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# Chicken α-Globin Switching Depends on Autonomous Silencing of the Embryonic π Globin Gene by Epigenetics Mechanisms

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## ABSTRACT

Switching in hemoglobin gene expression is an informative paradigm for studying transcriptional regulation. Here we determined the patterns of chicken α-globin gene expression during development and erythroid differentiation. Previously published data suggested that the promoter regions of α-globin genes contain the complete information for proper developmental regulation. However, our data show a preferential *trans*-activation of the embryonic α-globin gene independent of the developmental or differentiation stage. We also found that DNA methylation and histone deacetylation play key roles in silencing the expression of the embryonic π gene in definitive erythrocytes. However, drug-mediated reactivation of the embryonic gene during definitive erythropoiesis dramatically impaired the expression of the adult genes, suggesting gene competition or interference for enhancer elements. Our results also support a model in which the lack of open chromatin marks and localized recruitment of chicken MeCP2 contribute to autonomous gene silencing of the embryonic α-globin gene in a developmentally specific manner. We propose that epigenetic mechanisms are necessary for in vivo chicken α-globin gene switching through differential gene silencing of the embryonic α-globin gene in order to allow proper activation of adult α-globin genes. J. Cell. Biochem. 108: 675–687, 2009. 

**KEY WORDS:** DNA METHYLATION; SILENCING; HEMOGLOBIN SWITCHING

Hemoglobin switching represents the transition from embryonic to adult globin peptide synthesis in order to adjust oxygen requirements in the organism during development, and is accomplished through coordinated and differentially regulated globin gene expression. This process depends on a complex interplay between gene promoters, distal regulatory elements, nuclear dynamics and the epigenetic milieu [West and Fraser, 2005; Ragoczy et al., 2006]. Initially, Engel and collaborators proposed gene competition for long-distance regulatory elements in order to explain the developmentally regulated exclusive expression of the chicken adult $\beta^A$- and embryonic ε-globin genes, which share an enhancer residing between the two genes [Foley and Engel, 1992]. To date, the exact mechanism responsible for the switch remains unclear.

In addition to the genetic processes regulating globin gene expression, epigenetic regulation also plays a key role in globin switching [Goren et al., 2006; Demers et al., 2007; Song et al., 2007]. The human and murine embryonic and fetal β-like globin genes are

Abbreviations used: LCR, locus control region; MARE, Maf recognition element; EKLF, erythroid Krüppel-like factor; YY1, Ying Yang 1 nuclear factor; RBC, red blood cells; TSA, trichostatin-A; 5-azadC, 5-aza-2′-deoxycytidine.

Héctor Rincón-Arano and Georgina Guerrero contributed equally to this work.

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silenced via DNA methylation [Lavelle et al., 2006; Kransdorf et al., 2006; Mabaera et al., 2008]. Previous in vitro evidences has shown that methyl-CpG-binding proteins can bind methylated promoters; for example MBD2 interacts with the promoter region of the chicken β-globin ρ gene in definitive erythropoiesis [Singal et al., 2002; Kransdorf et al., 2006]. Furthermore, specific histone hypoacetylation of the embryonic ε and fetal γ β-like globin genes can be detected in adult stages. Depletion of open chromatin marks like H3K4me2 and H3K79me2 on the embryonic β-globin promoter are also evident during β-globin switching in baboons [Lavelle et al., 2006]. These studies support a role of epigenetic modifications in the hemoglobin switching processes during development.

The chicken globin genes have been widely used as model for studying transcriptional regulation during development and erythroid differentiation. Although the mechanisms that regulate the chicken β-globin genes have been addressed, the in vivo mechanisms involved in regulating α-globin switching remain more elusive. Unlike the chicken β-globin domain, the α-globin domain lies in a constitutive open chromatin context on minichromosome 14 and contains the embryonic π gene and adult $\alpha^D$ and $\alpha^A$ genes (Fig. 1A). Initial data suggested that the three chicken α-globin genes are constitutively expressed during embryogenesis with the gradual silencing of the embryonic gene in definitive erythropoiesis. Further analysis of methylation patterns showed the in vitro association of MeCP2 to the π gene promoter [Singal et al., 2002]. Here we investigated whether in vivo epigenetic processes contribute to chicken α-globin gene switching. Our gene expression profiles demonstrated a preferential expression of the π gene in embryonic stages with a tight transition in α-globin gene expression at day 7 of chicken development. After day 7, DNA methylation can be detected on the promoter region of the embryonic gene, which coincides with the expression of the adult $\alpha^D$ and $\alpha^A$ genes. Interestingly, this DNA methylation can only be detected after embryonic day 7 and is responsible for MeCP2 recruitment to the embryonic promoter upon differentiation. Consistent with these observations, MeCP2 knockdown induces de-repression of the embryonic gene in definitive erythrocytes. Our results also suggest a model in which a preferential *trans*-activation of the embryonic π gene promoter can occur in any developmental or differentiation stage by the 3′ α-globin enhancer; in contrast, $\alpha^D$ and $\alpha^A$ gene

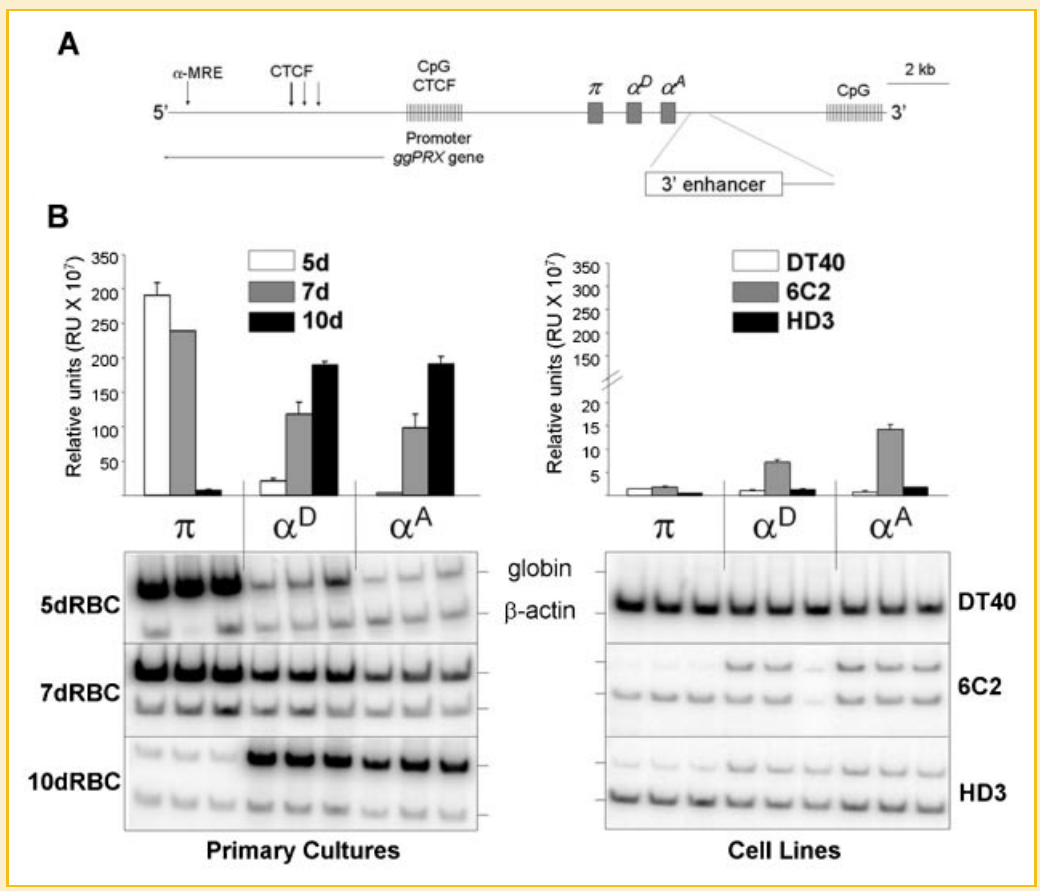


Fig. 1. Chicken α-globin gene expression patterns during erythroid differentiation and development. A: Scheme of the chicken α-globin domain. At the 5′ non-coding region there is a putative LCR element also known as α-MRE [Flint et al., 2001], three DNase I hypersensitive sites (vertical arrows), an in vivo CTCF binding site co-localizing with a group of three DNase I hypersensitive sites (vertical arrows) [Valadez-Graham et al., 2004], a CpG island which has been proposed to correspond to the transcription start region of the antisense and highly conserved *ggPRX* gene [Drissen et al., 2004; Klochkov et al., 2006]. The 3′-side enhancer is located 450 bp downstream of the adult $\alpha^A$ gene. B: Semi-quantitative gene expression evaluation of each one of the chicken α-globin genes by radioactive duplex PCR and β-actin was used for normalization. The plot represents the average value of two independent RT reactions and two series of PCR amplifications performed in triplicate.

promoters exhibit an erythroid differentiation-regulated activity. Therefore, we propose that in vivo epigenetic mechanisms negatively regulate the embryonic π gene promoter in order to establish the adult α-globin gene expression profile.

