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Molecular Hallmarks of Endogenous Chromatin Complexes Containing Master Regulators of Hematopoiesis▽†

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Combinatorial interactions among *trans*-acting factors establish transcriptional circuits that orchestrate cellular differentiation, survival, and development. Unlike circuits instigated by individual factors, efforts to identify gene ensembles controlled by multiple factors simultaneously are in their infancy. A paradigm has emerged in which the important regulators of hematopoiesis GATA-1 and GATA-2 function combinatorially with Scl/TAL1, another key regulator of hematopoiesis. The underlying mechanism appears to involve preferential assembly of a multimeric complex on a composite DNA element containing WGATAR and E-box motifs. Based on this paradigm, one would predict that GATA-2 and Scl/TAL1 would commonly co-occupy such composite elements in cells. However, chromosome-wide analyses indicated that the vast majority of conserved composite elements were occupied by neither GATA-2 nor Scl/TAL1. Intriguingly, the highly restricted set of GATA-2-occupied composite elements had characteristic molecular hallmarks, specifically Scl/TAL1 occupancy, a specific epigenetic signature, specific neighboring *cis* elements, and preferential enhancer activity in GATA-2-expressing cells. Genes near the GATA-2–Scl/TAL1-occupied composite elements were regulated by GATA-2 or GATA-1, and therefore these fundamental studies on combinatorial transcriptional mechanisms were also leveraged to discover novel GATA factor-mediated cell regulatory pathways.

Combinatorial interactions among *trans*-acting factors establish transcriptional circuits that control fundamental biological processes. In the context of metazoans, these interactions often occur at regulatory elements far from genes and within introns. Many genes require a complex collection of *trans*-acting factors, coregulator complexes, and long-range regulation, and therefore considerable challenges exist in forging general principles to explain combinatorial transcriptional control. We investigated combinatorial transcriptional mechanisms in the context of GATA factors, which interact with an assortment of regulatory factors to control differentiation, survival, and development (12, 42).

GATA-1 and GATA-2 have unique and essential roles to control hematopoiesis. GATA-2 is required for maintenance and expansion of hematopoietic stem cells (HSCs) (78, 79), while GATA-1 promotes the development of erythrocytes (20, 62, 63, 72), megakaryocytes (70), eosinophils (92), and mast cells (54). GATA-2 is also expressed in endothelial cells (17, 48, 56), and conditional GATA-2 expression in embryonic stem (ES) cells increases the genesis of hemangioblasts, precursors to hematopoietic and endothelial cells (50). GATA-2 deregulation is associated with early-onset coronary artery disease (15), atherosclerosis (69), and chronic myelogenous leukemia (94), whereas GATA-1 mutations cause megakaryoblastic leukemia (85) and additional blood disorders (16, 58).

Both GATA-1 and GATA-2 bind an identical DNA motif (WGATAR) (45, 52), but the majority of these motifs are unoccupied in cells (8, 26, 27, 34, 36, 51). Despite this shared binding specificity, GATA-1 and GATA-2 can exert distinct biological activities (21), indicating that each factor has certain unique targets and/or they differentially regulate common genes. GATA-1 and GATA-2 can occupy identical chromatin sites and induce opposite transcriptional outputs (9). However, they function redundantly to promote primitive erythroblast development (21). The modes by which GATA factors select target sites and mechanisms underlying their context-dependent functions are unresolved.

Context-dependent GATA-1 activity involves the capacity of GATA-1 to utilize diverse coregulators (5, 32, 68, 80) and the differential sensitivity of target loci to GATA factor levels (38). Combinatorial actions of GATA factors with other *trans*-acting factors are also important (42). A paradigm has emerged in which GATA-1 functions cooperatively with the E-box binding proteins Scl/TAL1 and E2A as well as LMO2 and LDB1 on WGATAR- and E-box (CANNTG)-containing composite elements in erythroid cells (47, 74, 83, 84, 89). In the context of naked DNA, these factors form a multimeric complex that preferentially recognizes such composite elements. Scl/TAL1 is expressed in GATA-1- and GATA-2-expressing hematopoietic cells (23, 24, 28), is induced by GATA-2 (13, 50), and is required for development of all hematopoietic cell types (66, 71), hematopoietic commitment of hemangioblasts (50), vasculogenesis (77), and angiogenesis (82).

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Relative to GATA-1, considerably less is known about mechanisms underlying GATA-2 function. Only a few direct GATA-2 target genes are known, including genes encoding Scl/TAL1 (13, 50), GATA-2 itself (27, 46), and BMP4 (bone morphogenetic protein 4) (50). Although transcriptional elements uniquely controlled by GATA-2, but not other GATA factors, are unknown, an E-box–WGATAR composite element residing within a *Gata2* intron (9.5 kb downstream of the transcription start site; hereafter referred to as the +9.5 kb site) confers strong enhancer activity in GATA-2-expressing cells in vitro and in the vasculature and fetal liver of mouse embryos (41, 88). The enhancer activity requires both WGATAR and E-box motifs (41, 88). Taken together with the paradigm that emerged from the finding that GATA-1 and Scl/TAL1 preferentially assemble a multimeric complex on composite elements in the context of naked DNA (84), one might predict that GATA-2 and Scl/TAL1 commonly co-occupy and function through such composite elements in vivo. However, chromosome-wide analyses revealed that the vast majority of conserved composite elements are not occupied by these factors. Mechanistic studies revealed specific molecular hallmarks that distinguished these unoccupied elements from a highly restricted subset of occupied composite elements. Furthermore, the occupied sites pinpointed novel GATA factor target genes that highlight new GATA factor-dependent cell regulatory pathways.

MATERIALS AND METHODS

Cell culture. GATA-1-null G1E cells resemble normal proerythroblasts, express endogenous GATA-2, and represent a powerful system for dissecting GATA factor mechanisms (26, 27, 29, 86, 87). G1E-ER–GATA-1 cells express an estrogen receptor ligand binding domain fusion to GATA-1, which interacts with chromatin similarly to endogenous GATA-1 (37). G1E-ER–GATA-1 cells were cultured in Iscove's modified Dulbecco's medium (GIBCO) containing 15% fetal bovine serum (Gemini Bio-Products), 1% penicillin-streptomycin (GIBCO), 2 U/ml erythropoietin, 120 nM monothioglycerol (MTG [Sigma]), and 0.6% conditioned medium from a kit ligand-producing CHO cell line. Puromycin (1 mg/ml) was included in the medium for growth of G1E cells stably expressing ER–GATA-1 (29, 37). GATA-1 was induced in G1E-ER–GATA-1 cells with 1 μM β-estradiol. Human umbilical vein endothelial cells (HUVECs [Cascade Biologics]) were maintained in medium 200 (Cascade Biologics) containing 1% penicillin–streptomycin (GIBCO) and low serum growth supplement (Cascade Biologics). Mouse erythroleukemia (MEL) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc.) containing 5% fetal bovine serum (Gemini Bio-Products), 5% bovine serum (GIBCO), and 1% antibiotic-antimycotic (GIBCO).

Inducible GATA-2 (iGATA-2) ES cells (50) were maintained on PMEF cells (Specialty Media-Chemicon, NJ) in DMEM with 15% preselected fetal calf serum (FCS), 2% leukemia inhibitory factor (LIF), 1% L-glutamine, 1% nonessential amino acids, and 4.5×10^{-4} M MTG. Differentiation of ES cells into embryoid bodies (EBs) has been described previously (61). Cells were differentiated in Iscove's modified Dulbecco's medium containing 15% differentiation-screened FCS, 1% L-glutamine, 50 μg/ml ascorbic acid, and 4.5×10^{-4} M MTG for the indicated number of days. Serum-free conditions substituted Knockout SR (GIBCO) for FCS and contained 5% PFHM II (GIBCO). GATA-2 was induced in iGATA2 cells with 0.3 μg/ml doxycycline (Dox) on day 2 of the culture to generate EBs.

Plasmid constructs. GATA-2 sequences were cloned from a murine 129SV bacterial artificial chromosome DNA isolated by Research Genetics/Invitrogen. Primers used to amplify genomic regions of *Gata2* for the creation of the constructs used herein are available upon request. The integrity of cloned sequences was confirmed by DNA sequence analysis. The pGL3basic luciferase reporter plasmid was obtained from Promega. For *LacZ* reporter constructs, sequences identical to the respective transient construct were cloned into the pSVβ vector (Clontech).

