MOLECULAR AND CELLULAR BIOLOGY, Mar. 2009, p. 1526–1537 Vol. 29, No. 6
0270-7306/09/$08.00+0 doi:10.1128/MCB.01523-08

Ikaros and GATA-1 Combinatorial Effect Is Required for Silencing of Human γ-Globin Genes▽

Stefania Bottardi,[1]† Julie Ross,[1]† Vincent Bourgoin,[1] Nasser Fotouhi-Ardakani,[1] El Bachir Affar,[1] Marie Trudel,[2] and Eric Milot[1,3]*

Maisonneuve Rosemont Hospital Research Center, Maisonneuve-Rosemont Hospital and Faculty of Medicine, University of Montreal, 5415 Boulevard l'Assomption, Montreal, Quebec H1T 2M4, Canada[1]; Institut de Recherches Cliniques de Montreal and Faculty of Medicine, University of Montreal, 110 Avenue des Pins Ouest, Montreal, Quebec H2W 1R7, Canada[2]; and Institute for Research in Immunology and Cancer, University of Montreal, C.P. Succursale Centre-Ville, Montreal, Quebec H3T 3J7, Canada[3]

Received 30 September 2008/Returned for modification 19 October 2008/Accepted 19 December 2008

During development and erythropoiesis, globin gene expression is finely modulated through an important network of transcription factors and chromatin modifying activities. In this report we provide in vivo evidence that endogenous Ikaros is recruited to the human β-globin locus and targets the histone deacetylase HDAC1 and the chromatin remodeling protein Mi-2 to the human γ-gene promoters, thereby contributing to γ-globin gene silencing at the time of the γ- to β-globin gene transcriptional switch. We show for the first time that Ikaros interacts with GATA-1 and enhances the binding of the latter to different regulatory regions across the locus. Consistent with these results, we show that the combinatorial effect of Ikaros and GATA-1 impairs close proximity between the locus control region and the human γ-globin genes. Since the absence of Ikaros also affects GATA-1 recruitment to GATA-2 promoter, we propose that the combinatorial effect of Ikaros and GATA-1 is not restricted to globin gene regulation.

During hematopoiesis, lineage commitment and differentiation are coordinated by activation, as well as repression, of specific genes. Gene regulation is highly dependent on the combinatorial effect of particular transcription factors, which contributes to the establishment of specific transcription factor networks (37, 45). Transcription factors are best known as transcriptional activators, but they can also be involved in gene repression. The variable influence of transcription factors can be explained mainly by their reciprocal interactions and capacity to recruit cofactors such as histone modifying and/or chromatin remodeling activities to gene regulatory regions. The occupancy of target gene promoters by specific combinations of transcription factors and cofactors has been shown to be critical for controlling the expression of several genes in a variety of experimental systems.

The human β-globin (huβ-globin) locus, being the best-defined mammalian multigenic locus, provides a useful model for exploring the combinatorial effects of transcription factors on tissue- and development-specific gene expression. The huβ-globin locus contains five developmentally regulated genes (ε, ^{G}γ, ^{A}γ, δ, and β). The locus control region (βLCR), which is located far upstream of the globin genes, provides high-level globin gene expression in erythroid cells. The βLCR is composed of five DNase I-hypersensitive sites (HSs), which are particularly rich in transcription factor binding sites (15, 24). In erythroid cells, the βLCR favors high-level transcription through close interaction with gene promoters and is a major determinant of locus chromatin conformation (7). Certain transcription factors and cofactors are critical for globin gene regulation and for locus organization. Among these, GATA-1 and its cofactor FOG-1 (for Friend of GATA-1) (52), EKLF (9), and NLI/Lbd1 (46) are required for efficient long-range chromatin interactions between βLCR and β-like globin genes, thereby promoting high-level globin gene expression. During human ontogeny, huβ-like globin genes undergo two important developmental switches, i.e., embryonic to fetal in early fetal life and fetal to adult (γ- to β-globin switching) just after birth (15). A number of transcription factors are known to play a critical role during γ- to β-globin switching. For instance, Ikaros, GATA-1, the orphan nuclear receptors TR2 and TR4, and NF-E3/COUP-TFII have been associated with huγ-gene silencing (16, 25, 26, 48, 50).

Ikaros is a hematopoietic transcription factor shown to physically interact with distinct histone-modifying and chromatin-remodeling activities such as BRG1, Mi-2, and the histone deacetylase HDAC1 (11, 47). In vitro evidence suggests that Ikaros binds to several regions across the huβ-globin locus and is the sequence-specific DNA-binding factor of the PYR complex, which binds a pyrimidine stretch (Pyr region) located 1 kb upstream of the huδ gene (26, 35, 36). In vivo, Ikaros is reported to significantly occupy βLCR HS3 (20). The Ikaros transcript is alternatively spliced to generate multiple isoforms (30). Ikaros-1 and Ikaros-2 are most abundantly expressed during development (10). Ikaros-null mice (Ik^{null}) are null for any Ikaros protein due to a deletion in the last exon, leading to protein instability (54). In Ik^{null} mice carrying a human minilocus, γ- to β-globin switching is delayed (26, 35), B and T lymphopoiesis and hematopoietic stem cell (HSC) activities are severely affected, and reduction in HSCs leads to decreased

* Corresponding author. Mailing address: Maisonneuve Rosemont Hospital Research Center, Maisonneuve-Rosemont Hospital and Faculty of Medicine, University of Montreal, 5415 Boulevard l'Assomption, Montreal, Quebec H1T 2M4, Canada. Phone: (514) 252-3551. Fax: (514) 252-3430. E-mail: e.milot.1@umontreal.ca.

† S.B. and J.R. contributed equally to this study.

▽ Published ahead of print on 29 December 2008.

BFU-E and CFU-E activities (33). Fetal-to-adult globin switching is also delayed in Ikaros^{Plastic} mice harboring a point mutation in the third zinc finger of Ikaros, which selectively disrupts DNA binding (20, 39). Although Ikaros^{Plastic} homozygosity is embryonically lethal due to the failure of normal erythroblast growth and differentiation (39), fetal erythropoiesis is reported to be unaffected in Ik^{null} mice (54). Ikaros has been associated with gene activation mediated by SWI/SNF-like complexes, and it has also been shown to control a number of hematopoiesis-specific genes. However, most of the Ikaros proteins in lymphoid cells are present in the repressive NuRD (for nucleosome remodeling and histone deacetylase) complex, and a small amount is associated with the corepressors Sin3A, Sin3B, and Sin3BSF, as well as CtBP, CtIP, and Rb (10).

To define the molecular mechanisms of Ikaros-mediated huγ-globin gene repression, we investigated Ikaros occupancy at βLCR HS2, HS3, and all the huγ and huβ promoters, which are critical *cis*-regulatory regions controlling fetal and adult globin gene expression. Using fetal liver erythroid cells isolated from transgenic mice carrying the whole huβ-globin locus, we demonstrate in vivo that binding of Ikaros to several regions across the huβ-globin locus modulates the recruitment of distinct cofactors to βLCR and globin gene promoters. In particular, our data strongly suggest that the contribution of Ikaros to γ- to β-globin switching (26) is mediated by Ikaros-dependent recruitment of Mi-2 and HDAC1 to huγ promoters, and by reduced efficacy of long-range chromatin interactions between βLCR and huγ promoters. In addition, we provide novel evidence that Ikaros interacts with GATA-1, a transcription factor essential for erythroid and megakaryocytic development (4), thereby modifying GATA-1 recruitment to HS3, huγ promoters, and Pyr region. Our results elucidate the role of Ikaros in the global repressive mechanism leading to huγ-globin gene silencing at the time of γ- to β-globin switching, hence clarifying an important aspect of this tissue- and development-specific process. We also provide the evidence that other hematopoiesis-specific genes can be regulated by the combinatorial effect of Ikaros and GATA-1.

MATERIALS AND METHODS

Mouse transgenic lines. Homozygous line 2 (ln2) mice (49) were bred with CD1 females, and ln2 12.5- or 14.5-day postcoitum (dpc) heterozygous ln2 fetal livers were isolated. Otherwise, heterozygous Ik^{null} (54) mice were crossed with homozygous ln2 heterozygous Ik^{null} mice, and 12.5- or 14.5-dpc heterozygous ln2 homozygous Ik^{null} (ln2-Ik^{null}) fetal livers were isolated. Animals were sacrificed by cervical dislocation. Fetuses were isolated, and fetal livers were dissected and then homogenized in phosphate-buffered saline (PBS) by vigorous pipetting. Bone marrow cells were isolated from the femurs and humerus of adult transgenic mice by flushing bones in PBS with a 30G needle. Cell clumps were mechanically dissociated by passing cells through a 22G needle. Animal experiments were conducted in accordance with the Canadian Council on Animal Care guidelines and approved by the Maisonneuve-Rosemont Hospital animal care committee.

