Methylation of α-type embryonic globin gene α\(^\text{\small\texttt{\textmu}}}\) represses transcription in primary erythroid cells

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The inverse relationship between expression and methylation of β-type globin genes is well established. However, little is known about the relationship between expression and methylation of avian α-type globin genes. The embryonic α\(^\text{\small\texttt{\textmu}}}\)-globin promoter was unmethylated, and α\(^\text{\small\texttt{\textmu}}}\)-globin RNA was easily detected in 5-day chicken erythroid cells. A progressive methylation of the CpG dinucleotides in the α\(^\text{\small\texttt{\textmu}}}\) promoter associated with loss of expression of α\(^\text{\small\texttt{\textmu}}}\)-globin gene was seen during development in primary erythroid cells. A 315-bp α\(^\text{\small\texttt{\textmu}}}\)-globin promoter region was cloned in an expression construct (α\(^\text{\small\texttt{\textmu}}}\)-GL3E) containing a luciferase reporter gene and SV40 enhancer. The α\(^\text{\small\texttt{\textmu}}}\)-GL3E construct was transfected into primary erythroid cells derived from 5-day-old chicken embryos. Methylation of α\(^\text{\small\texttt{\textmu}}}\)-GL3E plasmid and α\(^\text{\small\texttt{\textmu}}}\)-globin promoter alone resulted in a 20-fold and 7-fold inhibition of expression, respectively. The fully methylated but not the unmethylated 315-bp α\(^\text{\small\texttt{\textmu}}}\)-globin gene promoter fragment formed a methyl cytosine-binding protein complex (MeCPC). Chromatin immunoprecipitation assays were combined with quantitative real-time polymerase chain reaction to assess histone acetylation associated with the α\(^\text{\small\texttt{\textmu}}}\)-globin gene promoter. Slight hyperacetylation of histone H3 but a marked hyperacetylation of histone H4 was seen in 5-day when compared with 14-day erythroid cells. These results demonstrate that methylation can silence transcription of an avian α-type embryonic globin gene in homologous primary erythroid cells, possibly by interacting with an MeCPC and histone deacetylase complex.

Introduction

The developmental regulation of globin gene expression is an intensely studied problem because of its central importance in the understanding of many common inherited diseases caused by defects in globin gene expression such as thalassemia. The chicken globin families are a well-characterized set of developmentally regulated genes. The avian β-type globin cluster has 4 functional genes (\(5'\)-p, BH, βA, ε-3') while the α-type globin cluster has 3 functional genes (\(5'\)-α\(^\text{\small\texttt{\textmu}}}\), α\(^\text{\small\texttt{\textmu}}}\)-3'). Primitive erythroid cells express embryonic β-globin genes, β and ε, and the α-globin genes, α\(^\text{\small\texttt{\textmu}}}\), α\(^\text{\small\texttt{\textmu}}}\), and α\(^\text{\small\texttt{\textmu}}}\). Definitive cells express BH and βA as well as α\(^\text{\small\texttt{\textmu}}}\) and α\(^\text{\small\texttt{\textmu}}}\). Tissue-specific and developmental stage–specific regulation of both α- and β-globin gene families primarily involves the locus control regions (LCR), which are erythroid-specific enhancers located many kilobases upstream of each gene cluster. The inverse relationship between expression and methylation of β-type globin genes is well established. We have previously shown that methylation of the promoter or proximal transcribed region of the chicken β-type embryonic globin gene represses transcription in primary erythroid cells. However, little is known about the relationship between expression and methylation of avian α-type globin genes. A previous study examined certain specific sites of the chicken α-globin cluster in DNA from embryonic and adult erythroid cells using a methylation-sensitive restriction enzyme–based technique. Even though the methylation pattern at HpaII sites 5' to the α\(^\text{\small\texttt{\textmu}}}\)-globin gene correlated to some extent with expression, a detailed study of the methylation pattern was not possible because of technical limitations inherent in that technique. Another study has recently shown that the domain of the chicken α-globin genes is preceded by a CpG island that is heavily methylated in lymphoid cells and is either nonmethylated or undermethylated in erythroid cells.