## MATERIALS AND METHODS

### CELL LINES AND PRIMARY CELL CULTURE

The avian erythroblastosis virus-transformed and temperature-sensitive chicken erythroblast cell line LSCCHD3 (HD3) and CFU-E arrested 6C2 cell lines were grown as previously described [Boyes and Felsenfeld, 1996; Rincón-Arano et al., 2005]. The DT40 lymphoid cell line was grown in DMEM supplemented with 50 μM 2-mercaptoethanol, 10% tryptose phosphate broth, 10% fetal calf serum, and 5% chicken serum [Dieken et al., 1996]. Primary cultures of chicken red blood cells (RBCs) were obtained from 5-, 7-, 8-, 10-day embryos and 14-day-old terminally differentiated erythrocytes (TD-RBCs), and maintained in L15 medium supplemented with 5% fetal calf serum and 1% chicken serum at 37°C. Trichostatine-A (Sigma) and 5-aza-2′-deoxycytidine (Sigma) were used at 2.5 ng/ml and 3 μM, respectively.

### HD3 CELLS DIFFERENTIATION INDUCTION

HD3 cells are chicken cells transformed by a thermosensitive mutant of the avian erythroblastosis virus (AEV) expressing the v-erb oncogene [Beug et al., 1979; Iarovaia et al., 2001]. The HD3 cells correspond to chicken hematopoietic cells of the definitive red lineage arrested in early stages of differentiation and they can be induced to differentiate. To induce differentiation, the proliferating HD3 cells were incubated with 5% of anemic chicken serum at 42°C for 5, 10, and 12 days. Aliquots were collected from the cell suspension at different time intervals after the beginning of induction and the percentage of cells synthesizing hemoglobin was calculated using benzidine staining. Under these conditions after 5 days of induction we reached about 70% of cells producing hemoglobin.

### PLASMIDS

The pGαD plasmids and their associated versions, which include the core enhancer and the entire enhancer were previously described [Rincón-Arano et al., 2005]. The π and $\alpha^A$ promoters were PCR amplified from 10-day-old embryonic erythrocytes from *Bam*HI-digested *Gallus gallus* genomic DNA, using the following primers: ALFA-AF: 5′-CGGGATCCCTCCACCTGCACCAAGGCAGAC-C-3′; ALFA-AR: 5′-CGGGATCCGGTGCCCCTGAAGTCTGCGTG-3′, ALFA-PF: 5′-CGGGATCCGCTCAAAATCCATTGAAAGGCACG-3′ and ALFA-PR: 5′CGGGATCCCCAGAGCAGGTTGTACTGAG-3′. The DNA fragments were introduced into the pDrive cloning vector (Qiagen PCR cloning Kit) and sequenced. The promoters were cloned into the previously described vectors which contain the different versions of the α-globin enhancer (see above), leading to the following π promoter-containing plasmids: pGπ, pGπE, pGπE4; and the following $\alpha^A$ promoter-containing plasmids: pGαA, pGαAE, and pGαAE4.

### ANTIBODIES

The antibodies against MBD2 N-18 (Sc-9397), Sp1 (Sc-59), GATA-1(Sc-13053) and control IgG were purchased from Santa Cruz Biotechnology. The antibody against the activating subunit NF-E2p45 was kindly provided by James Shen (Academia Sinica, Taiwan). Antibodies against the global acetylated form of histone H3 (06-599) and H4 (06-866), and the antibody against dimethylation of lysine 4 (07-030) and MeCP2 antibody (07-013) were obtained from Upstate. The antibody against dimethylation of lysine 79 was donated by Fred van Leeuwen (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Thomas Jenuwein (IMP, Austria, Vienna) donated the antibodies against the trimethylated versions of lysines 9 and 20 of the histones H3 and H4, respectively.

### TRANSIENT TRANSFECTIONS AND KNOCKDOWN ASSAY

The conditions for HD3 and 6C2 cell transfection have been previously published [Rincón-Arano et al., 2005]. Five- and 10-day-old chicken erythrocyte transient transfections were performed as described previously [Rincón-Arano et al., 2005]. For *trans*-activation assays and co-transfection experiments, 2 μg of the test constructs were mixed with 200 ng of Renilla (pRL-TK, Promega) and 2 μg of chicken GATA-1 cDNA (kindly provided by Cecelia Trainor, NIH, USA). Chicken MeCP2-specific Stealth siRNAs oligoribonucleotides were prepared according to the manufacturer's instructions (Invitrogen). HD3 cells were transfected using lipofectamine 2000. Briefly, a mix of 500 nM of each stealth siRNA was transfected in $5 \times 10^5$ differentiated HD3 cells. After 24 h, a second round of transfection was performed. Twenty hours after the second transfection the cells were harvested and proteins and RNA were extracted. The oligoribonucleotide sequences were as follows: (353): 5′-CCGGGAAGUACGACGUCUAUCUCA U-3′; 5′-AUGA-GAUAGACGUCGUACUUCCCGG-3′, and (410): 5′-UGGACCU-GAUCGCGUACUUCGAGAA-3′; 5′-UUCUCGAAGUACGCGAUCAG-UUCCA-3′ (derived from the MeCP2 sequence from the NCBI, accession number Y14166) [Weitzel et al., 1997]. The following sRUAi's were used as a control, C353: 5′-CCGAAGUACGACGU-CUAUCUGGCAU-3′ and 5′-AUGCCAGAUAGACGUCGUACUUCG-G-3′.

### RNA PURIFICATION AND SEMI-QUANTITATIVE RT-PCR

Cells were resuspended in Trizol (Invitrogen) and RNA was isolated following the manufacturer's protocol. The RT-PCR was performed using the Reverse Transcription System (Promega), oligo(dT) and 5 μg of RNA were used for each reaction. The PCR was radioactively labeled with [$\alpha$-$^{32}$P]dCTP and the amplification fragments were resolved in an acrylamide gel, dried and exposed on a Kodak phosphoimager cassette. ImageQuant software was used for quantitation. Primers for the chicken α-globin genes were designed flanking the intron–exon boundaries. Primer sequences were derived from the sequence number AF098919 (*G. gallus*; gi: 20043262), as follows: PHIF: 5′-TCACTGGAGAGGCTTTTTGCC-3′, PHIR: 5′-GTGGGAAAGCAGCTTGAAGTT-3′, AlphaDF: 5′-GCTCTGACTAG-GATGTTCACC-3′, AlphaDR: 5′-CTGCGACAACAGCTTGAAATTG-3′, AlphaAF: 5′-CCCTGGAAAGGATGTTCACC-3′, AlphaAR: 5′-GGCCCAGGAGTTTGAAGTTG-3′. Primers for the β-actin gene were used for normalization. They are as follows: BActinF2:

5′-CCAGACATCAGGGTGTGATG-3′ and BActinR: 5′-GAACACGGTATTGTCACCAACTGG-3′.