Wright-Giemsa staining. Ten to twenty thousand fetal liver cells were centrifuged on clean glass slides for 7 min at 600 rpm on a Cytospin3 (Shandon) system. Slides were air dried, fixed in 100% methanol for 20 s at room temperature, and stained for 5 min at room temperature in Wright-Giemsa stain modified solution (Sigma). Slides were then extensively washed in distilled water and completely dried.

ChIP and quantitative PCR (qPCR) analyses. Chromatin immunoprecipitation (ChIP) assays were carried out according to the manufacturer's instructions (Upstate Biotechnology) starting with 10^6 fetal liver cells. Cells were fixed with 1% formaldehyde (HCHO) for 10 min at 37°C. HCHO/EGS (ethylene glycol-bis[succnimidyl succinate]) dual cross-linking was carried out at room temperature, first in 1.5 mM EGS for 30 min and then in 1% HCHO for 10 min. Reactions were quenched by the addition of ice-cold glycine (20 mM final) (57). Chromatin was reduced in size by sonication in order to obtain fragments of 400 to 600 bp in size. Antibodies were raised against acetylated (K9 and K14) histone H3 or HDAC1 (Upstate Biotechnology) and GATA-1 (N6), BRG1 (H-88), Ikaros (E-20), FOG-1 (M-20), Mi-2 (H-242), or p45/NF-E2 (C-19) (Santa Cruz). About 1/30 of immunoprecipitated and unbound (input) material was used as a template for qPCR with SYBR green (Invitrogen) on an iCycler iQ (Bio-Rad) system, using one primer set specific for the huβ-globin locus or mouse amylase 2.1y (Amy) promoter and another set specific for the mouse kidney-specific Tamm-Horsfall gene promoter (Thp), used as an internal control. Amy and Thp are two genes that are not expressed in erythroid cells. Quantification was carried out according to the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T$ is calculated as follows: (ChIP C_T − input C_T of the target region) − (ChIP C_T − input C_T of the reference region). C_T indicates the cycle threshold (56). To correctly interpret C_T values obtained by qPCR, the efficiency of all primer set was carefully checked and primer pairs displaying an amplification efficiency ranging from 95 to 102% were chosen. All data shown are the results of at least four independent ChIP experiments with qPCR from each ChIP performed in triplicate and averaged (standard deviation). All primer sets and qPCR conditions are available upon request.

Generation of epitope-tagged Ikaros-expressing K562 cells. This protocol was exactly as described by Nakatani and Ogryzko (32), but K562 cells were used instead of HeLa cells.

Protein IP. For immunoprecipitation (IP) of fetal liver cells, ten million cells were lysed in 1 ml of ice-cold radioimmunoprecipitation assay buffer (10 mM Tris [pH 8], 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% Nonidet NP-40, 0.1% sodium dodecyl sulfate [SDS]) containing protease inhibitors (protease inhibitor cocktail; Sigma). Samples were rocked for 15 min at 4°C and then centrifuged for 15 min at 15,800 × *g* at 4°C. Supernatants were precleared with protein G-agarose beads (Upstate). Antibodies or immunoglobulin-matched controls were added to precleared protein extracts, and samples were rocked overnight at 4°C. IP complexes were collected with protein G-agarose beads and then washed three times with 1 ml of ice-cold radioimmunoprecipitation assay buffer. Samples were recovered by boiling the beads in sample buffer containing β-mercaptoethanol. GATA-1 (N6), Ikaros (E-20) antibodies, and isotype-matched immunoglobulins were purchased from Santa Cruz Biotechnology. IP of pOZ-FH-N and Ikaros-FH K562-infected cells was performed as described by Nakatani and Ogryzko (32). Anti-FLAG-conjugated agarose beads, as well as antihemagglutinin (anti-HA) antibodies, were purchased from Santa Cruz Biotechnology.

Quantitative reverse transcription PCR (qRT-PCR). Total RNA isolated from 10^6 mouse fetal liver, thymus, or bone marrow cells was extracted with TRIzol (Invitrogen) and treated with DNase I-RNase-free (Invitrogen). Reverse transcription reactions were performed with oligo$(dT)_{15}$ primers and SuperScript reverse transcriptase (Invitrogen). qPCR was carried out on an iCycler iQ (Bio-Rad) system using: (i) SYBR green (Invitrogen) to detect mouse GATA-1 and mouse actin (used as internal control) cDNA or (ii) Qiagen QuantiTect (Qiagen) probes specific for huγ-globin or huβ-globin cDNA. To avoid genomic DNA contamination, primers were designed to span intron-exon junctions. All reactions were independently run at least in triplicate. The following equation (42), which takes into account primer efficiencies, was used to quantify huγ-globin, huβ-globin, or GATA-1 gene expression relative to mouse actin gene expression: $E_{target}^{\Delta CP target\ (control\ -\ sample)}/E_{ref}^{\Delta CP target\ (control\ -\ sample)}$, where E_{target} is the huγ- or huβ-globin PCR efficiency, E_{ref} is the mouse actin PCR efficiency, CP is the crossing point, ΔCP_{target} is the crossing-point deviation of ln2 − ln2-Ik^{null} of the huβ-gene or huγ-gene or GATA-1 transcript, and ΔCP_{ref} is the crossing-point deviation of ln2 − ln2-Ik^{null} of the mouse actin transcript. The data shown are the results of at least three independent experiments with qPCR reactions from each cDNA performed in triplicate with corresponding standard deviations. All primer sets and qPCR conditions are available upon request.

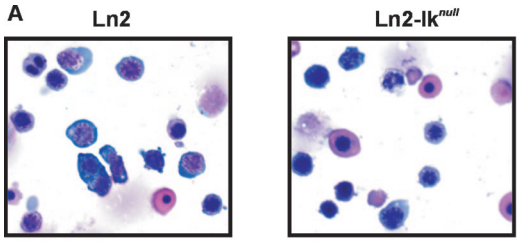
3C protocol. The chromosome conformation capture (3C) protocol was basically as previously described (6) with minor modifications. One 12.5-dpc ln2 or ln2-Ik^{null} fetal liver (1×10^6 to 2×10^6 cells, on average) was resuspended in 2 ml of Dulbecco modified Eagle medium--10% fetal bovine serum. Cells were collected by centrifugation, transferred into 2 ml of Dulbecco modified Eagle medium--10% fetal bovine serum, and fixed with 2% HCHO (38) for 10 min at room temperature. The reaction was quenched by the addition of ice-cold glycine (125 mM final), and the cells were centrifuged and washed once with ice-cold PBS. At this step, 6 million cells were pooled together, and nuclei were harvested by lysis of the cells in 5 ml of ice-cold lysis buffer (30 mM Tris-HCl [pH 8.0], 10 mM NaCl, 0.2% Nonidet NP-40) containing protease inhibitors (Sigma) and

then rocked for 30 min at 4°C. After centrifugation at 1,600 rpm for 15 min at 4°C, nuclei were resuspended in 1.1× EcoRI digestion buffer–0.3% SDS and incubated at 37°C for 1 h with shaking. Triton X-100 (1.8% final) was then added to sequester the SDS, and the nuclei were incubated as described above. Finally, to about 1/10 of the reaction, 625 U (10% in volume) of EcoRI (Invitrogen) was added, and the digestion was carried out at 37°C overnight, with gentle agitation. After digestion, EcoRI restriction enzyme was inactivated by the addition of SDS (1.6% final) and incubation at 65°C for 20 min. The sample was diluted 10 times in ligation buffer (30 mM Tris [pH 8.0], 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP) containing 1% Triton X-100 and then incubated at 37°C for 1 h, with gentle agitation. After incubation, 7500 cohesive end ligation unit of T4 DNA ligase (New England Biolabs) was added to the reaction, and ligation was carried out for 16 h at 16°C. The sample was then treated with proteinase K (Invitrogen) and incubated overnight at 65°C to reverse cross-links. Finally, after RNase I (Invitrogen) treatment (at 37°C for 10 min), DNA was purified by phenol-chloroform extraction and ethanol precipitation and resuspended in TE buffer. About 1/60 of each sample was used as a template for qPCR with SYBR green (Invitrogen).

Naked DNA control templates consist of the huβ-globin PAC clone PAC148γlox (18), together with the mouse β-actin BAC clone (BACe3.6-actin, BAC PAC resources CHORI), which both span the complete loci. Equimolar amounts of both clones were digested with EcoRI and ligated with T4 DNA ligase, and DNA was precipitated as described above. Naked huβ-globin locus and murine β-actin DNA were used to correct for the PCR amplification efficiency of each primer set (all primer efficiencies ranged between 91 and 102%) because these control templates provide all possible ligation products in equimolar amounts. The endogenous β-actin locus was used as additional control to correct for differences in quality and quantity of chromatin templates between different experiments. Brain cells were used as negative control for the 3C assay because globin genes are not expressed in these cells. Enrichment levels were calculated according to the $2^{-\Delta\Delta CT}$ method, wherein $\Delta\Delta C_T$ corresponds to (3C sample C_T − PAC C_T of the target region [globin locus]) − (3C sample C_T − PAC C_T of the reference region [actin]) (56). Enrichment levels were obtained from the average of at least three independent experiments. qPCR analyses were run in triplicate and averaged (standard deviation).

