MOLECULAR AND CELLULAR BIOLOGY, June 2007, p. 4551–4565 Vol. 27, No. 12
0270-7306/07/$08.00+0 doi:10.1128/MCB.00235-07

Dissecting Molecular Steps in Chromatin Domain Activation during Hematopoietic Differentiation▽

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Received 8 February 2007/Returned for modification 21 March 2007/Accepted 3 April 2007

GATA factors orchestrate hematopoiesis via multistep transcriptional mechanisms, but the interrelationships and importance of individual steps are poorly understood. Using complementation analysis with GATA-1-null cells and mice containing a hypomorphic allele of the chromatin remodeler BRG1, we dissected the pathway from GATA-1 binding to cofactor recruitment, chromatin loop formation, and transcriptional activation. Analysis of GATA-1-mediated activation of the β-globin locus, in which GATA-1 assembles dispersed complexes at the promoters and the distal locus control region (LCR), revealed molecular intermediates, including GATA-1-independent and GATA-1-containing LCR subcomplexes, both defective in promoting loop formation. An additional intermediate consisted of an apparently normal LCR complex and a promoter complex with reduced levels of total RNA polymerase II (Pol II) and Pol II phosphorylated at serine 5 of the carboxy-terminal domain. Reduced BRG1 activity solely compromised Pol II and serine 5-phosphorylated Pol II occupancy at the promoter, phenocopying the LCR-deleted mouse. These studies defined a hierarchical order of GATA-1-triggered events at a complex locus and establish a novel mechanism of long-range gene regulation.

Transcriptional networks involving coordinated actions of numerous regulatory factors ensure high-fidelity cellular differentiation and organogenesis. In the context of hematopoiesis, GATA factor networks are particularly important. The hematopoietic GATA factors (GATA-1, GATA-2, and GATA-3) have overlapping and unique expression patterns and both shared and unique biological activities (11). GATA-2 (41, 81, 87) is crucial for the integrity/function of multipotent hematopoietic precursors (72, 73) and regulates urogenital (86), central nervous system (15, 51), and pituitary (12) development. By contrast, GATA-1 (20, 74) promotes erythroid, megakaryocyte, and mast cell development (48, 50, 58, 59, 67–69, 79, 82), and GATA-3 (28) regulates lymphopoiesis (56, 70).

Analogous to many transcription factors, GATA-1 has a dual zinc finger domain (54) and binds the histone acetyltransferase CBP/p300 (7). GATA-1 binds the coregulator Friend of GATA-1 (FOG-1), which mediates activation and repression (16, 75, 76). GATA-1 recruits FOG-1 (24) and associated NuRD components (29, 37) to chromatin sites, where NuRD mediates repression of certain target genes (29). GATA-1 also binds PU.1 (52, 62, 83), Sp1 (46), and erythroid Krüppel-like factor (EKLF) (46) and forms diverse multiprotein complexes (29, 63). This complexity of protein-protein interactions might reflect unique requirements for regulation of distinct loci. Indeed, GATA-1 targets differ in their sensitivities to changes in GATA-1 levels/activities (37), and factors occupying GATA-1 targets can differ (63).

Analysis of β-like globin gene regulation led to the discovery of GATA-1 (20, 74). The murine β-like globin gene cluster contains genes expressed during embryonic/fetal development (*Ey* and β*H1*) and in the adult (β*major* and β*minor*) (8, 39). A far upstream locus control region (LCR) (21, 26) mediates high-level transcription of the β-like globin genes at all developmental stages (5, 19). Despite the presence of more than 200 GATA motifs (A/TGATAA/G) in the gene cluster, endogenous and conditionally active GATA-1 proteins fused to the estrogen receptor ligand binding domain (ER-GATA-1) occupy only a small subset of these motifs (32, 36). GATA-1 occupancy at additional chromosomal regions (23, 24, 33, 37, 45) further reveals an exquisite discrimination among GATA motifs.

GATA-1-null (G1E) cells expressing ER-GATA-1 (G1E-ER-GATA-1) have been an important system for dissecting GATA-1 function (25), as ER-GATA-1 activation in G1E cells recapitulates a normal window of erythropoiesis (80), ER-GATA-1 and GATA-1 occupy similar chromatin sites (23, 32, 36, 45), and the ER-GATA-1 level/activity can be titrated by varying the β-estradiol or tamoxifen concentration (24, 32, 37, 45). A low ER-GATA-1 level/activity preferentially occupies the LCR versus that in the adult β*major* promoter, and ER-GATA-1 occupies DNaseI hypersensitive sites (HSs) HS1, HS2, HS3, and HS4 of the LCR prior to the promoter. The elevation of diacetylated histone H3 (acH3) and binding of additional factors also occur at the LCR prior to the promoter (32). These results support a model involving GATA-1-dependent LCR complex assembly and subsequent GATA-1-dependent promoter complex assembly (32). Although this analysis segregated certain steps, the interrelationship and importance of individual steps are largely unknown. For example, CBP/p300, the BRG1 component of the SWI/SNF chromatin re-

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▽ Published ahead of print on 16 April 2007.

modeling complex, EKLF, and FOG-1 occupy the LCR (32), but it is unclear whether they are corecruited or independently recruited and whether they collectively or independently regulate Pol II recruitment.

The orders of events of transcriptional activation at endogenous loci appear to be highly context dependent (1, 14, 27, 47, 66). At the *Saccharomyces cerevisiae* HO endonuclease promoter, Swi5p promotes recruitment of SWI/SNF, followed by the histone acetyltransferase GCN5 and then activator binding (2, 14), whereas at the HeLa cell beta interferon promoter, an enhanceosome recruits GCN5, followed by histone acetylation at the promoter, CBP and Pol II recruitment, and then SWI/SNF recruitment (2, 14). The differing sequences of reactions might reflect context-dependent activation requirements. In this regard, GATA-1 regulates transcription via FOG-1-dependent activation (16), FOG-1-independent activation (16), FOG-1-dependent repression (16), FOG-1-independent repression (33), and FOG-1-independent activation coupled to FOG-1-dependent repression (37).

Unlike the HO and beta interferon promoters discussed above, regulation of the β-globin locus and other complex mammalian chromatin domains involves assembly of dispersed complexes over broad chromosomal regions (8, 17). The LCR HSs, which are all important for full transcriptional activation (6), form a higher-order "active chromatin hub" (71). Many questions regarding how dispersed complexes function at complex loci and how their activities are dynamically regulated during cellular differentiation remain unanswered. Herein, we demonstrate that GATA-1-mediated β-globin locus activation can be resolved into discrete molecular steps that provide fundamental insights into chromatin domain activation.

MATERIALS AND METHODS

Cell culture. G1E cells expressing ER-GATA-1 (32) or ER-GATA-1(V205G) were cultured in Iscove's modified Dulbecco's medium (GIBCO/BRL) containing 2% penicillin-streptomycin (GIBCO/BRL), 2 units/ml erythropoietin, 120 nM monothioglycerol (Sigma), 0.6% conditioned medium from a Kit ligand-producing CHO cell line, 15% fetal bovine serum (FBS; GIBCO/BRL), and 1 μg/ml puromycin (Sigma).

Quantitative ChIP assay. Real-time-PCR-based quantitative chromatin immunoprecipitation (ChIP) analysis was conducted as described previously (31). Before β-estradiol treatment, cells were grown for at least 24 h in medium containing 15% charcoal-stripped FBS to eliminate steroids. The cells were then cultured in medium containing 7.5% FBS-7.5% charcoal-stripped FBS with or without 1 μM β-estradiol (Steraloids Inc.) for 48 h at 25°C or 37°C. Single-cell suspensions were isolated from fetal livers of E12.5 $Brg1^{null/ENU1}$ embryos, which contain a null allele and a mutated allele (E1083G mutation in the catalytic ATPase domain) generated via ethylnitrosourea mutagenesis (9). The cells were cross-linked identically to G1E-ER-GATA-1 cells, except 4×10^6 cells were used per immunoprecipitation condition. DNA was analyzed by real-time PCR (ABI Prism 7000; PE Applied Biosystems). Primers amplified 50- to 150-bp amplicons, specific product was measured by SYBR green fluorescence, product was quantitated relative to a standard curve of input chromatin, and dissociation curves showed that PCRs yielded single products.

Quantitative RT-PCR. Total RNA was purified with TRIzol (GIBCO/BRL) from the same cultures as those used for ChIP. cDNA was prepared by annealing RNA (1 μg) with 250 ng of a 5:1 mixture of random and oligonucleotide (deoxyribosylthymine) primers preheated at 68°C for 10 min, followed by incubation with Moloney murine leukemia virus reverse transcriptase (RT; 50 units) (GIBCO/BRL) combined with 10 mM dithiothreitol (DTT), RNasin (Promega), and 0.5 mM deoxynucleoside triphosphates at 42°C for 1 h. Reaction mixtures were diluted to a final volume of 130 μl and heat inactivated at 98°C for 5 min. The reaction mixtures (15 μl) contained 2.0 μl of cDNA, 7.5 μl of SYBR green master mix (Applied Biosystems), and the appropriate primers. Product accumulation was monitored by SYBR green fluorescence. Control reactions lacking RT yielded little to no signal. Relative expression levels were determined from a standard curve of serial dilutions of cDNA samples and were normalized to *Gapdh* expression levels.

Protein analysis. For Western analysis, total cell lysates were prepared by boiling 1×10^6 cells for 10 min in sodium dodecyl sulfate (SDS) sample buffer (25 mM Tris, pH 6.8, 2% β-mercaptoethanol, 3% SDS, 0.05% bromophenol blue, and 5% glycerol). Lysates were resolved on SDS-polyacrylamide gels, and proteins were detected by Western blotting using ECL Plus (Amersham Pharmacia). To detect endogenous FOG-1, lysates were prepared in radioimmunoprecipitation assay buffer (phosphate-buffered saline [PBS] supplemented with 2 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin and containing 0.5% Triton X-100, 0.1% sodium deoxycholate, and 0.1% SDS). Lysates were cleared by centrifugation at 13,000 × *g* for 30 min at 4°C, divided into two aliquots, and immunoprecipitated with preimmune serum or rabbit anti-FOG-1 polyclonal antibody (24). Immune complexes were collected by adsorption to protein A-Sepharose and washed five times at 4°C with lysis buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on an 8% acrylamide gel and analyzed by Western blotting using ECL Plus.

