound Pol II (33, 34), and the transcripts are abrogated by Pol II elongation blockade with 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (33). GATA-1 increases Pol II occupancy at the HSs (32), and GATA-1 is crucial for Pol II recruitment to the βmajor promoter. Based on GATA-1-mediated Pol II recruitment to the β-globin LCR and the finding that GATA-1-mediated repression of Gata2 is associated with reduced Pol II at the 1S and 1G promoters (25), we investigated whether Pol II is recruited and dynamically regulated at Gata2 HSs.

We describe the localization and differential regulation of Pol II at Gata2 HSs. GATA-1-mediated repression abrogated Pol II occupancy at all sites of the locus except the −77-kb region and, to a lesser degree, the 1G promoter. TFIIIB occupancy persisted at most GATA switch sites upon repression, whereas TATA-binding protein (TBP) occupancy paralleled...
that of P-Ser-5 Pol II. These results support a model in which GATA-2-bound extragenic regulatory elements recruit Pol II and GATA-1-mediated displacement of GATA-2 instigates repression via generation of TFIIB-chromatin complexes that fail to support subsequent Pol II recruitment.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—G1E-ER-GATA-1 cells (25, 29, 35), which stably express GATA-1 as a fusion to the human estrogen receptor ligand-binding domain (supplemental Fig. S1), were maintained in Iscove’s modified Dulbecco’s medium (Invitrogen) containing 2% antibiotic-antimycotic (Invitrogen), 2 units/ml puromycin. NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Biofluids) containing 10% calf serum and 15% fetal bovine serum and treated with or without 1 μM tamoxifen. NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Biofluids) containing 10% calf serum and 15% fetal bovine serum and treated with or without 1 μM tamoxifen.

**Antibodies**—Rabbit polyclonal IgG anti-TBP (S-11, sc-273), anti-TFIIB (C-18, sc-225), and anti-Pol II (N-20, sc-899) antibodies were from Santa Cruz Biotechnology., Inc. Mouse monoclonal IgM anti-RNA Polymerase II H14 (MMS-134R), which recognizes the phosphoserine 5 variant of Pol II, was from Covance Research Products. AffiniPure (Jackson Immunoresearch) rabbit anti-mouse IgM, μ chain-specific antibody was used as the secondary antibody for the Phospho-Ser-5 Pol II ChIP analysis. Rabbit preimmune serum and mouse ascites fluid (IgM, λ M2521 from murine myeloma; Sigma) were controls.

**RNA Isolation and Reverse Transcriptase 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 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 RNAasin (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 98 °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 RT-PCR (5′-3′): glyceraldehyde-3-phosphate dehydrogenase, TGC CCCCATGGTTTGATG and TGTGGTCTAGAGCCTTC; GATA-2-exon3/4, GCAAGAGCAAGGCTCGC and CAGTTGACACACTCCC-GGC.

**Quantitative Chromatin Immunoprecipitation (ChIP) Assay**—Real-time PCR quantitative ChIP analysis was conducted as described (36). Cells were cross-linked with 1% formaldehyde. In Figs. 1, 3, 4, and 5, cells were grown in medium containing 15% fetal bovine serum and treated with or without 1 μM tamoxifen for 48 h. In Fig. 2, cells were treated for 16 h with 0, 8, and 200 nM β-estradiol; β-estradiol and tamoxifen activate ER-GATA-1 similarly. 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- to 150-bp amplicons and were based on GenBankTM accession number AB009272 and sequences in Ensembl (www.ensembl.org/Mus_musculus/;geneview?gene=ENSMUSG0000015053). Samples from three or more independent ChIP experiments 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 showed that PCR reactions yielded homogeneous products.

**RESULTS AND DISCUSSION**

**Cell Type-specific Pol II Occupancy at Extragenic HSs of the Active Gata2 Locus Is Abrogated upon Repression**—GATA switches at the −77, −39, −2.8, −1.8, and +9.5-kb regions of the Gata2 locus result in broad histone deacetylation of the locus and transcriptional repression (25–27). As Pol II occupies extragenic regulatory regions of the β-globin locus and GATA-1 increases Pol II occupancy at these sites (32, 33), it is possible that GATA factor-mediated Pol II recruitment to extragenic elements is a fundamental step in GATA factor-mediated transcriptional regulation. Thus, we tested whether Pol II localizes to the Gata2 switch sites of the endogenous Gata2 locus.

