Expression of the β-globin genes proceeds from basal to exceptionally high levels during erythroid differentiation in vivo. High expression is dependent on the locus control region (LCR) and coincides with more frequent LCR-gene contacts. These contacts are established in the context of an active chromatin hub (ACH), a spatial chromatin configuration in which the LCR, together with other regulatory sequences, loops toward the active β-globin-like genes. Here, we used recently established I/11 cells as a model system that faithfully recapitulates the in vivo erythroid differentiation program to study the molecular events that accompany and underlie ACH formation. Upon I/11 cell induction, histone modifications changed, the ACH was formed, and the β-globin-like genes were transcribed at rates similar to those observed in vivo. The establishment of frequent LCR-gene contacts coincided with a more efficient loading of polymerase onto the β-globin promoter. Binding of the transcription factors GATA-1 and EKLF to the locus, although previously shown to be required, was not sufficient for ACH formation. Moreover, we used knock-out mice to show that the erythroid transcription factor p45 NF-E2, which has been implicated in β-globin gene regulation, is dispensable for β-globin ACH formation.

The mammalian β-globin gene loci serve as a model system for studying developmental gene regulation. The murine β-globin locus contains four β-like globin genes that are arranged on the DNA in the order of their developmental expression. Expression of the β-like globin genes is restricted to the erythroid lineage. In erythroid progenitor cells, they are expressed at basal transcription levels, comparable with that of most other genes. However, at later stages of erythroid maturation they are expressed at exceptionally high levels. This high transcription rate is dependent on the β-globin locus control region (LCR)\(^2\), a cis-regulatory DNA element located upstream of the β-like globin genes that contains six erythroid-specific DNase I hypersensitive sites (HSs) (Fig. 1A).

Recently developed chromosome conformation capture (3C) technology provides insight into the spatial conformation of the β-globin locus. This technique involves quantitative PCR analysis of formaldehyde cross-links made between selected DNA fragments as a measure of their interaction frequency. 3C technology revealed that high expression of the β-globin genes at later stages of differentiation coincides with the formation of an active chromatin hub (ACH), a spatial configuration of the locus in which the LCR, together with additional HSs upstream and downstream of the locus, loops toward the active β-like globin genes (2). At early stages of erythroid differentiation a smaller chromatin hub (CH), is present. It is composed of contacts between the outer HSs of the locus and part of the LCR but does not contain the genes (3). The β-globin genes switch interaction with the ACH in relation to their transcriptional activity during development. The embryonic genes contact the LCR in embryonic blood, whereas the adult genes do so in fetal liver and adult bone marrow (3). Based on these observations, ACH formation was proposed to be crucial for the high expression rates of the β-globin genes: spatial clustering of cis-regulatory DNA elements ensures a high local concentration of transcription factors, required for efficient gene transcription (4). Although this is likely true for LCR-gene contacts, the functional significance of the outer HSs participating in the hub is still unclear. Deletion of these sites, or depletion of the transcription factor CTCF, which abrogates interactions with the outer HSs, has no measurable effect on β-globin gene expression (5, 6). Little is known about the molecular events that are involved in the establishment of contacts between the LCR and the genes as the locus proceeds from a CH to an ACH during erythroid differentiation.

Mice containing a targeted deletion of the β-globin LCR have provided insight into LCR-independent modifications of the locus (1). Although none of the β-globin genes express beyond basal levels in these mice, they appear to have similar chromatin properties as their wild-type counterparts that express 25–100-fold more efficiently. Thus, the promoters are hypersensitive, contain highly acetylated histones, and bind both basal transcription factors such as TATA-binding protein and tissue-specific transcription factors such as NF-E2 and GATA-1, albeit at
slightly reduced efficiency (7–10). RNA polymerase II (RNAP II) binding to the β-major promoter was reduced only 2-fold in the absence of the LCR, whereas a more dramatic reduction (5-fold) in binding was observed at the third exon of β-major, leading to the conclusion that the LCR primarily acts by enhancing the transition from initiation to elongation (9).