### METHYLATION SENSITIVE ENDONUCLEASE *MSP*I/*HPA*II ASSAY

Twenty micrograms of genomic DNA were digested with 100 U of *Msp*I or *Hpa*II. The digested DNA was purified by standard phenol extraction and ethanol precipitation and further digested with 20 U of *Bam*HI and *Hin*dIII. The digested DNA was resolved on a 2.5% agarose gel and transferred onto a nitrocellulose membrane (Hybond $N^+$). The promoter regions of the three α-globin genes were labeled with [α-$^{32}$P]dCTP by random priming and then hybridized.

### METHYLATION SENSITIVE-PCR

Genomic DNA from 6C2, HD3, and DT40 cell lines and from 5-, 8-, and 10-day-old embryonic erythrocytes were digested with *Eco*RI. For the time course study, 1 μg of digested DNA was denatured at 95°C for 5 min, chilled on ice, and incubated with 0.3 M NaOH in a 20 μl reaction volume at 37°C for 5 min. Freshly prepared solutions of sodium bisulfite (S-8890, Sigma), adjusted to pH 5.0 with NaOH, and hydroquinone (H-9003, Sigma) were added at final concentrations of 1.7 M and 0.5 mM, respectively, in a final volume of 240 μl. DNA solutions were mixed and incubated at 55°C for 16 h. Non-reacting bisulfite was removed by column chromatography (Wizard DNA Clean-Up System A7280 HR; Promega). Purified DNA samples were mixed with NaOH at a final concentration of 0.3 M, incubated at 37°C for 20 min, neutralized on the DNA Clean-Up System. Nested PCR reactions were performed in 50 μl reaction volumes using HotStart Taq DNA polymerase (Qiagen). For PCR amplification, 5 μl of bisulfite treated DNA was amplified using 20 pmol/μl of the following primers: $\alpha^D$ms1.1 (meCpG): (Forw) 5′-TATAGTTACGGTTTTTTCGTGC-3′, $\alpha^D$ms1.2 (CpG): (Forw)5′-GTATAGTTATGGTTTTTTTGTGT-3′, and the reverse primer, $\alpha^D$msR; 5′-CCAAATACCCCTAAACCTAA-3′. $\alpha^A$ms1.1(meCpG): (Forw) 5′-TAATATTAATTTTAGTTCGCGTC-3′, $\alpha^A$ms1.2(CpG): (Forw)5′-TAATATTAATTTAGTTTGTGTT-3′, and the reverse primer, $\alpha^A$msR; 5′-ATTTTAATAAAAATACCCTTAAC-3′. πms1.1(meCpG): (Forw) 5′-TTAGGTTATACGGTTAATAC-3′, πms1.2(CpG): (Forw) 5′-TTAGGTTATATGGTTAATAT-3′, and the reverse primer, πmsR; 5′GGATTGTTGTTTTAGTTTTTTT-3′.

### WESTERN BLOTS

Detection of GATA-1, MeCP2, β-actin and Lamin B was performed as previously described [Valadez-Graham et al., 2004].

### CHROMATIN IMMUNOPRECIPITATION ASSAY (CHIP) AND PCR REACTIONS

Chromatin immunoprecipitation was carried out as previously described [Valadez-Graham et al., 2004]. Duplex PCR was performed as previously described using the following primers [Rincón-Arano et al., 2007; Furlan-Magaril et al., 2009]: $\alpha^A$F3: 5′-CTGCACCAAGGCAGACCCTAACCTC-3′; $\alpha^A$R3: 5′-GCTGTCAGCCTTATATGCTGTGCTG-3′; $\alpha^D$F: 5′-CGGGATCCCCTGCATGCAGTGTGGAGC-3′, $\alpha^D$R: 5′-CGGGATCCGGGCTGGTGGCTGGTGG-3′; $\alpha^\pi$F: 5′-CGGGATCCCCTCAAAATCCATTGAAAGGCACG-3′; $\alpha^\pi$R: 5′-CGGGATCCCAGAGCAGGTTGTACTGAG-3′. The following primers were used as control and their sequences are located around 5 kb upstream of the π gene: SRF1: 5′-CGGGCAGGTCGCGCTCAGAG-3′ and SRR1: 5′-CGGGGCTGAGGTGTGAGGTG-3′.

## RESULTS

### CHICKEN α-GLOBIN GENE EXPRESSION DURING DEVELOPMENT AND ERYTHROID DIFFERENTIATION

Previously, it had been suggested in a qualitative manner that the chicken α-globin genes were expressed during the embryonic stages, with a gradual loss of the embryonic π gene expression during development and concomitant increase in adult gene expression [Minie et al., 1992]. To establish quantitatively the expression pattern of the α-globin genes, we analyzed globin mRNA levels in erythrocytes from 5-, 7-, and 10-day-old chicken embryos (Fig. 1B). Using semi-quantitative duplex RT-PCR, we found that in 5-day-old embryo red blood cells (5dRBCs), the embryonic π gene is dominantly expressed with a basal transcription of the adult genes. In 7-day embryo erythrocytes (7dRBCs), the embryonic π gene begins to be silenced and the adult genes $\alpha^D$ and $\alpha^A$ are expressed. Finally, in 10dRBCs, the expression of the embryonic gene is absent, and the adult $\alpha^D$ and $\alpha^A$ genes are transcribed at high levels. These results show the differential expression of the chicken α-globin genes during development demonstrating the existence of a transcriptional switching mechanism.

In order to establish the expression pattern of the α-globin genes during definitive erythroid differentiation we used several chicken cell lines that mimic different stages of differentiation (Fig. 1B) [Beug et al., 1979, 1982]. We used the transformed 6C2 cell line that is arrested in the pre-erythroblast stage, the HD3 cell line, which is arrested at the erythroblast stage, and the DT40 lymphoblast cell line as a representative non-erythroid cell [Dieken et al., 1996]. Our data show that the expression of the embryonic π gene and adult $\alpha^D$ and $\alpha^A$ genes is essentially absent in the different erythroid cell lines (Fig. 1B). This transcription profile is erythroid-specific due the absence of α-globin gene expression in the lymphoid DT 40 cell line. In summary, these results establish the differential developmental expression pattern of the chicken α-globin genes and there is basically no globin gene expression in definitive erythroid cell lines.

### AUTONOMOUS EMBRYONIC GENE SILENCING IN DEFINITIVE ERYTHROPOIESIS IS CAUSED BY DNA METHYLATION

Epigenetic mechanisms like DNA methylation correlate with the inactive transcriptional status of globin genes during development [Groudine and Weintraub, 1981; Goren et al., 2006; Kiefer, 2007]. CpG methylation of the embryonic π gene promoter occurs in differentiated 10dRBCs, in which the embryonic gene becomes transcriptionally inactive [Singal et al., 2002]. To evaluate the DNA methylation status of the α-globin gene promoters, we performed a methylation-sensitive restriction enzyme assay using *Msp*I/*Hpa*II endonucleases in the context of developmental erythropoiesis as well as by using differentiating erythroid cell lines (Fig. 2A). The digestion of genomic DNA from 5dRBC revealed an absence of CpG methylation at the three promoters in the early developmental stage. In later developmental stages, we detected DNA methylation at the π promoter in 8dRBC, a stage immediately subsequent to the switching