Restriction endonuclease accessibility assay. Untreated and β-estradiol-treated (48 h) G1E-ER-GATA-1 cells cultured at 25°C or 37°C were collected by centrifugation at 240 × *g* for 6 min at 4°C. The cells were washed with ice-cold PBS, resuspended in 1.5 volumes of lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.2% Nonidet P-40, and 10 mM DTT, pH 7.5), and incubated for 10 min at 4°C. Nuclei were collected by centrifugation at 600 × *g* for 2 min, resuspended in wash buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, and 10 mM DTT, pH 7.5), and collected again by centrifugation. Nuclei were resuspended in 1× New England Biolabs restriction enzyme buffer no. 2 at 2×10^7 nuclei/0.2 ml. Restriction enzyme was added to aliquots, which were incubated for 45 min at 37°C. Reactions were terminated with 10 mM Tris-HCl, 25 mM EDTA, and 1% SDS, pH 7.5; proteinase K (0.4 mg/ml) was added; and samples were incubated overnight at 37°C. DNA was purified, and equal amounts of DNA (15 μg) were digested with appropriate restriction enzymes. Samples were resolved on a 1.1% agarose gel, and fragments were detected by Southern blotting with high-specific-activity, randomly primed probes.

3C assay. Chromosome conformation capture (3C) analysis was conducted as described previously (24, 77). The following primers were used to analyze ligation products: HS2, ATGACTCAGCACTGCTGTGCTCAAGCC; β*major*, GGTGGAAGGGGGTATTATGAACATTCGG.

Immunofluorescence microscopy. Untreated and β-estradiol-treated (48 h) G1E-ER-GATA-1 cells cultured at 25°C or 37°C were attached to slides by cytospin centrifugation at 1,000 × *g* for 5 min at room temperature. The cells were fixed with 3.7% paraformaldehyde in PBS for 30 min at room temperature, permeabilized with 0.2% Triton X-100 for 15 min at room temperature, and blocked with PBS-Tween 20 (PBST) containing 10% normal fish serum (Aveslab) for 1 h at 37°C. The cells were then incubated with rabbit anti-estrogen receptor AB16 antibody (Lab Vision Corporation) in PBST (1:250 dilution) containing 1% normal fish serum at 4°C overnight. After being washed in PBST, the cells were incubated with Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG) antibody (Molecular Probes, Inc.) in PBST (1:500 dilution) containing 1% normal fish serum for 1 h at 37°C. Coverslips were mounted in VECTASHIELD (mounting medium containing DAPI [4′,6′-diamidino-2-phenylindole]) (Vector laboratories, Inc.). Samples were analyzed with a microscope (Axiovert 200 M; Carl Zeiss MicroImaging, Inc.) equipped with an α-plan-FLUAR 100× 1.45-numerical-aperture oil immersion objective. Fluorescence images were collected with a digital camera (AxioCam HRm; Carl Zeiss MicroImaging, Inc.). The images were deconvoluted with Axiovision 4.3 software (Carl Zeiss MicroImaging, Inc.). Similar results were obtained with an anti-GATA-1 polyclonal antibody (data not shown).

Antibodies. Rabbit anti-GATA-1 polyclonal antibody was described previously (32). Rabbit anti-Pol II (N-20, sc-899), anti-CBP (A-22, sc-369), and anti-BRG1 (H-88, sc-10768) antibodies were from Santa Cruz Biotechnology. Mouse monoclonal IgM anti-RNA polymerase II H14 (MMS-134R), which recognizes the phosphoserine 5 version 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. Anti-diacetylated histone H3 (06-599) antibody was from Upstate Biotechnology. Anti-murine FOG-1 antibody was raised in rabbits (24). Anti-EKLF monoclonal 7B2 was from James Bieker (Mt. Sinai School of Medicine, NY). Anti-p45/nuclear factor erythroid 2 (anti-p45/NF-E2) rabbit polyclonal antibody was described previously (49).

PCR primers. Oligonucleotide sequences are shown in Table 1.