GATA-1, EKLF, and NF-E2 are the best characterized tissue-specific transcription factors involved in β-globin gene transcription. All three factors bind to elements in the β-globin LCR and to promotors of the β-globin genes (11–14). GATA-1 is essential for the development of the erythroid lineage in mice (15), and restoration of GATA-1 activity in a GATA-1-null cell line leads to β-globin gene activation (16). EKLF is essential for adult β-globin gene expression in mice (17, 18). EKLF and GATA-1 both function in β-globin ACH formation; frequent contacts between the LCR and the genes are lacking in their absence (10, 19). The role of NF-E2 in β-globin gene regulation is less well understood. NF-E2 is composed of a ubiquitously expressed subunit, MafK (or p18 NF-E2), and an erythroid-specific transactivator subunit, p45 NF-E2 (20). NF-E2 binds most prominently to HS2 in the LCR but also to an element just downstream of the adult β-globin gene promoter (11, 12). Based on studies using a mouse erythroleukemia (MEL) cell line lacking the p45 subunit, NF-E2 is thought to be essential for β-globin gene transcription and to mediate the hyperacetylation of histones and the deposition of RNAP II at the β-globin gene promoters (21–24). However, mice lacking the erythroid-specific subunit p45 show normal erythropoiesis and express the β-globin genes at slightly reduced levels compared with wild type (25). The current view is that p45 NF-E2 is crucial in certain cultured erythroid cell systems but has redundant functions in β-globin ACH formation.

Here we analyzed the role of erythroid-specific transcription factors and characterize the molecular events that accompany activation of high β-globin gene expression rates at the late stages of erythroid differentiation. Careful analyses of recently established I/11 erythroid cells show that they provide a good model system for these studies, where upon maturation, β-globin gene expression increases to reach the high transcription levels observed in vivo. Increased transcription coincides with stabilized LCR-gene contacts and a more efficient loading of RNAP II onto the β-globin gene promotors. GATA-1 and EKLF bind their target sites in the β-globin locus prior to ACH formation, showing that although required, the mere binding of these factors is not sufficient for the establishment of frequent LCR-gene contacts. Similarly, NF-E2 already binds to the locus in erythroid progenitor cells prior to ACH formation. We used p45 knock-out mice to investigate the role of NF-E2 in β-globin gene regulation and demonstrate that although β-globin gene expression is slightly reduced in the absence of this factor, the β-globin ACH is formed normally. Thus, unlike GATA-1 and EKLF, NF-E2 is dispensable for long-range LCR-gene contacts in the β-globin locus.

EXPERIMENTAL PROCEDURES

Culturing I/11 Cells and Primary Fetal Liver Cells—To generate primary fetal liver cells, livers were isolated from B6 E12.5 embryos, resuspended by repeated pipetting and applied through a 40-μm cell strainer (BD Falcon). Next, primary E12.5 liver cells were cultured as described previously for I/11 cells (29, 30). In short, cells were cultured in serum-free stem cell medium (StemPro-34 SFM, Invitrogen) containing 0.5 units/ml erythropoietin (a kind gift from Ortho Biotech), 100 ng/ml stem cell factor, and 1 μM dexamethasone. Proliferating cells were expanded and kept at a density between 1.5 × 10⁶ and 4 × 10⁶ cells/ml. To induce differentiation, proliferating cells were washed twice with Hank's balanced salt solution and seeded at 2–3 × 10⁶ cells/ml in differentiation medium containing 5 units/ml erythropoietin and 1 mg/ml transferrin (Sigma-Aldrich). During differentiation, cells were kept at densities of 2–6 × 10⁶ cells/ml. Differentiation status was monitored by measuring cellular size distribution using an electronic cell counter (CASY-1, Schärfe-System). Differentiated cells were harvested when size distribution ranged between 6 and 7 μm, which typically took 24–32 h for primary fetal liver cells and 40–48 h for I/11 cells.