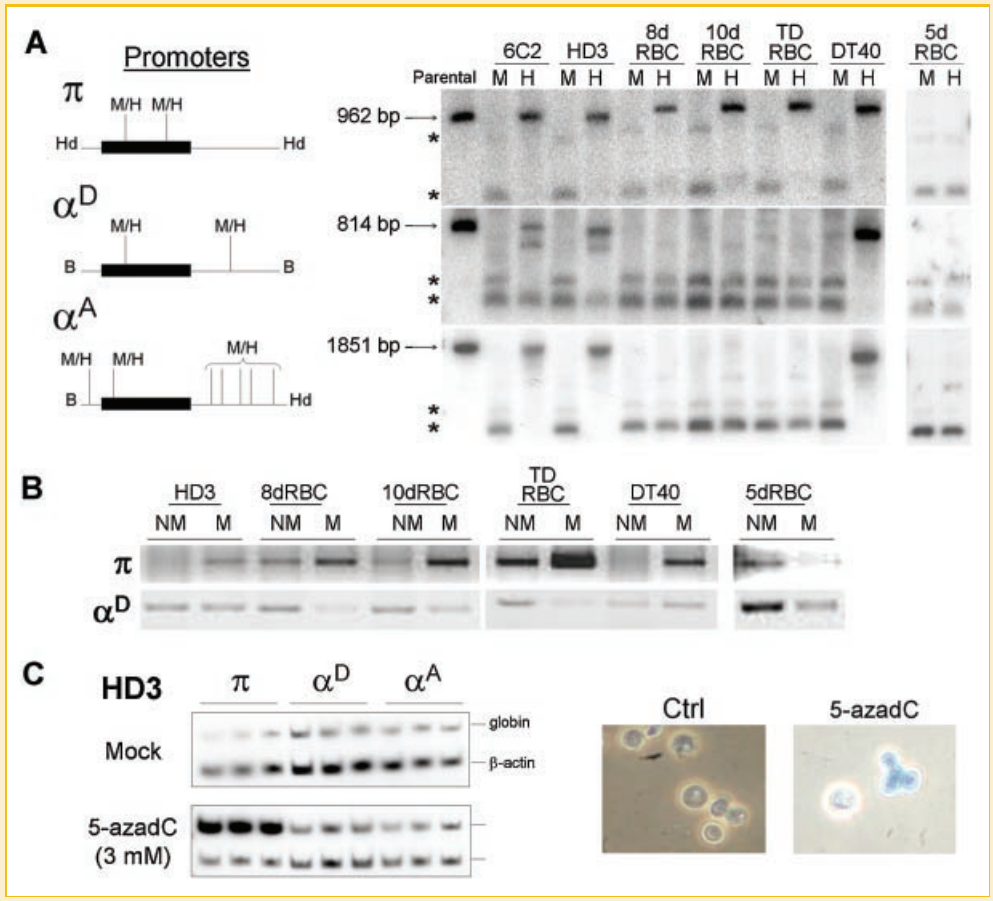


Fig. 2. DNA methylation status of the chicken α-globin gene promoters. A: Scheme of the *Msp*I/*Hpa*II (M/H) restriction enzyme recognition sites and Southern blot hybridized with probes corresponding to each promoter. The restriction sites for genomic digestions are indicated as: Hd: *Hin*dIII and B: *Bam*HI. TD-RBC, correspond to genomic DNA from 14-day-old chicken embryo terminally differentiated erythrocytes. For the embryonic π gene promoter a parental band of 962 bp is expected on the basis of the Hd-Hd digestion; M/H digestions generates DNA fragments of 655, 247 bp (asterisks), and 60 bp (not shown). For the $\alpha^D$ gene promoter 814 bp DNA fragment is expected as parental band from the B-B digestion; M/H digestions generates DNA fragments of 330, 278 bp (asterisks), and 206 bp (not shown). For the $\alpha^A$ gene promoter a parental band of 1,851 bp is observed after B-Hd genomic digestion; M/H digestions generates eight DNA fragments and due to the gel resolution only the 551 and 351 bp DNA fragments (asterisks) are shown. B: Methylation-sensitive PCR (MS-PCR) assay using primers from the π and $\alpha^D$ promoters. The primer pairs allowed amplification of either non-methylated (NM) or methylated (M) promoters. C: Reactivation of the embryonic gene expression using 5-aza-2′-deoxycytidine evaluated by duplex RT-PCR and benzidine staining.

transition between embryonic and adult gene expression (Fig. 1). Nonetheless, no DNA methylation was detected at the $\alpha^D$ and $\alpha^A$ adult gene promoters (Fig. 2A). These results are consistent with previous reports that suggested an inverse correlation between DNA methylation and transcriptional activity of the embryonic α-globin promoter [Singal et al., 2002]. Additionally, our result showed that in early adult pre-erythroblast and erythroblast stages, CpG methylation can be detected at the three α-globin promoters, confirming that this epigenetic mark is established over the α-globin domain in the hematopoietic precursors [Groudine and Weintraub, 1981]. Methylation status was also evaluated by methylation sensitive PCR showing similar results (Fig. 2B).

To evaluate whether DNA methylation participates in the final gene expression profile in definitive erythropoiesis, we treated the HD3 cell line were the α-globin genes are silenced with the DNA methylation inhibitor 5-aza-2′-deoxycytidine, and mRNA levels were evaluated by semi-quantitative duplex PCR. Interestingly, our results showed that the embryonic π gene could be exclusively reactivated in erythroblasts by the inhibitor, but adult genes remained unaffected in treated HD3 cells. By methylation-sensitive PCR analysis we confirmed that the embryonic π gene promoter was unmethylated after treatment (data not shown). This response can be observed at protein levels since cells positively stain with the hemoglobin dye benzidine (Fig. 2C) [Alev et al., 2008]. RBCs were not tested due to their lack of cell proliferation. Thus, our results confirm that π gene expression silencing is mediated by DNA methylation in erythroblast HD3 cells, and predicts that this methylation is maintained until terminal differentiation steps of erythropoiesis in order to allow autonomous and regulated adult gene expression.

## MECP2 IS DIFFERENTIALLY RECRUITED TO THE EMBRYONIC π GENE PROMOTER DURING DEFINITIVE ERYTHROPOIESIS

Our results suggested that DNA methylation targets the promoter region of the embryonic α-globin gene during chicken α-globin switching in definitive erythropoiesis (Fig. 2). $C^mpG$ methylation

represents a docking site for the recruitment of methyl-CpG-binding proteins [Clouaire and Stancheva, 2008]. Although previous reports had proposed the in vitro presence of $C^{m}pG$ binding proteins on the embryonic π promoter, confirmation that such a complex was bound and the identity of associated binding proteins involved remained elusive [Singal et al., 2002]. Thus, we decided to evaluate in vivo whether there is a regulated incorporation of methyl-CpG-binding proteins correlating with the status of DNA methylation (Fig. 3). Using chromatin immunoprecipitation, we determined the in vivo binding for MeCP2 and MBD2 methyl-CpG-binding proteins at the embryonic π and the adult $\alpha^{A}$ globin promoters during erythroid development and differentiation (Fig. 3A). We found that MeCP2 is specifically recruited to the embryonic promoter, exclusively in HD3 cells and 10dRBC, but not in embryonic 5dRBC (Fig. 3A). In contrast, we did not detect any significant incorporation of MBD2 at the embryonic π gene.

To obtain more direct evidence of the contribution of MeCP2 to the autonomous silencing of the embryonic π gene, we used RNA interference in HD3 cells, which can be terminally differentiated in vitro [Beug et al., 1979; Iarovaia et al., 2001]. In such condition the embryonic π gene is not transcribed because, as for the 10dRBCs and non-induced HD3 cells, the embryonic promoter remains hypermethylated (Fig. 2). In contrast, $\alpha^{D}$ and $\alpha^{A}$ transcription is robustly activated on induced HD3 cells mimicking the adult developmental stages (Fig. 3B). Two rounds of transient transfections were performed with two siRNAs against the amino terminal region of chicken MeCP2, and a scrambled siRNA was also transfected as a negative control (Fig. 3B) [Weitzel et al., 1997]. In differentiating HD3 cells, we observed that the previously silenced embryonic π gene is now partially reactivated consistent with our previous data (Fig. 2) These findings suggests that the embryonic π gene is autonomously silenced at least by DNA methylation mechanisms.