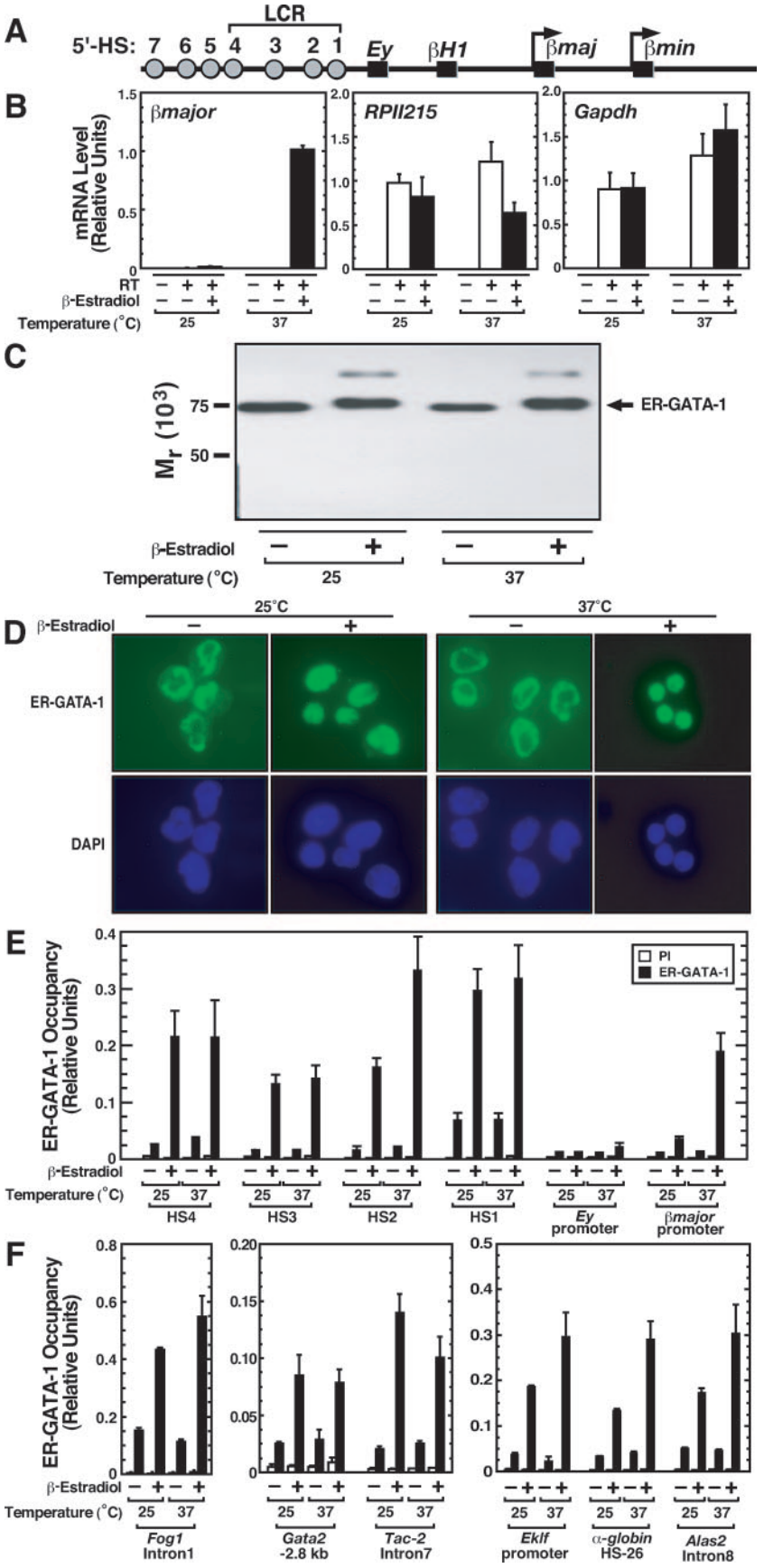
TABLE 1. Oligonucleotide primers

Designation	Forward and reverse sequences (5′–3′)
Primers for real-time RT-PCR	
Gapdh	TGCCCCCATGTTTGTGATG
	TGTGGTCATGAGCCCTTCC
RPII215	CGAATCCGCATCATGAACAG
	TGCATCGCAGGAAGACATCA
β*major*	TTTAACGATGGCCTGAATCACTT
	CAGCACAATCACGATCATATTGC
Ey	AAGGCTTCTTGTTGTGTACCCA
	CAAAAGCAGTCAGCACCTTCTT
Slc4a1	GGACAGATAGCATATAGAGACCTAACCA
	CGTAGTCTGTGGCTGTTTGCTC
Alas2	CCATCTTAAGGCAACCAAGGC
	ACAGCATGAAAGGACAATGGC
*Csf2r*β*2*	GCTAATCAACATGACCCTCCTCTATC
	GCACTCTGACCACATGAGTTTCTC
α-*globin*	GTGGATCCCGTCAACTTCAAG
	CAAGGTCACCAGCAGGCAGT
Ahsp	GATCTCCACAGGGATAAAGGAGTTT
	CAGTCATGAACCACAATCACCAT
Hebp1	GCCTTGGCAGGTTCTAAGCAC
	TTGTCTGTCACTTCCACAGTAGCA
Tac-2	TCAGCTTGGCTTGGACCTTC
	GACGGAGGCAGCTGATAGAGA
Epb4.9	CCAGCCAGCCAAGATAGAGACT
	TCTGCCCCCTTTCGAGATG
Fog1	CCTTGCTACCGCAGTCATCA
	ACCAGATCCCGCAGTCTTTG
Eklf	CACGCACACGGGAGAGAAG
	CGTCAGTTCGTCTGAGCGAG
c-*Kit*	AGCAATGGCCTCACGAGTTCTA
	CCAGGAAAAGTTTGGCAGGAT
c-*Myb*	TTACCAGGCACACAAGCGTCT
	GAATTCCAGTGGTTCTTGATAGCA
Sialy8	TGGCTCCACCATCTTCCAAC
	TTGACCACAGATACGTCACGTTC
Cpa3	AGTTAGTGAGAAGGAATCTCAGACCATC
	CTGTTTCTCTATCTCTTCTTGTAGGTCATG
Primers for quantitative ChIP analysis	
HS4	GAGATCCTGCCAAGACTCTGATAATACTA
	CCTGGCTTCCTGGTCCAGTAG
HS3	CTAGGGACTGAGAGAGGCTGCTT
	ATGGGACCTCTGATAGACACATCTT
HS2 (GATA)	AGGGTGTGTGGCCAGATGTT
	ACCCAGATAGCACTGATCACTCAC
HS1	CAGATCCTCAAACACTCTCCCATAA
	TGCCTTCTTTGTCCCATCATT
Ey promoter	ATGACCTGGCTCCACCCAT
	TCTTTGAAGCCATTGGTCAGC
β*major* promoter (GATA)	TGAGCAAATGCGTTGCCA
	AGCTCTGGGTACTCCCTCTGAATA
Fog1 intron 1	TGCAAGTCCCATCCTGATAAGA
	GCACGCCAGATAAGATCACAATT
Gata2 HS-2.8	GCCCTGTACAACCCCATTCTC
	TTGTTCCCGGCGAAGATAAT
Tac-2 intron 7	CAGTGCATAACGCTGGAGGA
	CCTGCGATGGCCAGATAATT
Eklf promoter	GATTTGAGGGGACTCCTTTTGC
	AGGAGTGGACCAGGAAGGATAGA
α-*globin* HS-26	AGCAGACCACTGTGGGATCTATG
	TGGCCTCTAGAAACTCTGAGTCATG
Alas2 intron 8	CCTGCTCCTGCCTATCTAGTCATC
	CCATGTTAGAGCCTTGCTTCTGT
necdin promoter	GGTCCTGCTCTGATCCGAAG
	GGGTCGCTCAGGTCCTTACTT
RPII215 promoter	GCGAATCTATAAAGGGCGTCACT
	TCGGCGCTTCTGAGGAGA

RESULTS AND DISCUSSION

Temperature sensitivity of GATA-1-mediated β-globin locus activation. We reasoned that reducing the cell culture temperature might slow the kinetics of the multiple steps involved in GATA-1-mediated β-globin locus (Fig. 1A) activation. Culturing G1E-ER-GATA-1 cells at 25°C almost completely ablated ER-GATA-1-mediated β*major* activation (Fig. 1B, left), while the expression levels of the constitutive *RPII215* and *Gapdh* genes were unaffected (Fig. 1B, middle, right). Impaired β*major* activation was not associated with reduced ER-GATA-1 levels (Fig. 1C), and β-estradiol induced ER-GATA-1 nuclear localization similarly at 25°C and 37°C (Fig. 1D). The smaller cell and nucleus size at 37°C reflects greater differentiation at 37°C.

We tested whether impaired β*major* activation at 25°C results from defective chromatin occupancy or postoccupancy steps. Quantitative ChIP analysis was used to measure ER-GATA-1 occupancy at the four HSs of the LCR, the *Ey* promoter, and the β*major* promoter 48 h after ER-GATA-1 activation (Fig. 1E). ER-GATA-1 occupied HS1, HS3, and HS4 similarly at 25°C and 37°C, whereas occupancy at HS2 was 51% lower at 25°C than at 37°C (Fig. 1E). ER-GATA-1 occupancy at the β*major* promoter was 82% lower at 25°C than at 37°C (Fig. 1E). No occupancy was detected at the *Ey* promoter (Fig. 1E), which is not expressed in G1E-ER-GATA-1 cells (data not shown) at 25°C or 37°C, with or without activated ER-GATA-1. In addition to normal ER-GATA-1 occupancy at HS1, HS3, and HS4, ER-GATA-1 occupied regulatory elements of additional loci (*Fog1* intron 1, the *Gata2* kb −2.8 region, and *Tac-2* intron 7) identically at 25°C and 37°C (Fig. 1F, left and middle). ER-GATA-1 occupancy at the *Eklf* promoter, α-*globin* HS-26, and *Alas2* intron 8 was ~2-fold lower at


A
LCR
5'-HS: 7 6 5 4 3 2 1
Ey
βH1
βmaj
βmin
B
βmajor
RPII215
Gapdh
mRNA Level (Relative Units)
RT
β-Estradiol
Temperature (°C)
25
37
C
Mr (10^3)
75
50
ER-GATA-1
β-Estradiol
Temperature (°C)
25
37
D
25°C
37°C
β-Estradiol
ER-GATA-1
DAPI
E
ER-GATA-1 Occupancy (Relative Units)
PI
ER-GATA-1
β-Estradiol
Temperature (°C)
HS4
HS3
HS2
HS1
Ey promoter
βmajor promoter
F
ER-GATA-1 Occupancy (Relative Units)
β-Estradiol
Temperature (°C)
Fog1 Intron1
Gata2 -2.8 kb
Tac-2 Intron7
Eklf promoter
α-globin HS-26
Alas2 Intron8

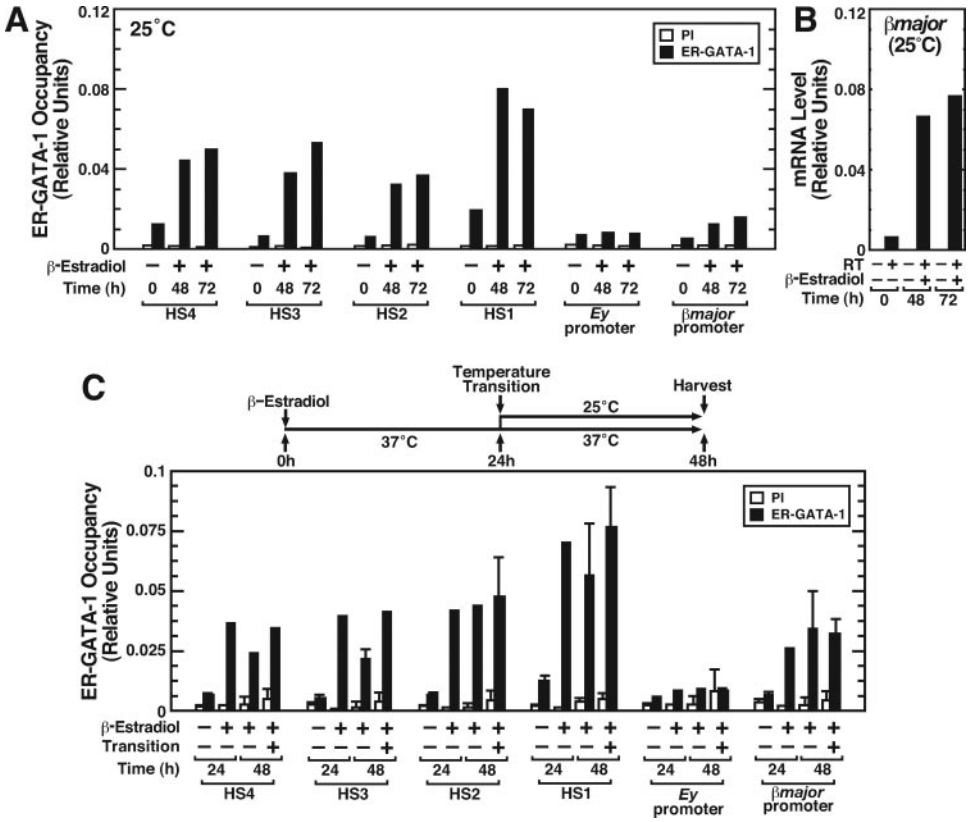


FIG. 2. Establishment, but not maintenance, of the active β-globin locus is temperature sensitive. (A) Quantitative ChIP analysis of ER-GATA-1 occupancy at the LCR, the *Ey* promoter, and the β*major* promoter in G1E-ER-GATA-1 cells cultured at 25°C for up to 72 h after β-estradiol treatment. (B) Real-time RT-PCR analysis of β*major* mRNA normalized to *Gapdh* mRNA levels in G1E-ER-GATA-1 cells under the same conditions (mean values from two independent experiments). (C) A schematic representation of the experimental strategy is shown at the top. Replicate G1E-ER-GATA-1 cultures were treated with β-estradiol at 37°C for 24 h, at which point one culture was continued at 25°C for 24 h, while the other culture was maintained at 37°C for 24 h. Results are shown for quantitative ChIP analysis of ER-GATA-1 occupancy at the LCR, the *Ey* promoter, and the β*major* promoter in untreated (37°C), β-estradiol-treated (37°C for 24 h or 37°C for 48 h), and β-estradiol-treated/temperature-shifted (37°C → 25°C) G1E-ER-GATA-1 cells (mean values from 2 independent experiments). PI, preimmune sera.

25°C than at 37°C (Fig. 1F, right). These results indicate that decreased ER-GATA-1 occupancy at the β*major* promoter at 25°C does not reflect a general loss of ER-GATA-1 chromatin binding activity, but rather, occupancy at the β*major* promoter is unusually sensitive to reduced temperature.

