Developmentally Regulated Demethylase Activity Targeting the β_A-Globin Gene in Primary Avian Erythroid Cells

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ABSTRACT: Differential expression of globin genes has provided an interesting model system for better understanding commonly inherited diseases such as thalassemia. In the avian β-type globin cluster (5′-ρ-β_H-β_A-ϵ-3′), silencing of the embryonic ρ-globin gene occurs concomitantly with the activation of the adult β_A-globin gene during embryonic development. DNA methylation is a dynamic process that regulates gene expression. We observed a progressive loss of methylation of β_A-globin gene, during avian embryonic development that was concurrent with the expression of the gene. The promoter and exon 1 regions of the template strand were completely demethylated, whereas residual methylation was retained in exons 2 and 3. Using a modified methylation-sensitive single-nucleotide primer extension (MS-SNuPE) assay, we observed stage-specific demethylase activity in the nuclear extracts of chicken red cells; activity in 5-, 8-, and 11-day-old erythroid cell nuclear extracts was 6, 76, and 24%, respectively. The demethylase targeted both hemimethylated and fully methylated substrates. Our findings demonstrate stage-specific demethylase activity in nuclear extracts from primary chicken erythroid cells that could target the fully methylated promoter of a developmentally regulated native gene.

The chicken globin family comprises a set of well-characterized, developmentally regulated genes. Primitive erythroid cells express only two members of the β-globin gene cluster, viz., ρ and ϵ, along with all three members of the α-globin gene family, whereas definitive cells express β_H, β_A, and α^A and α^D (*1*). The stage-specific regulation of the globin gene family is a complex mechanism involving many processes. DNA methylation has been shown to play a pivotal role in gene regulation during embryonic development (*2*, *3*). We have previously shown that methylation of the promoter or proximal transcribed region of the chicken β-type embryonic ρ-globin gene represses transcription in primary erythroid cells (*4*, *5*), whereas de novo methylation of the embryonic ρ-globin gene occurs in early definitive cells (*6*). An earlier study correlated the absence of DNA methylation at certain specific sites and expression of β_A-globin in primary erythroid cells (*7*). However, the study was limited to analysis of a few CpG dinucleotides on day 5 of embryonic development. Further, the temporal sequence of loss of methylation and transcriptional activation of the β_A-globin gene was not explained. There is still uncertainty with regard to the mechanism of DNA demethylation.

In this study, we have used bisulfite genomic sequencing to study the overall methylation pattern as well as that of individual DNA strands during development in primary chicken erythroid cells. Rapid loss of methylation along with the methylation pattern of individual DNA molecules suggested "demethylation" of the β_A-globin gene during development rather than the replacement of methylated sequences in primitive cells with unmethylated sequences in definitive cells. This was confirmed by the ability of the nuclear extracts of embryonic chicken red blood cells (RBCs)[1] to demethylate a methylated 70-mer oligonucleotide by up to 83%. Interestingly, the demethylase activity was stage-specific; i.e., maximal activity was observed on day 8 of embryonic development. Contrary to earlier reports indicating that replication-mediated priming was necessary for the initiation of enzymatic demethylation, we observed equal demethylase activity on both fully methylated synthetic oligonucleotide and the hemimethylated substrate.

MATERIALS AND METHODS

Eggs. Eggs were purchased from CBT Farms, Inc. (Chestertown, MD), and incubated in a Lyon Roll-X Automatic Incubator from Lyon Electric Co. (Chula Vista, CA) according to the manufacturer's instructions.

Cell Culture. K562 cells were grown as suspension cultures in RPM1 1640 supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine, 100 μg/mL streptomycin, and 100 units/mL penicillin.