## SILENCING OF THE EMBRYONIC π GENE EXPRESSION IN DEFINITIVE ERYTHROPOIESIS REQUIRES HISTONE DEACETYLATION

MeCP2-mediated silencing has been shown to require histone deacetylases [Jones et al., 1998; Nan et al., 1998]. Nevertheless, it has been suggested that chromatin modifications play a key role in the silencing of embryonic β-globin genes [Lavelle et al., 2006]. Thus, we asked whether a repressive chromatin configuration was the basis of π gene silencing. To define whether histone deacetylation regulates chicken α-globin expression, we performed reactivation studies employing the HDAC inhibitor Trichostatin-A (TSA). In 5dRBCs, TSA increased the mRNA level of the π gene, as well as the basal transcription of the $\alpha^{D}$ and $\alpha^{A}$ adult genes suggesting that chromatin structure relaxation modulates α-globin gene expression in embryonic RBCs (Fig. 4A). On the other hand, TSA treatment had an unexpected effect on chicken α-globin gene expression in definitive erythrocytes. As predicted, inhibition of histone deacetylation was able to reactivate the expression of the embryonic π gene in 10dRBCs, but adult gene expression showed a different expression trend (Fig. 4A). Both $\alpha^{D}$ and $\alpha^{A}$ genes demonstrated a reproducible reduction in the mRNA levels (Fig. 4A), contrary to the profile seen in 5dRBCs after TSA treatment. This observation

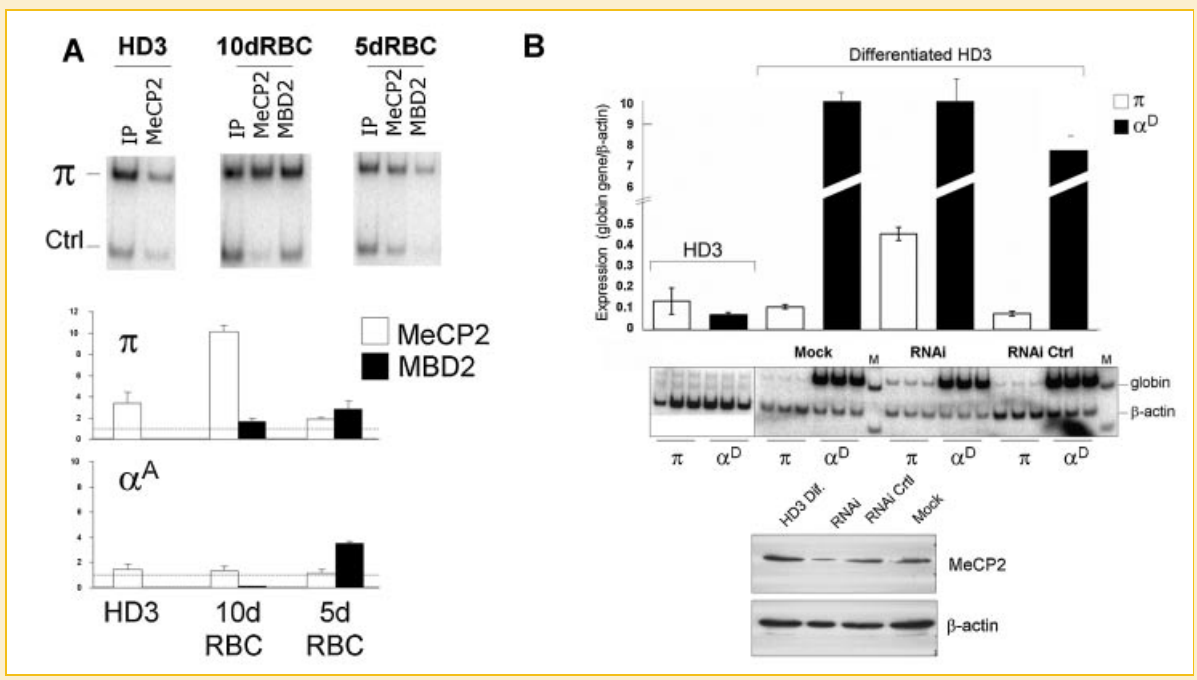


Fig. 3. Methyl-CpG-binding proteins and RNA interference reactivation at the embryonic π gene. A: Semi-quantitative chromatin immunoprecipitation to demonstrate the association of MeCP2 and MBD2 methyl-CpG binding proteins with the embryonic and adult gene promoters. Duplex PCR were performed using primers against an intergenic region located around 5 kb upstream of the embryonic π promoter (Ctrl) and IP represents the Input. The horizontal dotted line shows the Input value (normalized to 1). B: RNA interference against MeCP2 showing reactivation of the embryonic gene. This experiment was performed on induced to differentiation HD3 cells where the π gene remains silenced and $\alpha^{D}$ and $\alpha^{A}$ gene transcription is robustly induced (Mock and RNAi Ctrl). M, molecular marker. Western blot is presented to demonstrate the reduction in MeCP2 protein synthesis.

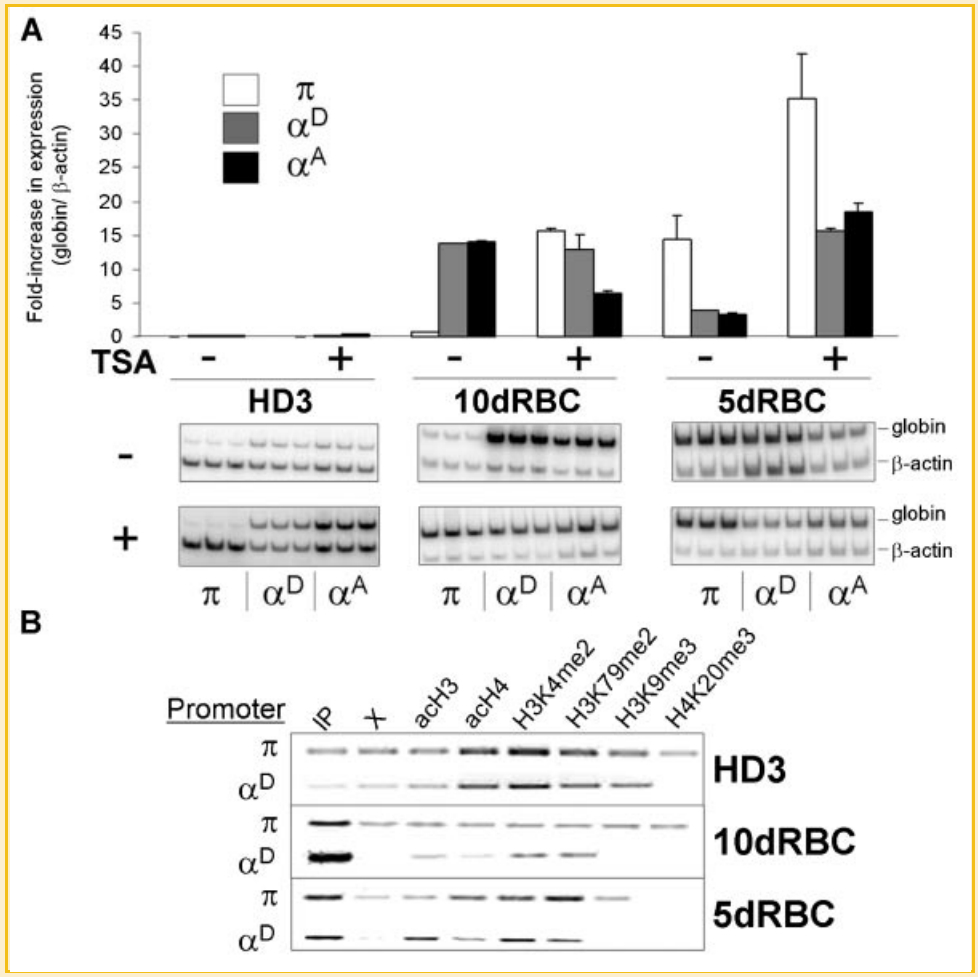


Fig. 4. Histone deacetylase inhibition and α-globin gene expression patterns. A: Cells were incubated with TSA and duplex RT-PCR was performed to evaluate the α-globin gene expression levels. Primers against β-actin were used for normalization. Data is presented as the fold-increase in gene expression levels. B: Qualitative evaluation by chromatin immunoprecipitation of different histone chromatin marks is shown. This is a representative figure of two independent ChIP assays. IP, represents the Input and X, the immunoprecipitation using an irrelevant antibody (IgG).

suggests that chromatin structure controls the final α-globin gene expression profile in definitive erythrocytes. Moreover, it also indicates that de-repression of the embryonic π gene affects the expression profiles of the $\alpha^D$ and $\alpha^A$ genes in definitive erythrocytes. The HDAC-dependent silencing of the embryonic gene is specifically established in definitive erythrocytes, as evidenced by the lack of drug-induced reactivation of the α-globin genes in HD3 erythroblasts even though, adult gene and β-actin are expressed at comparable levels (Fig. 4A).