High resolution quantitative ChIP analysis was conducted to determine where Pol II resides at the Gata2 locus (Fig. 1A). Treatment of GATA-1-null G1E cells stably expressing condi-
tionally active ER-GATA-1 (G1E-ER-GATA-1) with tamoxifen for 24 and 48 h strongly repressed Gata2 transcription from both 1S and 1G promoters (Fig. 1B). Pol II occupied the promoters, the open reading frame, and the four extragenic GATA switch sites (−77, −3.9, −2.8, and −1.8 kb) at the transcriptionally active Gata2 locus (Fig. 1C). Pol II occupancy overlapped with all previously mapped ER-GATA-1 and GATA-2 occupancy sites (27), except the −12-kb region, in which Pol II but no ER-GATA-1 occupancy was detected. The −12-kb region lacks known regulatory elements. To determine whether GATA switches regulate Pol II occupancy, occupancy was analyzed at the repressed locus 48 h post-tamoxifen treatment. Pol II occupancy was nearly abolished at the proximal GATA switch sites (−3.9, −2.8, and −1.8 kb), the 1S promoter, and the open reading frame. Analysis of Pol II occupancy at the −2.8-kb region and the 1S promoter with a distinct Pol II antibody yielded similar results (supplemental Fig. S2). However, Pol II occupancy persisted at the −77-kb region, and ~50% of the Pol II remained at the 1G promoter (Fig. 1D). The persistent Pol II at the −77-kb region post-ER-GATA-1 occupancy indicates that ER-GATA-1 occupancy is insufficient to displace Pol II from sites bound by ER-GATA-1. Taken together with the GATA-1 activity to recruit Pol II to the β-globin locus, GATA-1 regulates Pol II occupancy in a context-dependent manner. Furthermore, the nearly quantitative loss of Pol II occupancy at the 1S promoter with partial retention of Pol II at the 1G promoter, despite repression of both promoters (Fig. 1B, data not shown), suggests the 1S and 1G promoters are differentially regulated. Both promoters are transcribed in erythroid cells, whereas the 1G promoter has broader activity in certain non-hematopoietic cell types (13, 37).
Extragenic Pol II might localize to the promoters via long-range Pol II transfer (38) or tracking (39) or might generate regulatory transcripts (40). RNA transcripts are detected at β-globin upstream HSs bound by Pol II (33, 34) and at abundant intergenic sites within genomes (41). To determine whether Pol II at Gata2 extragenic sites is transcriptionally competent, we tested whether extragenic Pol II is phosphorylated at Ser-5 of the CTD (RNA Pol II carboxyl-terminal domain). Serine 5 phosphorylation is a key step in the transition of stalled elongating Pol II (42, 43). P-Ser-5 was measured by quantitative ChIP analysis with the H14 antibody (44) (Fig. 1E). The distribution of P-Ser-5 and total Pol II overlapped at all sites of the active locus except the −12-kb region, at which P-Ser-5 was absent. P-Ser-5 persisted at the −77-kb region and partially at the 1G promoter upon repression. As expected, P-Ser-5 was lost from exon 3 within the open reading frame (Fig. 1F). Pol II occupancy at the RPII215 promoter was unaffected by tamoxifen treatment, demonstrating the specificity of reduced Pol II occupancy at the Gata2 locus and indicating that reduced Pol II occupancy is not a consequence of cellular differentiation (Fig. 1G). The persistence of P-Ser-5 at the −77-kb site and partial retention at the 1G promoter suggested that Pol II remained in an elongation-competent form at restricted subregions of the repressed locus. Other examples in which P-Ser-5 occupancy precedes activation include paused Pol II at the 5′-end of the inactive hsp70 gene in Drosophila melanogaster (45) and at the mammalian c-myc locus (46–48). At the inactive peroxisome proliferator-activated receptor γ2 locus, Pol II associated with the basal transcriptional machinery does not initiate transcription until the SWI/SNF complex is recruited (49).

If extragenic Pol II at the active Gata2 locus is functionally important, one would expect Pol II to be absent from the locus in cells that never express Gata2. We tested whether Pol II occupied the Gata2 locus in murine 3T3 fibroblasts, which do not express Gata2 (data not shown). Pol II did not occupy the −77, −3.9, −2.8, and −1.8-kb regions or the promoters (Fig. 1H), whereas Pol II occupied the constitutively active RPII215 promoter in 3T3 cells (Fig. 1H, right panel). Thus, Pol II occupancy at the Gata2 locus is cell-type specific.