Analysis of Gene Expression—Total RNA was isolated from ~1 × 10⁶ cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. 1.5 μg of RNA was treated for 1 h with DNase I (Invitrogen) to remove genomic DNA contamination. cDNA synthesis was performed using Superscript II RNase H-reverse transcriptase (Invitrogen) according to the manufacturer’s instructions using either 500 ng of oligo(dT)₁₂–₁₈ or 200 ng of random hexamers as primers. Quantification of transcripts was performed on Opticon II real-time PCR machines (MJ Research) using Platinum TaqDNA polymerase (Invitrogen) and SYBR Green (Sigma-Aldrich). The following PCR program was used: 2 min at 94 °C, 45 cycles of 30 s at 94 °C, 1 min at 55 °C, 15 s at 72 °C (during which measurements were taken) followed by a 10-min chain extension. Primer sequences are listed in supplemental Table 1. Expression levels were normalized to levels measured for control genes (hypoxanthine guanine phosphoribosyltransferase (HPRT), ribonuclease/angiogenin inhibitor 1 (Rnh1), and 18S).

Chromatin Immunoprecipitation (ChIP)—ChIP analysis was performed as described in the Upstate protocol (www.upstate.com), except that cells were cross-linked using 2% formaldehyde for 5 min at room temperature. Real-time quantification of precipitated DNA sequences (with typical fragment sizes between 300 and 600 bp) was performed on Opticon II PCR machines (MJ Research) using Platinum TaqDNA polymerase (Invitrogen) and SYBR Green (Sigma-Aldrich) under the following cycling conditions: 2 min at 94 °C, 45 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 60 °C and 15 s at 75 °C (during which measurements were taken) followed by a 10-min chain extension. Primer sequences are listed in supplemental Table 1. Expression levels were normalized to levels measured for control genes (hypoxanthine guanine phosphoribosyltransferase (HPRT), ribonuclease/angiogenin inhibitor 1 (Rnh1), and 18S).
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**FIGURE 1.** Characterization of murine primary erythroid progenitor cells. A, schematic presentation of the mouse β-globin locus. Mouse olfactory receptor genes (MOR) are indicated by boxes; embryonic (ey, βH1) and adult (βmaj) and β minor β-globin genes are indicated by triangles. DNase I HSs are indicated by arrows. B, relative β-globin mRNA levels (as determined by quantitative RT-PCR) in non-induced proliferative primary erythroid progenitor cells (I–) and induced differentiated primary erythroid cells (I+) and E14.5 liver cells (set to 1). C, ChIP using an antibody against RNAP II. Plotted are relative enrichments measured for sites in the β-globin locus in non-induced and induced primary erythroid progenitor cells and E14.5 liver cells. Highest value is set to 1. D, quantitative 3C analysis on non-induced and induced primary erythroid cells. Shown are relative cross-linking frequencies with HS2. Highest value is set to 1.

MafK (C-16; sc-477), and anti-Nrf-2 (H-300; sc-13032) (all obtained from Santa Cruz Biotechnology); anti-acetyl histone H3 (catalog No. 06-599, from Upstate); anti-dimethyl histone H3 K9/K27 (ab7312) and anti-histone H3 (ab1791) (from Abcam). Anti-EKLF (5-V) was kindly provided by J. Philipsen, and anti-Bach1 antiserum (A1-5) was kindly provided by K. Igarashi (31). Primer sequences are listed in supplemental Table 1. Data were normalized to input, and enrichment was measured over control gene (necdin or amylase, giving identical results).

3C Analysis—3C analysis using HindIII as restriction enzyme was performed essentially as described (32). Real-time quantification of ligation products was performed on Opticon II PCR machines (MJ Research) using Platinum Taq (Invitrogen) and double-dye oligonucleotides (5'-FAM and 3'-TAMRA) as probes. The following PCR program was used: 2 min at 94 °C, 45 cycles of 15 s at 94 °C and 90 s at 60 °C. Primer and probe sequences are listed in supplemental Table 1. Data were normalized to interaction frequencies measured in the XPB (xero-derma pigmentosum type B) locus to account for differences in quality and quantity of DNA templates.