To confirm histone deacetylation at the embryonic promoter, we performed qualitative chromatin immunoprecipitation assays with antibodies against open and closed histone chromatin marks. We analyzed the following open chromatin marks: acetylation of histone H3 (acH3) and H4 (acH4) and methylation of H3K4me2 and H3K79me2 and the following repressive chromatin marks: H3K9me3 and H4K20me3 (Fig. 4B). In 5dRBCs, we predominantly found the presence of active marks in embryonic and adult α-globin promoters (Fig. 4B). In 10dRBCs, open chromatin marks over the embryonic π promoter were lost; but nevertheless, there was no gain of repressive marks, such as H3K9me3 and H4K20me3. In the adult $\alpha^D$ promoter, open chromatin marks correlate with its active transcriptional status. On the other hand, this differential chromatin configuration on embryonic and adult promoters was not observed in erythroblasts since both promoters have similar histone modifications (Fig. 4B).

The observed DNA methylation and histone covalent modifications suggest that an epigenetic silencing of embryonic genes is required to activate the expression of adult α-globin genes. Furthermore, local chromatin remodeling on embryonic and adult promoters is needed for the proper establishment of the α-globin gene expression profile along development.

### *TRANS*-ACTIVATION OF THE CHICKEN α-GLOBIN GENE PROMOTERS BY THE ENHANCER

Our results suggested that the chromatin structure modulates the chicken α-globin switching, but this regulation could be a consequence of developmentally expressed transcription factors. Thus, to address whether the promoters contain all the information

required for proper developmental and differentiation functions, we evaluated the ability of each chicken α-globin promoter to be *trans*-activated by a globin enhancer. The α-globin domain possesses two main regulatory elements: (1) a putative LCR-like element located approximately 18 kb upstream of the embryonic π gene and (2) the 3′-side enhancer (Fig. 1A) [Flint et al., 2001; Escamilla-Del-Arenal and Recillas-Targa, 2008]. We investigated whether differential α-globin gene expression is dependent on the selectivity between individual promoters and distal regulatory elements, with a particular focus on the 3′-side enhancer. We constructed a set of luciferase-containing plasmids under the control of each of the α-globin gene promoters in the presence or absence of the core enhancer or the entire enhancer, which incorporates a 120 bp DNA fragment that modulates its activity [Rincón-Arano et al., 2005; Escamilla-Del-Arenal and Recillas-Targa, 2008]. These plasmids were transiently transfected in cells corresponding to specific stages of development and adult erythroid differentiation (Fig. S1). In primary erythrocytes from early and late developmental stages, all three promoters can be *trans*-activated by the enhancer. On the other hand, the 120 bp DNA fragment works differentially during development, since its incorporation favors *trans*-activation in 5dRBCs, but its presence reduced *trans*-activation in 10dRBCs, confirming its modulator role [Rincón-Arano et al., 2005]. These results reveal that in a terminally differentiated environment, the enhancer has the ability to *trans*-activate all three α-globin promoters regardless of the developmental stage.

As well, we observed a different *trans*-activation profile during definitive erythroid differentiation. In HD3 cells transiently transfected with the same constructs, the embryonic π gene promoter was efficiently *trans*-activated by the enhancer (Fig. S1). However, the enhancer is incapable of positively influencing the adult promoters in the erythroblast stage, in spite of the fact that these cell lines mimic a definitive erythroid differentiation milieu (Fig. S1). These experiments were also performed in the pre-erythroblast 6C2 cell line, and similar results were obtained (Fig. S1). Additionally, individual promoters were not able to induce maximal expression of the reporter gene in any tested condition in the absence of the enhancer. We conclude that in early differentiation stages of definitive erythropoiesis, the machinery required to activate the embryonic π gene expression is still present. In contrast, adult $\alpha^D$ and $\alpha^A$ gene promoters require differentiation stage-specific transcription factors. Thus, the embryonic and adult gene promoters display a differential sensitivity to *trans*-activation by the 3′-side enhancer that depends on the developmental context.

### DIFFERENTIATION AND DEVELOPMENTALLY SPECIFIC TRANSCRIPTION FACTOR OCCUPANCY IN THE CHICKEN α-GLOBIN PROMOTERS AND THE ENHANCER

Globin gene transcription is usually regulated by a common set of erythroid-specific and ubiquitous transcription factors [Goodwin et al., 2001; Drissen et al., 2004; Bresnick et al., 2005; Vakoc et al., 2005]. Our results demonstrated a preferential *trans*-activation of the embryonic gene in embryonic and definitive erythropoiesis (Fig. S1). To determine whether the differential activity of α-globin promoters is consequence of the action of a specific combination of transcription factors, we performed chromatin immunoprecipitation (ChIP) assays using antibodies against GATA-1, YY1, Sp1, and the erythroid-specific NF-E2p45 (Fig. 5). By semi-quantitative duplex PCR, we observed that in 5dRBCs, GATA-1 and Sp1 are associated with the embryonic π gene promoter (Fig. 5A). Interestingly, the adult $\alpha^D$ promoter is bound by the four transcription factors we analyzed, even in undifferentiated erythroid cells where expression is basal (Fig. 1B). In 10dRBCs, GATA-1, YY1, NF-E2p45 and Sp1 are recruited to adult $\alpha^D$ and $\alpha^A$ promoters correlating with their transcriptionally active status. Therefore, our results reveal that the embryonic π gene promoter possesses a "simpler" organization than the adult genes, with the embryonic promoter requiring only GATA-1 and Sp1. In contrast, the adult $\alpha^D$ and $\alpha^A$ gene promoters are more complex and require additional transcription factors for being functional, including YY1 and NF-E2p45.

To assess the in vivo incorporation of transcription factors at the enhancer, we performed ChIPs using enhancer-specific primers (Fig. 5B). Three main observations emerged from our experiments. First, we found an association of GATA-1, YY1, and NF-E2p45 with the enhancer in 6C2 and HD3 cells despite the lack of transcription of the α-globin genes (see Fig. 1B). Furthermore, the incorporation of nuclear factors at each of the three promoters is marginal in HD3 cells (Fig. 5A). Secondly, in 5dRBCs, when the embryonic π gene is transcriptionally active, we observe only the binding of GATA-1 (Fig. 5A). Finally, the complete set of transcription factors is incorporated in 10dRBCs (Fig. 5B), when the adult genes are expressed.

These results illustrate that the apparently "simpler" regulation of the embryonic gene could be a consequence of a preferential regulation by long distal regulatory elements in an erythroid-specific environment. On the other hand, the redistribution and recruitment of transcription factors to the adult promoters is a requirement for their further specialized transcription [Escamilla-Del-Arenal and Recillas-Targa, 2008]. Therefore, to express the adult α-globin genes, a more complex combination of factors is needed at both individual promoters and enhancers to effect proper developmentally regulated activation.

### GATA-1 IS REQUIRED FOR ENHANCER *TRANS*-ACTIVATION OF THE EMBRYONIC π GENE PROMOTER

GATA-1 is a key regulator of erythropoiesis and globin gene expression. The protein level of GATA-1 increases during differentiation [Kinuko and Masayuki, 2002] and we have shown that this transcription factor is recruited to the π promoter region and required for the chicken α-globin enhancer function (Fig. 5A) [Minie et al., 1992; Escamilla-Del-Arenal and Recillas-Targa, 2008]. Therefore, we decided to address whether GATA-1 is responsible for π promoter expression in erythroblasts. To evaluate whether GATA-1 directly modulates the activity of the π promoter region and/or the enhancer we decided to transiently co-transfected in erythroblasts the π promoter-containing plasmids with the cDNA of GATA-1. GATA-1 over-expression has no effect on the basal promoter activity (Fig. 6A). The presence of the full 3′ α-globin enhancer increased reporter expression, which was slightly enhanced by GATA-1 over-expression. Thus, the GATA-1 dependent high-level expression of the embryonic gene is not mediated at the level of the promoter. Therefore, to test whether GATA-1 is responsible for transcriptional activation by the 3′-side enhancer, we transfected

