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The role of transcriptional activator GATA-1 at human β-globin HS2

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ABSTRACT

GATA-1 is an erythroid activator that binds β-globin gene promoters and DNase I hypersensitive sites (HSs) of the β-globin locus control region (LCR). We investigated the direct role of GATA-1 interaction at the LCR HS2 enhancer by mutating its binding sites within minichromosomes in erythroid cells. Loss of GATA-1 in HS2 did not compromise interaction of NF-E2, a second activator that binds to HS2, nor was DNase I hypersensitivity at HS2 or the promoter of a linked ε-globin gene altered. Reduction of NF-E2 using RNAi confirmed the overall importance of this activator in establishing LCR HSs. However, recruitment of the histone acetyltransferase CBP and RNA pol II to HS2 was diminished by GATA-1 loss. Transcription of ε-globin was severely compromised with loss of RNA pol II from the transcription start site and reduction of H3 acetylation and H3K4 di- and tri-methylation in coding sequences. In contrast, widespread detection of H3K4 mono-methylation was unaffected by loss of GATA-1 in HS2. These results support the idea that GATA-1 interaction in HS2 has a prominent and direct role in co-activator and pol II recruitment conferring active histone tail modifications and transcription activation to a target gene but that it does not, by itself, play a major role in establishing DNase I hypersensitivity.

INTRODUCTION

Transcriptional activators bind to enhancers and/or promoters, consequently initiating the transcription of a gene at the proper level. One of the roles of transcriptional activators is to recruit co-activators with chromatin modifying activities such as histone acetyltransferase (HAT) activity or ATP-dependent nucleosome remodelling activities that alter chromatin structure to a favourable status for transcription in regulatory regions and genes. The locus control region (LCR) of the β-globin genes is a complex transcriptional enhancer element (1,2). Numerous binding motifs for transcriptional activators including NF-E2 and GATA-1 cluster in the LCR DNase I hypersensitive sites (HSs) and are selectively occupied by the corresponding factors (3–5). Null mutations in murine erythroid cell lines indicate that these activators have important functions in chromatin remodelling and histone modification in the β-globin locus and transcription activation of the β-globin gene (6–8).

Gata-1 is essential for the maturation and differentiation of erythroid cells (9–11) likely through pleiotropic mechanisms. In mouse and human erythroid cells, GATA-1 occupies HS1–HS4 of the β-globin LCR and the promoters of actively transcribed globin genes (3,5). In murine erythroid G1E cells where the expression of GATA-1 protein can be regulated, transcription of the β-globin gene is strongly induced by restoration of GATA-1 function (4,7,8,12). The level of histone acetylation is also increased at the LCR HSs and the activated gene in this system and CBP, a co-activator HAT, associates with LCR HSs in a manner that is at least partially dependent on restoration of GATA-1 function (8,13). Moreover, experiments using this system indicate that GATA-1 function is required for loop formation (physical interaction) between the LCR and the β-globin gene which occurs upon transcription activation (14). Although these experiments support the idea that the effects of GATA-1 restoration are mediated directly through GATA-1 LCR (or promoter) occupancy, one cannot rule out the possibility that the effects are indirectly caused by other factors whose expression or function might be influenced by GATA-1 (7).

Individual NF-E2 or GATA-1 binding sites have not been ablated in their endogenous chromosomal setting. We have addressed the function of these activators at LCR sites by employing a model system in which LCR HS2 is linked to a complete ε-globin gene on chromatinized minichromosomes in erythroid K562 cells. HS2 activates minichromosomal ε-globin transcription to a very

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high level in this system. HS2 also induces an active chromatin structure across the 6 Kb model locus, including nucleosome remodelling in discrete regions and locus wide histone modification, faithfully recapitulating the chromatin structure at the endogenous locus (15,16). NF-E2, which binds to HS2, plays an essential role in the function of HS2. When NF-E2 binding sites are mutated, transcription of the linked globin gene is completely abolished and chromatin structure is not altered to an open configuration either in HS2 or at the linked gene. The complete inactivation resulting from inhibition of NF-E2 binding in HS2 has raised questions about which functions of HS2 are directly contributed by this activator and which functions might be contributed by other activators, such as GATA-1, whose interaction at HS2 might be precluded by loss of NF-E2. A study specifically mutating GATA-1 sites in HS2 could reveal the direct role of GATA-1 bound to this site.

To understand the role of GATA-1 interaction at the HS2 enhancer, we mutated the two GATA-1 motifs 3′ of the tandem NF-E2 motifs in HS2 on minichromosomes carrying a 6 Kb HS2/ε-globin locus. NF-E2 interacted normally with HS2 under these conditions. We found that GATA-1 was required for full function of the HS2 enhancer. Direct roles attributable to GATA-1/HS2 interaction included recruitment of CBP and RNA pol II to HS2 and of pol II to the ε-globin gene promoter. Hyperacetylation of H3 and di- and tri-methylation of H3 K4 in the ε-globin coding region depended on binding of GATA-1 to HS2. In contrast to loss of NF-E2 binding within HS2, loss of GATA-1 binding did not affect DNase I HS formation at HS2 or at ε-globin, even though gene transcription was severely compromised. The importance of NF-E2 to DNase I HS formation at the endogenous LCR in K562 cells was confirmed by reducing this factor using RNAi. These studies delineate different direct roles for NF-E2 and GATA-1 at HS2 in chromatin modification and transcription of human globin genes.