DNA Purification. Blood was collected with a sterile Pasteur pipet into room-temperature phosphate-buffered saline (PBS), washed twice with PBS, and spun at 320*g* for 5 min. RBCs were then resuspended in PBS and spun for 5 min at 720*g* to pellet cells. Cells were resuspended in 10 volumes of RNA STAT-60 (Tel-Test Inc., Friendswood, TX),

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[1] Abbreviations: RBC, red blood cell; RTPCR, reverse transcriptase polymerase chain reaction; MS-SNuPE, methylation-sensitive single-nucleotide primer extension; Dnmt, DNA methyltransferase; MBD, methyl binding domain.

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Table 1: Primers for Bisulfite Genomic Sequencing

Strand	Region amplified	Forward (5'-3')	Reverse (5'-3')	Position[a]	Product length
Sense	Promoter and Exon1	GGTATAGAGTTGTAGAGTTGGGGAAT	ACCACATTAACCTTACCCCAAAAAC	-225 to 141	366
	Exon2	GGTTGTTGATYGTTTATTTTTG	CTCCTCTTAAAAATACATTAAA	+260 to +533	273
	Exon3	GTTTTTGGGTGATATTTTTATTATTG	CAAATACATACAATAATTATAAATAC	+1293 to 1471	178
Antisense	Promoter and Exon1	GGTTATATTGATTTTGTTTTAGAGG	CAACACTAAAATAAACACAAAATACC	-287 to +141	428
	Exon2	GAGATGTATTGGGATGGGATTTAG	CCATCTCTCTACAAACTACTAATC	+246 to +533	287
	Exon3	CTACAAAAAATCCTCCTATTCC	GGAGTATTTTTGTAGGTGTATG	+1266 to 1484	218

[a] With respect to the transcription start site.

and after RNA extraction, DNA was extracted using DNA STAT-60 (Tel-Test, Inc.) according to the manufacturer's protocol.

Bisulfite Genomic Sequencing and Methylation Analysis. Bisulfite conversion of the DNA was carried out as previously described (*4*). In brief, after bisulfite treatment, the DNA template was amplified with primers (Table 1). Sequencing of the PCR-amplified product was performed using the forward and reverse primers. The α-^{33}P-labeled ddNTP terminator kit (USB Corp., Cleveland, OH) was used for sequencing. The sequencing gel was dried and exposed to a phosphorimager screen (Bio-Rad, Hercules, CA). Methylation analysis was carried out by quantitating the intensity of C and T bands using Quantity One (Bio-Rad) and calculating the percentage of C/C + T bands, as previously described (*6*).

Cloning and Sequencing. DNA extracted from RBCs harvested from 3-, 5-, and 7-day-old eggs was treated with bisulfite and amplified by PCR, and the promoter and exon 1 regions were amplified using sense strand primers (Table 1). PCR products were cloned using a Topo TA cloning kit (Invitrogen, Carlsbad, CA) by following the manufacturer's instructions. Positive colonies were screened for the presence of the PCR product. The cloned PCR products were sent to the DNA Sequencing Facility at Iowa State University (Ames, IA) for sequencing.

Plasmids. A PCR product corresponding to a 233 bp fragment of the β_A-globin promoter was amplified with the forward primer 5′-GAGCTCACGGATCTGGGCACCTTG-3′, which contains a SacI restriction site, and the reverse primer 5′-AAGCTTGGAGGGTAGCGTGTGGTT-3′, which contains a HindIII site. The amplified fragment was digested with restriction enzymes to create sticky ends and cloned into the pGL3-Basic vector (Promega) to yield β-pGL3. A 351 bp fragment of the 3′ enhancer of the β_A-globin gene was amplified by PCR using the forward primer 5′-CGCGGATCCCAATGGGGCGATGTCTGTAG-3′ containing a BamHI restriction site and the reverse primer 5′-CGCGTCGACGTTTGCATGCAAATGACACC-3′ containing a SalI site. The amplified fragment was then cloned into β-pGL3 to yield the β-promoter−pGL3-3′ β-enhancer. Methylation was accomplished with *Sss*I 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 assessed by digestion with methylation-sensitive restriction enzymes.