Transgenic mice. Transgenic mice harboring the (−77)1SLacZ reporter construct were generated by standard procedures by the University of Wisconsin Transgenic Animal Facility. DNA constructs for F_0 transgenic analysis were linearized, purified with an Elutip-d column (Schleicher & Schuell), and microinjected into fertilized mouse oocytes. To identify embryos containing LacZ transgenes, yolk sac genomic DNA was analyzed by PCR with LacZ-specific primers. For whole-mount analysis, 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-Gal [Sigma]) staining was performed with embryonic day 11.5 (E11.5) embryos as described previously (59, 88). Embryos were fixed with 2% formaldehyde, 0.2% glutaraldehyde, and 0.02% Nonidet P-40 (Sigma) in phosphate-buffered saline (PBS) for 2 h at 4°C. Embryos were washed twice with PBS and incubated overnight at 37°C in 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 0.5 mg/ml X-Gal in PBS. Embryos were washed twice with PBS and postfixed with 4% formaldehyde overnight at 4°C. For tissue sections, the postfixed embryos were dehydrated through progressive washes in 50, 70, 85, 95, and 100% ethanol. Paraffin-embedded embryos were dried overnight at room temperature, and the sectioned embryos (10 μm) were counterstained with 0.1% nuclear fast red staining solution in 5% aluminum sulfate.

Transient transfection assay. G1E and MEL cell transfections were conducted as described previously (27). HUVECs were plated 1 day prior to transfection and were ~60 to 70% confluent at the time of transfection. An equal amount of each plasmid (2 μg) was added to 100 μl of Opti-MEM (Invitrogen) reduced serum medium, incubated with Lipofectin reagent (6 μl/1 μg of DNA [Invitrogen]) for 15 min at room temperature, and then added to the cells. Cells were incubated with the transfection mixture for 3 h before the readdition of medium 200. Cell lysates were harvested 48 h posttransfection and assayed for luciferase activity using the Promega luciferase assay system. The luciferase activity of each sample was normalized to the protein concentration of the lysate, as determined by a Bradford assay using gamma globulin as a standard. At least two independent preparations of each plasmid were analyzed.

Quantitative ChIP assay. Quantitative chromatin immunoprecipitation (ChIP) analysis was performed as described previously (33). Samples were cross-linked with 1% formaldehyde. Anti-GATA-2 or anti-Scl/TAL1 rabbit polyclonal antibodies were used with protein A-Sepharose (Sigma) to adsorb immune-specific complexes (27). Preimmune serum was used as a control. Samples were analyzed by real-time PCR (ABI Prism 7000) using primers designed by PrimerExpress1.0 software (PE Applied Biosystems) to amplify regions of 50 to 150 bp that overlap with the appropriate motif. Product was measured by Sybr green fluorescence in 20-μl reaction mixtures, and the amount of product was determined relative to a standard curve generated from a titration of input chromatin. Analysis of dissociation curves postamplification showed that primer pairs generated single products.

RNA isolation and quantitative RT-PCR. Total RNA was purified with TRIzol (GIBCO/BRL). cDNA was prepared from 1.5 μg of purified total RNA. Reverse transcription-PCR (RT-PCR) mixtures (20 μl) contained 2 μl of cDNA solution with the appropriate primers. Product was measured by Sybr green fluorescence. mRNA levels were normalized to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) within the same sample, and changes (fold) in expression after iGATA-2 or ER–GATA-1 induction were quantitated by the ΔΔ threshold cycle ($\Delta\Delta C_T$) method.

Primers and antibodies. Anti-GATA-2 rabbit polyclonal antibody was generated against a purified glutathione *S*-transferase (GST) fusion to amino acids 120 to 235 of mouse GATA-2 (27). The Scl/TAL1 antibody was described previously (25). Antibodies recognizing diacetyl histone H3 (acH3), tetraacetyl histone H4 (acH4), histone H3 dimethyl lysine 4 (H3-dimeK4), histone H3 dimethyl lysine 36 (H3-dimeK36), and histone H3 trimethylated at lysine 9 were purchased from Upstate. The primers used in this article are available upon request.

RESULTS

Stringent molecular constraints for GATA-2-mediated combinatorial transcriptional control. The far upstream GATA-1- and GATA-2-binding region of the *Gata2* locus (−77 kb) (27) resembles the +9.5 kb site in containing a conserved WGATAR and neighboring E-boxes (Fig. 1A). We tested whether the −77 and +9.5 kb sites function similarly in vivo using a *LacZ* vector identical to that used in our analysis of the +9.5 kb element (88). The +9.5 kb vector contained a minimal *Gata2* promoter fused to *LacZ* and was active in vascular endothelium, endocardium, and the fetal liver (88). Despite the common E-box and WGATAR motifs in both the +9.5 kb and the −77 kb elements, the −77 kb site failed to activate the *Gata2* promoter-*LacZ* transgene in 12 out of 12 E11.5 F_0 transgenic embryos (Fig. 1B). In addition to its enhancer function in mouse embryos, the +9.5 kb site activates a

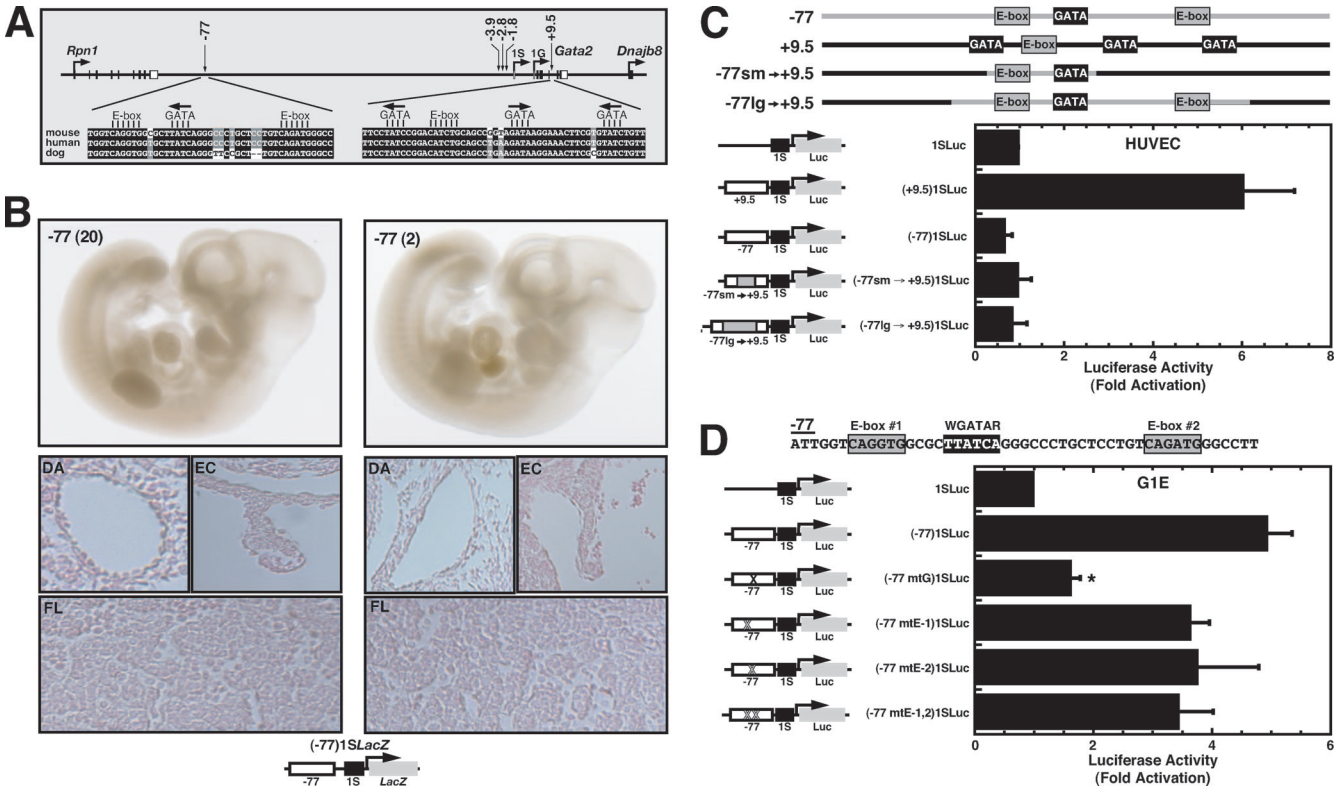


FIG. 1. A conserved WGATAR motif with nearby E-boxes is insufficient for autonomous enhancer activity in chromatin. (A) *Gata2* locus organization. Open and filled boxes depict noncoding and coding exons, respectively. The sequence conservation of the E-box- and GATA motif-containing core modules of the −77 and +9.5 kb sites is shown below. Arrows above the GATA motifs identify the orientation of each motif with respect to forward and reverse strands. (B) Representative photographs of whole-mount and transverse sections of two (left and right columns) E11.5 embryos harboring a transgene containing the *Gata2* −77 kb site upstream of the *Gata2* 1S promoter fused to *LacZ* [(−77)1SLacZ]. For embryos containing (−77)1SLacZ, histological sections show complete lack of endothelial staining in the dorsal aorta (DA) and endocardium (EC) and also in the fetal liver (FL). −77(20) and −77(2) are two representative transgene-positive embryos. Note that the transgene lacks activity in these and 10 additional embryos tested. (C) Analysis of core module activities via generation of chimeric regulatory elements. HUVECs were transiently transfected with reporters derived from pGL3luc containing the *Gata2* 1S promoter cloned upstream of luciferase (1SLuc). The plot depicts the average luciferase activities of the cell lysates normalized by protein concentrations (at least three independent experiments). In each experiment, transfections were performed in triplicate. (D) G1E cells were transiently transfected with reporters derived from pGL3luc containing the *Gata2* 1S promoter cloned upstream of luciferase (1SLuc). The plot depicts the average luciferase activities of cell lysates normalized by the protein concentrations (at least three independent experiments). In each experiment, transfections were performed in triplicate. *, $P < 0.05$ with respect to (−77)1SLuc.