Semiquantitative RT-PCR. Semiquantitative RT-PCR was carried out exclusively to study GATA-2 gene expression in fetal liver samples. Total RNA isolated from 10^6 mouse fetal liver cells was extracted with TRIzol (Invitrogen) and treated with DNase I-RNase-free (Invitrogen). Reverse transcription reactions were performed with oligo$(dT)_{15}$ primers and SuperScript reverse transcriptase (Invitrogen). PCR were carried out with primer sets specific for mouse GATA-2 or actin cDNAs. PCRs were resolved on a 2% agarose gel, and band intensities were quantified by using a Fuji LAS-3000 system and a MultiGauge 2.0 program. GATA-2 expression levels were calculated according to the following formula: [(GATA-2/actin) ln2-Ik^{null}/(GATA-2/actin) ln2]. The results were obtained with three independent experiments, and PCRs from each cDNA sample were performed in triplicate and averaged (standard deviation). The primer sets and PCR conditions are available upon request.

NE and EMSAs. Nuclear extracts (NE) were prepared as described in Dignam et al. (8) from mouse erythroleukemia (MEL) cells or as described in Andrews and Faller (1) from 12.5-dpc fetal liver cells. For electrophoretic mobility shift assay (EMSA) reactions, 50 ng of sense strand oligonucleotides were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). Then, 55 ng of complementary antisense oligonucleotides was added, and the samples were heated at 95°C for 5 min and then allowed to cool to room temperature. Labeled probes were purified by gel filtration through Sephadex G-50 column (Pharmacia), and 10^4 cpm of labeled probe were used for each EMSA reaction. EMSA binding reactions (20 μl) were incubated at room temperature for 20 min; 4 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 10% Ficoll) was then added, and samples were resolved on 5% polyacrylamide gels. Electrophoresis was carried out at 200 V, at room temperature, in 0.5× Tris-borate buffer. Gels were finally dried and analyzed by using a phosphorimager. Binding reactions for the 44-bp ApaI-AvaII probe were carried out as described by Liu et al. (25). EMSA reactions for the 45-bp ^{A}γ exon 1-intron 1 junction of huγ-globin genes (Huγ probe) contained 20 mM HEPES-KOH (pH 7.9), 100 mM KCl, 10 μM $ZnCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol. For both probes, 1 μg of nonspecific competitor poly(dI-dC), 5- to 15-μg portions of NE and, when required, a 100-fold molar excess of cold specific competitor oligonucleotides and 1 to 2 μg of antibodies (for supershift assays) were used. The oligonucleotide sequences are available upon request.



B

	Ln2 (%)	Ln2-Ik^{null} (%)
Non-erythroid cells	4 ± 1	5 ± 1
Proerythroblasts	11 ± 4	12 ± 3
Basophilic erythroblasts	48 ± 3	47 ± 5
Polychromatic erythroblasts	31 ± 1	30 ± 2
Orthochromatic erythroblasts	6 ± 1	6 ± 2

FIG. 1. Morphological analysis of ln2 and ln2-Ik^{null} fetal livers. (A) Wright-Giemsa staining of ln2 (left panel) or ln2-Ik^{null} (right panel) cells. (B) Detailed counting of the different cellular elements in the two genetic backgrounds. The percentages represent the averages from three independent experiments with the corresponding standard errors of the mean.

RESULTS

Ikaros binds to the huβ-globin locus in erythroid cells. It has been shown that Ik^{null} adult mice display anemia due to increased destruction of red cells (26), while erythropoiesis is normal at the fetal stage of development (54). Since the Ik^{null} background is extensively used in the present study, we sought to confirm that the absence of Ikaros does not alter erythroid differentiation in fetal livers. For this purpose, ln2 fetal liver erythroid cells were compared to ln2-Ik^{null} cells. ln2 transgenic mice carry a 70-kb DNA fragment containing the entire huβ-globin locus, express the human globin genes in a developmental-specific manner, undergo γ- to β-globin switching around day 12 postcoitum (49), and display normal erythroid cell differentiation (data not shown). Wright-Giemsa staining of ln2 or ln2-Ik^{null} fetal livers revealed that on average 95% of fetal liver population is composed of erythroid cells and that ln2-Ik^{null} samples contain typical proportion of erythroid precursors with no evidence of abnormal red cell morphology (Fig. 1). These results, which are the average of three independent experiments, indicate that, as previously reported (54), the absence of Ikaros does not preclude normal fetal erythroid cell differentiation and thus is not expected to induce stress erythropoiesis at the fetal stage of hematopoietic differentiation.

Previous studies have shown that Ikaros binds the huβ-globin locus in vitro and, when overexpressed in K562 erythroleukemia cells, which express huγ- but not huβ-globin upon induction, Ikaros-1 binds to βLCR HS3 (20, 35). However, the physiological role of Ikaros and the molecular mechanisms modulated by Ikaros during globin switching remain to be defined. In order to better delineate the role of Ikaros, specifically during globin switching, we used 12.5-dpc fetal liver erythroid cells where huγ- and huβ-globin are transcribed and Ikaros isoforms are expressed at physiological levels. Using the TGGGAA Ikaros DNA-binding consensus sequence, which is also found at TCR-α and TCR-β enhancers and at the CD4

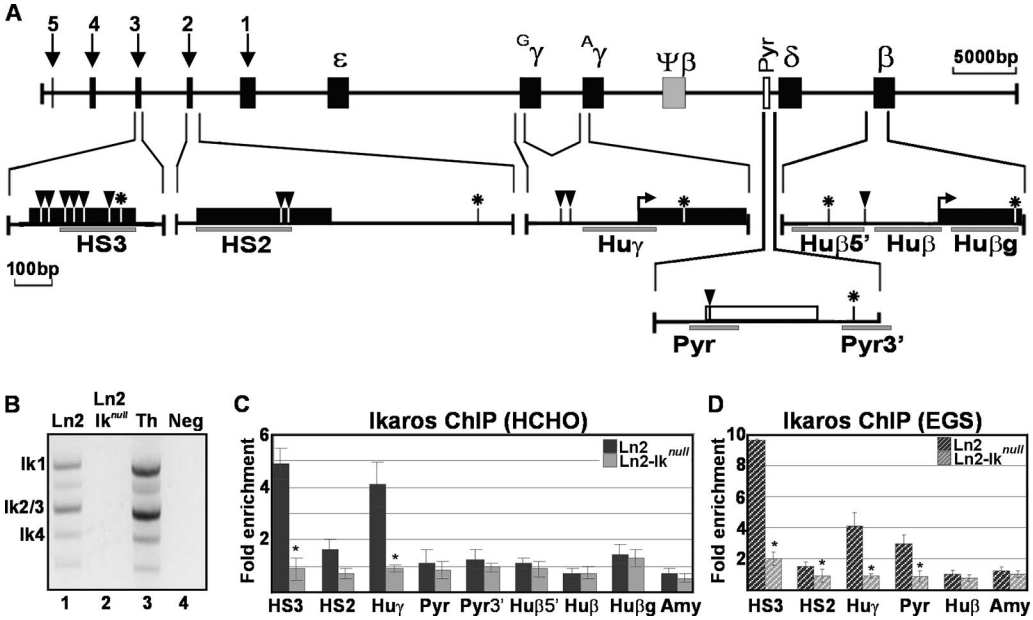


FIG. 2. Ikaros recruitment to the human β-globin locus in ln2 and ln2-Ik^{null} 12.5-dpc fetal liver cells. (A) Map of the huβ-globin locus. The locations of the βLCR HSs are indicated by arrows; genes are indicated as black boxes and Pyr region as a white box. Black arrowheads mark GATA-1 binding sites; asterisks indicate potential Ikaros binding sites according to the TGGGAA hexanucleotide consensus sequence. Amplicons for ChIP analysis are represented by gray lines depicted underneath each genomic region. (B) RT-PCR performed on equal amounts of RNA purified from ln2 (lane 1) or ln2-Ik^{null} (lane 2) 12.5-dpc fetal liver cells. Th, thymus control (lane 3); Neg, negative control (lane 4). Ikaros cDNA spliced variants are indicated on the left side of the panel. (C and D) Ikaros ChIP. Immunoprecipitated and unbound (input) chromatin samples were used as templates in qPCR analyses with primers specific for amylase 2.1y (Amy) promoter or the huβ-globin regions βLCR HS3 and HS2, the huγ promoters (Huγ), the proximal (Pyr) and distal (Pyr3′) Pyr regions, the proximal (Huβ5′) and distal (Huβ) huβ-promoter regions, and the huβ-gene region (Huβg). Quantification was carried out according to the $2^{-\Delta\Delta CT}$ method, using mouse kidney-specific Thp protein promoter as an internal control, since this gene is not expressed in erythroid cells. Mouse Amy/Thp control is included to confirm that no enrichment was observed at regulatory regions of nonhematopoietic genes. Enrichment levels are represented by bars, with their corresponding standard deviations. A value of 1 indicates no enrichment. *, $P \leq 0.05$ (Student t test). All data shown are the results of at least four independent ChIP experiments, with qPCR from each ChIP performed in triplicate and averaged (standard deviation). (C) HCHO-fixed cells. (D) EGS-fixed cells.