**RESULTS**

I/11 Cells as a Model System for Studying LCR-mediated β-Globin Gene Expression—Primary erythroid cells isolated directly from mouse fetal liver can be both expanded in vitro and induced to undergo synchronous differentiation to fully mature, enucleated erythrocytes (33). We found that they express the adult β-major gene at basal levels when brought into culture under conditions that enrich for progenitor cells (Fig. 1B). Differentiation of these cells in vitro resulted in an increase in steady-state β-globin messenger RNA levels to amounts similar to that observed in vivo (Fig. 1B). Differentiation was accompanied by an increase in RNAP II-binding to the promoter and third exon of the β-major gene (Fig. 1C) and an increase in interaction frequency between the LCR (HS2) and the β-major gene (Fig. 1D), as determined by an improved 3C strategy that uses Taqman probes for a more quantitative analysis of interaction frequencies (6). However, primary progenitor cells often differentiate spontaneously, which precludes the collection of large homogeneous cell populations required for large-scale ChIP and 3C (34) experiments. We therefore compared I/11 erythroid progenitors (29) with these primary erythroid cells with respect to β-globin gene expression characteristics during erythroid differentiation. I/11 cells are erythroid progenitor cells established from fetal livers of p53−/− mice. The cells can be expanded indefinitely in vitro, and upon exposure to physiologically relevant stimuli such as erythropoietin, they undergo terminal differentiation into enucleated erythrocytes in a synchronized manner (29, 30). Detailed analysis of β-globin gene expression showed that differentiation of I/11 cells was accompanied by an ∼20-fold increase in steady-state levels of β-major transcripts, reaching levels comparable with those observed in vivo (Fig. 2A). Also, RNAP II-binding to the promoter and third exon of the β-major gene increased and reached levels equal to those observed in vivo (Fig. 2B). Finally, more frequent LCR-gene contacts were observed upon differentiation of I/11 cells (Fig. 2C) (3) in a manner similar to that seen in primary erythroid cells (Fig. 1D). Therefore, we conclude that I/11 cells faithfully recapitulate the later stages of erythroid differentiation not only morphologically (29) but also with respect to the transcriptional profile of the β-globin genes. Moreover, we note that non-induced pro-
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**Histone Modifications Accompanying ACH Formation**—Next, ChIP experiments were performed on I/11 cells to investigate whether ACH formation and transcriptional enhancement are accompanied by changes in histone modifications at the regulatory sites of the β-globin locus. Acetylation of histone H3 (AcH3) is a mark for active chromatin. The murine β-globin locus is characterized by high levels of erythroid-specific AcH3 at the cis-regulatory sites present in the LCR and at the promoters of the active genes (35, 36). We found that in non-induced I/11 cells, histones at the HSs of the LCR and the promoter of the active β-major gene, but not at the inactive βh1 gene, were already hyperacetylated. In differentiated I/11 cells, H3 acetylation levels increased maximally 3.5-fold at sites within the LCR and less than 2-fold at the β-major promoter (Fig. 3A). The observation that histone H3 at the β-major promoter is already hyperacetylated prior to ACH formation is in agreement with the fact that promoter hyperacetylation occurs also in the absence of the LCR (8). The data show that a strong increase in β-major transcription activity that takes place upon erythroid differentiation is accompanied by a small increase in the levels of H3 acetylation at the promoter; we therefore investigated whether local abundance of a repressive histone modification also changes upon I/11 differentiation.