Transient Transfections. Using the Amaxa nucleofection device, K562 cells were transfected with 2 μg each of methylated and mock-methylated β-promoter−pGL3-3′ β-enhancer contructs. As controls, 2 μg of pGL3 basic and 2 μg of pGL3 control vector (Promega) were transfected. To correct for transfection efficiencies, 100 ng of pRLTK thymidine kinase *Renilla* luciferase vector (Promega) was cotransfected. We checked for optimal transfection efficiency by transfecting the pmaxGFP (Amaxa) encoding the enhanced green fluorescent protein eGFP (not shown). The transfections were carried out in duplicate. The cells were plated in 12-well dishes.

Luciferase Assays. Luciferase assays were performed using the dual luciferase reporter assay system from Promega following the manufacturer's protocol for single-sample luminometers. Seventy-two hours post-transfection, K562 cell suspensions were spun down; pellets were washed twice with PBS and then resuspended in 500 μL of 1× passive lysis buffer (Promega). The cell lysates were cleared by centrifugation for 2 min at 4 °C and then transferred to fresh 1.5 mL tubes and stored at −80 °C. Luciferase and *Renilla* activity was 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.

Nuclear Extracts. Blood was collected from 5-, 8-, and 11-day-old chicken embryos, and nuclear extracts were prepared according to the protocol of Dignam et al. (*8*).

Demethylase Assay. Substrates for the demethylase assay were custom-synthesized (Sigma Genosys, St. Louis, MO) 70-mer oligonucleotide sequences derived from a CpG rich region of the β-globin promoter (Table 2). The complementary strands were annealed in 1× annealing buffer [10 mM Tris (pH 7.5−8.0), 50 mM NaCl, and 1 mM EDTA] following the manufacturer's protocol. Nuclear extracts (240 μg of protein) from 5-, 8-, or 11-day-old chicken embryonic RBCs were incubated with 10 μg of the substrate oligonucleotides in a total reaction volume of 100 μL containing

Table 2: 70-mer Oligonucleotide Substrate Derived from the β_A-Globin Promoter Region (positions −120 to −51)

5′cctcccc**CG**gggtgccaaggctgggggcccctc**CG**gagatgcagccaattg**CG**gggtgcc**CG**gggaagag3′ 3′ggagggg**GC**cccacggttccgacccccggggag**GC**ctctacgtcggttaac**GC**cccacgg**GC**cccttctc5′	β_A-U/U
5′cctcccc**CG** gggtgccaaggctgggggcccctc**CG**gagatgcagccaattg**CG** gggtgcc**CG**gggaagag3′ 3′ggagggg**GC**^{m}cccacggttccgacccccggggag**GC**^{m}ctctacgtcggttaac**GC**^{m}cccacgg**GC**^{m}cccttctc5′	β_A-U/M
5′cctcccc^m**CG**gggtgccaaggctgggggcccctc^m**CG**gagatgcagccaattg^m**CG**gggtgcc^m**CG**gggaagag3′ 3′ggagggg **GC**cccacggttccgacccccggggag**GC**ctctacgtcggttaac **GC**cccacgg**GC**cccttctc5′	β_A-M/U
5′cctcccc^m**CG**gggtgccaaggctgggggcccctc^m**CG**gagatgcagccaattg^m**CG**gggtgcc^m**CG**gggaagag3′ 3′ggagggg**GC**^{m}cccacggttccgacccccggggag**GC**^{m}ctctacgtcggttaac**GC**^{m}cccacgg**GC**^{m}cccttctc5′	β_A-M/M

25 mM Hepes (pH 7.5), 0.5 mM EDTA, 0.1 mM $ZnCl_2$, 40 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 20 mM creatine phosphate, 0.5 mM ATP, and 1 mM dNTPs (*9*) for 18 h at 37 °C. The reaction was stopped by adding 150 μL of 20 mM EDTA. The oligonucleotide was extracted twice with phenol and chloroform, precipitated in absolute ethanol, and washed with 70% ethanol. The dried pellet was then resuspended in 18 μL of nuclease free water.