promoter (30), we identified other potential Ikaros binding sites across the huβ-globin locus (data not shown). To validate Ikaros consensus sequences at critical *cis*-regulatory regions implicated in γ- to β-globin switching (Fig. 2A), we carried out ChIP assays on 12.5-dpc fetal livers isolated from ln2 transgenic mice. Endogenous Ikaros, expressed in fetal liver erythroid cells (Fig. 2B, lane 1), is efficiently recruited to HS3 and exon 1-intron 1 junction of huγ-globin genes (hereafter referred to as huγ promoters [Huγ]), as revealed by qPCR on immunoprecipitated material. On the other hand, no significant binding could be detected at the Pyr region (Pyr), huβ promoter (Huβ), and amylase 2.1y (Amy) promoter used as a negative control (Fig. 2C). To confirm the absence of relevant Ikaros occupancy at the Pyr and huβ-globin regions, we tested three additional amplicons: a downstream Pyr region (Pyr3′), a proximal huβ-promoter region (Huβ5′), and the huβ-gene exon2 (Huβg). As shown in Fig. 2C, the absence of significant binding at these adjacent regions confirms that Ikaros is not recruited to the Pyr region and to the huβ promoter in vivo. However, since in vitro Ikaros binds the Pyr region (35) and Ikaros either directly binds DNA or acts as a cofactor (10), we carried out ChIP analysis using EGS in combination with HCHO as cross-linking agents. EGS is a chemical cross-linker with a longer spacer arm than HCHO (16.1 Å versus 2 Å). Since EGS is a protein-protein cross-linker, it is useful for detection of proteins indirectly associated with DNA (57). Upon chromatin treatment with HCHO and EGS, Ikaros is detected at HS3, huγ promoters, and the Pyr region, whereas no significant binding is evident at the huβ and Amy promoters (Fig. 2D). To control for specificity, ChIP assays with Ikaros antibodies were carried out on ln2-Ik^{null} 12.5-dpc fetal liver cells. As expected, Ikaros is not detected at the tested regions (Fig. 2C and D). These results suggest that in 12.5-dpc fetal liver erythroid cells, among the regions tested, Ikaros binds HS3 and huγ promoters, whereas it seems indirectly recruited to the Pyr region.

Ikaros direct interaction with the DNA at the huγ promoters was confirmed by EMSAs. A 45-bp oligonucleotide spanning the Ikaros consensus binding site at the exon 1-intron 1 junction of huγ-globin genes (Huγ probe) was end labeled and incubated with 15 μg of MEL NE. The huγ probe shows one clear gel mobility shift band (Ik), which is efficiently competed by cold huγ oligonucleotide (Huγ comp) but not by an oligonucleotide containing a mutated (TTGGAA instead of TGG GAA) Ikaros consensus binding site (Huγ comp^{mut}) (Fig. 3). The presence of Ikaros in the retarded protein complex was confirmed by supershift assays with antibodies specific for the C- or N-terminal region of Ikaros protein. As expected, supershifts are seen with both antibodies (Fig. 3, lane 2 versus lanes 5 and 6), indicating that Ikaros can indeed directly bind its consensus site at the huγ promoter region.

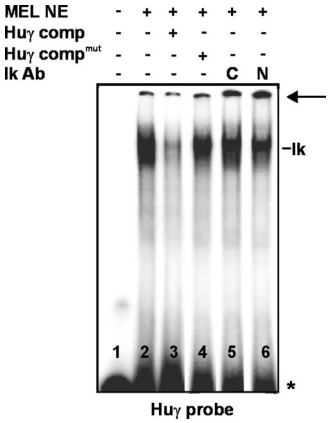


FIG. 3. Binding of Ikaros to the exon 1-intron 1 junction of huγ-globin genes. EMSA of exon 1-intron 1 junction of huγ-globin genes (Huγ probe) was performed with 15 μg of NE from MEL cells. An Ikaros-specific retarded band (Ik) is competed out by a 100-fold molar excess of cold double-stranded oligonucleotide (Huγ comp, lane 3) but not by an oligonucleotide containing a mutated (TTGGAA instead of TGGGAA) Ikaros consensus binding site (Huγ comp^{mut}, lane 4). Supershift experiments were carried out with antibodies (Ik Ab) raised to the C (C, lane 5) and N (N, lane 6) termini of the Ikaros protein. The antibody-shifted complexes are indicated by an arrow; an asterisk indicates free, labeled probe.

Ikaros modulates HDAC1, BRG1, and Mi-2 recruitment to specific regions of the huβ-globin locus. Biochemical analyses in lymphoid and erythroid cells have demonstrated that Ikaros can interact with chromatin modifying and remodeling proteins, including BRG1, HDAC1, and Mi-2 (21, 35, 36, 47). Therefore, we investigated whether recruitment of these cofactors to the huβ-globin locus in 12.5-dpc fetal liver cells is modulated by Ikaros. By ChIP assays, we observed that BRG1 is equally recruited to HS2, huγ, and huβ promoter in ln2 and in ln2-Ik^{null} fetal liver cells (Fig. 4A). However, BRG1 is recruited less efficiently to HS3 in ln2-Ik^{null} than in ln2 cells ($P <$ 0.05). HDAC1 is recruited more efficiently to huγ promoters and less efficiently to huβ promoter in ln2 than in ln2-Ik^{null} cells (Fig. 4B). The level of histone H3 acetylation is as predicted, consistent with HDAC1 distribution, since histone H3 acetylation level is lower at huγ promoters and higher at the huβ promoter in ln2 than in ln2-Ik^{null} cells (Fig. 4C). Finally, Mi-2 is detected at huγ promoters in ln2 but not in ln2-Ik^{null} cells (Fig. 4D). These results reveal that Ikaros binding to the huβ-globin locus is important for the recruitment of chromatin modifying and remodeling activities to βLCR (BRG1) and to huγ and huβ promoters (HDAC1 and Mi-2). Thus, Ikaros is likely to contribute to local chromatin conformational changes occurring at the time of γ- to β-globin switching.

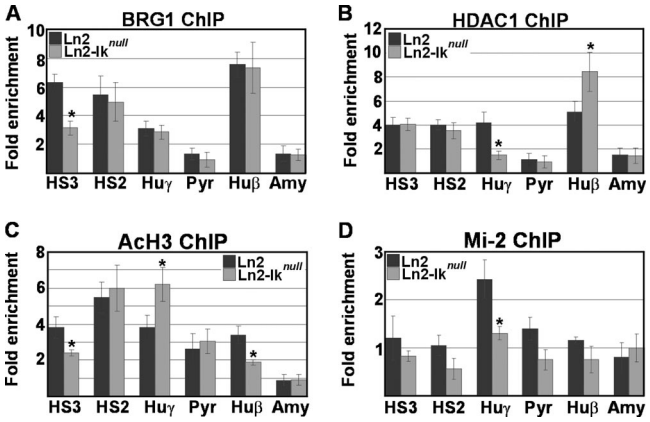


FIG. 4. Recruitment of chromatin modifying and remodeling activities to the human β-globin locus in ln2 and ln2-Ik^{null} 12.5-dpc fetal liver cells. ChIP analysis of ln2 and ln2-Ik^{null} cells was performed. Analysis and quantification of the immunoprecipitated samples were as described in Fig. 2C and D. *, $P \leq 0.05$ (Student t test). The antibodies used are indicated at the top of each panel. AcH3, anti-acetylated (K9 and K14) histone H3 antibodies. Black bars indicate ln2 cells; gray bars indicate ln2-Ik^{null} cells.

Ikaros modulates GATA-1 binding at specific sites across the huβ-globin locus. GATA-1 and Ikaros consensus binding sequences are found in close proximity at several regions across the huβ-globin locus, including HS3, the huγ and huβ promoters, and the Pyr region (Fig. 2A). To verify whether GATA-1 binding to these chromosomal regions can be influenced by Ikaros, we carried out ChIP assays with GATA-1 antibodies on ln2 and ln2-Ik^{null} 12.5-dpc fetal liver cells. As expected, GATA-1 is detected at HS3, HS2, the huγ and huβ promoters, and the Pyr region, but not at the Amy promoter (Fig. 5A). Most interestingly, the absence of Ikaros, which by itself does not modify GATA-1 expression (Fig. 5B) (26), affects GATA-1 binding at HS3 and Pyr region and reduces to background level GATA-1 recruitment to huγ promoters ($P \leq$ 0.05; Fig. 5A). GATA-1 binding at HS2 ($P = 0.172$) and the huβ promoter ($P = 0.275$) does not significantly change between ln2 and ln2-Ik^{null} cells (Fig. 5A). The influence of Ikaros on GATA-1 direct binding to DNA was further investigated by EMSA, using 5 μg of ln2 or ln2-Ik^{null} fetal liver NE and a labeled ApaI-AvaII DNA fragment of the hu^{A}γ promoter, which contains two GATA-1 binding motifs and one Oct-1 binding motif (ApaI-AvaII probe) (25). As previously reported (25), both Oct-1 (Fig. 5C, band A) and GATA-1 (Fig. 5C, band B) proteins can bind this DNA fragment. However, when equal amounts of NE are used in EMSAs, GATA-1 but not Oct-1 binding is affected in ln2-Ik^{null} relative to ln2 samples (Fig. 5C, compare lanes 2 and 4 with lanes 6 and 8). Altogether, these results strongly suggest that Ikaros contributes to GATA-1 recruitment to huγ promoters and contributes to GATA-1 recruitment and/or stability at HS3 and Pyr region at the time of γ- to β-globin switching.