Liberating I/11 progenitor cells express the β-globin genes at levels comparable with the basal β-globin gene expression levels observed in transgenic mice lacking the LCR (1). Non-induced cells also lack the frequent β-globin LCR-gene contacts observed in induced I/11 cells, suggesting that proliferating I/11 progenitor cells represent a stage of erythroid differentiation where transcription of the β-globin-like genes is mostly LCR-independent. Indeed, when I/11 cells were analyzed at different time intervals after induction of differentiation, increased expression rates of β-major, as measured by the amount of nascent transcripts (Fig. 2D), coincided well with increased HS2/β-major interaction frequencies (Fig. 2E), supporting the idea that the LCR regulates transcription by contacting the gene. We therefore conclude that I/11 cells are a good model system to study the molecular mechanisms behind LCR-mediated activation of high β-globin gene expression levels.
obtained with antibodies against acetylated and methylated H3 truly reflect differentiation-dependent changes in histone H3 modifications. We conclude that the transition from basal to highly activated \( \beta \)-globin gene expression during erythroid differentiation is accompanied by an increased ratio of positive versus negative chromatin modifications at the \( \beta \)-major promoter and the HSs of the LCR.

GATA-1 and EKLF Are Required, but Not Sufficient, for ACH Formation—Erythroid-specific \( \beta \)-globin gene expression is in part regulated through theaction of lineage-restricted transcription factors such as GATA-1 and EKLF that bind to specific motifs found at regulatory sequences throughout the locus. Recent insight into the regulatory role of these transcription factors has come from EKLF knock-out mice and GATA-1-null cells containing inducible versions of the corresponding proteins. For both transcription factors it was shown that induction of their activity resulted in more frequent contacts between the LCR and the \( \beta \)-major gene and increased \( \beta \)-major expression (10, 19). Without EKLF, the \( \beta \)-globin locus adopted a spatial configuration reminiscent of the CH present in erythroid progenitor cells (19). Thus EKLF and GATA-1 appear to be required for the conformation of the \( \beta \)-globin locus to proceed from a CH containing only the outer HSs of the locus and part of the LCR to an ACH containing also the entire LCR and the actively transcribed gene. The experimental systems used for these studies precluded investigating whether binding of EKLF and/or GATA-1 to the \( \beta \)-globin locus is also sufficient for ACH formation. We therefore analyzed whether EKLF and GATA-1 are bound to the \( \beta \)-globin locus in non-induced I/11 progenitor cells that do not show ACH formation. ChIP experiments on non-induced I/11 cells revealed strong binding of GATA-1 to its target sites in the \( \beta \)-globin locus (Fig. 4A and B). The same was true for its heterodimerization partner, MafK (Fig. 4D). Recruitment of p45 NF-E2 to the \( \beta \)-major promoter in progenitor cells lacking frequent LCR-gene contacts seemed to be in agreement with the observation in mice lacking the LCR that

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![Figure 3](image3.png)

**FIGURE 3.** Differentiation of I/11 cells is accompanied by an increased ratio of positive versus negative chromatin modifications at the LCR and \( \beta \)-major promoter. A–C, ChIP using an antibody against acetylated histone H3 (Ach3) (A), dimethylated lysine 9 and 27 of histone H3 (2MeH3K9/K27) (B), and the C terminus of histone H3 (C). Plotted are relative enrichments measured for sites in the \( \beta \)-globin locus in non-induced (I-11) and induced (I+) I/11 cells. In all panels, S.E. is indicated.

![Figure 4](image4.png)