MATERIALS AND METHODS

Minichromosome construction and cell culture

Minichromosomes containing HS2 and the ε-globin gene have been described previously (16). The GATA-1 binding motifs in HS2 were mutated by site-directed mutagenesis in minichromosomes (17). The minichromosomes were transfected into K562 cells, and multiple individual clones were selected and grown in RPMI 1640 medium containing 10% FBS and hygromycin as a selective marker. Minichromosome copy numbers were typically 10–15 per cell in these clones. Thus, the contribution of the endogenous signal to chromatin immunoprecipitation results is minimal and all observed changes can be attributed to the mutation introduced into the minichromosomes.

Reverse Transcription-PCR (RT-PCR)

RNA was prepared from 5×10^6 K562 cells carrying minichromosomes by use of the PUREscript kit (Gentra). Before reverse transcription, 1 μg of RNA was treated with RNase free-DNase I. Purified RNA (0.5 μg) was reverse transcribed with random hexamers using the Superscript III first-strand synthesis system (Invitrogen). cDNA was diluted to 400 μl, and 2.5 μl of cDNA was amplified in a 12.5 μl reaction volume by real-time qPCR using the ABI Prism 7900 (PE Applied Biosystems). The amount of cDNA was compared with that of a genomic DNA standard purified from K562 cells carrying minichromosomes and then corrected by the amount of actin cDNA compared with the genomic DNA.

DNase I sensitivity assay

Nuclei were prepared from K562 cells containing minichromosomes as described previously (15). Aliquots of 3×10^6 nuclei were digested with 10 to 80 U DNase I in a 100 μl volume for 10 min at room temperature. DNA was purified and run on a 1% agarose gel to visualize the level of digestion. The digested DNA was quantitatively compared with undigested DNA by real-time qPCR with normalization to the actin signal (18).

Chromatin immunoprecipitation (ChIP)

ChIP assays for nonhistone proteins and histone proteins was carried out using chromatin cross-linked with 1% formaldehyde (19). Nuclei were prepared from 2×10^7 K562 cells after fixation and subjected to MNase digestion and sonication to generate chromatin of primarily mono- and di-nucleosome size. The fragmented chromatin was reacted with antibody in 800–1000 μl volume, immunoprecipitated with protein A or G agarose beads and washed in five times before eluting DNA as described (19). The input and immunoprecipitated DNA were quantitatively compared by real-time qPCR. The relative intensity for each primer pair was determined by comparing the amount of target sequence in 1.25% of immunoprecipitated DNA to the amount of target sequence in 0.01% of input DNA.

Antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for GATA-1 (sc-1233), CBP (sc-369), NF-E2 (sc-291) and Pol II (sc-899), from Abcam (Cambridge, UK) for mono-methylated (K4) histone H3 (ab8895), and from Upstate Biotechnology (Lake Placid, NY, USA) for di-acetylated (K9, K14) histone H3 (06-599), di-methylated (K4) histone H3 (07-030) and tri-methylated (K4) histone H3 (07-473). Normal rabbit Ig G (sc-2027) was purchased from Santa Cruz Biotechnology.

Lentivirus production and transduction

The lentiviral shRNA vector was constructed in plasmid pLV-TH (Dr. Didier Trono, University of Geneva, Geneva, Switzerland). Target sequences for knockdown of NF-E2 were: NF-E2 shRNA (GATCCCCAGGATT ATCCCTCAACTA), mutant NF-E2 shRNA (GATCC CCAGCATTAACCCTCAACTA). Virus stock was generated by transfecting each lentiviral vector into 293T/17 cells using a standard calcium phosphate precipitation technique. The appropriate lentiviral vector plasmid (10 μg) and the packaging vector plasmids (3.5 μg of pMD.G and 6.5 μg of pΔ8.2) were co-transfected into 80% confluent 293T/17 cells. Viral supernatants were

harvested at 48 and 72 h after transfection and filtered through a 0.45 μm pore size filter. Viral supernatants were concentrated to high titer by ultracentrifugation for 90 min at 50 000 × *g* in a Sorvall centrifuge and the viral particles were then resuspended in RPMI1640 containing 10% FBS. Transduction of K562 cells was performed in the presence of 8 μg/ml polybrene (Sigma Chemicals). Knockdown cell lines were monitored using EGFP expression under fluorescence microscopy and sibselection. Knock down of NF-E2 protein was confirmed by western blot analysis.

Real-time PCR analysis, primers and TaqMan probes

DNA obtained from reverse transcription, DNase I digestion and ChIP assay was analysed by quantitative real-time PCR (ABI Prism 7900) using TaqMan probes and primers (Primer Express 1.0, PE Applied Biosystems). Amplification was carried out with 200 nmol of TaqMan probes and 900 nmol of primers in a 12.5 μl reaction volume. Data were collected at the threshold where amplification was linear and analysed by the comparative Ct method. Sequences of primers and TaqMan probes have been described previously (16) and the locations of all amplicons in the minichromosomal locus are indicated in Figure 1A.