Gata2 promoter-luciferase reporter in GATA-2-expressing endothelial (HUVEC) and hematopoietic (G1E) cells, which requires WGATAR and E-box motifs (88). Since the −77 kb site can activate the *Gata2* promoter reporter in G1E cells, we tested whether it also functions in HUVECs. In contrast to the +9.5 kb site, the −77 kb site lacked activity (Fig. 1C).

The +9.5 kb site enhancer activity in endothelial cells requires an E-box–WGATAR-containing core module and regulatory modules containing additional *cis* elements (88), whereas the core module suffices for activity in GATA-2-expressing hematopoietic cells. The functional difference between +9.5 and −77 kb sites in HUVECs might therefore arise from the lack of −77 kb regulatory modules or differences in their core modules. To distinguish between these possibilities, chimeric elements were generated in which the WGATAR- and E-box-containing −77 kb core was substituted for the +9.5 kb core. The −77 kb core was incapable of reconstituting activity of the core-deleted +9.5 kb site, and a larger −77 kb core fragment containing the WGATAR motif and two flanking E-boxes also did not reconstitute activity (Fig. 1C). Thus, the −77 kb core differs from the +9.5 kb core, which confers GATA-2-dependent activation in endothelial cells.

Since the +9.5 and −77 kb cores are functionally distinct, presumably their *cis*-element compositions or configurations differ. The +9.5 kb core critically requires WGATAR and E-box motifs for activity in HUVEC and G1E cells (88). Although the −77 kb site lacks enhancer activity in HUVECs (Fig. 1C), it is active in G1E cells, and we tested whether this activity is WGATAR and E-box dependent. While mutation of WGATAR abrogated activity, mutation of the two E-boxes individually or collectively only slightly reduced activity (Fig. 1D). The −77 kb site activity therefore requires WGATAR, but the conserved E-boxes are largely unimportant in G1E cells. These results illustrate how WGATAR motifs suffice to mediate GATA factor function in certain contexts, while requiring additional *cis*-elements in other contexts.

Although both the +9.5 and −77 kb cores contain WGATAR and E-box motifs, they differ in their cell-type-specific enhancer

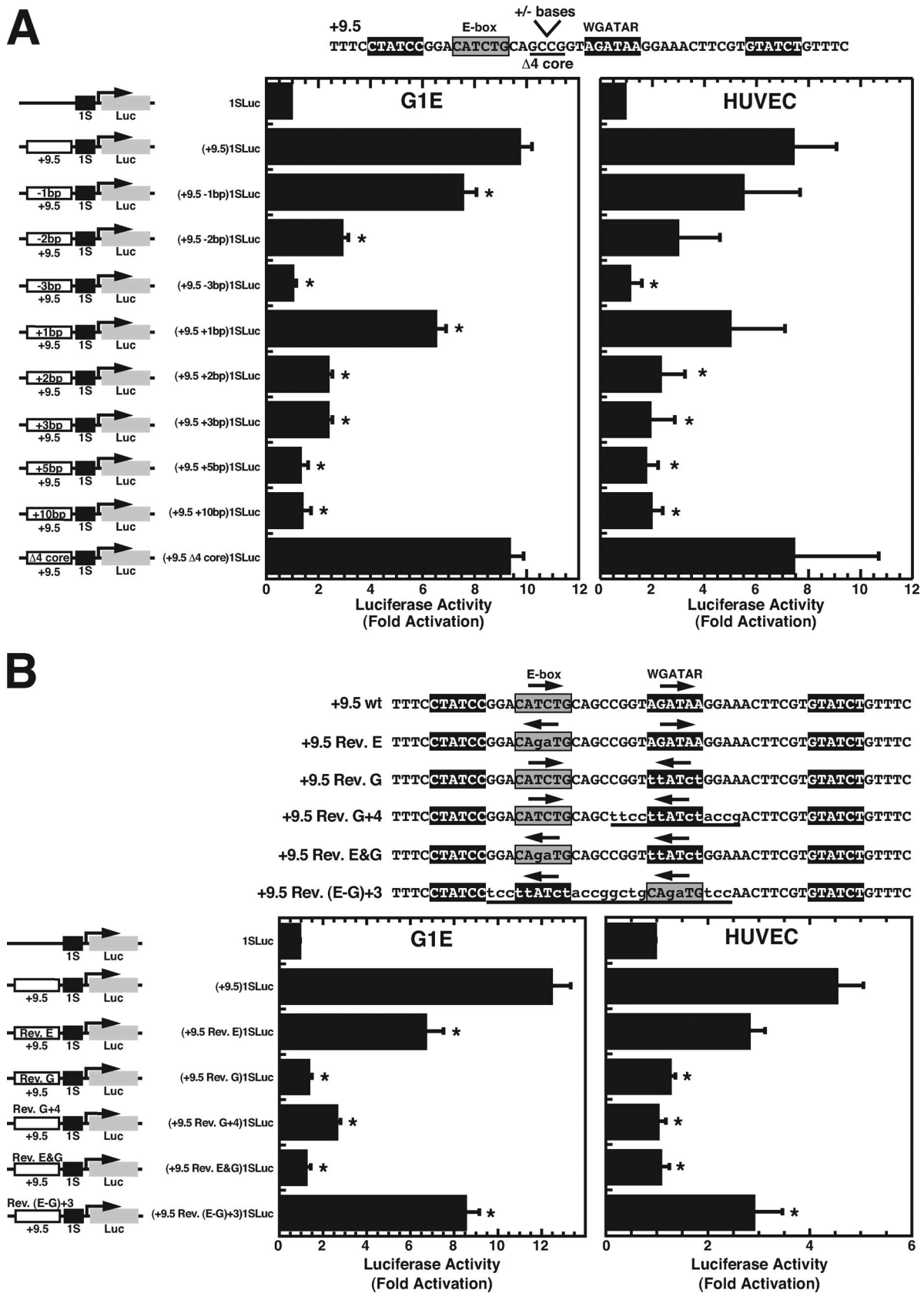


FIG. 2. Strict architectural constraints for GATA factor-mediated combinatorial transcriptional control. (A) *cis*-element spacing requirements. Mutant plasmids were generated in which nucleotides between the E-box and WGATAR motifs were either deleted (−1, −2, and −3) or added

activities and E-box utilization. Their WGATAR motifs have distinct orientations and spacing relative to neighboring E-boxes. To test whether spacing constraints exist for +9.5 kb activity in G1E cells and HUVECs, 1, 2, or 3 bp were deleted, and 1, 2, 3, 5, or 10 bp were inserted between the WGATAR motif and E-box. Whereas 1-bp deletions or insertions were tolerated with only 22 to 33% decreases in enhancer activity (Fig. 2A), deletions or insertions of ≥2 bp severely reduced activity. Scrambling 4 bp of intervening sequence (Δ4 core) did not affect activity, indicating that the specific intervening sequences are not essential. If the deletions or insertions inhibit activity by altering the helical orientation of the WGATAR and E-box with respect to each other, a 10-bp insertion that maintains the configuration should be inconsequential. However, the 10-bp insertion mutant lacked activity, consistent with a critical spacing constraint rather than a precise helical geometry.

To determine if the linear orientation of WGATAR relative to the E-box is important, the WGATAR and E-box orientations were reversed (Fig. 2B). While reversing the orientation of the E-box reduced enhancer activity by 38 and 46% in HUVEC and G1E cells, respectively, reversing the orientation of WGATAR, with or without 3 bp of flanking sequence, abrogated activity. Reversing the orientation of both WGATAR and the E-box was also devastating, whereas reversing the orientation of the full core and 3 bp of flanking sequence, which maintains the WGATAR orientation relative to the E-box, only slightly reduced activity. These results provide evidence for a stringent orientation constraint in which CANNTG resides upstream of WGATAR on the same strand of DNA. It is instructive to compare these findings to a site selection analysis with erythroleukemia cell extracts and randomized 26-bp oligonucleotides (84). This analysis used LMO2, Scl/TAL1, and E2A antibodies to select bound oligonucleotides containing an E-box 8 to 10 bp upstream of a GATA motif. Although the importance of spacing, helical geometry, and motif orientation on naked DNA binding, enhancer activity, and chromatin occupancy were not evaluated, our results on constraints for enhancer activity mirror those obtained from the site selection analysis. Thus, combinatorial regulation of enhancer activity with nonchromosomal templates might reflect GATA-2–E-protein nucleoprotein complex assembly, analogous to the proposed mechanism for GATA-1 (84).