We then investigated whether Ikaros could influence the recruitment of FOG-1 (3) to the same genomic sites. ChIP analysis on 12.5-dpc fetal liver cells revealed that FOG-1 recruitment is slightly decreased at the huγ promoters and the Pyr region in ln2-Ik^{null} (versus ln2), hence reflecting the GATA-1 binding profile at these sites (Fig. 5A and D). These results imply that Ikaros contributes to efficient binding of GATA-1 and FOG-1 to huγ promoters and the Pyr region and indicate that, as observed at the mouse locus (22), GATA-1 binding to HS3 is FOG-1 independent.

In order to investigate whether reduced GATA-1 binding in ln2-Ik^{null} cells is specifically ascribed to lack of Ikaros or is merely a reflection of a more general alteration of cooperative transcription factor binding to DNA, we analyzed p45/NF-E2 occupancy at HS3, HS2, and the huγ promoters. We observed that the absence of Ikaros does not alter p45/NF-E2 binding to

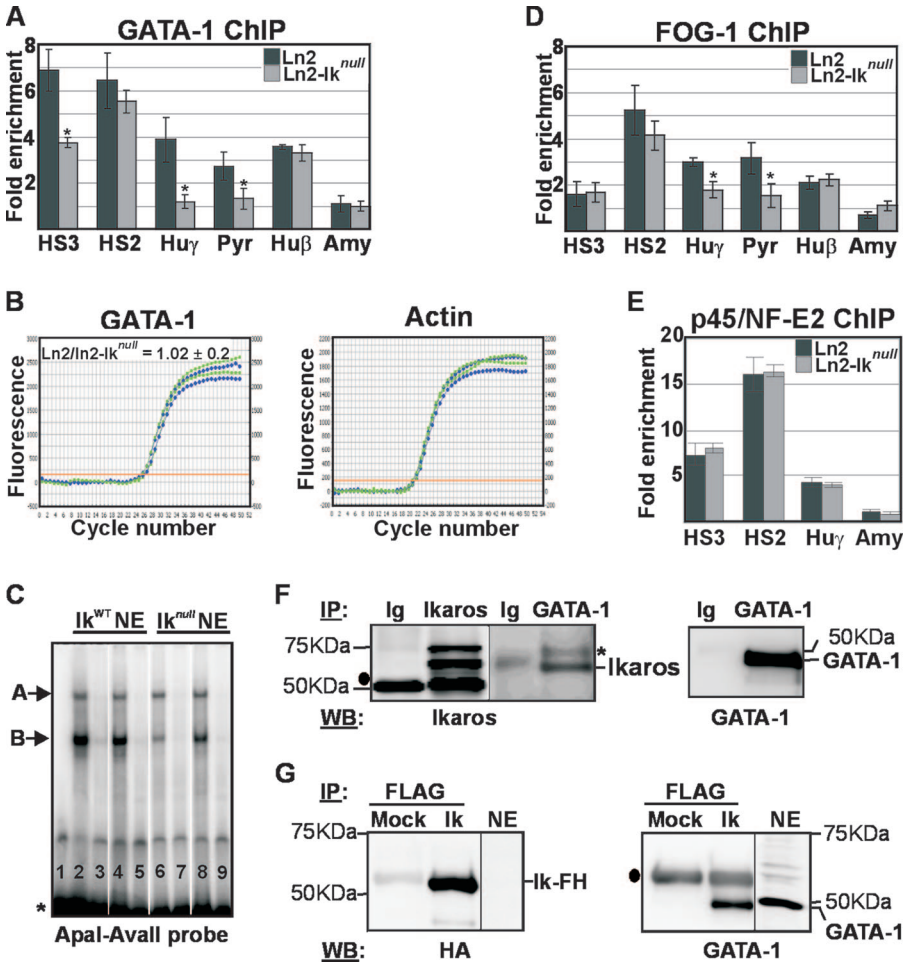


FIG. 5. Ikaros-GATA-1 interaction. (A, D, and E) ChIP analysis of ln2 and ln2-Ik^{null} cells. Analysis and quantification of chromatin immunoprecipitated samples are as described for Fig. 2C and D. *, $P \leq 0.05$ (Student t test). (B) GATA-1 gene expression. Representative examples of qRT-PCR carried out on ln2 (blue circles) and ln2-Ik^{null} (green squares) 12.5-dpc fetal liver cells. GATA-1 (left panel) expression levels in ln2 relative to ln2-Ik^{null} cells were calculated according to the method of Pfaffl (42) (see also Materials and Methods) using mouse actin (right panel) as an internal control, and they are expressed as the ln2/ln2-Ik^{null} ratio. y axis, derivative of SYBR green fluorescence. (C) EMSA of huγ-promoter AvaI-ApaII fragment (25) (ApaI-AvaII probe) was carried out with 5 μg of NE from ln2 (Ik^{WT} NE) or ln2-Ik^{null} (Ik^{null} NE) fetal liver cells. Lane 1, no NE; lanes 3, 5, 7, and 9, competition with a 100-fold molar excess of cold ApaI-AvaII oligonucleotide. Arrow A, Oct-1-specific retarded band; arrow B, GATA-1-specific retarded band; *, free, labeled probe. (F and G) Representative examples of protein IP on whole-cell extracts prepared from ln2 12.5-dpc fetal liver cells (F) or pOZ-FH-N or Ikaros-FH K562-infected cells (G). The antibodies used for IP or WB assays are indicated at the top and the bottom of the panels, respectively. Ikaros (Ikaros and Ik-FH)- and GATA-1-specific bands are indicated on both sides of the panels. Filled circles represent contaminating immunoglobulin heavy chain band. A higher-molecular-weight Ikaros-1-specific band is indicated by an asterisk. Ig, isotype-matched immunoglobulin control; Mock, pOZ-FH-N K562-infected cells; Ik, Ikaros-pOZ-FH-N K562-infected cells; NE, wild-type K562 NE.

any of these regions (Fig. 5E), further supporting the proposal that the effect of Ikaros on GATA-1 binding is specific. These results indicate that Ikaros modulates GATA-1 binding at precise regions across the huβ-globin locus, suggesting a possible interaction between these two proteins. This prompted us to investigate by protein IP whether Ikaros indeed binds to GATA-1 in ln2 12.5-dpc fetal liver erythroid cells. Proteins were immunoprecipitated with antibodies specific for GATA-1, Ikaros, or isotype-matched immunoglobulin controls. Western blot (WB) membranes were probed with GATA-1 or Ikaros antibody. In three distinct IP experiments, Ikaros was immunoprecipitated by Ikaros and GATA-1 antibody but not by isotype-matched immunoglobulin (Fig. 5F). It is worth noting that another band of ~70 kDa is observed upon Ikaros or GATA-1 IPs and Ikaros WB detection (Fig. 5F, asterisk). This band is likely to correspond to Ikaros posttranslational modifications such as phosphorylation (12). The reciprocal IP (IP with Ikaros antibody and WB with GATA-1 antibody) did not allow the detection of any specific band (data not shown). To better characterize Ikaros–GATA-1 interaction, K562 cells were infected with a Moloney murine leukemia virus-based pOZ-FH-N vector, which contains a bicistronic transcriptional unit that allows expression of double epitope-tagged (FLAG and HA; FH) proteins from single transcripts. Notably, the expression levels of pOZ-FH-N epitope-tagged proteins are comparable to the endogenous ones (32). Epitope-tagged Ikaros can be detected by protein IP with anti-FLAG antibody, followed by WB detection with HA antibody