RESULTS

Transcriptional inactivation of the ε-globin gene by inhibition of HS2 GATA-1 binding

GATA-1 binds to LCR HSs (HS1, 2 and 4) in the endogenous human β-globin locus in K562 cells (5). Mutation of a canonical GATA-1 binding motif in core HS2 (374 bp) in the context of minichromosomes stably maintained in K562 cells decreased transcription of a linked embryonic ε-globin gene to 20% of the level obtained with a wild type HS2 (20). More recently, we have studied the mechanism by which the HS2 enhancer remodels chromatin and activates transcription in this model locus using a longer 1.46 Kb HS2 fragment containing the core and flanking sequences (Figure 1A) (16). A more extensive mutation including an imperfect GATA-1 motif near the canonical site was created within this longer HS2 fragment (Figure 1B) (17).

We verified that mutation of the GATA-1 motifs resulted in loss of GATA-1 binding by performing ChIP assays using an antibody against GATA-1 in K562 cells carrying minichromosomes. The K562 cells were cross-linked using formaldehyde and chromatin was fragmented into mono and di-nucleosome size fragments by MNase digestion and sonication and reacted with antibodies. DNA was extracted from the immunoprecipitated chromatin and analysed by quantitative real-time PCR with TaqMan probes. The HS2 core region was strongly immunoprecipitated by GATA-1 antibodies in K562 cells carrying unmutated HS2 minichromosomes, but the immunoprecipitation was greatly reduced in cells carrying GATA-1 mutant minichromosomes (Figure 1C) as expected (17).

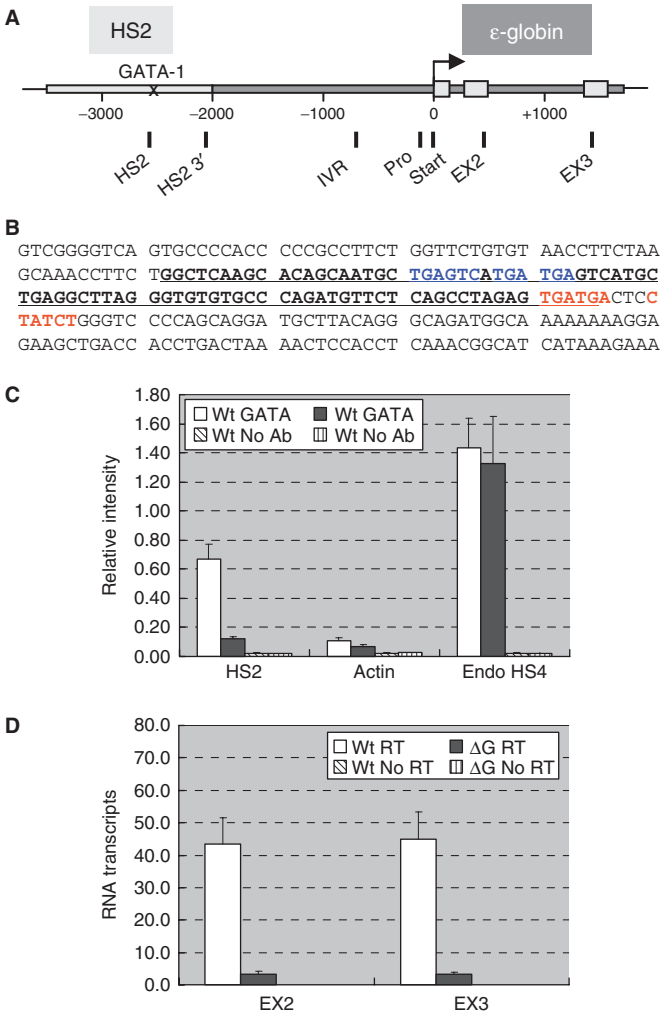

Figure 1. Inhibition of GATA-1 binding and ε-globin transcription on minichromosomes containing HS2 and the ε-globin gene. (**A**) LCR HS2 (1.46 kb) was linked to the ε-globin gene (3.7 kb) in the minichromosome locus. A GATA-1 mutant locus was created in which GATA-1 motifs in HS2 were destroyed by clustered point mutations. Amplicons used in real-time PCR are indicated by vertical bars below the diagram and named. (**B**) NF-E2 (blue) and GATA-1 (red) motifs in HS2 and the HS2 TaqMan amplicon (bold and underlined) are indicated in the wild type sequence of the HS2 core region. Imperfect GATA-1 motif TGATGA and canonical GATA-1 motif CTATCT (inverted motif) were changed to TTCATA and CGCGAT, respectively. (**C**) ChIP was performed with an antibody specific to GATA-1 in K562 cells carrying minichromosomes for the wild type (Wt) or GATA-1 mutant (ΔG) loci. Relative intensity was determined by quantitatively comparing input with immunoprecipitated DNA for the indicated amplicons (see Materials and Methods). Actin and endogenous HS4 (Endo HS4) served as negative and positive controls, respectively. No Ab, no antibody. The results of three independent experiments ± SEM are graphed. (**D**) cDNA was prepared from RNA isolated from K562 cell containing minichromosomes and then amplified by real-time PCR using primers and probes for exon 2 and 3 of the ε-globin gene. Control samples were generated without reverse transcriptase (No RT). The results of four independent experiments ± SEM are graphed.