Chromosome-wide analysis of GATA-2 occupancy at conserved composite elements: the vast majority of composite elements are unoccupied. Endogenous GATA-1 and an estrogen receptor ligand binding domain fusion to GATA-1 (ER–GATA-1) occupy a small percentage of WGATAR motifs in chromatin (8). Our studies at multiple loci revealed GATA-1 occupancy at a small subset (<10%) of conserved WGATAR motifs (8). GATA-1 and GATA-2 share many chromatin sites, but differences can exist (51). Whereas FOG-1 increases GATA-1 occupancy at certain sites (49, 60), other parameters governing occupancy are undefined. Whether positioning an E-box near a WGATAR motif influences the probability of GATA factor occupancy is unknown.

As E-box–WGATAR composite element function in GATA-2-expressing cells requires a precise geometry (Fig. 1 and 2), this geometry might facilitate GATA-2 chromatin occupancy or enhance GATA-2 function postoccupancy. We conducted quantitative ChIP analysis in GATA-1-null G1E cells to test whether GATA-2 preferentially occupies composite elements in a configuration that is optimal for enhancer activity (Fig. 2) versus WGATAR motifs lacking E-boxes within 20 bp of WGATAR. GATA-2 occupancy was analyzed at 63 conserved WGATAR motifs lacking E-boxes on chromosome 6 (Fig. 3A) and at all conserved composite elements on chromosomes 1, 6, and 7 (Fig. 3B to D). Amplicons encompassed WGATAR motifs or composite elements in which the WGATAR motif, E-box, and the intervening spacing, are conserved (mice to humans). To minimize gross differences in chromosomal positions and to ensure that ChIP signals did not overlap, we analyzed conserved WGATAR motifs lacking nearby E-boxes that were 3 to 50 kb from the corresponding composite elements on chromosome 6. GATA-2 occupied 4.8% (3/63) (Fig. 3A) and 9.8% (16/164) (Fig. 3B to E) of WGATAR motifs lacking E-boxes and composite elements, respectively. Statistical analysis using a *z*-test for two proportions indicated that the E-box does not significantly ($P = 0.342$) increase the probability of GATA-2 occupancy. The vast majority of both composite and WGATAR sites tested are unoccupied.

Diagnostic molecular hallmarks of GATA-2-occupied composite elements. Scl/TAL1 and GATA-1 preferentially assemble a complex on oligonucleotides containing E-box–WGATAR composite elements (84). As Scl/TAL1 is also expressed in GATA-2-expressing multipotent hematopoietic precursors (23, 28), we reasoned that Scl/TAL1 might reside at certain GATA-2-occupied composite elements. Quantitative ChIP analysis in G1E cells revealed little to no signal at the *necdin* and *Ey* promoters that lack composite elements, at multiple conserved WGATAR motifs within the *Gata2* and *Fmod-Btg2* loci lacking E-boxes, and at conserved composite elements not occupied by GATA-2 (Fig. 4). In contrast, Scl/TAL1 occupied all GATA-2-occupied composite elements, and therefore Scl/TAL1 occupancy at the composite elements was absolutely predictive of GATA-2 occupancy.

Another potentially important hallmark of GATA-2-occupied composite elements is the local chromatin environment. The chromatin environment surrounding large numbers of WGATAR motifs or composite elements has not been described. Intriguingly, the occupied and unoccupied conserved composite elements had distinct epigenetic signatures. Epigenetic marks that often signify active chromatin, specifically di-acetylated H3, tetra-acetylated H4, and H3-dimeK4 (8),

(+1, +2, +3, +5, and +10). In the (+9.5 Δ4core)1SLuc plasmid, 4 bp between the E-box and WGATAR motifs were scrambled. G1E cells and HUVECs were transiently transfected with the indicated reporter plasmids. The plot depicts the average luciferase activities of the cell lysates normalized by protein concentrations (at least three independent experiments). In each experiment, transfections were performed in triplicate. *, $P < 0.05$ with respect to (+9.5)1SLuc. (B) *cis* element orientation requirements. G1E cells and HUVECs were transiently transfected with the indicated reporters. The plot depicts the average luciferase activities of the lysates normalized by protein concentrations (at least three independent experiments). In each experiment, transfections were performed in triplicate. *, $P < 0.05$ with respect to (+9.5)1SLuc.

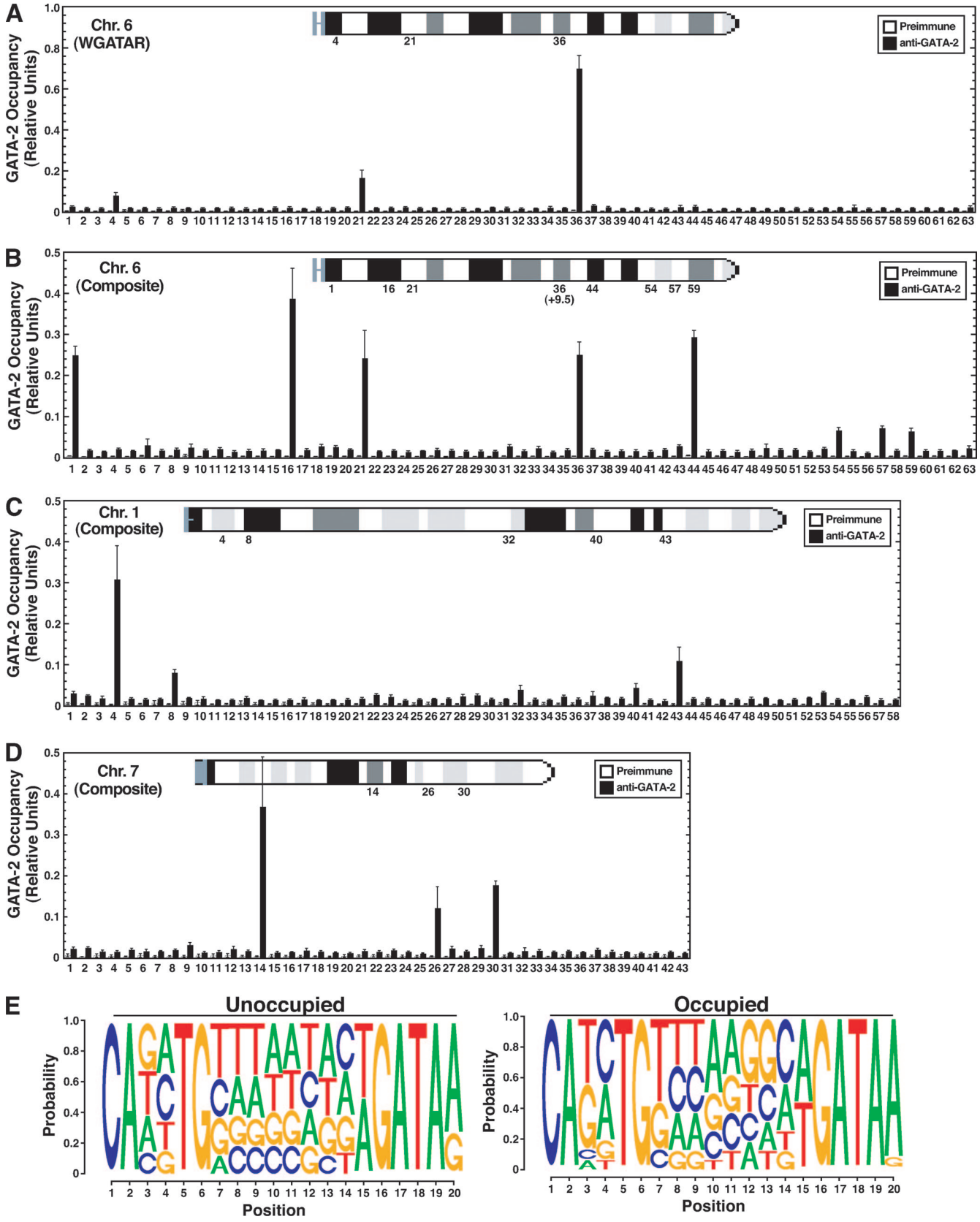


FIG. 3. Chromosome-wide GATA-2 occupancy at conserved WGATAR motifs and E-box-WGATAR composite motifs. (A) Quantitative ChIP analysis of GATA-2 occupancy at 63 conserved WGATAR motifs (within 3 to 50 kb of the corresponding conserved composite motifs of panel B) across mouse chromosome (Chr.) 6 in G1E cells (mean ± standard error from three independent experiments). The numbers on the *x* axis