A previous analysis of the β-globin locus demonstrated that lower ER-GATA-1 activity is sufficient to occupy GATA motifs at the HSs of the LCR versus the βmajor promoter (50). As ER-GATA-1 occupancy at the LCR initiates factor recruitment and histone modifications, which are followed by ER-GATA-1 occupancy at the promoter and additional molecular events, the differential usage of GATA motifs appears to underlie the multistep activation mechanism. Similarly, Pol II loss at the GATA switch sites might not occur concomitantly, but rather Pol II at certain sites might be preferentially sensitive to ER-GATA-1. We tested whether Pol II at the HSs of ER-GATA-1 occupancy, exhibits greater sensitivity to ER-GATA-1 versus Pol II at the promoters that are not occupied by ER-GATA-1. G1E-ER-GATA-1 cells were treated with 0, 8, or 200 nM β-estradiol for 16 h. The concentration-dependent reductions in Pol II occupancy at the sites were similar, indicating that Pol II occupancy at GATA switch sites and sites lacking GATA factors is equally sensitive to ER-GATA-1 under the experimental conditions (Fig. 2). ER-GATA-1 activation resulted in slightly greater Pol II retention at the 1G promoter versus other sites, consistent with the partial Pol II retention at the repressed 1G promoter following a 48-h treatment with 1 μM tamoxifen (Fig. 1D).

**GATA-1-mediated Repression Yields Persistent TFII-B-Chromatin Complexes**—Although the basal transcription factors TFII-D and TFII-B are crucial for recruiting Pol II to promoters (51–54), the role of these factors in recruiting intergenic Pol II is unknown. We tested whether intergenic Pol II colocalizes with TFII-B and the TFII-D component TBP and, if so, whether Pol II and basal transcription factor occupancy are differentially regulated. Using a highly specific TFII-B antibody (Fig. 3A), quantitative ChIP analysis at the Gata2 locus in untreated G1E-ER-GATA-1 cells revealed TFII-B occupancy at extragenic sites at least as high if not higher than at the 1S and 1G promoters (Fig. 3B). TFII-B occupancy overlapped with P-Ser-5 occupancy and was not detected at numerous other Gata2 sites (Fig. 3B). The low enrichments at extragenic sites away from the promoters resembled the low enrichments at the inactive Necdin promoter (Fig. 3B, center panel), whereas the active RPII215 promoter yielded strong enrichments (Fig. 3B, right panel).

Because Pol II occupancy at multiple regions of the Gata2 locus is abrogated upon repression, we asked whether ER-GATA-1 binding to the chromatin similarly affects TFII-B occupancy. By contrast with Pol II, TFII-B occupancy was largely constant throughout the locus (compare left panels in Fig. 3, B and C) with one exception, the −1.8-kb region in which TFII-B occupancy was abrogated. Following the GATA switch, only low level ER-GATA-1 (and GATA-1) occupancy is detected at the −1.8-kb region, whereas considerably higher occupancy occurs at other GATA switch sites, and the −1.8-kb HS is lost (26). TFII-B occupancy at the Necdin and RPII215 promoters was unaffected by ER-GATA-1 (Fig. 3C, center and right panels). As expected from the lack of Pol II, TFII-B was not...
detected at \( Gata2 \) in 3T3 cells (Fig. 3D) but was detected at the \( RPII215 \) promoter (Fig. 3D, right panel).