To confirm the importance of GATA-1 interaction at HS2 to transcription of the linked ε-globin gene, we measured transcripts using RT real-time PCR. RNA was purified from the K562 cells carrying either wild type or

GATA mutant loci and cDNA was generated using random hexamers after RNase-free DNase I treatment. Figure 1D shows that the GATA motif mutations reduced ε-globin transcription to almost undetectable levels (7% of the wild type level), as expected (17). This modified locus provides a highly appropriate tool with which to analyse the direct role of GATA-1 interaction at HS2 in recruitment of co-activators and chromatin remodelling of a target globin gene.

Non-reciprocal influence of GATA-1 and NF-E2 HS2 interaction

When NF-E2 binding was inhibited at core HS2 in minichromosomes, the occupancy of histone acetyltransferase CBP at HS2 was severely reduced (16,21). However it was not clear from these experiments whether NF-E2 directly recruited CBP, because the HS2 DNase I HS fails to form in the absence of NF-E2 binding and this might preclude interaction at HS2 of other activators such as GATA-1 that might play a direct role in the recruitment. To examine this possibility, we used ChIP assays and real-time qPCR to analyse GATA-1 occupancy at HS2 after mutation of the tandem NF-E2 sites. Figure 2A shows that the robust detection of GATA-1 in HS2 is severely reduced when the NF-E2 sites are mutated. However, the reverse is not true. Figure 2B shows that mutation of the GATA motifs in HS2 has no effect on NF-E2 occupancy, consistent with results in G1E cells (3). Thus, the effect of the loss of GATA-1 from HS2 can be independently assessed in this model system. These data also indicate that NF-E2 does not substantially interact with the embryonic ε-globin promoter as it does with the adult β-globin gene promoter (3,22).

GATA-1 at HS2 is required for CBP and RNA pol II recruitment to HS2

In murine G1E cells, restoration of GATA-1 leads to increased detection of CBP and of RNA pol II at LCR HS2 (3,13). We analysed CBP and pol II occupancy at HS2 using ChIP assays and real-time PCR when the GATA-1 motifs were mutated. CBP occupancy at HS2 was reduced to 30% of normal by inhibition of GATA-1 binding at HS2 and CBP was no longer detected above background levels at ε-globin (Figure 3A). A 60% reduction in pol II recruitment was observed at HS2 in the absence of GATA-1 interaction there (Figure 3B) while the very high levels of pol II recruited to ε-globin were strongly reduced by the HS2 GATA mutation. Overall, these results show that inhibition of GATA-1 binding in HS2 recapitulates in reverse the effects of restoration of GATA-1 function in G1E GATA-1 null erythroid cells (4,7,8,12,13). This is consistent with the effects of GATA-1 on CBP and pol II recruitment being mediated directly through binding in HS2.

The formation of DNase I hypersensitive sites is independent of the association of GATA-1 with HS2

The HS2 core region is hypersensitive to DNase I in the endogenous globin locus and in the context of minichromosomes. The formation of the hypersensitive site in