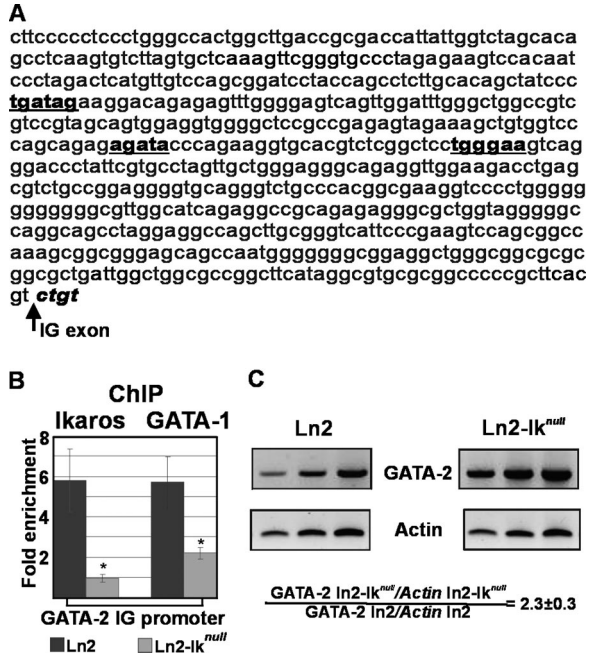


FIG. 6. Ikaros and GATA-1 recruitment to the GATA-2 IG promoter in ln2 and ln2-Ik^{null} 12.5-dpc fetal liver cells. (A) Schematic overview of GATA-2 IG promoter region. In boldface and underlined are the GATA-1 and Ikaros DNA consensus binding sites; in boldface italics is the beginning of exon IG (29). (B) ChIP analysis of ln2 and ln2-Ik^{null} cells with Ikaros and GATA-1 specific antibodies. Analysis and quantification of immunoprecipitated samples are as described in Fig. 2C and D. *, $P \leq 0.05$ (Student t test). (C) Representative example of semiquantitative RT-PCR performed on equal amounts of RNA purified from ln2 or ln2-Ik^{null} 12.5- dpc fetal liver cells. Top panel, mouse GATA-2 cDNA; bottom panel, mouse actin cDNA, used as a control. Band intensities were quantified with the MultiGauge 2.0 program, and the relative levels of GATA-2 gene expression were quantified according to the formula depicted underneath the panels.

(Fig. 5G). As expected, after IP of epitope-tagged Ikaros with anti-FLAG antibody, GATA-1 is readily detected by WB, indicating a physical interaction between GATA-1 and Ikaros (Fig. 5G). This result is consistent with the ChIP data and supports the notion that the interaction between Ikaros and GATA-1 contributes to huγ gene repression at the time of γ- to β-globin switching.

In the attempt to verify whether Ikaros–GATA-1 cooperative binding might occur at other gene regulatory regions, we studied the murine GATA-2 gene promoter, which is regulated by GATA-1 (13). GATA-2 is required for expansion of hematopoietic progenitor cells and is downregulated in erythroid cells (3). The proximal (IG) promoter controls GATA-2 expression in various tissues, including erythroid cells (29). The distal (IS) promoter specifically controls GATA-2 expression in hematopoietic progenitors (13). Since DNA sequence analysis of the GATA-2 IG promoter reveals the presence of nearby GATA-1 (TGATAG; AGATA) and Ikaros (TGG GAA) consensus DNA-binding sites (Fig. 6A), we verified the in vivo recruitment of GATA-1 and Ikaros to GATA-2 IG promoter by ChIP analysis with Ikaros- and GATA-1-specific antibodies. As shown in Fig. 6B, both proteins can be detected at the GATA-2 IG promoter in vivo in ln2 erythroid cells, whereas ChIP analysis in ln2-Ik^{null} cells revealed that the absence of Ikaros significantly reduces GATA-1 binding. Finally, as observed at huγ-globin genes, reduced Ikaros and GATA-1 recruitment to GATA-2 promoter affects GATA-2 silencing in erythroid cells, as indicated by a 2.3-fold increase GATA-2 gene expression in ln2-Ik^{null} cells relative to ln2 cells (Fig. 6C).

Ikaros contributes to huγ gene silencing at the time of γ- to β-globin switching while precluding efficient interaction between βLCR and huγ promoters. Since (i) Ikaros affects γ- to β-globin switching (20, 26), (ii) GATA-1 can repress huγ gene expression when bound at position −175 of the hu^{A}γ promoter (25), and (iii) GATA-1 binding/stability at huγ promoters is decreased in ln2-Ik^{null} fetal liver cells (Fig. 5A and C), we evaluated the huγ and huβ gene expression levels in ln2 and ln2-Ik^{null} cells by qRT-PCR. Total RNA isolated from fetal liver cells was used for cDNA synthesis. Real-time qPCR was performed with Qiagen QuantiTect probes specific for huβ-globin or huγ-globin cDNA. Mouse actin cDNA was used as a control. The averages of three independent experiments were as follows: for fetal liver cells at 12.5 dpc, the ln2-Ik^{null}/ln2 values were 2.2 ± 0.4 and 0.6 ± 0.1 for huγ and huβ genes, respectively, and for fetal liver cells at 14.5 dpc, the ln2-Ik^{null}/ln2 values were 3.7 ± 0.4 and 0.5 ± 0.05 for huγ and huβ genes, respectively. Thus, at 12.5 dpc, huγ-gene expression is 2.2-fold higher ln2-Ik^{null} than in ln2 cells, and huβ-gene expression decreases to 0.6-fold in ln2-Ik^{null} cells. To investigate whether Ikaros participates in huγ-gene silencing also by affecting long-range interactions at the globin locus at the time of γ- to β-globin switching, a 3C assay (6) was applied to ln2 or ln2-Ik^{null} erythroid cells (Fig. 7). With this assay, it is possible to determine the physical proximity between chromosomal regions that are normally located far apart in vivo. Chromatin was digested with EcoRI and randomly ligated with T4 DNA ligase, and the proximity between βLCR and downstream regulatory regions was assessed by qPCR with primer sets designed to span several site pairs formed upon EcoRI restriction enzyme digestion. This restriction enzyme was chosen based on previous reports of equal nuclear digestion among different tissues, specifically fetal livers and brains (38). The same report also demonstrated that the βLCR HS2-HS4 "fixed" fragment (hereafter referred to as HS2-4) is appropriate to investigate how βLCR holocomplex and the distal active genes come in close proximity in fetal liver nuclei. Analysis of the huβ-globin locus in 12.5-dpc fetal liver reveals significant cross-linking frequency between the HS2-4 fragment and the active huγ, huδ, and huβ genes ($^{G}\gamma^{A}\gamma$, $^{A}\gamma$, δ, and β regions). The highest cross-linking efficiencies between HS2-4 and ε or iε-γ closest fragments results from direct correlation between spatial proximity and distance along the linear DNA template rather than productive nuclear chromatin interactions (6). Importantly, relative to ln2, ln2-Ik^{null} cells display higher amplification frequencies between HS2-4/$^{G}\gamma^{A}\gamma$, as well as HS2-4/$^{A}\gamma$ fragments. These results indicate that in erythroid cells lacking Ikaros proteins, the βLCR preferentially contacts fetal rather than adult globins at a developmental stage where huγ gene expression should progressively be extinguished and huβ-gene expression should progressively increase. Surprisingly, the low cross-linking frequency between HS2-4 and the EcoRI fragment enclosing the Pyr region (iγ-δ) illustrates that this region

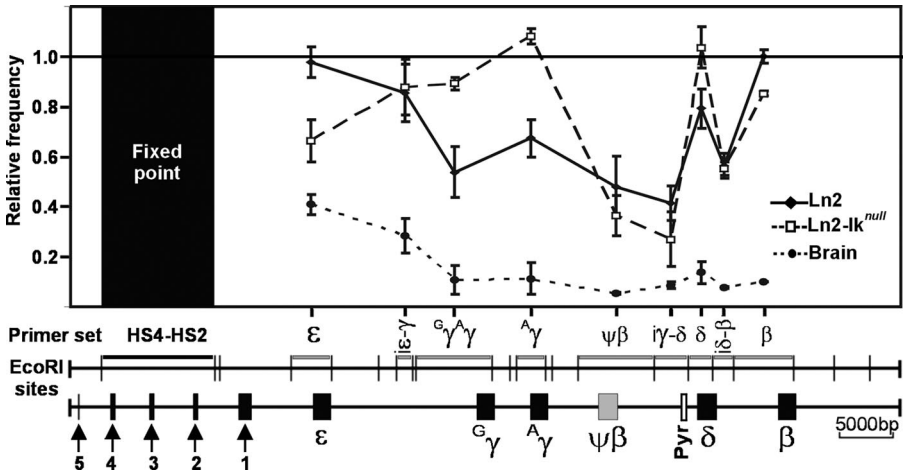


FIG. 7. Physical proximity between βLCR and globin gene promoters in ln2 and ln2-Ik^{null} 12.5-dpc fetal liver cells. 3C was applied on HCHO-fixed ln2 or ln2-Ik^{null} 12.5-dpc fetal liver cells. Nuclei were digested with EcoRI, and genomic DNA was ligated and subjected to qPCR with SYBR green. βLCR HS2-HS4 (HS2-4) EcoRI fragment was used as a "fixed" fragment, and specific primer sets were designed in order to amplify the genomic regions corresponding to the ε gene (ε), inter-ε-γ region (iε-γ), $^{G}\gamma^{A}\gamma$ genes ($^{G}\gamma^{A}\gamma$), $^{A}\gamma$ gene ($^{A}\gamma$), ψβ region (ψβ), inter-γ-δ region (iγ-δ), δ gene (δ), inter-δ-β region (iδ-β), and β gene region (β). Relative cross-linking frequencies (*y* axis) of the fixed fragment with globin fragments were defined using naked DNA encompassing the whole huβ-globin as a control and normalized to endogenous mouse actin. A value of 1 was attributed to the highest cross-linking frequency obtained with ln2 samples. Error bars represent standard deviations. The *x* axis indicates the position across the locus.

does not efficiently contact the βLCR in 12.5-dpc fetal liver cells isolated from ln2 or ln2-Ik^{null} mice.