Titrating ER-GATA-1 levels/activity with increasing β-estradiol concentrations, and also analyzing the kinetics of ER-GATA-1 chromatin occupancy, revealed ER-GATA-1 occupancy at the LCR prior to the β*major* promoter (32). Defective β*major* activation at 25°C might therefore result from a further reduction in the kinetics of promoter occupancy. To test this possibility, G1E-ER-GATA-1 cells were treated with β-estradiol for up to 72 h at 25°C, and ER-GATA-1 occupancy was measured. Extending the β-estradiol treatment time to 72 h did not significantly increase occupancy at promoter or LCR sites (Fig. 2A), and β*major* mRNA did not increase at 72 h compared to the level at 48 h (Fig. 2B), indicating that the temperature-sensitive step(s) mediates establishment of the active β*major* promoter. As steps regulating establishment and maintenance of an active locus might be shared, we tested whether maintenance is temperature sensitive. G1E-ER-GATA-1 cells were treated with β-estradiol for 24 h at 37°C to

FIG. 1. Temperature sensitivity of GATA-1-mediated β-globin locus activation. (A) Murine β-globin locus organization. DNaseI HSs are depicted as filled circles, and embryonic (*Ey* and β*H1*) and adult (β*maj* and β*min*) globin genes are depicted as boxes. (B) Quantitative RT-PCR analysis of β*major*, *RPII215*, and *Gapdh* mRNA transcripts in G1E-ER-GATA-1 cells cultured at 25°C or 37°C for 48 h after β-estradiol treatment (means ± standard errors for three independent experiments). β*major* and *RPII215* mRNA levels were normalized to *Gapdh* levels. (C) Western blot analysis of ER-GATA-1 expression in untreated and β-estradiol-treated (48 h) G1E-ER-GATA-1 cells. (D) Immunofluorescence staining of ER-GATA-1 in G1E-ER-GATA-1 cells under the same conditions as those for panel B (×100 magnification). Representative images from two independent experiments are shown. (E) Quantitative ChIP analysis of ER-GATA-1 occupancy at HS4, HS3, HS2, HS1, the *Ey* promoter, and the β*major* promoter in untreated and β-estradiol-treated (48 h) G1E-ER-GATA-1 cells under the same conditions as those for panel B (means ± standard errors for four independent experiments). (F) ChIP analysis of ER-GATA-1 occupancy at *Fog1* intron 1, *Gata2* HS-2.8, *Tac-2* intron 7, the *Eklf* promoter, α-*globin* HS-26, and *Alas2* intron 8 under the same conditions as those for panel B (means ± standard errors for three independent experiments). The symbols are identical to those defined in panel E. PI, preimmune sera.

confer maximal β*major* activation and subsequently incubated at 25°C for 24 h. If maintenance of the active state is temperature dependent, the temperature transition should decrease promoter-bound ER-GATA-1. However, once ER-GATA-1 occupied the promoter, occupancy was insensitive to a subsequent 25°C incubation (Fig. 2C), indicating that establishment, but not maintenance, of the active locus is temperature sensitive.

Dissecting steps in chromatin domain activation: LCR complex assembly. The failure of ER-GATA-1 to strongly activate β*major* at 25°C, despite nearly normal LCR occupancy, represents a unique system that allows dissection of the interrelationship of the multiple steps in activation. Since ER-GATA-1 occupancy at the LCR for up to 20 h is associated with modestly increased acetylated histone H3 (acH3) at the LCR (32, 43), we compared acH3 levels in untreated and β-estradiol-treated G1E-ER-GATA-1 cells at 25°C and 37°C. The ~2-fold increase in acH3 at HS3 after 48 h of ER-GATA-1 activation (Fig. 3A) is consistent with that described previously after 20 h (32). ER-GATA-1 activation for 48 h did not affect acH3 at HS2 and HS4, whereas small increases were observed previously at 20 h (32). Importantly, highly enriched acH3 levels at HS2, HS3, and HS4, relative to values for acH3 at the *Ey* promoter and the control antibody, were indistinguishable at the two temperatures, indicating that loss of this transcriptionally permissive epigenetic mark does not underlie strongly impaired β-globin locus activation.

GATA-1 physically interacts with CBP and regulates CBP occupancy at certain chromatin sites (7, 43). Activated ER-GATA-1 increased CBP occupancy at HS2, HS3, and HS4 1.5- to 3-fold at 37°C but not at 25°C (Fig. 3B).

GATA-1-mediated stimulation of erythropoiesis requires binding of GATA-1 to FOG-1 (16). FOG-1 occupies all GATA-1-bound chromatin sites tested (24, 37, 57) and facilitates GATA-1 chromatin occupancy at certain sites (42, 57). We tested whether ER-GATA-1 and FOG-1 similarly colocalize at the LCR at 25°C and 37°C. FOG-1 occupied HS2, HS3, and HS4 only at 37°C (Fig. 3C). The ~2-fold decrease in ER-GATA-1 occupancy at HS2 (Fig. 1E) without FOG-1 occupancy is consistent with reports that FOG-1 facilitates ER-GATA-1 occupancy at HS2 but not at other HSs (42, 57).

At least as much Pol II resides at the LCR HSs as is present at the active β*major* promoter (34–36, 78). GATA-1 modestly increases, but is not essential for, Pol II occupancy at HS2 and HS3 (36). Since ER-GATA-1 occupies the LCR at 25°C, we tested whether ER-GATA-1 occupancy is sufficient to recruit Pol II. Western blotting revealed that Pol II levels were indistinguishable at 25°C and 37°C (data not shown). Active ER-GATA-1 increased Pol II occupancy at HS2 and HS3 ~2-fold at 37°C (Fig. 3D). ER-GATA-1 did not increase Pol II occupancy at HS2 at 25°C; occupancy in untreated cells was equivalent to that in cells containing activated ER-GATA-1. Pol II occupancy at HS4 decreased ~2-fold upon ER-GATA-1 activation at 37°C, whereas Pol II occupancy at HS4 was constant at 25°C. Importantly, despite the differences in regulation of Pol II occupancy at HS4 and HS2, LCR-bound Pol II persisted at 25°C.

GATA-1 functions with additional hematopoietic transcription factors, including p45/NF-E2 and EKLF, to regulate the expression of the β-like globin genes (10). Thus, the role of these factors in GATA-1-mediated chromatin domain regulation needs to be considered. Similar to the scenario described above in which Pol II occupies the LCR of the inactive β-globin locus at 25°C, Pol II occupies the LCR, but not the promoter, in CB3 erythroleukemia cells (44) lacking p45/NF-E2 (3, 53), in which β*major* transcription is silenced (34, 35). Although p45/NF-E2 is not required for Pol II occupancy at the LCR (34, 35), p45/NF-E2 induces Pol II recruitment to the β*major* promoter (34, 36) and transcriptional activation (40, 44). p45/NF-E2 physically and functionally interacts with CBP/p300 (22, 30), and therefore, reduced LCR-associated CBP at 25°C might result from loss of p45/NF-E2 occupancy. Both p45/NF-E2 levels (data not shown) and occupancy (Fig. 3E) at HS2 were ~2-fold lower at 25°C than at 37°C. Thus, normal ER-GATA-1 and half-maximal p45/NF-E2 occupancy suggest that loss of CBP occupancy is not explained by the absence of these factors. The persistent Pol II occupancy and acH3 at the LCR at 25°C indicate that these factors are insufficient for CBP occupancy.

ER-GATA-1 activation increases EKLF expression and chromatin occupancy (32). Activated ER-GATA-1 increased EKLF occupancy at HS2 and HS3 at 37°C (Fig. 3F), consistent with the report that ER-GATA-1 activation for up to 20 h increased EKLF occupancy at these sites (32). No EKLF occupancy was detected at these sites or at HS4 at 25°C. EKLF binds BRG1 (38, 84), but EKLF occupancy and BRG1 occupancy at the murine β-globin locus do not correlate precisely (32). Similar to those of EKLF and CBP, BRG1 occupancy at the LCR was considerably lower at 25°C than at 37°C (Fig. 3G). Western blotting revealed similar levels of both EKLF and BRG1 at 25°C and 37°C (data not shown).

The persistent acH3, Pol II, and p45/NF-E2 occupancy at HS2 at 25°C, indicative of an LCR subcomplex, suggests that loss of chromatin accessibility does not underlie the incomplete LCR complex assembly. The terms "complexes" and "subcomplexes" refer to groups of factors assembled at a common chromatin site, and there is no evidence that stable complexes exist prior to chromatin occupancy. Analysis of HaeIII site accessibility at HS2 (Fig. 3H) revealed similar degrees of cleavage at 25°C and 37°C (~60% of templates cleaved). Thus, despite the incompletely assembled LCR at 25°C lacking FOG-1, CBP, EKLF, and BRG1, chromatin accessibility persists.

Dissecting steps in chromatin domain activation: promoter complex assembly. Deletion of the LCR via homologous recombination strongly reduces β*major* transcription (5, 19) but does not prevent all factors from occupying the β*major* promoter. p45/NF-E2 (65), GATA-1 (77), and EKLF (85) occupy the promoter of an LCR-deleted allele in splenic samples from phenylhydrazine-treated mice. In addition, 50% of the Pol II associated with the promoter of a wild-type allele occupies the promoter of the mutant allele (65). These findings suggest that LCR complex assembly is not required for the assembly of at least certain components of the promoter complex in the context of an LCR-deleted allele.