The persistence of TFIIB occupancy at sites in which Pol II occupancy declines to an undetectable level raises the question of whether other basal transcription factors remain in the TFIIB-chromatin complex. As the interaction of TBP with TFIIB is important for Pol II recruitment (54), we tested whether TBP colocalized with Pol II and TFIIB. Quantitative ChIP analysis with an anti-TBP antibody validated by Western blotting (Fig. 4A) revealed TBP occupancy at the \(-77\)-kb region, the HSs, and the promoters of the active \( Gata2 \) locus in G1E-ER-GATA-1 cells (Fig. 4B, left panel). No TBP occupancy was detected at the inactive \( Necdin \) promoter (Fig. 4B, right panel). TBP occupancy persisted at the \(-77\)-kb region upon repression, remained \(-2\)-fold greater than the control antibody at the 1G promoter, and was abrogated at other sites (Fig. 4C, left panel). The P-Ser-5 and TBP distributions within the active and repressed locus overlapped, both before and after repression. By contrast to ER-GATA-1-mediated \( Gata2 \) repression, ER-GATA-1-mediated \( \beta\)-globin locus activation involved dynamic changes in TFIIB occupancy. ER-GATA-1 strongly induced TFIIB (Fig. 4D), TBP (Fig. 4E), and Pol II (Fig. 4F) occupancy at the \( \beta\) major promoter. Thus, despite persistent TFIIB occupancy upon \( Gata2 \) repression and the established TFIIB activity to interact directly with Pol II (52), apparently TFIIB lost the capacity to efficiently recruit Pol II following ER-GATA-1 occupancy. Although we are unaware of a scenario in which TFIIB complexes exist on chromatin in the absence of TBP, potential explanations for this result include: the failure to cross-link TBP to the repressed chromatin; direct TFIIB-DNA interactions, because TFIIB has sequence-specific DNA binding activity (55, 56); or TFIIB tethering to the chromatin via protein-protein interactions.
GATA-1 Antagonism of Pol II Recruitment with TFIIB Retention, a Common Repression Mechanism—We tested whether GATA-1-mediated abrogation of Pol II recruitment with TFIIB retention occurs at additional GATA-1-repressed loci. GATA-1 represses c-Myb and c-Kit genes via direct interactions with promoter and intronic regulatory elements, respectively (57, 58). Quantitative ChIP analysis revealed that ER-GATA-1-mediated repression of c-Myb and c-Kit involves strongly reduced Pol II occupancy (Fig. 5 A) at their promoters with little to no change in TFIIB occupancy (Fig. 5 B). Thus, reduced Pol II recruitment with persistent TFIIB-chromatin interactions at extragenic sites and promoters is a hallmark of repression at three GATA-1-repressed genes.

The work described herein demonstrates Pol II and basal transcription factor complexes at extragenic GATA switch sites and Gata2 promoters, which are dynamically regulated via GATA switches. Several lines of evidence suggest that these complexes are functionally important. First, Pol II, TFIIB, and TBP at the extragenic sites are at least as high as that present at the 1S and 1G promoters. Second, the complexes were only detected in erythroid cells. Third, the complexes are dynamically regulated in response to ER-GATA-1 chromatin occupancy. ER-GATA-1-mediated repression remodels the complexes such that Pol II and TBP are no longer detected, whereas TFIIB persists at most sites (Fig. 6). Complexes at certain sites are differentially regulated, e.g. TFIIB occupancy is selectively lost from the −1.8-kb region upon repression and complexes at the −77-kb region are insensitive to ER-GATA-1. Lastly, Pol II resides at the −77-kb site independent of Pol II occupancy at the 1S promoter of the repressed locus, strongly suggesting that
the structure and regulation of spatially distinct complexes at functional transcripts. The results described herein, defining Pol II transfer to the promoters or the generation of Pol II occupancy at the associated promoters (32, 33). Collectively, these results strongly suggest that Pol II has important functions at Gata2 extragenic sites. Such functions might include Pol II transfer to the promoters or the generation of functional transcripts. The results described herein, defining the structure and regulation of spatially distinct complexes at the endogenous locus, provide a strong foundation for further dissecting the underlying mechanisms.

Regardless of the function of the extragenic Pol II, our mechanistic analysis revealed Pol II occupancy of the full ensemble of sites only at the active locus, whereas TFIIIB-chromatin interactions persist at all but the −1.8-kb region upon repression. In this regard, basal transcription factors can occupy heterochromatin in S. cerevisiae (59) and D. melanogaster (60) and can occupy repressed loci in mammalian mitotic chromatin (61). It is attractive to propose that once GATA-1 binding expels Pol II from the Gata2 locus, the persistent TFIIIB complex retains an important function. It seems unlikely that TFIIIB persistence reflects that of a poised transcriptionally competent promoter, as once GATA-1 represses Gata2 no evidence exists to suggest that Gata2 is subsequently reactivated. Of considerable interest is why the TFIIIB complex persists at a locus that is not destined for reactivation and how the persistent complex lacks the capacity to efficiently recruit Pol II. We anticipate that the answers to these questions will have broad implications for understanding the dynamic disassembly of transcriptional complexes during development.

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