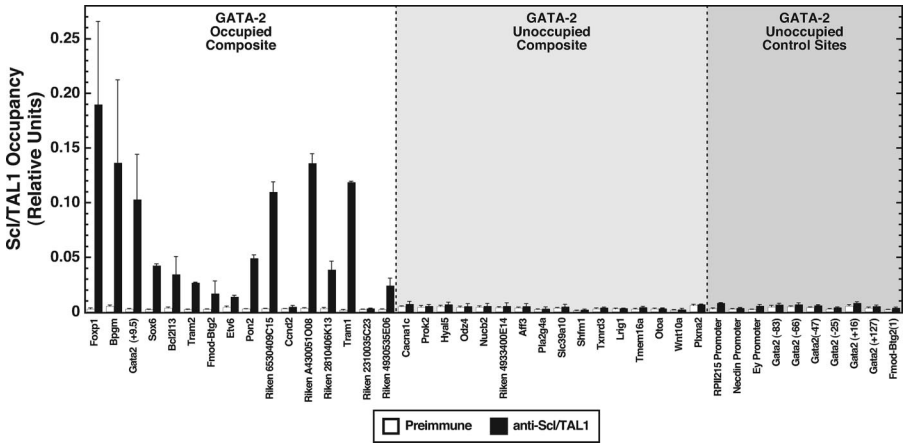


FIG. 4. Scl/TAL1 occupancy at conserved composite elements occurs exclusively at GATA-2-occupied elements. Quantitative ChIP analysis was conducted in G1E cells to measure Scl/TAL1 occupancy at GATA-2-occupied, conserved composite elements, GATA-2-unoccupied, conserved composite elements, and control sites lacking composite elements (mean ± standard error from three independent experiments).

were selectively enriched at the occupied sites (Fig. 5). H3-dimeK36, whose distribution in functionally distinct chromatin regions is less well defined, was also enriched at the occupied sites (Fig. 5). In contrast, H3-trimeK9, which is often, but not always, present at repressed chromatin sites (8, 81), was selectively enriched at half of the unoccupied sites and was enriched at only 1 of 16 occupied sites. Considering the combinations of epigenetic marks, enrichments of acetylated H3 and H4, H3-dimeK4, and H3-dimeK36 combined with a deficiency of H3-trimeK9 are highly predictive of GATA-2 and Scl/TAL1 occupancy at conserved composite elements. As a sole predictive parameter, H3-trimeK9 was least useful, which might relate to the fact that this marker is enriched at certain repressed and active chromatin sites (81).

To reveal additional potential molecular hallmarks of GATA-2–Scl/TAL1 occupancy, rigorous statistical analysis was conducted to identify sequences that may discriminate between occupied and unoccupied composite elements. We tested whether sequences within the composite element correlate with occupancy by using a logistic regression model with occupancy status as the outcome and nucleotide compositions in each of the 12 degenerate positions (positions 3, 4, 7 to 15, and 20) within the 20 positions of the composite element as explanatory variables. This model also allowed two-way interactions of these positions. Neither variations among nucleotide compositions of the individual positions (Fig. 3E) nor their two-way interactions significantly correlated with occupancy.

Since multiple *trans*-acting factors interact with GATA factors (42) and certain factor (e.g., Ets) are required for GATA-2-dependent transcription in specific contexts (65), we tested whether their cognate motifs reside near the composite elements and correlate with occupancy. Consensus motifs for Ets factors, Sp1, EKLF, ZBP89, NF-E2/AP1, AML1/Runx1, and Gfi1b were identified in sequences flanking occupied and unoccupied composite elements (± 50, 100, 150, 250, or 500 bp from the composite element). We tested whether these sites discriminate between the two groups of composite elements. Only the Sp1 consensus [GT][GA]GGC[GT][GA][GA][GT] was a significant discriminator, which appeared in 6/16 occupied composite elements and only 14/148 unoccupied elements, when considering ±250 bp of flanking sequence (P = 0.0056; Fisher's exact test of the corresponding 2-by-2 table). When the flanking sequences are extended to ±500 bp, 9/16 and 37/148 of the regions in occupied and unoccupied groups, respectively, have at least a copy of this motif within their flanks (P = 0.016). Thus, the Sp1 consensus, which binds multiple factors (64), some of which interact with GATA-1 (53), and cooperates with WGATAR and additional *cis*-elements to establish DNase I hypersensitivity (22), is significantly enriched in occupied versus unoccupied regions.

De novo analysis of sequences flanking the composite elements (± 50, 100, 150, 250, or 500 bp) using MEME (3) and COSMO (4) algorithms identified a highly significant differential enrichment of a novel motif [TC][CT][CT]TG[GT][GC][CG][AT]G[TG] in occupied versus unoccupied groups. This motif occurs within ±100 bp of the composite elements in 10/16 occupied regions and only 7/148 unoccupied regions (P = 4.109e−08), and has not been implicated in protein binding or transcription. The positions of the novel and Sp1 motifs with respect to occupied composite elements are shown

correspond to nearest-neighbor genes listed in Table S1 in the supplemental material. The numbers of GATA-2-occupied motifs are also shown at their specific chromosomal locations. (B to D) Quantitative ChIP analysis of GATA-2 occupancy at all conserved E-box–WGATAR composite motifs across mouse chromosome 6 (B), chromosome 1 (C), and chromosome 7 in G1E cells (mean ± standard error from three independent experiments). The numbers on the *x* axis correspond to nearest-neighbor genes listed in Table S1 in the supplemental material. The numbers of GATA-2-occupied motifs are also shown at their specific chromosomal location. (E) Sequence composition of 148 unoccupied (left) and 16 occupied composite elements (right). The *x* axis depicts the nucleotide position within the composite element, and the *y* axis represents the relative frequencies of the nucleotides.