As noted above, LCR components appear to assemble prior to the promoter complex in G1E (32) and CB3 (34) cells. In the context of the wild-type locus, certain steps at the LCR might be required for subsequent steps at the promoter, or LCR and promoter complex assembly might represent parallel pathways. Since

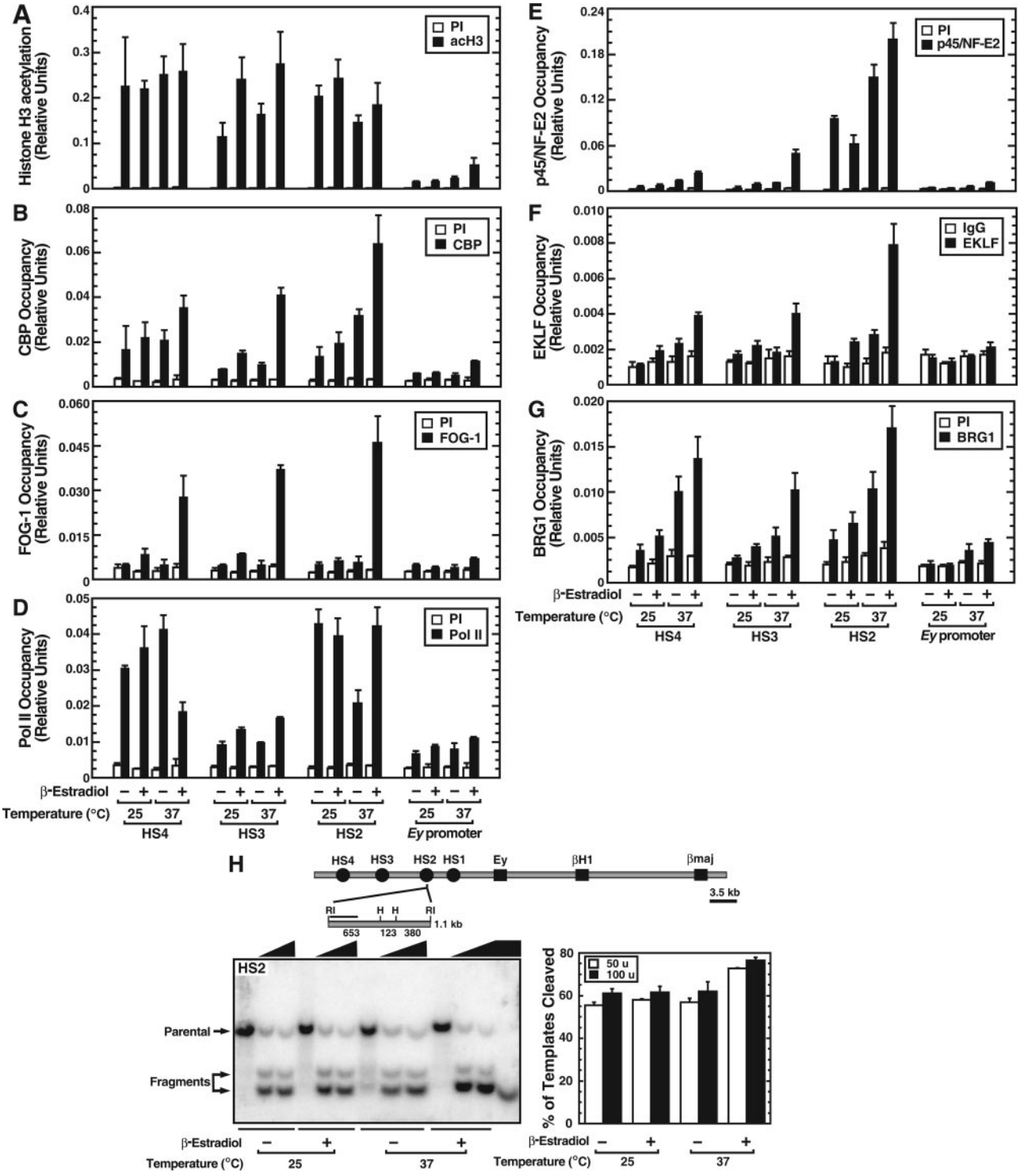
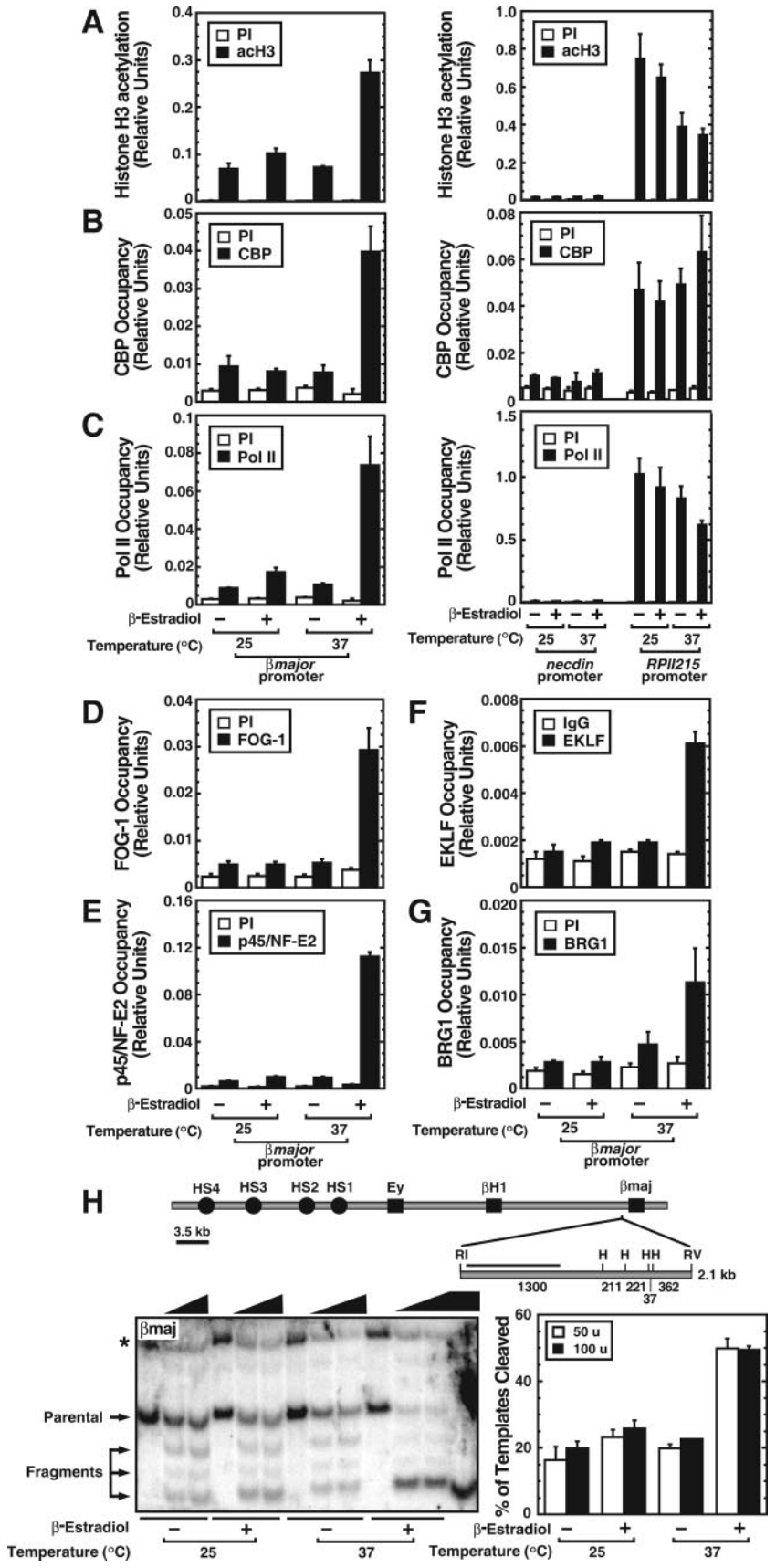


FIG. 3. An LCR subcomplex assembles at 25°C. (A to G) Quantitative ChIP analysis of acH3 (A), CBP (B), FOG-1 (C), Pol II (D), p45/NF-E2 (E), EKLF (F), and BRG1 (G) chromatin occupancy at HS4, HS3, HS2, and the *Ey* promoter in G1E-ER-GATA-1 cells cultured at 25°C or 37°C for 48 h after β-estradiol treatment (means ± standard errors for three or four independent experiments). (H) Chromatin accessibility at HS2. Nuclei from untreated and β-estradiol-treated (48 h) G1E-ER-GATA-1 cells cultured at 25°C or 37°C were incubated with 0, 50, or 100 units of HaeIII for 45 min at 37°C, and cleavage was measured by Southern blotting. The Southern blotting strategy is shown at the top. β-globin genes, HSs, and probes are depicted as boxes, circles, and black bars, respectively. RI, EcoRI; H, HaeIII. A representative Southern blot is shown (lower left). Parental fragments and cleavage products are indicated by arrows. Results for PhosphorImager analysis are shown (lower right) (mean values from two independent experiments). Open bars, 50 units; filled bars, 100 units. PI, preimmune sera.


A
Histone H3 acetylation (Relative Units)
PI
acH3
B
CBP Occupancy (Relative Units)
PI
CBP
C
Pol II Occupancy (Relative Units)
PI
Pol II
β-Estradiol
Temperature (°C)
25
37
βmajor promoter
necdin promoter
RPII215 promoter
D
FOG-1 Occupancy (Relative Units)
PI
FOG-1
F
EKLF Occupancy (Relative Units)
IgG
EKLF
E
p45/NF-E2 Occupancy (Relative Units)
PI
p45/NF-E2
G
BRG1 Occupancy (Relative Units)
PI
BRG1
H
HS4
HS3
HS2
HS1
Ey
βH1
βmaj
3.5 kb
RI
H
H
HH
RV
2.1 kb
1300
211
221
362
37
βmaj
*
Parental
Fragments
% of Templates Cleaved
50 u
100 u

assembly of certain LCR components is impaired at 25°C, we tested whether the promoter complex assembles under these conditions. ER-GATA-1 induced acH3 (Fig. 4A, left), CBP (Fig. 4B, left), Pol II (Fig. 4C, left), FOG-1 (Fig. 4D), p45/NF-E2 (Fig. 4E), EKLF (Fig. 4F), and BRG1 (Fig. 4G) occupancy at the promoter at 37°C but not at 25°C. Analysis of cells cultured at 25°C (at which point the LCR subcomplex forms) and then for 12 h at 37°C revealed subcomplex maturation and promoter complex assembly (data not shown). acH3 occupancy (Fig. 4A, right), CBP occupancy (Fig. 4B, right), and Pol II occupancy (Fig. 4C, right) at the active *RPII215* promoter were similar at 25°C and 37°C and undetectable at the inactive *necdin* promoter.

Though the incompletely assembled LCR was not associated with reduced chromatin accessibility at HS2 (Fig. 3H), promoter accessibility was low under conditions in which ER-GATA-1-induced promoter complex assembly was blocked at 25°C (Fig. 4H). ER-GATA-1 increased β*major* promoter accessibility only at 37°C (Fig. 4H), indicating that the Pol II-, p45/NF-E2-, and ER-GATA-1-containing LCR subcomplex is insufficient to induce any steps in promoter complex assembly.