To better define the role of Ikaros for huγ-gene silencing during fetal erythropoiesis, huγ-globin gene expression was studied in 14.-dpc fetal liver erythroid cells. As shown above, huγ-gene expression is 3.7-fold higher in ln2-Ik^{null} than ln2 cells, strongly suggesting that Ikaros is necessary for appropriate huγ-gene silencing at the time of globin switching and later on during the fetal stage of definitive erythropoiesis.

DISCUSSION

Ikaros–GATA-1 protein interaction. We show here for the first time that Ikaros and GATA-1 do interact and that in erythroid cells Ikaros contributes to GATA-1 recruitment and/or stability at HS3, the huγ promoters, and the Pyr region. We also provide evidence that this effect is not limited to the globin locus since Ikaros is recruited to the GATA-2 IG promoter, where it affects GATA-1 occupancy and GATA-2 gene expression. Ikaros–GATA-1 interaction has been hypothesized but never reported, perhaps because (i) biochemical purification of Ikaros-associated complexes has been generally carried out in lymphoid cells (10) and, most importantly, because (ii) beside Ikaros interaction with Helios, it is known that Ikaros forms low-stability complexes with other proteins in vivo, which might not resist high-stringency washes (47). Accordingly, the majority of Ikaros interacting partners have been identified or confirmed by IP of overexpressed chimeric proteins carrying epitope tags.

It has been shown that HS3 and HS2 are important for GATA-1-mediated βLCR/β major long-range chromatin interactions at the mouse globin locus and that submaximal concentration of GATA-1 can still trigger βLCR/βmaj proximity (52). This could explain why, even though GATA-1 binding to HS3 is affected in ln2-Ik^{null} cells (Fig. 5A) the βLCR retains the capacity to be in close proximity with the huβ promoter (Fig. 7). On the other hand, lack of Ikaros leads to reduced GATA-1 binding at huγ promoters, enhanced chromosomal proximity between the βLCR and huγ regions, and delayed huγ-gene silencing. Thus, these results suggest that Ikaros and GATA-1 act as transcriptional repressors of huγ genes at the time of γ- to β-globin switching and that their combinatorial effect at huγ promoters impairs long-range interactions between βLCR and huγ promoters. Accordingly, it has been shown that GATA-1 binding at positions −173 (25) and −566 (16) of the huγ promoters contributes to huγ-gene silencing in adult erythroid cells. Analysis of the −566 genomic region reveals the presence of a related Ikaros consensus binding sequence (TGGGAG) (30). It has been proposed that this, as well as many other low-affinity Ikaros consensus sites, does not bind Ikaros proteins very well. However, low-affinity binding sites can be occupied by Ikaros when present in multiplicity and in proximity. Furthermore, it has been suggested that combination of low- and high-affinity binding sites across a given gene regulatory region might control the global DNA-binding affinity of Ikaros (30). It is therefore possible that the low-affinity Ikaros site at position −310, together with the high-affinity Ikaros site at the exon 1-intron 1 junction of huγ-globin genes, might influence GATA-1 binding to huγ-regulatory regions during development, particularly at the time of γ- to β-globin switching.

In contrast to the Ikaros-dependent GATA-1 binding at HS3 and huγ promoters, GATA-1 recruitment and/or stability at HS2 and the huβ promoter appears to occur independently of Ikaros. This site selectivity might depend on the interaction of Ikaros and/or GATA-1 with other factors. For instance, both Ikaros and "GATA-1-FOG-1" can functionally interact with chromatin remodeling complexes that generally either promote gene activation (SWI/SNF or the SWI/SNF-related

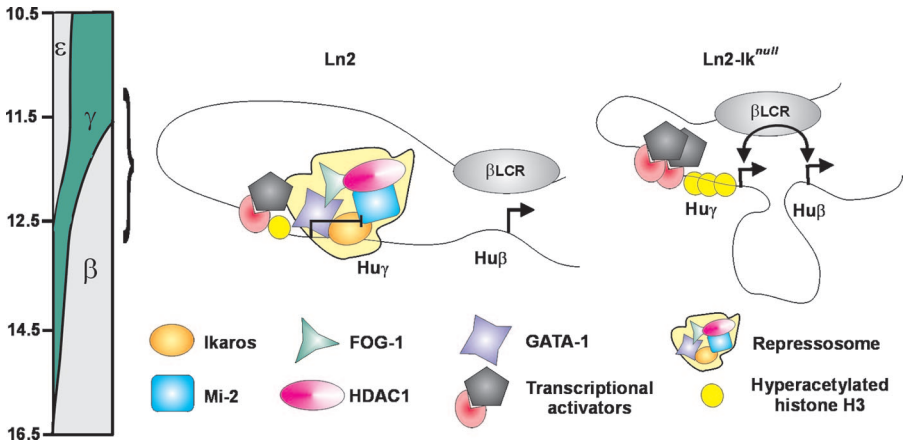


FIG. 8. Model of hypothetical Ikaros-dependent repressosome nucleation leading to huγ-gene repression at the time of γ- to β-globin switching. A hypothetical model of huγ-globin gene repression mediated by the Ikaros/GATA-1/FOG-1/Mi-2/HDAC1 repressosome (for simplicity, only one of the two huγ genes is depicted). Repressosome nucleation at the huγ promoters requires the presence of Ikaros. In ln2 cells, chromatin conformation at the huγ-region limits transcriptional activator recruitment to huγ promoters. Thus, huγ genes are progressively and efficiently silenced. However, in ln2-Ik^{null} cells (ln2-Ik^{null}), the repressosome is formed less efficiently, and chromatin at the huγ promoters maintains an accessible conformation, which sustains recruitment of transcriptional activators and coactivators hence, higher huγ-gene expression. Repressosome nucleation, by reducing chromatin accessibility, progressively decreases the frequency of productive interactions between βLCR and huγ promoters. At the same time, several transactivators (such as EKLF) and chromatin-modifying activities (such as that of the SWI/SNF related complex, E-RC1), gathered to the huβ promoter, contribute to chromatin activation and facilitate βLCR/huβ over βLCR/huγ long-range interactions.

ACF complex) or repression (NuRD) (10, 44). Recently, Naito et al. (31) demonstrated that during development and lineage specification, Ikaros can promote recruitment of either negative or positive transcriptional regulators to the CD4 silencer. Similarly, it has been shown that GATA-1 and EKLF, two zinc-finger proteins that physically interact, might co-occupy or bind independently the murine globin locus (17, 27).

Even though Ikaros is expressed in almost all hematopoietic cells, distinct cell lineages are affected more or less severely in Ik^{null} mice. The variable phenotypes suggest that Ikaros proteins may carry out specific functions in different hematopoietic cells, perhaps in association with lineage-restricted partners. This is the case for two Ikaros-interacting proteins, Aiolos and Helios, which are predominantly expressed in B cells and HSC, respectively (19, 53). Accordingly, the interaction with GATA-1 could provide Ikaros with erythroid-specific functions by targeting chromatin-modifying and -remodeling activities to the huβ-globin locus and to other loci/genes, including the GATA-2 IG promoter.

Recruitment of Ikaros, GATA-1, FOG-1, Mi-2, and HDAC1 to huγ promoters at the time of γ- to β-globin switching. Binding of Ikaros to the huβ-globin locus has been observed in vitro (20, 35). More recently, recruitment of Ikaros to HS3 has also been shown in K562 erythroleukemia cells overexpressing Ikaros-1 (20). However, since (i) K562 cells can be induced to produce huγ- but not huβ-globins, (ii) imbalance between Ikaros isoforms (as observed when only one isoform is overexpressed) can modify Ikaros-target gene transcriptional regulation (10), and (iii) Ikaros overexpression is reported to arrest the cell cycle at the G_1-to-S-phase transition (2), as well as favor apoptosis in adult erythroid cells (43), we verified the physiological role of Ikaros in primary erythroid cells. We show that in freshly isolated erythroid cells, endogenous Ikaros (i.e., when expressed at physiological levels) binds in vivo to the huβ-globin locus and to the GATA-2 IG promoter. Most importantly we shed light on the molecular mechanisms of Ikaros-mediated huγ-gene repression at the time of γ- to β-globin switching, showing that Ikaros may favor recruitment to the huγ promoters of a repressosome-like complex containing GATA-1, FOG-1, and the NuRD complex components Mi-2 and HDAC1 (Fig. 8). Due to the presence of Mi-2 and HDAC1, two well-known Ikaros interacting partners (47), this complex could locally transform transcriptionally active chromatin into chromatin refractory to transcription. Accordingly, analysis of ln2-Ik^{null} cells revealed that Ikaros favors HDAC1 recruitment to huγ promoters, hence reducing the histone acetylation level and contributing to huγ-gene silencing at the time of γ- to β-globin switching. Support for this model also derives from the observation that histone deacetylase inhibitors can reactivate huγ-gene expression in adult erythroid cells (28), suggesting that histone acetylation level is important for huγ-gene transcriptional regulation. The fact that at adulthood γ- to β-globin switching is eventually completed without the contribution of Ikaros (data not shown) indicates that Ikaros-mediated repression is an early event leading to huγ-gene silencing at the fetal stage of development and that, in erythroid cells lacking Ikaros, huγ-gene silencing eventually occurs by other compensatory mechanisms or by the participation of additional transcription factors (48, 50).