Direct binding of specific transcriptional repressors to methylated DNA appears to be a major mechanism of transcriptional repression. Of the 5 proteins that have the methyl-CpG-binding domain, 4 (MBD1, MBD2, MBD3, and MeCP2) are implicated in transcriptional repression. MBD2 is a component of the methyl cytosine-binding protein 1 (MeCP1) complex, together with histone deacetylases HDAC1 and HDAC2. MBD3 is a component of the Mi2/NuRD deacetylase complex. MBD1 binds selectively to methylated DNA and represses transcription from a naked methylated promoter in vitro. MBD4 is a thymidine glycosylase repair enzyme and is not associated with transcriptional inactivation. We have shown that chicken MeCPC, which contains MBD2 and HDAC1, binds to the methylated ρ-globin gene in vitro. Globin gene clusters offer an interesting system to study the interaction between methylation and histone deacetylation. Despite varying methylation patterns of individual globin genes, the α- and β-globin loci are in an open
chromatin configuration. At the β-globin locus, even though methylation correlates inversely with expression, both embryonic and adult genes were highly acetylated at histones H3 and H4 in 5-day and 15-day erythrocytes.\textsuperscript{16} A recent study showed that an erythroid-specific domain of histone acetylation encompassing the α-globin genes has been conserved across several species.\textsuperscript{17}

In this study, we show that methylation represses the expression of the αα-globin gene in chick primary erythroid cells, possibly through the formation of a MeCPC. We also show that the αα-globin gene is hyperacetylated for histones H3 and H4 in 5-day cells where it is expressed, in comparison to 14-day erythroid cells where it is transcriptionally silent.

Materials and methods

Blood collection

Fertilized eggs were obtained from Truslow Farms (Chestertown, MD) and incubated to the desired stage of development in a Lyon Roll-X Automatic Incubator (Lyon Electric, Chula Vista, CA) according to the manufacturer’s instructions. Blood was collected with a sterile Pasteur pipette into room temperature phosphate-buffered saline (PBS), washed twice with PBS, and spun at 320g for 5 minutes. Red blood cells (RBCs) were then resuspended in PBS and spun for 5 minutes at 720g to pellet cells.

RNA/DNA purification

Cells were resuspended in 10 volumes of RNA Stat-60 or DNA Stat-60 (Tel-Test, Friendswood, TX), and RNA or DNA was extracted following the manufacturer’s protocol. Briefly, the cells were homogenized, the lysates were extracted with chloroform, and the nucleic acids were precipitated with isopropanol. Nucleic acids were resuspended in nuclease-free water (Ambion, Austin, TX) and quantitated on a Beckman DU series 64 spectrophotometer (Beckman Instruments, Fullerton, CA).

RT-PCR

Primers for αα-globin were designed so as to flank the intron-exon boundaries (accession no. V00408). PiRTR1 (5’TCACTGAGAG-GCTTTTTGCC-3’) corresponded to positions 466 to 477 and 1055 to 1063. PiRTR1 (5’TGGGGAAGCAGCTGTAAGGTC-3’) corresponded to positions 1565 to 1554 and 1259 to 1251. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the Reverse Transcription System from Promega (Madison, WI) following the manufacturer’s protocol except that 5 μg RNA was used to make cDNA. αα-globin cDNA was made using the specific primer PiRTR1; for β-actin, cDNA was made with the random primers included in the kit. The αα-globin cDNA was then amplified using PiRTF1 and PiRTR1; β-actin was amplified using the primers BactinRT1 (5’CGCTCGTTGTTGACATTGGCTCT-3’) and BactinRT2 (5’CCAGTTGGTGACAATTACCCTGTC-3’).

Bisulfite conversion and methylation analysis

RBCs were collected from 4-, 5-, 6-, 8-, 11-, and 14-day embryos and bisulfite treated as previously described.\textsuperscript{6,11} Primers for the amplification of the αα-globin promoter region were designed with the help of a Microsoft Word macro program.\textsuperscript{19} Bisulfite-treated DNA was amplified using the primers PiBisulfF (5’TCTTTAGCTTTTTGTTAAATTATTTTGGAAGG-3’, corresponding to positions –322 to –304 relative to the transcription start site) and PiBisulfR (5’ATAAACACCCAAAAACAAATATACAC-3’, corresponding to positions +22 to –3 relative to the transcription start site). Sequencing of the PCR-amplified product was performed using the forward and reverse primers. The αα-globin promoter fragment was digested with restriction enzymes and purified from agarose gels, run approximately 3 hours at 150 V in 0.5×TBE (Tris-borate-EDTA), and then dried and analyzed using a Cyclone phosphorimager (Packard Instrument).