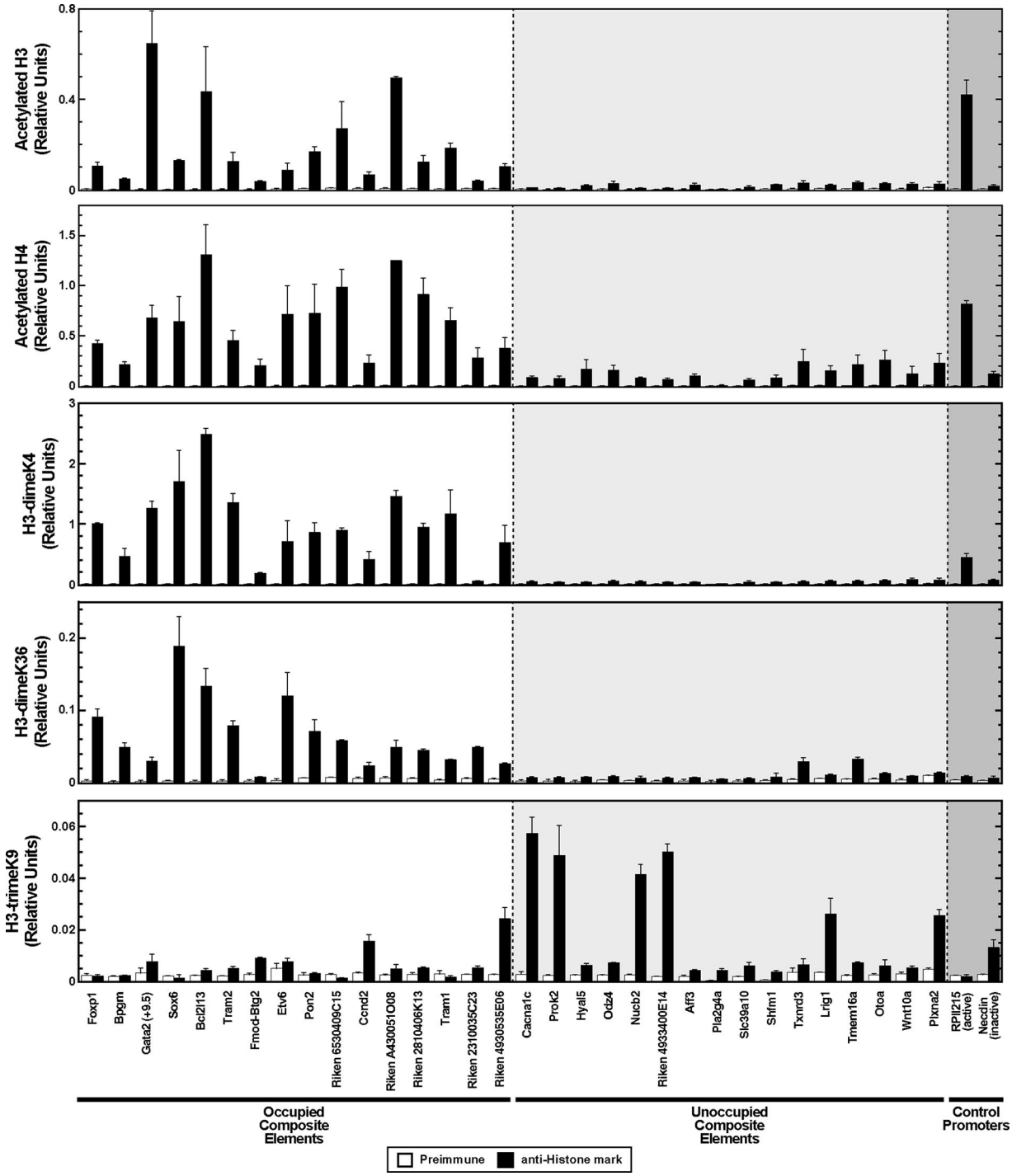


FIG. 5. GATA-2–Scl/TAL1-occupied and -unoccupied conserved composite elements have diagnostic epigenetic signatures. Quantitative ChIP analysis was conducted in G1E cells to measure the indicated epigenetic marks at GATA-2-occupied, conserved composite elements, GATA-2-unoccupied, conserved composite elements, and active (*RPII215*) and inactive (*necdin*) promoters lacking composite elements (mean ± standard deviation from two independent experiments).

in Fig. S1 in the supplemental material. A combinatorial rule, in which the Sp1 and/or the novel motif resides at a locus, identifies 14/16 occupied sites as occupied and only 21/148 unoccupied sites as occupied ($P = 5.596e{-}08$). Neither the novel nor the Sp1 motif significantly associates with occupied WGATAR sequences lacking nearby E-boxes. Collectively, the Scl/TAL1 occupancy, the epigenetic patterns, and the statistical distribution of specific *cis* elements establish diagnostic molecular hallmarks of Scl/TAL1–GATA-2 complexes on composite elements in hematopoietic cells.

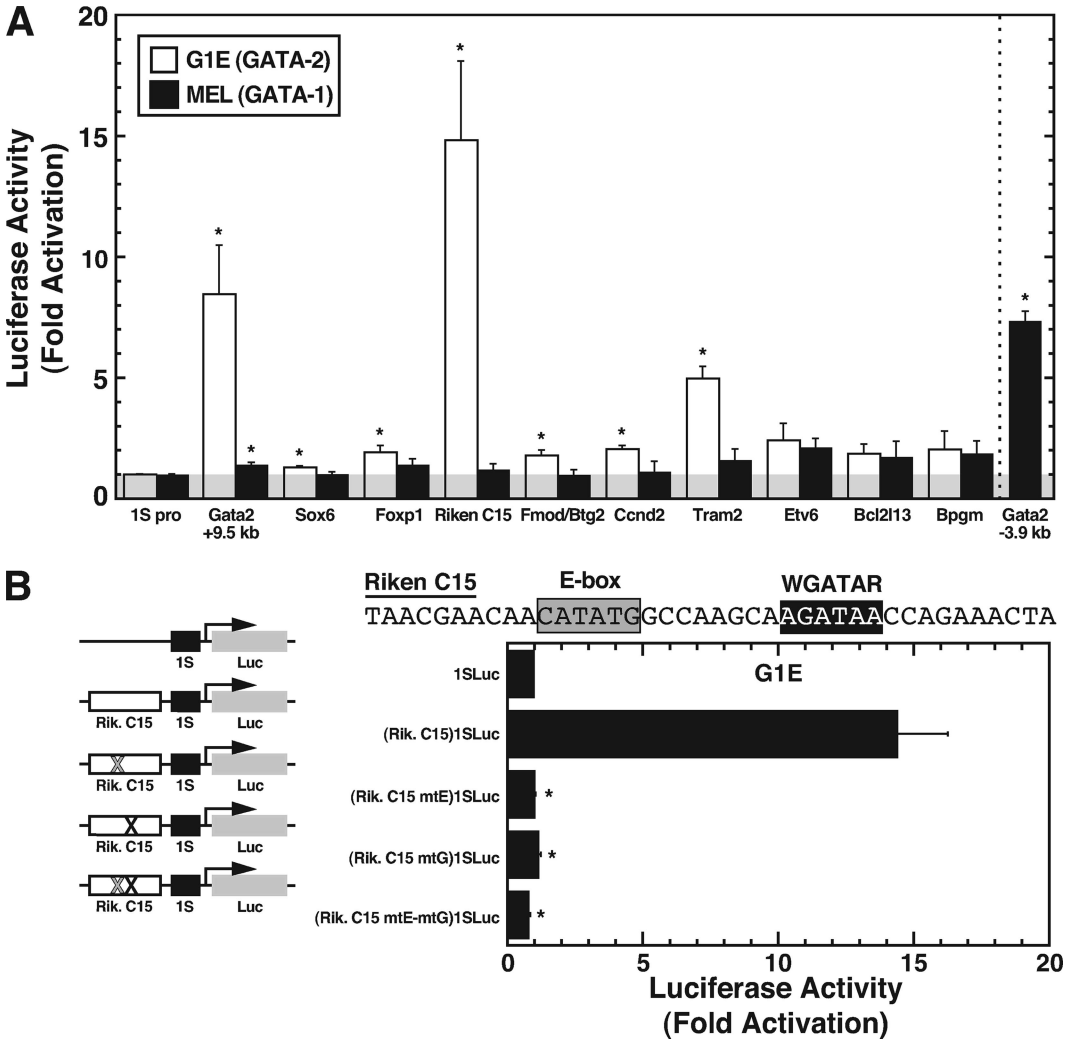


FIG. 6. GATA-2–Scl/TAL1-occupied composite elements function as enhancers in GATA-2- but not GATA-1-expressing cells. (A) G1E and MEL cells were transiently transfected with reporter constructs containing the indicated composite elements as well as 10 bp of upstream and downstream flanking sequence. The plot depicts the average luciferase activities of the cell lysates normalized by protein concentrations (mean ± standard error from at least three independent experiments). In each experiment, transfections were performed in triplicate. *, $P < 0.05$ with respect to 1SLuc. The horizontal gray bar delineates the 1.0 value of the 1SLuc construct. (B) G1E cells were transiently transfected with reporter constructs containing the wild type (sequence depicted on top of the graph), E-box mutant, WGATAR mutant, and E-box–WGATAR double mutant of the Riken 6530409C15 (Riken C15) composite element with 10 bp of upstream and downstream flanking sequence. The plot depicts the average luciferase activities of the cell lysates normalized by protein concentrations (mean ± standard error from three independent experiments). In each experiment, transfections were performed in triplicate. *, $P < 0.05$ with respect to (Rik. C15)1SLuc. (Rik. C15)1SLuc is the 1SLuc plasmid containing the Riken C15 composite element; (Rik. C15 mtE)1SLuc is the 1SLuc plasmid containing the Riken C15 composite element with a scrambled E-box (CATATG→GAATTC); (Rik. C15 mtG)1SLuc is the 1SLuc plasmid containing the Riken C15 composite element with a scrambled WGATAR motif (AGATAA→GAGCTC); (Rik. C15 mtE-mtG)1SLuc is the 1SLuc plasmid containing the Riken C15 composite element with a scrambled E-box and scrambled WGATAR motif.

Based on the molecular hallmarks described above, we reasoned that Scl/TAL1–GATA-2 complexes assembled at composite elements would have the capacity to regulate transcription. To test whether occupied composite elements function as GATA factor-dependent enhancers, we analyzed the activities of the respective elements with 10 bp of flanking sequence on the 5′ and 3′ ends in a transient transfection assay (Fig. 6A). While 7 out of 10 occupied composite elements conferred statistically significant enhancer activity in GATA-2-expressing G1E cells, with three exhibiting particularly notable activity, only 1 out of 10 composite elements (*Gata2* +9.5 element) had significant activity in GATA-1-expressing MEL cells, and this activity was low. Similar results were obtained in DMSO-induced MEL cells (data not shown). In contrast, the −3.9 kb region of the *Gata2* locus, which lacks a composite element but is activated by GATA-1 via a WGATAR motif (51), was highly active in MEL cells. Thus, a subset of the occupied composite elements function as enhancers, preferentially in GATA-2-expressing cells. To test whether the novel enhancers that function in G1E cells indeed require E-box and WGATAR motifs, we generated reporter constructs containing the Riken 6530409C15 (Riken C15) composite element in

which these motifs were mutated (Fig. 6B). Mutation of the E-box, WGATAR, or both motifs abrogated enhancer activity (Fig. 6B).