It has been shown that Ikaros physically interacts with Mi-2 and HDAC1 (47). However, it cannot be excluded that reduced recruitment of Mi-2 and HDAC1 to huγ promoters in ln2-Ik^{null} cells be also an indirect effect due to reduced GATA-1–FOG-1 occupancy because GATA-1 and FOG-1 are found in a complex also containing Mi-2 and HDAC1 (44). Nonetheless, impaired GATA-1 and FOG-1 binding in ln2-Ik^{null} cells supports the hypothesis that Ikaros can be required for

development-specific nucleation of a repressosome at huγ promoters.

Even though in vitro Ikaros binds the Pyr region (35, 36), in vivo Ikaros association with this region appears to be indirect (Fig. 2C and D). At the time of γ- to β-globin switching, the Pyr region does not interact with the βLCR (Fig. 7). In addition, in contrast to what was observed at HS3 and the huγ and huβ promoters, histone acetylation is not modified at Pyr region in ln2-Ik^{null} cells (Fig. 4C). These results, along with the fact that we could not reveal BRG1, HDAC1, or Mi-2 occupancy at the Pyr region (Fig. 4A, B, and D), suggest that the recruitment of the PYR complex to this region in 12.5-dpc fetal liver cells might not be a major factor for γ- to β-globin switching. Indeed, the PYR complex has been purified in MEL cells, which are proerythroblast-like cells expressing only adult hemoglobin, and PYR activity has been found in 14.5-dpc mouse fetal liver cells (expressing adult globin genes) but not in yolk sac primitive erythroid cells (expressing embryonic globin genes). Thus, without excluding the possibility that the Pyr region could be important for Ikaros-dependent globin gene regulation at specific developmental stages, our results suggest that this region is not a critical target of Ikaros activity at the time of γ- to β-globin switching.

In vitro, PYR complex DNA-binding activity copurifies with few SWI/SNF complex subunits (35). Nevertheless, we could not demonstrate any clear involvement of Ikaros as component of a SWI/SNF complex capable of directly contributing to huβ-gene activation at the time of γ- to β-globin switching. However, we show that in ln2-Ik^{null} cells, BRG1 recruitment and histone H3 acetylation levels are reduced at βLCR HS3 (Fig. 4A and C). HDAC1 levels are similar in ln2 and ln2-Ik^{null} cells; hence, it is likely that HS3 hypoacetylation in ln2-Ik^{null} cells results from reduced recruitment of histone acetyltransferase activities. Interestingly, it has been shown that GATA-1 favors BRG1 (17) and CBP (23) acetyltransferase occupancy at βLCR HS3, and here we provide evidence that lack of Ikaros proteins significantly affects GATA-1 recruitment to HS3. Therefore, it is possible that reduced BRG1 recruitment and histone H3 acetylation at this region result from the combinatorial effect of Ikaros and GATA-1. Interestingly, even though CBP and BRG1 are recruited less efficiently to HS3, βLCR chromatin organization and long-range chromosomal interactions are not profoundly affected (Fig. 7) and huγ genes are efficiently expressed, suggesting that decreased recruitment of these activities does not preclude the formation of active chromatin conformation at HS3.

It is known that several transcription factors and cofactors contribute to chromatin conformation across the βLCR and a few among them, such as p45/NF-E2 and EKLF, can influence GATA-1 occupancy at βLCR and vice versa. However, we show that the absence of Ikaros does not alter p45/NF-E2 occupancy at HS3, HS2, and huγ promoters (Fig. 5E). This result and the fact that BRG1 recruitment to huγ promoters is not modified in ln2-Ik^{null} cells (Fig. 4A) suggest that the effect of Ikaros on GATA-1 DNA binding is specific and is not due to a general modification of chromatin conformation due to the absence of Ikaros.

Ikaros and chromatin looping. Long-range chromatin interaction is the mechanism by which β-like globin genes are highly expressed in a development- and tissue-specific manner (5, 38, 51). Efficient long-range interactions between βLCR and adult globin genes requires GATA-1 with FOG-1 (52), EKLF (9), NLI/Lbd1 (46), and possibly other as-yet-unidentified transcription factors. Nonetheless, little is known about the mechanisms and chromatin-associated proteins that actively contribute to the impairment of productive long-range interactions between βLCR and globin promoters. Here, we show that Ikaros, by binding to the huγ promoters, can reduce βLCR/huγ close proximity at the time of γ- to β-globin switching. This indirectly suggests that huγ-promoter chromatin organization contributes substantially to efficient long-range chromatin interaction with the βLCR and that local chromatin changes are critical for development-specific transcriptional silencing of huγ genes. During development, progressive reduction of βLCR/huγ proximity, together with the action of several transactivators (like EKLF) gathered to the huβ promoter, favor huβ over huγ promoters for long-range interactions with the βLCR. In the absence of Ikaros, the switching is delayed and the active chromatin conformation at huγ promoters appears to favor efficient βLCR/huγ chromatin contacts for longer periods (Fig. 7).

Gene expression studies by microarray have shown that expression of the erythroid transcription factor EKLF decreases to 0.6-fold in Ik^{null} relative to wild-type 14.5-dpc fetal liver cells (26). This level of expression is similar to the level observed in EKLF heterozygous null background mice (EKLF$^{+/-}$), which are viable and appear normal in terms of adult globin gene expression (34, 41). EKLF is required for adult globin gene expression in both mice (34, 41) and humans (55) during fetal and adult erythropoiesis. It has been shown that the decrease in EKLF affects the huγ/huβ ratio during globin switching with decreased huβ-gene and increased huγ-gene expression. However, by 14.5 dpc, both huγ- and huβ-gene expression in mouse fetal livers returns to normal levels (40, 55). Since (i) in 14.5-dpc fetal liver cells huγ-gene silencing is delayed in ln2-Ik^{null} mice, whereas it is not affected in EKLF$^{+/-}$ animals and since (ii) βLCR/huβ-gene chromatin interactions are profoundly altered in 12.5-dpc fetal livers isolated from EKLF-deficient mice (9), whereas no major changes are observed in 12.5-dpc ln2-Ik^{null} fetal livers (Fig. 7), it is unlikely that the results obtained in ln2-Ik^{null} erythroid cells are the mere consequence of a reduced level of EKLF gene expression. Instead, our data suggest that Ikaros can exert a direct and specific effect on huγ-gene regulation in fetal liver erythroid cells.

In conclusion, we demonstrate that Ikaros, together with GATA-1, contributes to development-specific silencing of huγ genes. The absence of Ikaros delays huγ-gene silencing and alters long-range chromatin interactions across the locus, favoring more prolonged productive contacts between βLCR and huγ genes. Based on our results, we propose that Ikaros-dependent nucleation of a repressosome-like complex contributes to progressive reduction of βLCR/huγ chromatin interactions by affecting huγ-promoter organization (i.e., influences transcription factor and cofactor recruitment/stability). Finally, molecular analyses carried out at GATA-2 IG promoter suggest that the Ikaros–GATA-1 combinatorial effect is not limited to huβ-globin gene regulation, but it also affects the transcriptional regulation of other hematopoietic genes. Interestingly, Ikaros and GATA-1 take part in transcriptional regulation of the interleukin-4 gene (14). Thus, by influencing

promoter organization and long-range chromatin interactions, Ikaros and GATA-1 combinatorial effects might represent an important mechanism of gene regulation during hematopoiesis.

ACKNOWLEDGMENTS

We thank E. Drobetsky for critical reading of the manuscript, K. Georgopoulos for the Ik^{null} mouse line, H. Beauchemin for technical advice, and G. D'Angelo for Wright-Giemsa staining analysis.

This study was supported by a grant from the Leukemia and Lymphoma Society of Canada and from the Canadian Institutes of Health Research (CIHR) held by E.M. M.T. is supported by a CIHR grant, J.R. is supported by a Fond de la Recherche en Santé du Québec (FRSQ) Doctoral Training Award, and E.B.A. and E.M. are scholars of the FRSQ.

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