Plasmids

A PCR product corresponding to a 327-bp fragment of the αα-globin promoter was amplified with the primers PiClonF1 (5’TGTGAGCT-CAAAATCCATGGAAGACG-3’), which contains a SacI restriction site, and PiClonR1 (5’GAGAAGCTTACGATGCGCCTC-3’), which contains a HindIII site. The amplified fragment was digested with restriction enzymes to create sticky ends and cloned into pGL3-Basic and pGL3-Enhancer vectors (Promega) to yield αα-globin and αα-globin-3L vectors, respectively. Methylation was accomplished with SsI methylase from New England Biolabs (Beverly, MA); the unmethylated control plasmid was treated similarly but without the addition of S-adenosylmethionine. Completion of the reaction was determined by digestion with methylation-sensitive restriction enzymes. For the probes used in electrophoretic mobility shift assays (EMSA), the entire plasmid was methylated, and then the fragment was excised and labeled. For transient transfections, in certain experiments, the fragment was excised, methylated, and religated into the reporter plasmid.

Transient transfections

RBCs were resuspended to a final volume of 100 μL per transfection and then spun down. The pellets were resuspended in 1 mL filter-sterilized NH4Cl (pH 7.2-7.3), freshly made, and incubated at room temperature for 2 hours with periodic mixing, spun down, and resuspended in 0.5 mL transfaction medium (66.6% [vol/vol] L-15 [Sigma Chemical, St. Louis, MO]; 50 mM Tris (tris(hydroxymethyl)aminomethane)-HCl, pH 7.45; 300 μg/mL diethylaminoethyl [DEAE]-Dextran) containing the 2 μg of the DNA construct to be transfected and 200 ng of the plasmid pRL-TK as a control for transfection efficiency. All transfections were done in triplicate. The transfections were incubated at room temperature for 10 minutes, then at 37°C for 10 minutes, and then spun down. Pellets were washed with 800 μL chicken culture medium (74% L-15, 22% chicken serum [Gibco, Grand Island, NY], 3.5% fetal bovine serum [FBS] [Gibco], 0.5% penicillin-streptomycin solution [Sigma]) and then resuspended in 500 μL culture medium and added to culture flasks containing 5 mL medium. Cells were grown for 48 hours and then harvested.

Luciferase assays

Luciferase assays were performed using the Dual Luciferase Reporter Assay system from Promega following the manufacturer’s protocol for single sample luminometers. The cell suspensions were spun down, and the RBC pellets were washed twice with PBS and then resuspended in 500 μL 1× passive lysis buffer (Promega). The cell lysates were cleared by centrifugation for 2 minutes at 4°C and then transferred to fresh 1.5 mL tubes and stored at −80°C. Luciferase and Renilla activity were measured on a TD-20/20 Luminometer from Turner Designs (Sunnyvale, CA). Results were reported in terms of the ratio of luciferase to Renilla activity and expressed as the percentage of the value of unmethylated αα-globin promoter-driven activity.

Electrophoretic mobility shift assays

RBC nuclear extracts from 14-day embryos were prepared according to a modified Dignam procedure as described previously.\textsuperscript{5,7} The probes used were in vitro–methylated and mock-methylated αα-globin promoter excised from αα-globin and labeled with [α-32P]dATTP (dCTP) (New England Nuclear, Boston, MA) using the Klenow fragment of DNA polymerase I. Assay conditions were as previously described.\textsuperscript{5,7} Two micrograms of sonicated Micrococcus lysodeikticus DNA was used as a nonspecific competitor in all assays. Assays were performed on 2% agarose gels, run approximately 3 hours at 150 V in 0.5×TBE (Tris-borate-EDTA), and then dried and analyzed using a Cyclone phosphorimager (Packard Instrument).

For antibody/supershift ablation experiments, EMSA was performed with or without the addition of one of the following antisera (1.5 μL) or antibodies (800 ng): anti-MBD2 antisera, anti-MBD2 IgG (Upstate Biotechnology, Lake Placid, NY), anti-MBD1, anti-MBD3, and anti-MBD4 IgG (Santa Cruz Biotechnology, Santa Cruz, CA).
Chromatin immunoprecipitation (ChIP) assays were carried out with a kit from Upstate Biotechnology using the manufacturer’s protocol and reagents except that the reactions were scaled down 10-fold. Briefly, 2 × 10^7 cells were incubated in 1% formaldehyde for 10 minutes to cross-link bound proteins, washed, lysed in sodium dodecyl sulfate (SDS) lysis buffer, and sonicated to 100–500-bp lengths; 10 μL chromatin was mixed with 90 μL dilution buffer and pre-cleared with protein A–agarose, and then the chromatin was incubated with antibody overnight at 4°C. Thirty μL protein A–agarose beads were added, and the chromatin was immunoprecipitated 2 hours at 4°C. The supernatant (unbound chromatin) and beads (bound chromatin) were separated. The beads were washed 5 times with the buffers provided, and then the chromatin was eluted twice in 1% SDS in 0.1 M NaHCO₃. Both bound and unbound chromatin fractions were de-cross-linked by the addition of 5 M NaCl and incubation at 65°C for at least 4 hours. Proteins were digested by protease K, and then chromatin was extracted with phenol/chloroform. DNA was ethanol precipitated and dissolved in 100 μL water. The experiment was repeated on 3 separate occasions.