**FIGURE 4.** GATA-1, EKLF, and p45 NF-E2 are already bound to their cognate binding sites prior to ACH formation. A–E, ChIP using an antibody against GATA-1 (A), EKLF (B), p45 NF-E2 (C), MafK (D), and Bach1 (E). Plotted are relative enrichments measured for sites in the \( \beta \)-globin locus in non-induced (I-) and induced (I+) I/11 cells. In all panels, S.E. is indicated. Note that the scale on the y axis differs between the panels.
NF-E2 binding to the promoter is LCR-independent (9). Upon differentiation of I/11 cells, binding of both p45 NF-E2 and MafK to HS2 and the β-major promoter increased (up to 2.5-fold) (Fig. 4, C and D), as observed also for GATA-1 and EKLF. The small subunit of NF-E2, MafK, which contains a DNA-binding domain, cannot only form a complex with the transcription activator protein p45 NF-E2 but also with the repressive factor Bach1, and the exchange of binding partners from Bach1 to p45 NF-E2 is reported to be a key step in the activation of β-globin gene transcription during MEL cell differentiation (38). Bach1 indeed associates with HS2, but not significantly with the β-major promoter, in non-induced I/11 cells, and its binding to HS2 was lost upon I/11 differentiation (Fig. 4E). Thus, Bach1 dissociates from HS2, whereas p45 NF-E2 binding to this site and the β-major promoter increases as cells progress through the later stages of erythroid differentiation. However, the exchange of MafK binding partners that accompanies the induction of high levels of β-globin gene expression during I/11 cell differentiation is not as absolute as observed during MEL cell differentiation, because we found that p45 NF-E2 was already abundantly present at HS2 and the β-major gene promoter before induction of differentiation. Based on this result we conclude that, like GATA-1 and EKLF, NF-E2 binding to its target sites in the β-globin locus is not sufficient for ACH formation and the induction of high levels of β-globin gene expression.

p45 NF-E2 Is Dispensable for β-Globin ACH Formation—To further investigate the role of p45 NF-E2 in β-globin ACH formation and gene activation, we used mice deficient for this transcription factor (39). We first performed ChIP experiments to confirm that p45 NF-E2 was absent from the β-globin locus in E14.5 livers from NF-E2 knock-out embryos. No recruitment of the protein to HS2 or the β-major promoter was found with an antibody recognizing the C terminus of p45 NF-E2. The same result was obtained using an antibody against the N-terminal part of p45 NF-E2, showing that also no truncated versions of the protein associated with the β-globin locus (Fig. 5A). We then analyzed β-globin gene expression in these mice. Previously this had been analyzed by measuring steady-state messenger RNA and protein levels (25). We used intron-specific primers in a real-time quantitative PCR assay and found that in the absence of p45 NF-E2, the rate of β-major transcription was reduced to ~65% of wild-type levels (Fig. 5B). In agreement, ChIP analyses showed that RNAP II binding to the gene was also reduced to 60–70% in the absence of p45 NF-E2. This reduction was found both at the promoter and the third exon of the β-major gene but not at HS2 of the LCR (Fig. 5C). Thus, depletion of p45 NF-E2 in mice has a mild but significant effect on β-globin gene expression, in agreement with the presence of a mild erythroid defect in these mice (25). Next, we asked whether p45 NF-E2 is required for β-globin ACH formation. Locus-wide 3C technology was applied to samples obtained from E14.5 livers and revealed that interactions of the β-major gene with regulatory DNA elements elsewhere in the locus were not affected by the loss of p45 NF-E2. Thus, the β-globin ACH, containing the outer HSs (HS-85, HS-62/-60, and 3’-H51), the LCR, and the active β-major gene, was formed normally in p45 knock-out mice (Fig. 5D). We noticed that β-major interaction frequencies with HS2 and HS1, both target sites of NF-E2 (11, 37), increased compared with wild type. Subsequently, we investigated whether p45 NF-E2 depletion influenced the binding of its complexing partner MafK to the locus. We found that binding of MafK to the β-major promoter was almost completely abolished in p45 NF-E2 knock-out mice. In contrast, MafK binding to HS2 was still considerable, albeit 2-fold reduced (Fig. 5E). This raised the possibility that MafK associates with other proteins at HS2. Indeed, binding of Bach1 increased 2-fold, and some Bach1 was also found associated with the β-major promoter in the absence of p45 NF-E2 (Fig. 5F). A more dramatic increase in recruitment however was observed when we analyzed Nrf-2 binding. Binding of this factor to HS2 increased 6-fold in p45 knock-out mice compared with wild type (Fig. 5G). No increase in binding efficiency of Nrf-2 was observed at the β-major gene promoter in p45 knock-out liver cells, consistent with the almost complete absence of MafK at this site. Thus, other MafK dimerization partners, most prominently Nrf-2, appear to physically replace p45 NF-E2 at HS2, whereas these complexes are almost completely absent at the β-major promoter in p45 NF-E2 knock-out mice. Collectively, these data show that the transcription factor p45 NF-E2, unlike GATA-1 and EKLF, is not required for the formation of the β-globin ACH.