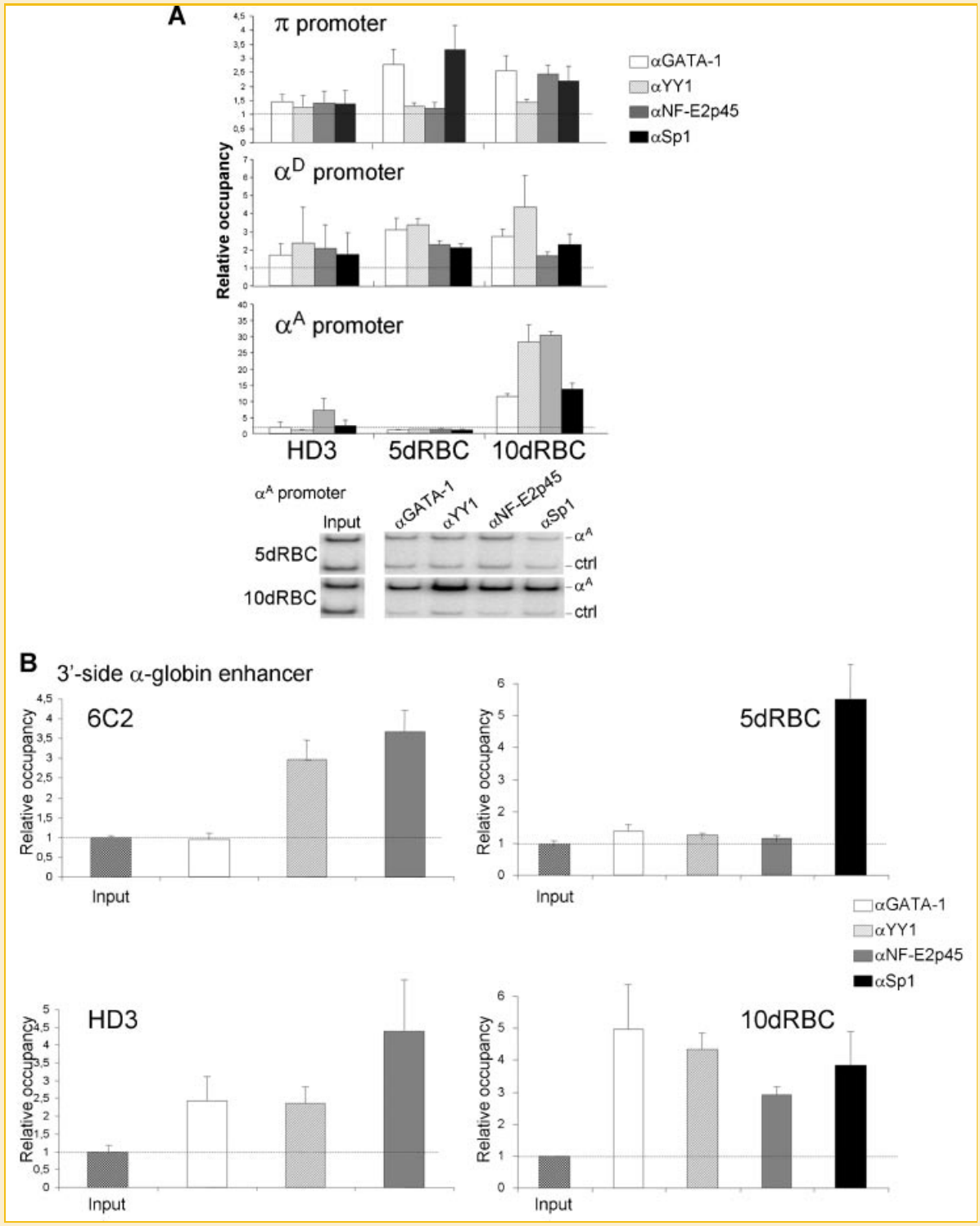


Fig. 5. In vivo nuclear factors associated with the α-globin gene promoters and the enhancer. A: Semi-quantitative chromatin immunoprecipitation assays using primers covering the core promoter of each gene. A representative semi-quantitative duplex PCR is shown using primers for the $\alpha^A$ promoter and for normalization we used primers from the intergenic region located around 4 kb upstream of the π gene. B: The same experiment as in A, employing a pair of primers spanning the enhancer. The graph summarizes the results of two independent chromatin immunoprecipitations, and for each one, at least triplicate duplex PCR reactions were carried out. The horizontal dotted line shows the Input value (normalized to 1).

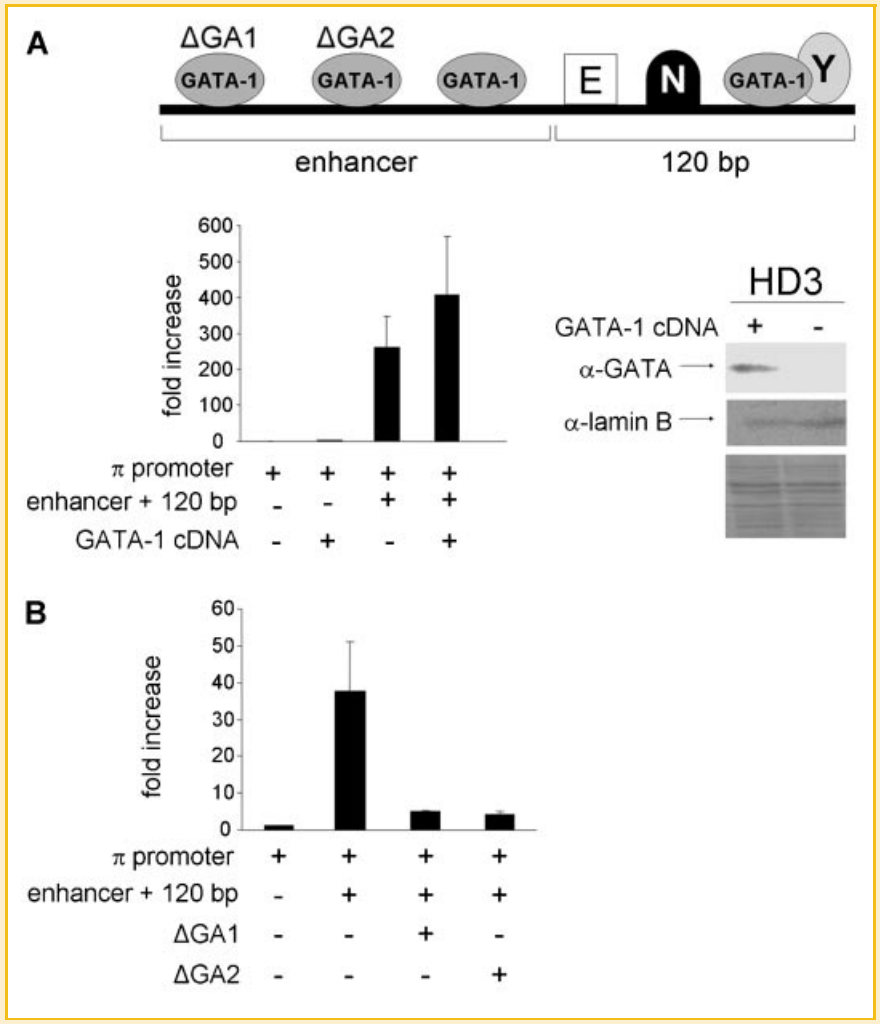


Fig. 6. Contribution of GATA-1 to promoter activity of the embryonic π gene. A: Scheme of the enhancer and associated nuclear factors, E: EKLF; N: NF-E2 and Y: YY1. *trans*-activation assay with co-transfecting the chicken GATA-1 factor cDNA and a representative Western blot confirming the over-expression of GATA-1. B: Transient transfections using the luciferase gene as reported and ΔGA1 and ΔGA2, which correspond to the first and second GATA-1 binding site mutations located at the core enhancer. These mutations were previously tested for their lack of GATA-1 binding by gel shift [Escamilla-Del-Arenal and Recillas-Targa, 2008].

luciferase expressing constructs under the regulation of the π promoter in the presence of the entire enhancer and mutated versions into the HD3 cell line (Fig. 6B) [Escamilla-Del-Arenal and Recillas-Targa, 2008]. Our results showed that the mutation of the GATA-1 binding sites in the enhancer abrogate the activation of the π promoter. These results support the idea that, in erythroblasts, transcriptional activation of the π gene requires a GATA-1 modulated enhancer during erythropoiesis.