Results

Expression of the chicken α^+^-globin gene during development in primary erythroid cells

We examined the expression of avian α-type embryonic α^+^-globin during development in primary erythroid cells using RT-PCR. α^+^-Globin mRNA is easily detected in 4- to 5-day primitive embryonic erythroid cells but is barely detectable by day 11 of embryonic development (Figure 1). These results are consistent with the earlier studies that examined the expression of avian α-type globin genes during development in erythroid cells.  

Methylation analysis of the chicken α^+^-globin gene during development in primary erythroid cells

We have previously shown that the chicken β-type embryonic globin gene (p-globin) promoter is completely unmethylated in primitive erythroid cells and completely methylated in erythroid cells from adult chickens.  

Methylation of a luciferase reporter construct driven by α^+^-globin promoter represses expression in transient transfection assays

To determine whether methylation of the α^+^-globin promoter alone can inhibit transcription, a construct of a 315-bp α^+^-globin promoter region was cloned into the luciferase expression vector pGL3. This construct was transfected into primary erythroid cells derived from 5-day-old chicken embryos. Methylation of the α^+^-globin promoter construct was determined by bisulfite genomic sequencing and methylation analysis of the α^+^-globin promoter sequences resulted in a 7-fold inhibition of expression.
to detect an MeCP1-like complex as described. On autoradiography, a complex was observed with methylated \( \alpha^2 \)-globin probe, as shown in Figure 4A, and this complex was effectively competed by a 25-fold molar excess of cold methylated \( \alpha^2 \)-globin but not by a 200-fold excess of cold unmethylated \( \alpha^2 \)-globin fragment (Figure 4B). However, unlike the complex observed with \( p \)-globin gene proximal transcribed sequence, this complex could not be depleted with MBD2 antibodies or MBD2 antiserum (data not shown). We also performed EMSA using antibodies against other known methylcytosine binding proteins (MBD1, MBD3, MBD4, and MeCP2). No depletion or supershift of the complex was observed. This could be due to either failure of the antibodies (raised against human proteins) to recognize chicken homologs or due to the presence of yet unknown proteins present in the complex. Further efforts to determine the composition of this complex are underway. Interestingly, this complex was also seen with 5-day chicken red cell nuclear extract (data not shown), suggesting that the proteins that bind to methylated \( \alpha^2 \)-globin promoter are present throughout development.

Histone H3 and H4 acetylation pattern of the \( \alpha^2 \)-globin promoter in day 5 and day 15 chicken primary erythroid cells

Histone acetylation and deacetylation play important roles in transcriptional regulation. Methylated cytosines are important in guiding histone deacetylases to specific DNA sequences. This suggests that methylation represses transcription by recruiting
HDAC activity, resulting in hypoacetylation of histones in methylated DNA. We measured the relative level of histone acetylation of the α-globin promoter in day 5 and 14 erythroid cells using a chromatin immunoprecipitation assay. Formaldehyde cross-linked chromatin was immunoprecipitated with antibodies against acetylated histones H3 and H4. The antibody-bound DNA was analyzed for the α-globin gene using a quantitative real-time PCR approach. There are many advantages to quantifying gene sequences using this technology, the foremost being sensitivity and precision. ChIP assays have previously been based on qualitative or semiquantitative analysis usually involving the examination of PCR products at a fixed cycle number, followed by densitometry of the radioactive band or ethidium bromide-stained DNA.

Slight hyperacetylation of histone H3 but a marked hyperacetylation of histone H4 was seen in 5-day when compared with 14-day erythroid cells (Figure 5B). These results are consistent with the recruitment of histone deacetylase-containing complexes by methylated DNA, resulting in a localized deacetylation.