Novel cell regulatory pathways derived from analysis of chromatin targets. As GATA-2 occupied composite elements near genes not known to be GATA factor target genes, we tested whether Dox-mediated induction of GATA-2 during mouse ES cell differentiation into EBs (50) alters expression of genes neighboring the occupied composite elements (Fig. 7A). We also compared gene expression in *Gata2*$^{-/-}$ and wild-type EBs. Addition of Dox on day 2 of the culture greatly increased *Gata2* mRNA in day 3, 4, 6, and 8 EBs derived from iGATA-2 ES cells (Fig. 7B). Similarly, Dox significantly induced the established GATA-2 target (50) Scl/TAL1 (12-fold), and the novel GATA-2 targets *Sox6* and *Fmod* (4.1- and 68-fold, respectively) (Fig. 7B). Sox6 promotes chondrogenesis (73), represses embryonic β-like globin transcription in definitive erythroid cells (91), and functions cell autonomously to promote proliferation, survival, and differentiation of erythroid cells (18). Fibromodulin is a small, leucine-rich proteoglycan that suppresses signaling mediated by transforming growth factor β (TGF-β) and related cytokines (90), and the related protein biglycan binds BMP4, suppressing BMP4 signaling (57). Dox significantly decreased *Btg2* expression (2.1-fold), while expression of other genes changed <2-fold. Btg2 (1) functions downstream of p53 to promote cell cycle arrest (6), suppresses Ras-induced transformation (6), and is deregulated in breast and renal cancer (40, 76).

Fmod was highly downregulated in *Gata2*-null EBs (Fig. 7C). Taken together with the striking Dox-dependent *Fmod* induction (68-fold) and the endogenous GATA-2 interaction with the *Fmod* locus, these results establish *Fmod* as a bona fide GATA-2 target gene (Fig. 7D). The magnitude of the GATA-2-mediated *Fmod* induction is considerably greater than that for any other GATA-2 target gene reported. *Fmod* is the most highly overexpressed gene in B-cell chronic lymphocytic leukemia (CLL) (35, 44, 55), but mechanisms underlying its normal transcriptional control and deregulation in CLL are unknown. Fibromodulin is secreted from CLL cells and resides intracellularly (55). As fibromodulin suppresses signaling by TGF-β and related proteins (90), biglycan suppresses BMP4 signaling (57), proteoglycans suppress signaling by BMP factors in *Drosophila melanogaster* (7), and BMP4 induces *Gata2* expression (19, 50), *Fmod* overexpression in CLL might disrupt a circuit (Fig. 7D) in which BMP4 induces and/or sustains *Gata2* expression in the hematopoietic niche. The role of these factors in hematopoiesis and leukemogenesis is unexplored.

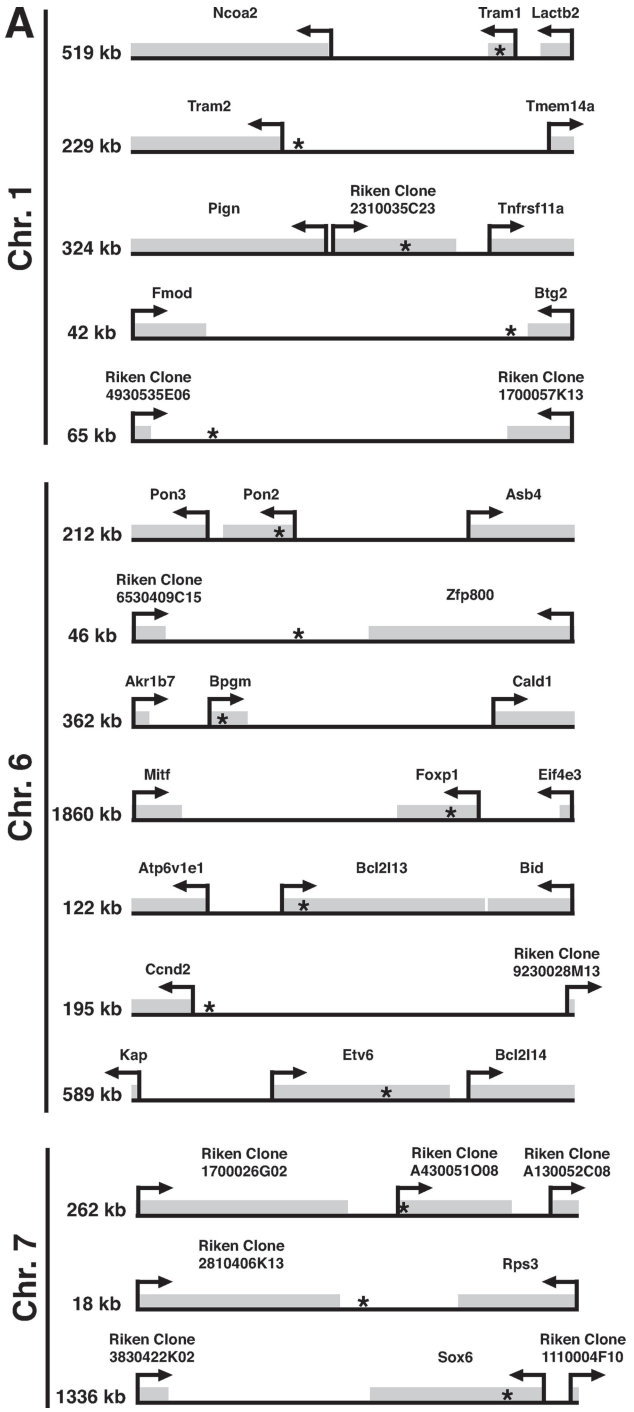
Expression of the additional genes in wild-type versus *Gata2*$^{-/-}$ EBs differed by less than twofold (Fig. 7C), although it cannot be ruled out that GATA-2 regulates these genes redundantly with other factors and/or nonredundantly in distinct contexts. We also conducted complementation analysis in GATA-1-null proerythroblast-like G1E cells stably expressing ER–GATA-1 (26, 27, 29, 34, 43, 51, 60). β-Estradiol-mediated ER–GATA-1 activation instigates GATA-2 displacement at chromatin sites, and "GATA switches" increase or decrease transcription (9). ER–GATA-1-mediated activation or repression can result from disruption of GATA-2-mediated transcriptional control. ER–GATA-1 activation in G1E-ER–GATA-1 cells repressed *Gata2* (76-fold) (Fig. 7B, right), as expected (26), and also repressed *Sox6*, *Tram2*, and *Etv6* (9.1-, 21-, and 3.2-fold, respectively). *Tram2* is a BMP and Runx2 target gene in osteoblasts (67) and regulates protein translocation in the endoplasmic reticulum (75). Etv6 (Tel), a member of the Ets transcription factor family, is a key regulator of adult HSCs that is frequently disrupted via leukemogenic chromosomal translocations (30, 93). ER–GATA-1 induced *Btg2*, *Bpgm*, *Foxp1*, and *Bcl2l13* (323-, 15-, 8.5-, and 6.0-fold, respectively). *Bpgm* encodes bisphosphoglycerate mutase, which catalyzes synthesis of the major allosteric regulator of hemoglobin (11). The Forkhead transcription factor Foxp1 is a prognostic factor in diffuse large B-cell lymphoma (10), but whether it functions in hematopoiesis is unclear. The proapoptotic factor Bcl2l13 (39) is a prognostic factor in B-lineage acute lymphocytic leukemia (31). *Fmod* was expressed at almost undetectable levels in untreated and estradiol-treated G1E-ER–GATA-1 cells, and considering the ES cell data, GATA-2 is necessary but insufficient to confer high-level *Fmod* expression.

DISCUSSION

We have demonstrated that GATA-2 occupies a highly restricted subset of conserved composite elements, and this subset exhibited unique molecular hallmarks relative to the highly abundant unoccupied elements. GATA-2-occupied sites were co-occupied by Scl/TAL1 and had both a distinctive epigenetic signature and specific neighboring *cis* elements. These parameters offer considerable predictive value and provide important mechanistic insights vis-à-vis GATA-2 recognition of target sites in chromatin.

The GATA-2–Scl/TAL1 occupancy at all GATA-2-occupied composite elements strongly suggests that these factors function combinatorially at common targets in the genome. As our quantitative comparison of GATA-2 occupancy at composite elements versus isolated WGATAR motifs did not reveal significant differences, it seems unlikely that the composite element serves to increase the probability of GATA-2 occupancy. Thus, it is attractive to propose that both factors function collectively post-chromatin occupancy to recruit requisite coregulators, thereby conferring combinatorial transcriptional control.