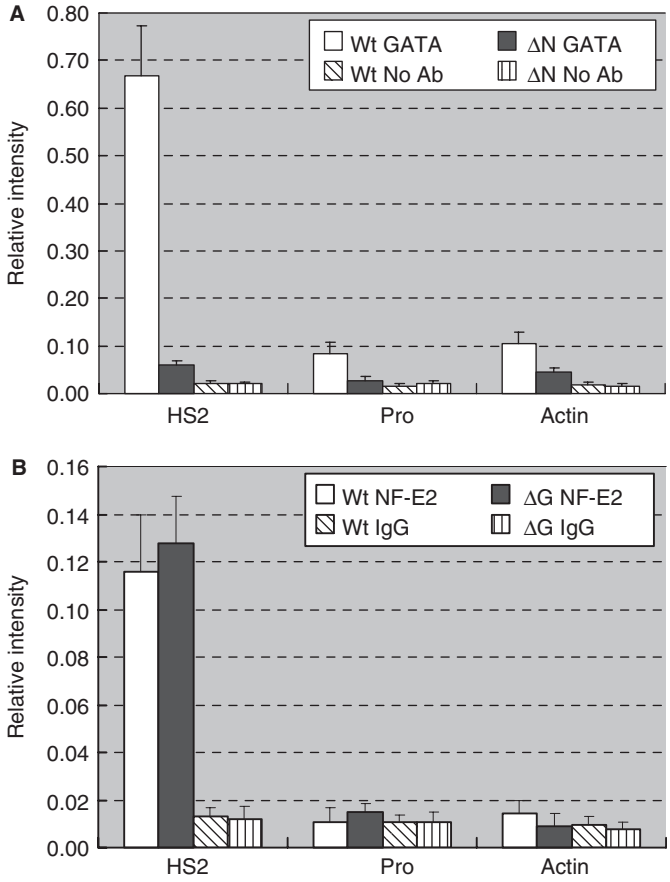

Figure 2. Interaction of NF-E2 at HS2 in the absence of GATA-1 binding there. (**A**) ChIP was performed with an antibody specific to GATA-1 in K562 cells carrying minichromosomes for wild type (Wt) or NF-E2 (ΔN) mutant loci. Relative intensity was determined as described in Materials and Methods. Actin served as negative controls. No Ab, no antibody. The results of two or three independent experiments ± SEM are graphed. (**B**) ChIP was performed with an antibody specific to NF-E2 in K562 cells carrying minichromosomes for wild type or GATA-1 mutant loci. Actin and normal rabbit IgG (IgG) served as experimental controls. The results of three independent experiments ± SEM are graphed.

HS2 is dependent on the interaction there of NF-E2 on minichromosomes (15,16). To ask whether GATA-1 binding contributes to the formation of HS2 chromatin structure, DNase I sensitivity of the minichromosomal globin locus was quantitatively measured in K562 cells containing normal and GATA site mutant HS2 minichromosomes. Nuclei were prepared and digested with DNase I at increasing concentrations (Figure 4A). DNA was purified from the digests and quantitatively analysed by comparison to undigested control DNA of the same cells using real-time PCR (Figure 4B).

HS2 was similarly sensitive to DNase I in wild type and mutant templates similar to results with G1E cells in the absence of GATA-1 function (3). Surprisingly, and in contrast to earlier data obtained with a smaller HS2 enhancer on minichromosomes (20), the analysis showed that DNase I hypersensitivity at the ε-globin promoter was not affected by loss of GATA-1 binding in HS2. In addition, other regions across the locus such as between HS2

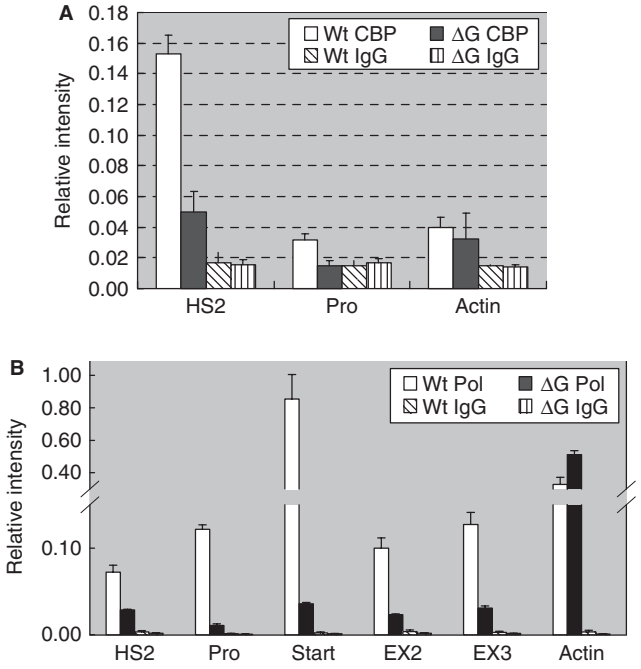

Figure 3. GATA-1 at HS2 influences CBP and pol II recruitment. Chromatin was immunoprecipitated with antibodies specific to (**A**) CBP or (**B**) RNA pol II. Input and immunoprecipitated DNA were analysed by real-time PCR with the indicated amplicons. Normal rabbit IgG (IgG) served as experimental control. The results of two or three independent experiments ± SEM are graphed.

and the linked gene and coding sequences were similarly sensitive in both loci. Thus, ε-globin transcription but not promoter hypersensitivity depends on GATA-1 interaction in HS2. We conclude that in the minichromosomal context both the promoter and HS2 enhancer hypersensitive sites form independently of GATA-1 binding in HS2. Promoter hypersensitivity in the absence of GATA-1 binding in HS2 is consistent with the retention of some pol II occupancy at ε-globin in the absence of GATA-1 interaction within HS2 (Figure 3B) and with maintenance of an open chromatin structure at the β^{maj} promoter in LCR deleted mice (23).

As we have seen, NF-E2 occupancy at HS2 was unaffected by loss of GATA-1 binding there. We had previously observed an important role for NF-E2 within HS2 in remodelling of the linked ε-globin gene promoter (15). To validate this observation in the endogenous globin locus, we knocked down expression of NF-E2 in K562 cells using RNA interference. NF-E2 protein was strongly reduced in NF-E2 shRNA cells (53% of WT) and expression of ε-globin RNA was reduced, while expression of a control shRNA had no effect (Figure 4C and data not shown). Reduction of NF-E2 had a widespread influence on the DNase hypersensitive sites in the globin LCR reducing sensitivity to about 40% compared to that observed with the control RNAi (Figure 4D). These data confirm the importance of NF-E2 for formation of the LCR HSs.