MS-SNuPE for Quantitation of Methylation. The oligos after demethylase treatment were treated with sodium bisulfite as described previously (*4*). MS-SNuPE reactions were carried out using ~0.2 μg of oligo template in a 25 μL reaction mixture containing 1× PCR buffer (Sigma), 1 μM SNuPE primer, 1 μCi of ^{32}P-labeled dATP or ^{32}P-labeled dGTP, and 1 unit of Taq polymerase (Sigma) (*10*). The 5′-CCAACCTTAACACCCC-3′ primers were used in this assay for the detection of the first CpG on the top strand. The symmetrical CpG groups on the top and bottom strands were analyzed using the primers 5′-AATTAACTACATCTCC-3′ and 5′-AACTAAAAACCCCTCC-3′, respectively. Oligos not subjected to the demethylase reaction were used as a control in the assay.

RESULTS

Expression of the avian β_A-globin gene during development is regulated by methylation. CpG methylation has been reported to play a central role in gene regulation during embryonic development (*3*). We have previously shown that the avian β-type ρ-globin and α^π-globin genes are silenced in the adult chicken erythroid cells by progressive DNA methylation during development (*6*, *11*). Via RT-PCR, we have demonstrated that the expression of the β_A-globin gene during the development of chicken embryos was concomitant with the silencing of the ρ-globin gene in primary chicken erythroid cells (*6*). To determine if methylation has a role in the regulation of the β_A-globin gene during development, we employed the bisulfite genomic sequencing method to determine the methylation pattern of the CpG dinucleotides (*12*, *13*). CpGs of the promoter, exons 1−3, were studied. Figure 1A depicts the distribution of CpG dinucleotides along the β_A-globin gene. Dense CpG islands are present in the promoter and exon 1 region. Exon 2 also has a fairly high CpG density, whereas exon 3 has a normal density of CpGs. We observed almost complete methylation in the different

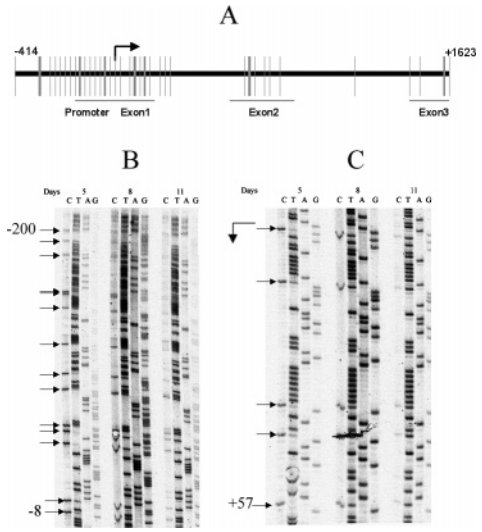

FIGURE 1: (A) Distribution of CpG dinucleotides in the chicken β_A-globin gene. The arrow indicates the transcription start site. CpG dinucleotides in the promoter and exons 1−3 are denoted with horizontal lines. Positions marked are with respect to the transcription start site. In vivo methylation pattern of CpG dinucleotides of the promoter (B) and exon 1 (C) in the chicken β_A-globin gene. The methylation pattern in 5-, 8-, and 11-day-old chicken embryonic erythroid cells was determined using bisulfite genomic sequencing. Arrows denote methylated cytosines. Positions indicated are relative to the transcription start site.

regions of the gene on day 5 of development. A progressive decrease in the level of methylation is seen by day 8 and by day 11, and the β_A-globin gene is largely unmethylated (Figure 1B,C). Studies from our laboratory on the strand-specific methylation patterns of the ρ-globin gene have shown that de novo methylation follows a specific pattern, starting in exon 1 and proceeding to exon 3 and then into the promoter region (*6*). To study the loss of methylation in different regions of the β_A-globin gene, the temporal methylation pattern on the coding and template DNA strands of the β_A-globin promoter and exons 1−3 was examined by bisulfite genomic sequencing (Figure 2), and quantitation of bands was accomplished using Quantity One (Bio-Rad). A significant loss of methylation is observed by day 8 (>50% of day 5), and by day 11, the promoter and exon 1 are almost completely demethylated (Figure 2). Progressive loss of methylation was observed in all four regions of the β_A-globin gene during chicken embryonic development.