DISCUSSION

We explored the use of I/11 cells as a model system to study differentiation-dependent β-globin gene activation. I/11 cells are erythroid progenitor cells that upon induction by physiologically relevant stimuli faithfully recapitulate the terminal erythroid differentiation program to mature into enucleated erythrocytes (29, 30). We have shown by several criteria that differentiated I/11 cells do express the β-like globin genes at the very high levels observed in vivo, whereas expression prior to induction is ~20-fold lower, comparable with that observed in mice lacking the LCR. This suggests that upon differentiation of I/11 cells, β-globin gene expression proceeds from an LCR-independent to an LCR-dependent mode. In agreement with this idea is the observation that I/11 cell induction coincides with the establishment of frequent LCR-gene contacts. We show that ACH formation and the induction of high β-globin transcription rates is accompanied by an increased ratio of positive (AcH3) versus negative (2MeH3K9/K27) histone modifications at the cis-regulatory sites of the β-globin locus. Importantly, we also show increased recruitment of RNAP II to the promoter of the active β-major gene. Previously, ChIP experiments have shown that deletion of the LCR caused only a 2-fold reduction of RNAP II at the β-major promoter and a more dramatic reduction (5-fold) at the third exon of β-major, leading to the conclusion that the LCR primarily acts by enhancing the transition from initiation to elongation (9). However, we would argue that this is difficult to conclude from ChIP experiments, because this assay cannot provide a measure for transcription reinitiation. ChIP involves the fixation of cells, which usually takes 5–10 min. RNAP II reloading onto the β-major promoter occurs multiple times during this time frame, but ChIP fails to appreciate this frequency. The fact that we find increased RNAP II recruitment to the promoter once LCR-
gene contacts are established reopens the possibility that the LCR also functions to efficiently recruit RNAP II to the active β-globin genes. NF-E2, GATA-1, and EKLF are the three lineage-restricted transcription factors most prominently associated with β-globin gene regulation. Previous work has demonstrated that the latter two factors are required for the stabilization of long-range DNA contacts between the β-globin LCR and the active adult β-globin-like genes (10, 19). Here, we demonstrate that GATA-1 and EKLF already bind to cognate sites in the β-globin locus at a stage of erythroid differentiation that precedes ACH formation. This shows that binding per se of these factors to the locus is not sufficient for ACH formation. We found that the efficiency of their recruitment was higher in differentiated cells that do form an ACH, which raises the possibility that binding levels are important for GATA-1 and EKLF to mediate ACH formation. An alternative and not mutually exclusive explanation for the higher recruitment in ACH-forming cells is that these cells may have greater nuclear import capabilities. This is currently under investigation.