## DISCUSSION

It has been suggested that hemoglobin switching is a process that involves autonomous silencing of the embryonic promoter and competition for regulatory elements [Stamatoyannopoulos, 2005]. Here, we demonstrate that the promoter of the embryonic gene is in vivo CpG methylated, which correlates with its silencing and the subsequent expression of the adult genes (around days 7–8) (Figs. 2 and 3). However, we found that the machinery responsible for activating the expression of the embryonic gene is still present during definitive erythropoiesis (Figs. 4 and 5). In a non-integrated context, we show that the α-globin enhancer can induce the activity of the three α-globin gene promoters at any stage of the development (Fig. S1). Interestingly, in erythroblasts, *trans*-activation by the enhancer is restricted to the embryonic promoter gene. This result suggests that during the erythroid differentiation the domain must be primed to activate the expression of the embryonic π gene. Alternatively, the use of a simple set of transcription factors to activate π gene transcription could increase the probability of *trans*-activation by enhancers. We propose that the "simplicity" of the π promoter gene facilitates in vivo its transcriptional *trans*-activation making it the default α-globin promoter during early erythroid differentiation. During differentiation in adult tissues, DNA methylation and other chromatin

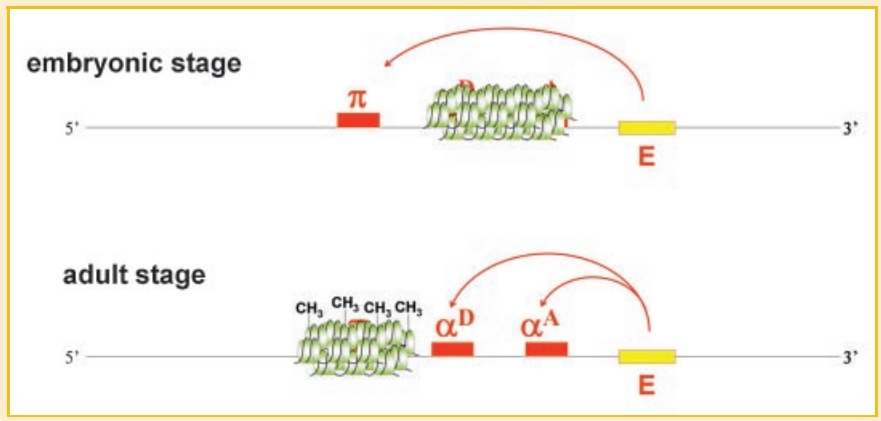


Fig. 7. Model in which autonomous silencing by epigenetic mechanisms is required for differential regulation of α-globin gene expression. In this model, once development proceeds the embryonic gene is epigenetically silenced. We propose that the enhancer is able to target the stage-appropriate promoter via the mechanism demonstrated. In addition, we showed that none of the genes are methylated in early developmental stage but in contrast, our TSA reactivation assay suggests that, at least, histone deacetylation seems to be present over adult genes. This model do not discard the possibility that other 5′ and 3′ distal erythroid regulatory elements may contribute to the differential chicken α-globin gene expression like the upstream Major Regulatory Element or α-MRE.

modifications are established along the three α-globin promoters in erythroblasts, despite the fact that that they are not typical CpG islands (Figs. 2–4) [Recillas-Targa and Razin, 2001]. However, upon differentiation, this epigenetic mark is exclusively erased from the adult promoters. Reactivation experiments with 5-aza-2′-deoxycytidine showed that erythroblasts can exclusively activate the expression of the embryonic π gene, favoring the idea that during erythropoiesis the π gene is transcribed by default (Fig. 2). Although the π gene promoter is not GATA-1 sensitive, the presence of an enhancer increases the transgene expression in a GATA-1-dependent manner (Fig. 6). Therefore, DNA methylation must interfere with GATA-1 dependent activation in the early stages of erythroid differentiation.

More recently, an HMG family member, Sox6, has been associated with the autonomous silencing of the embryonic ε gene during erythroid cell maturation [Yi et al., 2006]. Therefore, recruitment of developmental stage-specific transcription factors to target promoters seems to be necessary, but not sufficient for regulation of globin expression during development. Developmentally specific repressors or co-repressors must be required for silencing the expression of embryonic genes in adult tissues. In this context, we found that MeCP2 is recruited to the embryonic promoter in a differentiation-specific manner, altering the chromatin structure over the π gene promoter. The mechanism regulating MeCP2 recruitment to the embryonic α-globin promoter is still unknown. Nevertheless, MeCP2 is a dynamic repressor and binds methylated DNA near promoter regions and some imprinted genes, maintaining silencing through recruitment of the Sin3a–HDAC co-repressor complex [Chen et al., 2003; Martinowich et al., 2003] or compacting chromatin directly [Woodcock, 2006]. Interestingly, the presence of MeCP2 seems to be supplemented with the recruitment of histone deacetylases at the embryonic π promoter in terminally differentiated erythrocytes. TSA treatment reverses silencing of the π gene and, importantly, impairs the expression of the adult genes (Fig. 4). This result suggests that complementary epigenetic mechanisms are required to silence the embryonic gene promoter in order to avoid any interference with the expression of the adult genes (Fig. 7). Therefore, we believe that MeCP2 recruitment to the embryonic promoter generates a local chromatin compaction and removal of active histone marks via MeCP2-interacting partners, thereby silencing the embryonic gene during definitive erythropoiesis and allowing activation of the adult $\alpha^A$ and $\alpha^D$ genes.

During differentiation, the globin domain is already organized in a poised configuration to ensure the expression of the adult genes. This idea was previously proposed for both the β- and α-globin domains [Groudine and Weintraub, 1981]. There is evidence supporting that long distal regulatory elements such as the β-globin LCR are primed with specific epigenetic marks like methylation of H3K4 and acetylation of histone H3 in hematopoietic stem cells [Szutorisz et al., 2005; Demers et al., 2007]. We detect active chromatin marks including histone acetylation and methylation of H3K4 in the erythroblast and erythrocyte stages over the embryonic and adult promoters (Fig. 4B), but these epigenetic marks along with other open chromatin marks are specifically erased from the embryonic promoter in the adult erythrocyte, suggesting that local and dynamic chromatin changes are required for adequate regulation of developmentally regulated genes.

Another regulatory element that should be taken into consideration is the highly conserved chicken α-globin locus control region, also known as the upstream Major Regulatory Element (α-MRE) which is located around 20 kb in the 5′ non-coding region in relation to the embryonic π gene [Flint et al., 2001; Hughes et al., 2005]. It is then important to incorporate the α-MRE and its long distance action to our model based on its probable contribution to the differential α-globin gene expression and chromatin domain configuration. This view is recently supported by differential intrachromosomal contacts, defined by the chromosome conformation capture assay, between the α-MRE, the −9, −4 kb DNase I hypersensitive sites and the adult $\alpha^D$ gene promoter region forming an optimal spatial configuration that correlated with adult globin gene expression [Gavrilov and Razin, 2008]. A complementary

component emerge from the presence of the housekeeping and antisense *ggPRX* gene (also known as *CGTHBA* or *C15orf35* gene) that initiates its transcription at the CpG island located at the −4 kb DNase I hypersensitive site [Vyas et al., 1995; Klochkov et al., 2006]. We postulate that this antisense transcript may also participate in the chromatin remodeling of the chicken α-globin domain in early stages of erythroid differentiation.

In summary, DNA methylation, MeCP2-binding and histone deacetylation direct embryonic gene silencing to allow regulated transcriptional activation of the adult chicken α-globin genes. These epigenetic components are in vivo most likely recruited by stage specific transcription factors to the embryonic gene during the latter stages of the definitive erythropoiesis. Moreover, promoter-enhancer communication may not be sufficient for differential gene activation, and our results suggest that a repressive chromatin configuration of the embryonic gene promoter is required for successful hemoglobin switching.

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