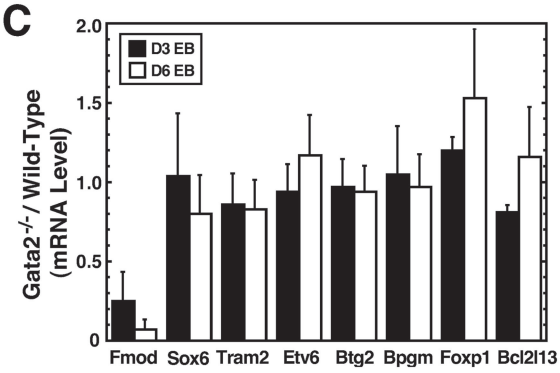
Since GATA-1 functionally interacts with Scl/TAL1 in the context of composite elements (84), we assumed that the GATA-2- and Scl/TAL1-occupied composite elements would function as GATA-1- and perhaps GATA-2-responsive enhancers. However, while a subset of these elements functioned as enhancers in GATA-2-expressing cells, 9 out of 10 were not significantly active in GATA-1-expressing cells (Fig. 6A). Thus, it is instructive to compare our results with those of studies implicating GATA-1 in functioning through DNA sequences containing E-box and WGATAR motifs. In gel-shift assays using MEL cell nuclear extracts, GATA-1, the E-proteins Scl/TAL1 and E2A, and their interacting proteins Lmo2 and Ldb1 assemble a complex on oligonucleotides containing E-box and WGATAR or GATA motifs (83, 84, 89). Sequences containing both E-box and WGATAR motifs, but considerably larger than the 40-bp composite elements that we analyzed, activate reporter genes in transiently transfected MEL cells (2, 14, 89) and in erythroid cells in vivo (83). The 1.7-kb P4.2 promoter, which contains two E-box–WGATAR composite



B

	iGATA-2			ER-GATA-1		
	Day 3/4 EB	Day 6 EB	Day 8 EB	24 h	48 h	Controls
Gata2	290 (+/- 32)	69 (+/- 13)	18 (+/- 2.2)	-42 (+/- 17)	-76 (+/- 44)	
Scl/TAL1	9.6 (+/- 3.0)	12 (+/- 3.0)	5.3 (+/- 1.7)	1.1 (+/- 0.2)	3.9 (+/- 0.4)	
Fmod	12 (+/- 5.5)	65 (+/- 20)	68 (+/- 19)	1.0 (+/- 0.0)	1.0 (+/- 0.0)	
Sox6	3.3 (+/- 0.7)	4.1 (+/- 1.2)	1.1 (+/- 0.1)	-9.1 (+/- 2.8)	-6.9 (+/- 3.6)	
Tram2	1.3 (+/- 0.2)	1.6 (+/- 0.3)	1.4 (+/- 0.2)	-7.7 (+/- 1.6)	-21 (+/- 6.6)	
Etv6	1.2 (+/- 0.2)	1.4 (+/- 0.2)	1.3 (+/- 0.1)	-3.2 (+/- 0.4)	-3.1 (+/- 0.7)	
Btg2	1.2 (+/- 0.2)	-1.4 (+/- 0.2)	-2.1 (+/- 0.3)	38 (+/- 3.1)	323 (+/- 130)	
Bpgm	1.2 (+/- 0.1)	1.3 (+/- 0.1)	-1.4 (+/- 0.2)	2.3 (+/- 0.6)	15 (+/- 4.4)	
Foxp1	1.0 (+/- 0.1)	1.5 (+/- 0.1)	1.4 (+/- 0.3)	1.5 (+/- 0.1)	8.5 (+/- 4.0)	
Bcl2l13	1.3 (+/- 0.1)	1.3 (+/- 0.1)	1.0 (+/- 0.1)	2.0 (+/- 0.4)	6.0 (+/- 0.9)	

GATA-2-activated
GATA-1-repressed
GATA-2-activated, GATA-1-repressed
GATA-1-activated



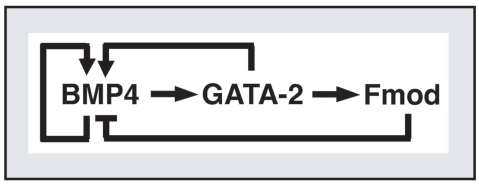


FIG. 7. Occupied composite elements reside at and near novel GATA factor target genes. (A) Diagrams of nearest-neighbor genes surrounding GATA-2-occupied E-box–GATA motifs on mouse chromosomes (Chr.) 1, 6, and 7. Asterisks denote the locations of conserved E-box–WGATAR motifs, arrows denote transcription start sites, and shaded boxes indicate the coding regions of the genes. (B) The table summarizes changes (fold) in the GAPDH-normalized expression of selected genes surrounding GATA-2-occupied E-box–WGATAR composite elements in day 3/4, 6, and 8 EBs derived from mouse ES cells following Dox-mediated GATA-2 induction (mean ± standard error from nine independent experiments for day 3/4 and 6 EBs and from six independent experiments for day 8 EBs) and also in G1E-ER–GATA-1 cells after estradiol-mediated activation of ER–GATA-1 (mean ± standard error from three independent experiments). mRNA levels were quantitated by real-time RT-PCR. (C) The graph depicts the GAPDH-normalized expression of genes surrounding GATA factor-occupied E-box–WGATAR composite elements in day 3 and 6 EBs derived from *Gata2*$^{-/-}$ ES cells divided by their expression in day 3 and 6 EBs derived from wild-type ES cells, respectively (mean ± standard error from three independent experiments). mRNA levels were quantitated by real-time RT-PCR. (D) Model of BMP4–GATA-2–fibromodulin regulatory circuit.

elements, functions as an enhancer in MEL cells (89). Furthermore, smaller sequences containing an E-box–E-box–WGATAR motif (14) or a WGATAR–E-box–WGATAR motif (2) function as enhancers in MEL cells (approximately twofold and approximately fivefold, respectively). A 1.1-kb *Gata1* regulatory element (HS1) containing a WGATAR motif 9 bp downstream from an E-box activates *LacZ* expression in primitive erythroid cells in the yolk sac at E10.5 and in definitive erythroid cells in the fetal liver of E14.5 transgenic mouse embryos (83). In contrast, a smaller HS1 derivative (62 bp) containing only the WGATAR motif without the E-box retains activity, and therefore the E-box is not required for activity of this sequence in erythroid cells. Our study, which represents the first to analyze E-box–GATA composite motifs with defined spacing/orientation, demonstrates striking differences in their responsiveness to GATA-1 versus GATA-2 and also different intrinsic activities in GATA-2-expressing cells. Thus, it is attractive to propose that *cis* elements neighboring the composite elements influence their efficacy as enhancers and also specificity vis-à-vis different GATA factors.

Intriguingly, a specific epigenetic signature was one of the molecular hallmarks deduced that distinguishes occupied versus unoccupied composite elements. This specific chromatin modification state might be a prerequisite for GATA-2–Scl/TAL1 complex assembly at composite elements in chromatin, or the complex might establish this signature post-chromatin occupancy. Since GATA-2 is expressed in HSCs, and no tractable systems exist to examine chromatin structure at target sites prior to the physiological appearance of GATA-2, distinguishing between these models will be challenging. In principle, one could ask whether knocking down GATA-2 in G1E cells reconfigures the epigenetic signature. However, we have not achieved efficacious GATA-2 knockdowns in this system. Even if a satisfactory degree of knockdown can be achieved, persistence of the epigenetic signature might reflect its establishment prior to complex assembly or a GATA-2 requirement for establishment, but not maintenance, of the signature. Nevertheless, our results provide the first example in which GATA-2-occupied target sites, or target sites occupied by any GATA factor or master regulator of hematopoiesis, exhibit a specific epigenetic signature that provides a unique foundation for conducting subsequent mechanistic analysis.

The GATA-2–Scl/TAL1-co-occupied target sites pinpointed neighboring genes that are GATA-2 and/or GATA-1 responsive. Thus, the functional genomic strategy established novel links between GATA-2 and proteins involved in important biological processes, including leukemogenesis. Other than *Btg2*, which was described in an analysis of GATA-1 target genes (87), GATA factors had not been linked to the regulation of genes identified in our screen. Furthermore, no prior reports have identified GATA-2 target genes using the multiple approaches described herein: endogenous GATA-2 occupancy of endogenous loci, regulation of the endogenous genes upon conditional expression of GATA-2 in physiologically relevant ES cells, and also regulation of the endogenous genes in *Gata2* knockout cells. The novel targets (Fig. 7B) define five transcriptional modes: (i) GATA-2 activated, (ii) GATA-2 activated and GATA-1 repressed, (iii) GATA-1 activated, (iv) GATA-1 repressed, and (v) neither GATA-1 nor GATA-2 regulated. It will be particularly instructive to assemble the genome-wide genetic network collectively instigated by GATA-2 and Scl/TAL1 at composite elements to gain a comprehensive view of how stem cells give rise to diverse lineages of blood cells, how perturbations of this network causes leukemia, and, more fundamentally, how two cell-type-specific *trans*-acting factors selectively recognize their targets and function at a highly restricted subset of composite elements in a complex genome.

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