β-Globin locus activation is associated with a higher-order chromatin transition that positions the LCR near the adult β-like globin genes (71). EKLF (18) and GATA-1 (77) promote loop formation. We used 3C analysis to test whether the ER-GATA-1-containing LCR subcomplex induces looping. Despite similar cleavage efficiencies at 25°C and 37°C (Fig. 5A and B, left), HS2 proximity to the β*major* promoter increased ~5-fold upon ER-GATA-1 activation only at 37°C (Fig. 5B, right; C; and D). Unlike the results shown in Fig. 4, in which ER-GATA-1 increased HaeIII accessibility at the promoter, the 3C BglII digest involved a prolonged incubation with high BglII levels, which would not be expected to reveal differential sensitivities.

FOG-1-dependent GATA-1 target genes are preferentially temperature sensitive. Defective looping and reduced ER-GATA-1 occupancy at HS2 are reminiscent of phenotypes seen in G1E cells expressing V205 mutants of ER-GATA-1, which are defective in FOG-1 binding (42, 77). Active ER-GATA-1 induced FOG-1 at 37°C, with little to no induction at 25°C (Fig. 6A). The equivalent ER-GATA-1-mediated inductions of FOG-1 mRNA at 25°C and 37°C (Fig. 6C) indicate that the failure of ER-GATA-1 to elevate FOG-1 protein levels at 25°C represents a posttranscriptional defect. Under conditions in which ER-GATA-1 is activated at 25°C and FOG-1 protein levels remain largely constant, FOG-1 chromatin occupancy at diverse target genes (*Fog1*, *Gata2*, *Tac-2*, *Eklf*, α-*globin*, and *Alas2*) was almost undetectable (Fig. 6B).

Because steady-state FOG-1 levels in cells containing activated ER-GATA-1 were lower at 25°C than at 37°C, we reasoned that FOG-1-dependent GATA-1 target genes would be preferentially temperature sensitive. However, temperature-sensitive β*major* activation might be multifactorial, and FOG-1-independent GATA-1 targets might also be dysregulated. FOG-1-independent GATA-1 targets were identified from studies of V205 mutants of GATA-1 (16) and by ER-GATA-1 expression in FOG-1-null cells (33, 37).

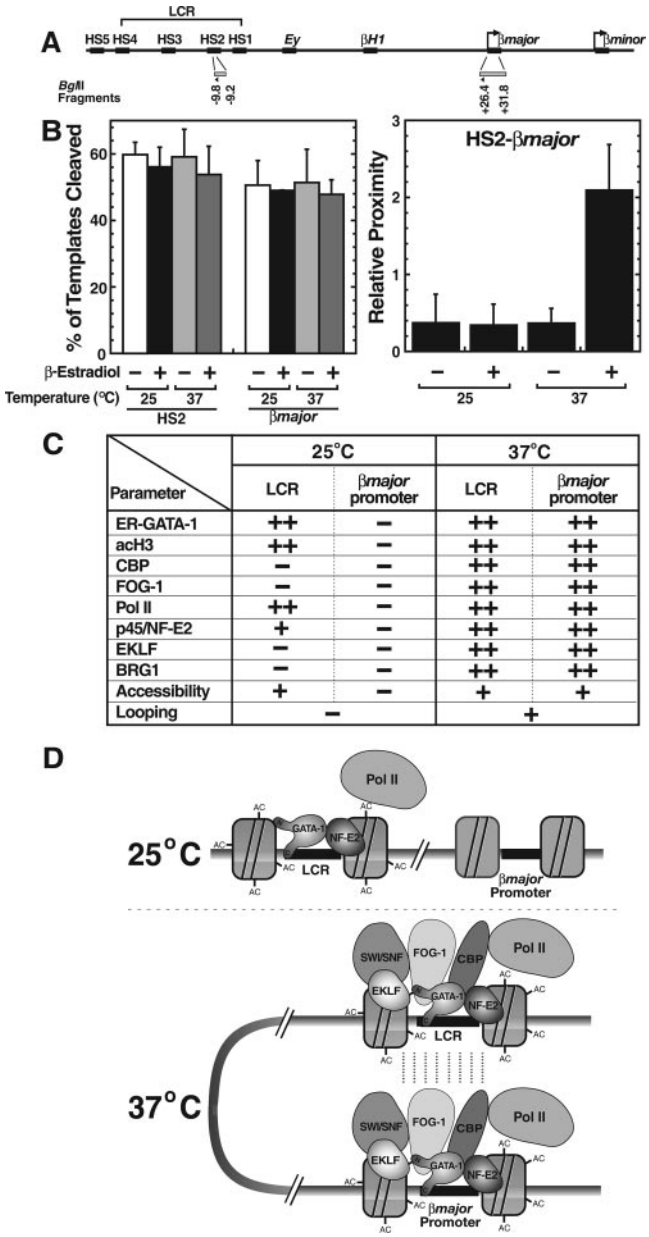
Expression of GATA-1-activated and -repressed genes was analyzed in G1E-ER-GATA-1 cells treated with β-estradiol for 48 h at 25°C or 37°C. ER-GATA-1 activation of β*major*, *Slc4a1*, *Alas2*, *Csf2r*β*2*, α-*globin*, *Ahsp*, and *Hebp1* was strongly repressed at 25°C, whereas *Tac-2*, *Epb4.9*, *Fog1*, and *Eklf* activation levels at 25°C were almost as high as, if not higher than, those at 37°C (Fig. 6C). ER-GATA-1 strongly repressed *Kit* and *Myb* at 37°C, but no significant repression was apparent at 25°C (Fig. 6C). By contrast, ER-GATA-1 repressed *Cpa3* and *Sialy8* 13- and 8.6-fold, respectively, albeit at a lower level than at 37°C. GATA-1 target genes were therefore relatively temperature insensitive or exhibited various degrees of temperature sensitivity.

As activation of the temperature-insensitive genes *Eklf*, *Fog1*, *Epb4.9*, and *Tac-2* (Fig. 6C) is FOG-1 independent (16, 37), we tested whether temperature-sensitive and -insensitive genes are exclusively FOG-1 dependent and independent, respectively. Gene expression was analyzed in β-estradiol-treated (24 h) G1E lines stably expressing ER-GATA-1 or ER-GATA-1(V205G) at levels differing by no more than two-fold (data not shown). ER-GATA-1(V205G) activated β*major*, *Slc4a1*, *Alas2*, *Csf2r*β*2*, α-*globin*, *Ahsp*, and *Hebp1* considerably less than ER-GATA-1 (Fig. 6C). By contrast, ER-GATA-1(V205G) activated *Tac-2*, *Epb4.9*, *Fog1*, and *Eklf* at least as much as ER-GATA-1 (Fig. 6C). FOG-1 suppresses the magnitude of GATA-1-mediated *Tac-2* activation (37). ER-GATA-1(V205G) repressed *Cpa3* and *Sialy8* but not *Kit* and *Myb* (Fig. 6C). The ratio of expression for cells containing activated ER-GATA-1 at 25°C or 37°C (25°C/37°C) correlated ($r = 0.947$) with the ratio of expression for cells containing activated ER-GATA-1(V205G) or ER-GATA-1 (V205G/WT) at 37°C (Fig. 6D), suggesting that impaired FOG-1 synthesis and/or function is a key component of temperature sensitivity (Fig. 6E).

BRG1 ensures maximal Pol II occupancy at the β*major* promoter. The SWI/SNF chromatin remodeling complex facilitates EKLF-dependent β-globin promoter activation in vitro (4). Since BRG1 occupies the LCR without ER-GATA-1 activity and ER-GATA-1 increases BRG1 recruitment at certain β-globin locus sites (32), we tested whether LCR and promoter complex assembly require BRG1.

Fetal liver cells were isolated from E12.5 mouse embryos

FIG. 4. All steps of promoter complex assembly are temperature sensitive. (A to G) Quantitative ChIP analysis of acH3 (A), CBP (B), and Pol II (C) chromatin occupancy at the β*major* (left), *necdin*, and *RPII215* (right) promoters and FOG-1 (D), p45/NF-E2 (E), EKLF (F), and BRG1 (G) chromatin occupancy at the β*major* promoter in G1E-ER-GATA-1 cells cultured at 25°C or 37°C for 48 h after β-estradiol treatment (means ± standard errors for three or four independent experiments). (H) Chromatin accessibility at the β*major* promoter. Nuclei from untreated and β-estradiol-treated (48 h) G1E-ER-GATA-1 cells cultured at 25°C or 37°C were incubated with 0, 50, or 100 units of HaeIII for 45 min at 37°C, and cleavage was measured by Southern blotting. The Southern blotting strategy is shown at the top. β-Globin genes, HSs, and probes are depicted as boxes, circles, and black bars, respectively. RI, EcoRI; RV, EcoRV; H, HaeIII. A representative Southern blot is shown (lower left). Parental fragments and cleavage products are indicated by arrows. Results for PhosphorImager analysis are shown (lower right) (mean values from two independent experiments). Open bars, 50 units; filled bars, 100 units. PI, preimmune sera.