FIGURE 5. p45 NF-E2 is dispensable for β-globin ACH formation. A, ChIP using antibodies against either the N-terminal or C-terminal part of p45 NF-E2. Plotted are relative enrichments measured for sites in the β-globin locus in E14.5 liver of p45 NF-E2 knock-out embryos (p45−/−) and wild-type (WT) littermates. B, relative β-globin primary transcript levels (as determined by quantitative RT-PCR) in E14.5 liver of p45 NF-E2 knock-out embryos and wild-type littermates (set to 1). C, ChIP using an antibody against RNAP II. Plotted are relative enrichments measured for sites in the β-globin locus in E14.5 liver of p45 NF-E2 knock-out mice and wild-type littermates. Highest value is set to 1. D, quantitative 3C analysis on E14.5 liver of p45 NF-E2 knock-out embryos and wild-type littermates. Shown are cross-linking frequencies between a β-major restriction fragment and several other fragments across the β-globin locus. Data were normalized against cross-linking frequencies measured for two XPB restriction fragments, and the highest value was set to 1. Size and position of restriction fragments analyzed are indicated by vertical gray shading; black shading represents the fixed fragment β-major. The highest value set to 1. E–G, ChIP using an antibody against MafK (E), Bach1 (F), and Nrf-2 (G). Plotted are relative enrichments measured for sites in the β-globin locus in E14.5 liver of p45 NF-E2 knock-out embryos and wild-type littermates. In all panels, S.E. is indicated.
exclusive possibility would be that at later stages of erythroid differentiation the proteins contain different modifications and/or are recruited as parts of different protein complexes to mediate β-globin ACH formation (40). The role of NF-E2 in the spatial conformation of the β-globin locus has not been investigated previously. The factor is reported to be absent from HS2 and the β-major gene in non-induced MEL cells (38) but is present at the human β-globin locus in primary multipotent hematopoietic progenitor cells (41). We also found that p45 NF-E2 is already (abundantly) present at the LCR and the β-major promoter in 1/11 progenitor cells that do not show frequent LCR-gene contacts and still express the β-major gene at basal levels. Thus, binding of p45 NF-E2 per se is not sufficient to confer high expression to the β-like globin genes. We show that in p45 NF-E2 knock-out mice long-range contacts in the β-globin locus are formed normally, and hence we conclude that p45 NF-E2 is dispensable for β-globin ACH formation. p45 NF-E2 is a member of the CNC (cap ‘n collar) subfamily of basic leucine zipper (bZIP) transcription factors, to which Nrf-1, Nrf-2, Nrf-3, Bach1, and Bach2 also belong. They can form heterodimers with the small Maf proteins that contain a basic DNA-binding domain but lack a transactivation domain (42). The Bach factors lack a canonical transactivation domain and act as repressors in reporter assays (43), but the Nrf factors are all potential candidates to compensate functionally for the loss of p45 NF-E2. However, Nrf-1-/-, -2, and -3 knock-out mice each show normal β-globin gene expression (26–28, 44, 45). Moreover, although the early embryonic lethality of Nrf-1-null mice compromises the combination of p45 NF-E2 and Nrf-1 deficiencies, mice lacking both p45 NF-E2 and Nrf-2, or p45 NF-E2 and Nrf-3, did not show an erythroid phenotype beyond that seen with the loss of p45 NF-E2 alone (26–28). Based on these findings, it was suggested that Nrf-2 and Nrf-3 do not complement p45 NF-E2 function. Here we demonstrate however that Nrf-2 binding to HS2 strongly increases when p45 is absent. This was not the case at the β-major promoter, where MafK binding was also lost in the absence of p45 NF-E2. This shows that different heterodimers have different affinities for DNA target sites. Whether the physical replacement of p45 NF-E2 by Nrf-2 at HS2 also has functional consequences for β-globin gene regulation remains an open question. In this respect it should be mentioned that deletion of HS2 from the endogenous mouse β-globin locus, which removed the most prominent p45 NF-E2-binding sites in the locus, caused a drop in β-globin gene expression similar to that seen in p45 NF-E2 knock-out mice (46). This may suggest that the absence of a dramatic effect on β-globin gene expression is not the consequence of related factors compensating functionally for the loss of p45 NF-E2. However, compound knock-out mice or knockdown cells lacking all NF-E2-related factors may need to be studied to address this issue unambiguously.

Acknowledgments—We thank Dr. R. Shvidasani for providing p45 NF-E2 knock-out mice, Dr. K. Igarashi for providing the anti-Bach1 antiserum, Dr. J. Philippen for providing the EKLF antibody and critical reading of the manuscript, and M. Vendello for assistance with experiments.

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