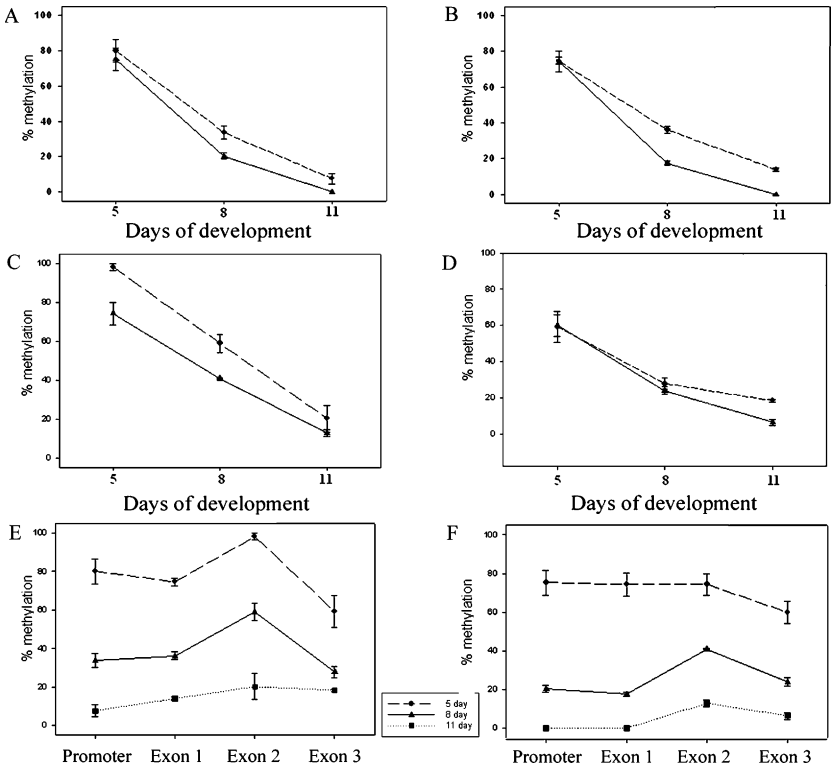

FIGURE 2: Loss of methylation in the different regions of the β_A-globin gene during development. Methylation pattern of the promoter (A), exon 1 (B), exon 2 (C), and exon 3 (D),of the chicken β_A-globin gene during different days of development. Solid lines represent data for the template strand and dotted lines data for the coding strand. Panels E and F show the demethylation pattern of the different regions of the chicken β_A-globin gene during development. Panel E represents the coding strand and panel F the template strand. The percent methylation was calculated using the formula $[C/(C + T)] \times 100$. The graph was plotted taking the standard error of the mean of the values.

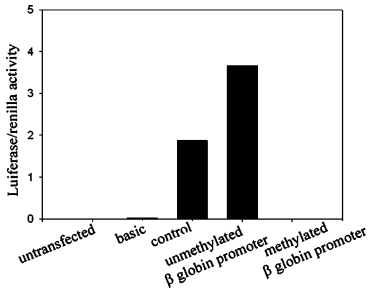

FIGURE 3: Methylation of the β_A-globin promoter-driven reporter vector represses transcription. K562 cells were transfected with the mock-methylated and fully methylated β-promoter−pGL3-3′ β-enhancer construct along with the pRLTK vector. As controls, pGL3 basic (negative control) and pGL3 control vector (positive control) were transfected. Mock-transfected cells were also used as a negative control. The ratio of luciferase to *Renilla* activity was plotted. This experiment is representative of three independent experiments.