Parameter	25°C		37°C	
	LCR	βmajor promoter	LCR	βmajor promoter
ER-GATA-1	++	−	++	++
acH3	++	−	++	++
CBP	−	−	++	++
FOG-1	−	−	++	++
Pol II	++	−	++	++
p45/NF-E2	+	−	++	++
EKLF	−	−	++	++
BRG1	−	−	++	++
Accessibility	+	−	+	+
Looping	−		+	

FIG. 5. ER-GATA-1-containing LCR subcomplex is insufficient to promote formation of a higher-order chromatin loop. (A) The diagram depicts the 3C strategy. BglII fragments and primers are depicted as shaded and white rectangles, respectively. (B) The graph depicts BglII cleavage efficiencies at HS2 and the β*major* promoter, determined by real-time PCR (left). The graph depicts a 3C analysis of the relative proximity of β-*globin* HS2 and the β*major* promoter in untreated and β-estradiol-treated (48 h) G1E-ER-GATA-1 cells cultured at 25°C or 37°C (means ± standard errors for three independent experiments) (right). (C) Summary of nucleoprotein parameters analyzed at 25°C and 37°C. Factor occupancy: ++, maximal occupancy; +, submaximal occupancy; −, no occupancy. HaeIII site accessibility: +, maximal accessibility; −, low accessibility. 3C analysis: +, looping; −, no looping. (D) Model of ER-GATA-1-dependent chromatin domain activation at 25°C and 37°C. (Upper) ER-GATA-1 occupancy at the LCR at 25°C initiates assembly of an LCR subcomplex involving increased acH3, chromatin accessibility, and p45/NF-E2 and Pol II occupancy at the LCR, whereas β*major* promoter complex assembly is completely blocked. (Lower) ER-GATA-1 occupancy at the LCR and the β*major* promoter at 37°C initiates multiple events, including increased acH3 accessibility; FOG-1, CBP, EKLF, p45/NF-E2, Pol II, and BRG1 occupancy; and looping.

containing a *Brg1* hypomorphic allele (Fig. 7A) that have reduced β*major* expression (9). The E1083G mutation within the ATPase domain does not affect ATPase activity, SWI/SNF complex assembly, or chromatin binding (9) (Fig. 7B). While there was little to no difference in *RPII215* and *Ey* expression (Fig. 7C, middle and right), β*major* expression was 91% lower in mutant cells than in wild-type cells (Fig. 7C, left). GATA-1 (Fig. 7D), p45/NF-E2 (Fig. 7E), and Pol II (Fig. 7F) occupied HS4, HS3, and HS2 in wild-type and mutant cells similarly. This result is consistent with ER-GATA-1, p45/NF-E2, and Pol II occupancy at the LCR, without BRG1 occupancy, at 25°C (Fig. 1 and 3). GATA-1 and p45/NF-E2 occupied the β*major* promoter normally in the mutant cells (Fig. 7D and E). Pol II occupancy at the β*major* promoter, but not the *RPII215* promoter, was significantly lower (51%; $P = 0.026$) in mutant cells than in wild-type cells (Fig. 7F). Pol II occupancy at β*major* exon 3 was 58% lower ($P = 0.002$) in mutant cells than in wild-type cells (0.095 ± 0.014 and 0.223 ± 0.023, respectively) (data not shown).

An LCR-deleted allele of the β-globin locus is characterized by ~50% lower Pol II occupancy in the β*major* promoter than in the wild-type allele and a more severe decrease in serine 5-phosphorylated Pol II (65). Pol II phosphorylation at serine 5 is an essential step in the transition from a stalled to an elongating polymerase (60). Given the similar magnitudes of decreased Pol II occupancy at the β*major* promoter in *Brg1* mutant mice (Fig. 7F) and mice containing the LCR-deleted allele (65), we tested whether the more severe defect in serine 5-phosphorylated Pol II was also a hallmark of the locus in *Brg1* mutant mice. Serine 5-phosphorylated Pol II at the β*major* promoter was 71% lower in *Brg1* mutant mice than in wild-type mice, whereas its levels were indistinguishable at HS3 and the *RPII215* promoter (Fig. 7G). Thus, two hallmarks of the molecular phenotypes associated with the LCR deletion and the BRG1 mutation are reduced Pol II, and there is a more severe reduction in serine 5-phosphorylated Pol II at the β*major* promoter (Fig. 7H and I).

Mechanistic insights arising from analysis of trapped molecular intermediates. Although studies have analyzed the coordinated recruitment of transcription factors and coregulators to chromatin at complex mammalian loci (13, 55), little is known about whether initial steps permit subsequent steps to proceed or whether subsequent steps occur independently. We used two systems (temperature sensitivity and *Brg1* hypomorphic mice) to address the interrelationship and importance of individual steps in chromatin domain activation.

Analysis of temperature sensitivity revealed novel molecular intermediates in chromatin domain activation. One intermediate present prior to ER-GATA-1 activation was characterized by normal acH3 and Pol II at the LCR, submaximal p45/NF-E2 at the LCR, and an absence of ER-GATA-1 and FOG-1. A second intermediate present after ER-GATA-1 activation at 25°C consisted of LCR-associated acH3, Pol II, ER-GATA-1, and submaximal p45/NF-E2 but lacked FOG-1. Loss of LCR-associated BRG1, EKLF, FOG-1, and CBP at 25°C indicates that these factors are not required to maintain acH3, p45/NF-E2, and Pol II at the LCR. Since looping is blocked at 25°C (Fig. 5), establishment of acH3, p45/NF-E2, and Pol II at the LCR precedes looping, and their maintenance does not require looping.

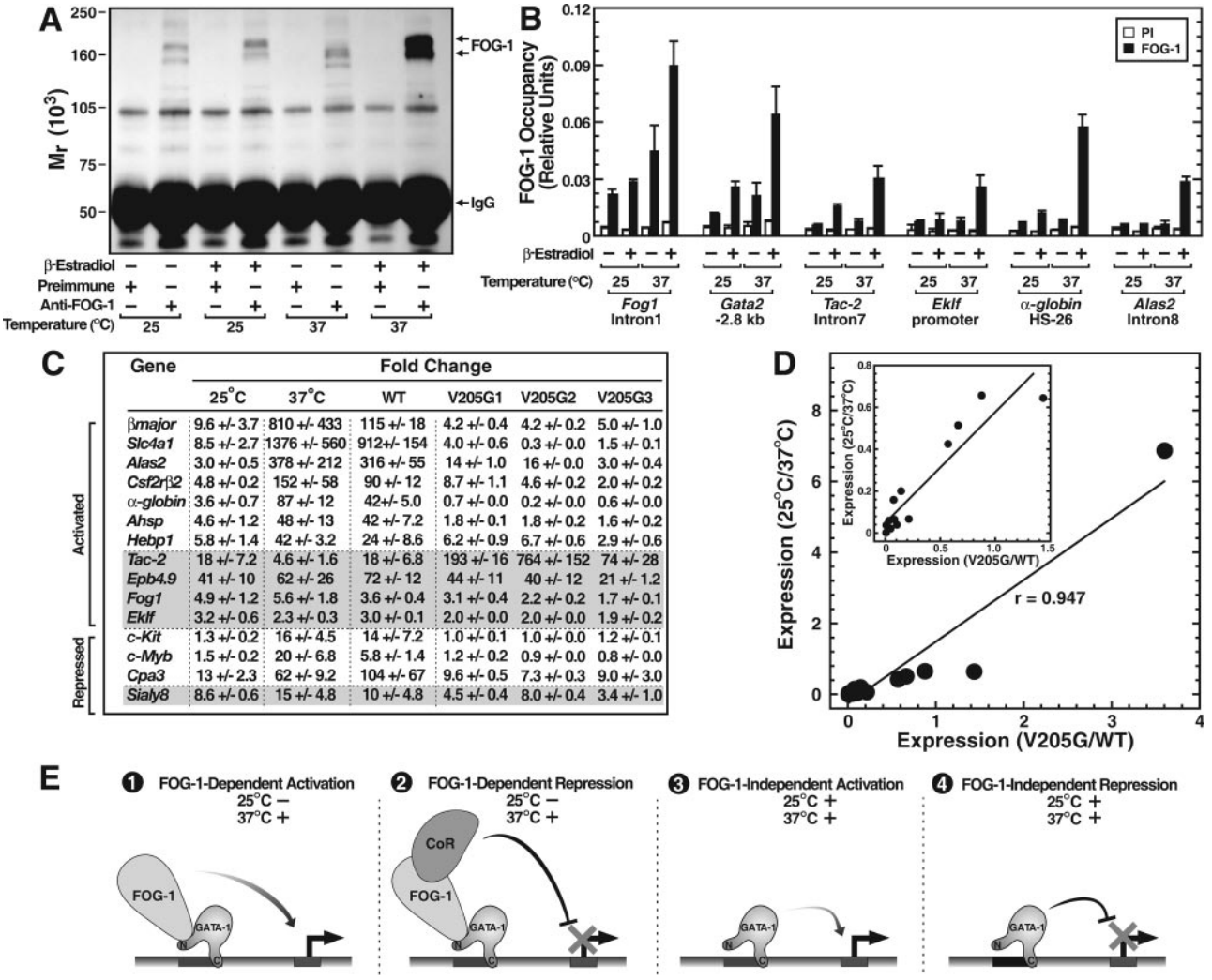


FIG. 6. FOG-1 is limiting at 25°C, and perturbation of FOG-1 function underlies the temperature sensitivity. (A) Western blot analysis of immunoprecipitated endogenous FOG-1 (arrow) in untreated and β-estradiol-treated (48 h) G1E-ER-GATA-1 cells cultured at 25°C or 37°C. The result is representative of five independent experiments. (B) ChIP analysis of FOG-1 occupancy at *Fog1* intron 1, *Gata2* HS-2.8, *Tac-2* intron 7, the *Eklf* promoter, α-*globin* HS-26, and *Alas2* intron 8 in G1E-ER-GATA-1 cells under the same conditions as those for panel A (means ± standard errors for three independent experiments). PI, preimmune sera. (C) Real-time RT-PCR quantitation of mRNA levels from GATA-1 target genes in G1E-ER-GATA-1 cells cultured at 25°C or 37°C for 48 h after β-estradiol treatment and wild-type ER-GATA-1 (average for two clones) and three V205G clones following treatment with 1 μM β-estradiol for 24 h (means ± standard errors for three independent experiments). The values represent ER-GATA-1-mediated activation or repression relative to values for cells containing inactive ER-GATA-1. Values for FOG-1-independent target genes are highlighted with a gray background, but certain target genes designated FOG-1 independent, e.g., *Sialy8*, are not fully regulated by ER-GATA-1(V205G). (D) Linear regression analysis of changes (*n*-fold) plotted as 25°C/37°C and V205G/wild-type (WT) ratios. The inset graph represents an expansion of the lower values. (E) Multiple modes of GATA-1 transcriptional regulation. The analysis described herein shows that limiting FOG-1 levels at 25°C correlate with defective transcriptional regulation of certain but not all GATA-1 target genes. FOG-1-dependent activated and repressed genes are not appropriately regulated at 25°C, whereas ER-GATA-1 retains the capacity to regulate FOG-1-independent activated and repressed genes at 25°C.