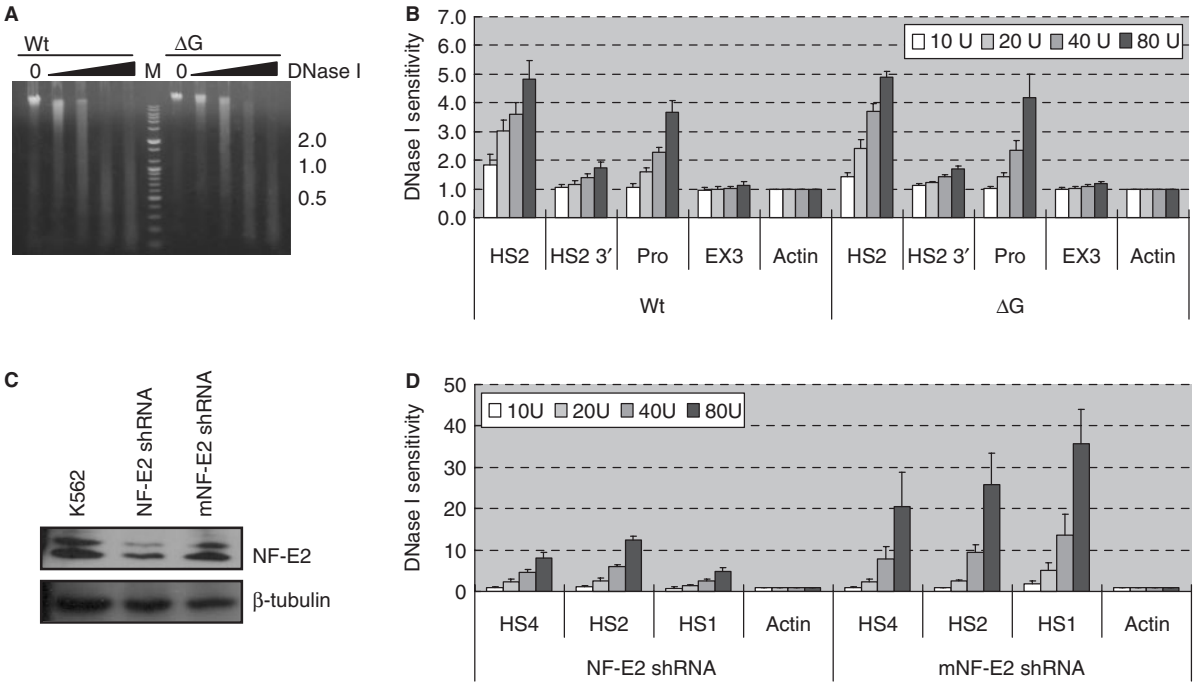

Figure 4. DNase I sensitivity independent from GATA-1 binding. (**A**) Nuclei of K562 containing minichromosomes for wild type and GATA-1 mutant loci were digested with 10 to 80 U DNase I. DNA extracted from the digest was run on 1% agarose gel. (**B**) DNase I sensitivity was determined by quantitatively comparing digested DNA with undigested DNA with normalization to the sensitivity at the endogenous actin gene at each concentration. The TaqMan primer/probe combinations were the same as for ChIP analysis (Figure 1A). The results are averages of four or five chromatin preparations ± SEM. (**C**) Western blotting was performed using an NF-E2 antibody and protein extract from K562 cells stably expressing NF-E2 short hairpin RNA (NF-E2 shRNA) or a control shRNA (mNF-E2 shRNA). Blotting with β-tubulin antibody was used an experimental control. (**D**) DNase I sensitivity was determined at HS1, HS2 and HS4 of the endogenous LCR in wild and mutant NF-E2i K562 cells as described in B.

H3 acetylation and K4 methylation in the ε-globin open reading frame are variably affected by GATA-1 interaction at HS2

Acetylation of histone H3 N-terminal tails is observed in active chromatin domains (24). On minichromosomes, the entire HS2-globin gene locus is marked by this modification and the acetylation is dependent on NF-E2 interaction at HS2 (16). GATA-1 independent HS2 formation allowed us to define independently the direct role of GATA-1 in histone acetylation in the locus. Mono- and di-nucleosomes were reacted with antibodies and small amplicons were used in real-time qPCR, generating high resolution results at the nucleosome level. As shown in Figure 5A, histones were markedly less acetylated in the ε-globin gene coding region in the absence of GATA-1 association at HS2. However at HS2 and in the intervening region between HS2 and the gene there were no major alterations in H3 acetylation. We conclude that binding of GATA-1 at HS2 is required for histone acetylation of the ε-globin gene.

H3 K4 di-methylation accompanies H3 acetylation in active chromatin regions (25). K4 can be modified at the levels of mono-, di- or tri-methylation. We had observed prominent K4 mono-methylation in the LCR and K4 tri-methylation in the highly transcribed γ-globin genes of the endogenous globin locus in K562 cells (26). To understand the role of GATA-1 in H3 K4 methylation we determined the levels of mono-, di- and tri-methylation using ChIP and real-time PCR in normal and GATA-1 mutant loci.