Discussion

The distribution of methylated and unmethylated CpG dinucleotides in vertebrates conforms to a generalized pattern. About 70% to 80% of CpG sites contain methylated cytosines. Promoter region CpG islands are usually unmethylated in all normal tissues regardless of the transcriptional activity of the gene. The inverse relationship between expression and methylation of β-type globin genes is well established. We have shown that, in the case of the developmentally regulated p-globin gene, methylation of both the CpG-dense (promoter and proximal transcribed region) and CpG-poor (distal transcribed region) regions correlates inversely with the stage-specific expression in avian erythroid cells. In this study we have shown that methylation of the chicken embryonic α-type globin gene correlates inversely with the stage-specific expression in primary erythroid cells. Further, methylation of the α promoter alone at the exact CpGs that are methylated in vivo resulted in transcriptional repression in a transient transfection assay. The repressive effect was more pronounced when the whole plasmid was methylated. This is likely to be related to the methylation of the luciferase gene and enhancer, because previous studies have shown that methylation of the transcribed region alone can mediate transcriptional repression.

Direct binding of specific transcriptional repressor complexes to methylated DNA appears to be a major mechanism of transcriptional repression. MeCP1 binds to DNA containing multiple symmetrically methylated CpGs and migrates as a large complex on EMSA. MeCP1 has been shown to repress transcription from densely methylated genes, and cells deficient in MeCP1 show much reduced repression of methylated genes. We have recently shown that a novel tissue- and sequence-specific methyl CpG binding protein complex MeCPC exists in chicken erythrocytes. This complex contains MBD2 and binds to sequences in the methylated but not the unmethylated p-globin gene proximal transcribed region. In the present study we have shown that in chicken erythroid cell nuclear extracts MeCPC binds to methylated but not to unmethylated α-globin gene promoter sequences. Interestingly, in contrast to the MeCPC observed with p-globin gene proximal transcribed region, the one observed with α promoter sequences could not be supershifted or ablated with MBD2 antibodies, suggesting that different methylated sequences may interact with different components of MeCPC. MBD2, but not MeCP2, has been shown to bind to methylated p16 or p14 promoters in colon carcinoma cell lines. Interestingly, no significant binding of MBD2 to methylated Alu elements that are located between p16 and p14 was detected.

Recent evidence suggests that acetylation of the aminoterminal tails of H3 and H4 may be a principal regulator of transcription factor access to nucleosomal DNA. The underlying patterns of methylated cytosines are important in guiding histone deacetylases to specific DNA sequences. This suggests that methylation represses transcription by recruiting HDAC activity, resulting in hypoacetylation of histones in methylated DNA. Consistent with this hypothesis, we found the α-globin promoter to be enriched in acetylated H3 and H4 in chromatin derived from day 5 erythroid cells when compared with day 14 erythroid cells. Even though a domain of histone acetylation exists across the α-globin gene cluster, our results indicate that the acetylation pattern of the α-globin varies with developmental stages. Similarly, the acetylation pattern of murine β-globin genes was found to vary at different developmental stages. Interestingly, the degree of deacetylation at the α-globin promoter was more prominent for histone H4 than for H3. For the β-globin genes, other studies have shown a more prominent acetylation for H3 for the active e-globin promoter or β-promoter. Individual activators or repressors may confer distinct patterns of histone acetylation on target promoters. In a recent study, mice were produced with transgene-induced methylation at the paternal allele of an imprinted gene, U2af1-rs1. In these mice, H3 was underacetylated across both the parental U2af1-rs1 alleles, whereas H4 acetylation was unaltered. In contrast, Rett syndrome mutations involving MeCP2 in clonal cell cultures resulted in no intact MeCP2 protein and hyperacetylated H4, but not H3. It is likely that methylated sequences repress transcription by interacting with distinct MBD proteins and HDAC complexes, resulting in a differential histone acetylation pattern. This may also account for the reactivation of some methylated genes, but not others, with histone deacetylase inhibitors.

Figure 5. ChIP/quantitative real-time PCR for analyses of the acetylated histones H3 and H4 at the α-globin promoter in day 5 and day 14 embryonic chicken erythroid cells. (A) Standard curve generated using varying amounts of chicken genomic DNA. X-axis shows log ng of chicken genomic DNA used as template, and y-axis shows the threshold cycle (Ct) value. Based on the standard curve, a linear regression equation was determined. This equation was used to calculate the amount of input DNA from Ct values. (B) Bound-unbound ratio for control (chromatin undergoing all the steps in ChIP assay but without addition of an antibody), acetylated histone H3, and acetylated histone H4. Results for acetylated histones H3 and H4 have been normalized for control. Error bars indicate results obtained with 3 independent experiments. For each experiment, real-time PCR was performed in triplicate, and a mean of Ct values was used for calculation of the input DNA.
References