A distinct molecular intermediate was revealed from the analysis of *Brg1* mutant mice. Despite severely impaired β*major* activation in *Brg1* mutant mice, only Pol II and serine 5-phosphorylated Pol II occupancy at the promoter and open reading frame was inhibited. One cannot unequivocally rule out a BRG1 requirement for other steps, as residual BRG1 activity in the mutant mice might fulfill such a requirement. Importantly, the data establish that BRG1 confers maximal Pol II recruitment, perhaps resembling the SWI/SNF activity for mediating late events in peroxisome proliferator-activated receptor γ2 promoter activation during adipogenesis (64).

LCR complex assembly prior to promoter complex assembly was a common theme that emerged from our studies. It is therefore instructive to consider why deleting the LCR from the mouse β-globin locus does not abrogate all molecular events at the β*major* promoter; p45/NF-E2, GATA-1, Pol II, and EKLF occupy the β*major* promoter of an LCR-deleted allele (65, 77, 85). Thus, contact between the LCR and the

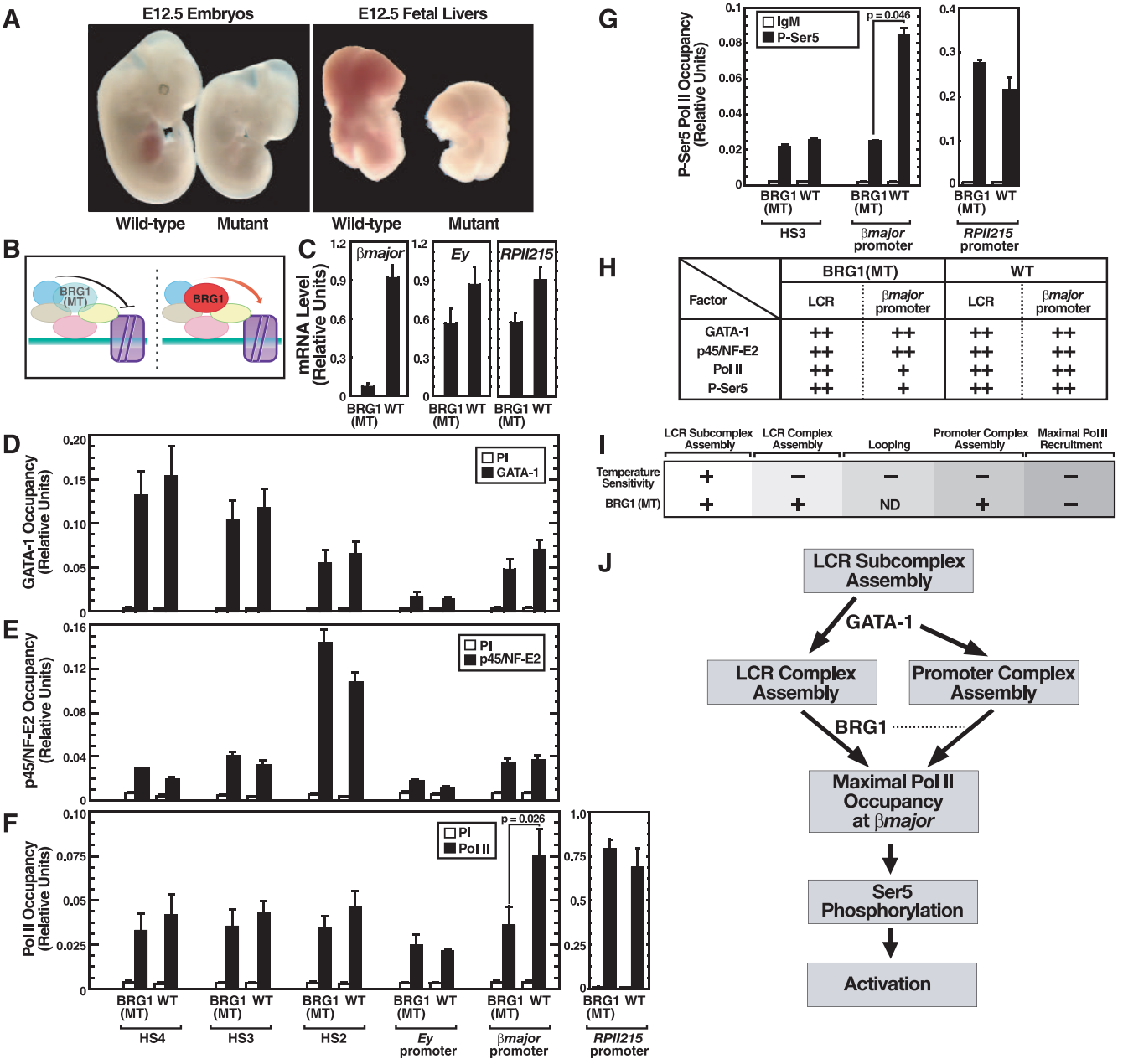


FIG. 7. BRG1 activity is required for maximal Pol II occupancy at the β*major* promoter but not the LCR. (A) Bright-field photographs of wild-type (left) and *Brg1* mutant (right) E12.5 embryos and fetal livers. (B) Schematic representation of wild-type (WT) and BRG1 mutant (MT)-containing SWI/SNF complexes. (C) Quantitative real-time RT-PCR analysis of β*major*, *Ey*, and *RPII215* mRNA transcripts in WT or BRG1 MT E12.5 fetal liver cells. β*major*, *Ey*, and *RPII215* mRNA levels were normalized to *Gapdh* levels. Each graph depicts the relative expression levels of a given gene (*Ey*, β*major*, or *RPII215*) in wild-type versus mutant samples. Thus, one cannot compare the absolute signals of *Ey* and β*major*. (D to G) Quantitative ChIP analysis of GATA-1 (D), p45/NF-E2 (E), Pol II (F), and serine 5-phosphorylated Pol II (G) occupancy at β-globin locus sites and the *RPII215* promoter in WT or BRG1 MT E12.5 fetal liver cells (means ± standard errors for three or four independent experiments). (H) Features of the molecular intermediate revealed by analysis of the *Brg1* hypomorphic allele. The table summarizes GATA-1, p45/NF-E2, total Pol II, and serine 5-phosphorylated Pol II occupancy at the HSs and the promoter of the β-globin locus in BRG1 MT (left) and WT (right) E12.5 fetal liver cells. ++, maximal occupancy detected; +, lower-level occupancy detected. (I) Summary of chromatin domain assembly in G1E cells expressing activated ER-GATA-1 at 25°C and in the fetal livers of BRG1 mutant mice. Steps that occur and are blocked are indicated by + and −, respectively. ND, not determined. (J) Model of GATA-1-mediated β*major* activation. An LCR subcomplex assembles in a GATA-1-independent manner. GATA-1 induces both LCR and promoter complex assembly, but since the LCR deletion does not abrogate promoter complex assembly (65), it is assumed that GATA-1-mediated LCR and promoter complex assemblies involve parallel pathways. Looping is not depicted, since our analyses did not segregate looping from promoter complex assembly. Both BRG1 (Fig. 7F) and the LCR (65) are required to achieve maximal Pol II at the promoter, and therefore, the LCR might utilize BRG1 to confer maximal Pol II recruitment. However, an important BRG1 function through the promoter cannot be ruled out. Finally, the model depicts the reported LCR-enhanced occupancy of the promoter by serine 5-phosphorylated Pol II (65). PI, preimmune sera.

promoter via looping is not required for assembly of at least these components at the promoter. As ~50% and ~70% of promoter-bound Pol II and serine 5-phosphorylated Pol II, respectively, are lost from the LCR-deleted allele (65), and β*major* transcription is strongly reduced (5, 19), both resembling the locus in *Brg1* mutant mice (Fig. 7), it is attractive to propose that the LCR utilizes BRG1 to confer maximal Pol II recruitment to the promoter (Fig. 7J). Defective BRG1 utilization might therefore underlie the LCR deletion phenotype. Since the LCR deletion changes the subnuclear localization of the β-globin locus (61), it will be informative to determine if BRG1 regulates the subnuclear localization of the locus.

Since ~50% of Pol II persists at the promoter of an LCR-deleted allele (65) and in *Brg1* mutant mice (Fig. 7F), and β*major* expression is severely reduced with the LCR-deleted allele (5, 19) and in *Brg1* mutant mice (Fig. 7C), the relationship between Pol II occupancy and mRNA accumulation is clearly not linear. Serine 5 phosphorylation of the Pol II carboxy-terminal domain was ~70% lower in the LCR-deleted allele than in the wild-type allele, and therefore, it was proposed that serine 5 phosphorylation can be limiting (65) (Fig. 7J). The 71% decrease in serine 5-phosphorylated Pol II in *Brg1* mutant mice, which resembles the ~90% decrease in β*major* mRNA, indicates that both the LCR and BRG1 function to ensure maximal serine 5-phosphorylated Pol II at the promoter. Further dissecting how the molecular intermediates described herein assemble and disassemble and the BRG1 activity for selectively maximizing Pol II at the promoter will provide additional fundamental insights into the function of regulatory complexes dispersed over a long distance on a chromosome.

ACKNOWLEDGMENTS

We acknowledge support from NIH grants DK50107 (E.H.B.) and DK55700 (E.H.B.) and an American Heart Association Predoctoral Fellowship (S.-I.K.).

We thank Jim Bieker for providing the EKLF antibody and Kirby Johnson for generating V205G clonal lines.

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