Mono-methylation at H3 K4 was widely observed in the minichromosome globin locus, and was more prominent in HS2 and the intervening region between HS2 and ε-globin than in the coding region (Figure 5B). This pattern across the locus was not affected by GATA-1 binding at core HS2. H3 K4 di-methylation decreased modestly in the ε-globin coding region when GATA-1 was lost from HS2 and increased K4 di-methylation was detected between HS2 and ε-globin (Figure 5C). In contrast, K4 tri-methylation was almost completely lost from the gene coding region in the HS2 GATA-1 mutant locus (Figure 5D). The decreases of K4 di- and tri-methylation in ε-globin coding sequences correspond to the loss of gene transcription in the absence of GATA-1 interaction at HS2. That mono-methylation of K4 across the locus is unaffected by loss of GATA-1 suggests that this modification is either targeted to the locus independent of GATA-1 or is the result of random, untargeted events.

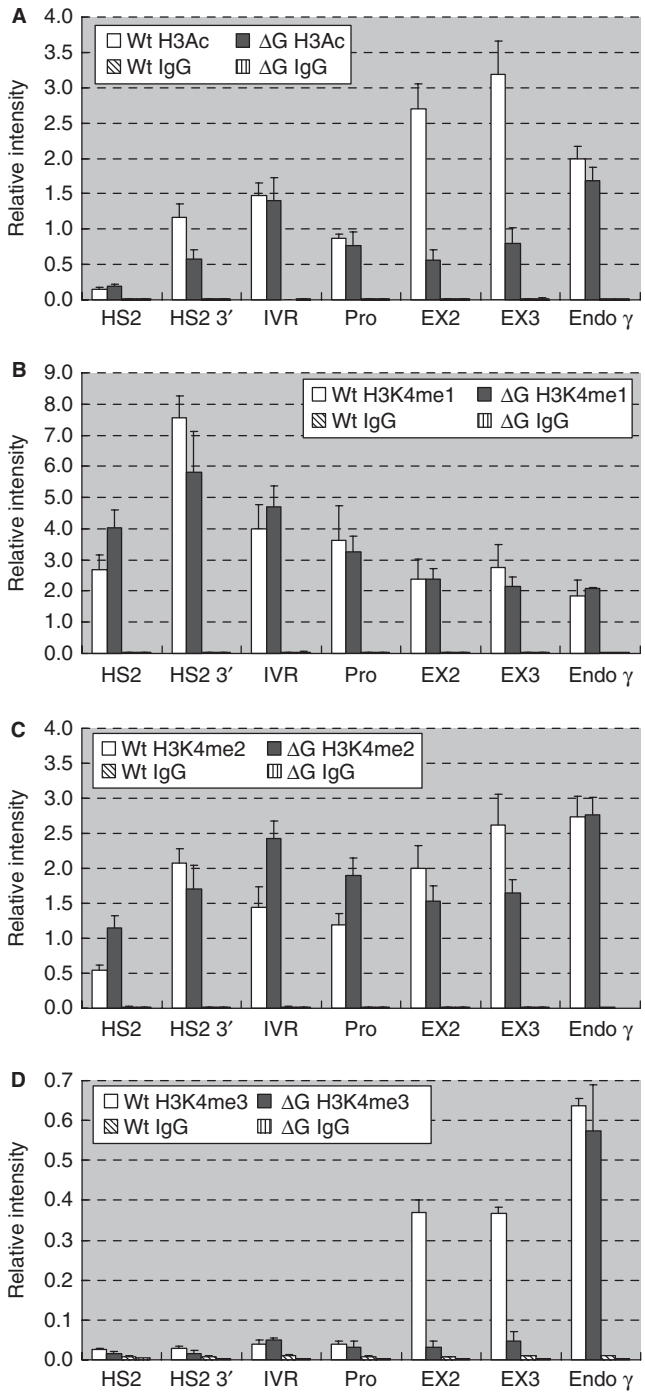

Figure 5. GATA-1 interaction at HS2 increases H3 acetylation in ε-globin but variably affects H3 K4 methylation. Chromatin was immunoprecipitated with antibodies specific to (**A**) diacetylated H3 (K9 and K14), (**B**) mono-methylated H3 K4, (**C**) di-methylated H3 K4 and (**D**) tri-methylated H3 K4. Relative intensity was determined as described in Materials and Methods. Controls include normal rabbit IgG (IgG) and endogenous γ-globin (Endo γ) where histone modifications are not affected by deletion of the minichromosomal HS2 site. The results of two to four independent experiments ± SEM are graphed.

DISCUSSION

This study addressed the roles of erythroid activator GATA-1 mediated by direct interaction at the LCR HS2 enhancer in activating chromatin structure and transcription of the embryonic ε-globin gene. Recruitment of a second erythroid activator NF-E2 to HS2 was independent of GATA-1 binding, but interaction of both factors was required for full enhancer function. Through use of a model HS2/ε-globin locus we were able to distinguish distinct roles for these two activators in chromatin modification and gene activation.

GATA-1 in histone modifications

In murine erythroid GATA-1 null cells, CBP recruitment and histone acetylation require the restoration of GATA-1 (4,8). We show that these properties of GATA-1 result directly from its binding in HS2 and not from an indirect effect on other activators. GATA-1 bound to HS2 might facilitate histone acetylation by interacting with CBP as observed *in vitro* (27). However GATA-1 alone does not appear to be sufficient for recruiting CBP to LCR HSs, because in the endogenous human β-globin LCR GATA-1 is strongly detected in HS1, HS2 and HS4, but CBP is substantially detected only at HS2 (5). NF-E2, which is specifically enriched specifically at HS2, is likely to be also required for optimal CBP recruitment at endogenous and minichromosomal HS2. The physical interaction of the NF-E2 p45 subunit with CBP *in vitro* and *in vivo* supports the idea that NF-E2 has a role in CBP recruitment to HS2 (28). Therefore both GATA-1 and NF-E2 appear to be required for full CBP recruitment to HS2.

We found that methylation of H3 K4 was variably related with GATA-1 interaction at HS2. K4 mono-methylation across the model locus was unaffected by loss of GATA-1 binding at HS2, indicating that this modification is not directly targeted by GATA-1. Mono-methylation might be a mark for potentially active chromatin since it is observed across the endogenous globin locus in K562 cells (26). K4 di-methylation, which is also a mark for active chromatin, was modestly affected by the GATA-1 binding. Interestingly, the intervening region between the enhancer and gene was marked by mono and di-methylation in the wild and mutated model loci. The presence of these marks might relate to intergenic transcription in this locus or be indicative of a poised state of the locus as has been suggested (29) and these events might be GATA-1 independent. In contrast, tri-methylation of H3 K4 was lost within the ε-globin coding region when GATA-1 binding in HS2 was lost. The strong GATA-1 dependence of tri-methylation at H3 K4 was also observed in GATA-1 null cells (4). However it is difficult to conclude that GATA-1 plays a direct role in tri-methylation at H3 K4, because the human histone methyltransferase sub-unit Ash2L binds to HS2 independent of GATA-1 (5). We suggest that the loss of K4 tri-methylation relates to failure to recruit pol II to ε-globin and loss of transcription since this modification is known to be linked to the transcriptional apparatus (30). The role of GATA-1 at HS2 in these events may reflect its required role in LCR-gene looping (14).

Distinct roles of GATA-1 and NF-E2 in chromatin modification and Pol II recruitment

The present studies using quantitative methods indicate that DNase I hypersensitive sites form at HS2 and the ε-globin promoter on minichromosomes without GATA-1 binding in HS2. Earlier work showed that these sites HS failed to form in the absence of NF-E2 binding in HS2 and our RNAi study indicates the widespread influence of NF-E2 loss on globin locus LCR HSs formation (16). Over all, these results suggest that formation of HSs is strongly influenced by NF-E2 and not GATA-1 consistent with the ability of NF-E2 to remodel chromatin in the absence of a SWI/SNF remodelling activity (31). That GATA-1 is dispensable for HS2 formation is consistent with high accessibility to restriction enzymes at HS2 in murine GATA-1 null cells (4). However, GATA-1-independent HS formation at ε-globin contrasts with the results using G1E cells and also with earlier work using a different globin minichromosome with a truncated HS2 (20). In addition, another series of studies using stable transfections indicated that both NF-E2 and GATA-1 contributed to HS formation (32–34). The reasons for these varying results are unclear. However, we note that residual pol II within ε-globin after loss of the HS2 GATA-1 sites is consistent with an open chromatin structure there. Furthermore, in LCR-deleted mice the β-globin gene promoter remained a DNase sensitive structure, although it was transcribed at a very low level, similar to our findings here (23).

The importance of GATA-1 to pol II recruitment to the promoter of the β-major globin gene was observed in murine erythroid GATA-1 null cells (3,13) and the present studies suggest that this is the direct result of lack of GATA-1 at HS2. Despite an open chromatin structure and a normal TATA box, only a low level of pol II (and GATA-1) was detected at a linked ε-globin when the HS2 GATA-1 sites were mutated. These studies using null cells and mutant minichromosomes support a role for GATA-1 in pol II recruitment to active globin genes. However another recent study performed in murine erythroid progenitor cells established from foetal liver reported that the binding of GATA-1 and NF-E2 was not sufficient for pol II recruitment and active chromatin hub formation with close contact between HS2 and β-globin (35). Nevertheless, the occupancy of HS2 by these activators is substantially increased by induction, with ensuing pol II recruitment and active chromatin hub formation in the erythroid cells.

In summary, our results show that inhibition of GATA-1 binding in HS2 mirrors in reverse the effects of restoration of GATA-1 function in G1E GATA-1 null erythroid cells (4,7,8,12,13) indicating that these effects are directly attributable to the HS2/GATA-1 interaction. Furthermore, it appears from the two kinds of studies that GATA-1 function with respect to the adult β-globin and embryonic ε-globin gene is substantially similar. This is interesting in light of the known role of GATA-1 in looping between the LCR HSs and the β-globin gene and the proposal that the ε-globin may instead be regulated by a tracking mechanism (36).

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