Methylation of the β_A-Globin Promoter Represses Luciferase Reporter Activity in Transient Transfection Assays. To determine if methylation of the β_A-globin promoter represses transcription in primary erythroid cells, a 240 bp β_A-globin gene promoter and 351 bp 3′ β-enhancer regions were cloned upstream and downstream of the luciferase reporter gene, respectively, to yield a β-promoter−pGL3-3′ β-enhancer construct. The methylated and mock-methylated plasmids were then transfected into human erythroleukemia cell line K562. Methylation of the construct resulted in nearly complete inhibition of expression of luciferase (Figure 3).

Methylation Pattern of Individual DNA Molecules. The loss of methylation of the β_A-globin gene may simply reflect dilution of methylated DNA in primitive cells by unmethylated strands in definitive cells rather than "active demethylation" of the β_A-globin gene. Earlier reports on the demethylation process hypothesized a passive mechanism by DNA replication, and other reports suggested that after one round of replication, active demethylation takes place (*14*, *15*). However, progressive demethylation that we observed by bisulfite genomic sequencing is suggestive of a predominant active demethylation process. We hypothesized that by examining the methylation pattern of the β_A-globin gene in several DNA strands in early definitive cells, we may encounter a methylation pattern that is intermediate between primitive and definitive cells. We performed bisulfite treatment on DNA derived from day 3, 5, and 7 embryos. Bisulfite-treated DNA was amplified with primers designed for amplifying the β_A-globin gene; the PCR product was cloned, and several colonies were sequenced. The sequences were carefully checked to confirm the conversion of non-CpG cytosines to thymidine, indicative of a successful bisulfite conversion reaction. Sequencing of several clones from day 3 embryos demonstrated that the single strands are largely methylated on most of the CpG dinucleotides (Figure 4). Day 5 embryos exhibited a pattern similar to that of day 3 embryos (clones 5.1, 5.4, 5.5, and 5.10). In addition, however, several strands (clones 5.2, 5.9, 5.11, and 5.13) exhibited an "intermediate" pattern. Day 7 embryos demonstrated several unmethylated strands (clones 7.13, 7.14, 7.18, and 7.20), a strand likely from primitive erythroid cells (clone 7.6), and other strands with a mixed methylation pattern (7.15, 7.16, 7.17, 7.19, and 7.21) (Figure 4). These results suggest that demethylation of the β_A-globin gene occurs in early definitive erythroid cells.

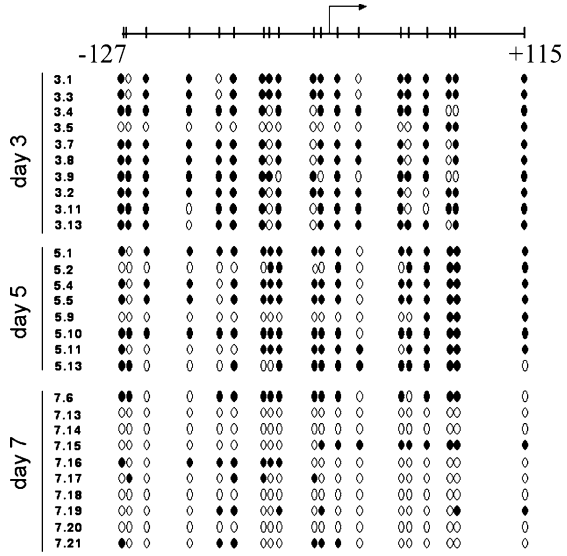

FIGURE 4: Analysis of CpG dinucleotides of individual DNA strands of β_A-globin during chicken embryonic development. The CpG dinucleotides located between positions −127 and 115 (with respect to the transcription start site) were analyzed. Bisulfite sequencing analysis of the individual strands showed an intermediate pattern of methylation. Marked demethylation was observed on day 7.

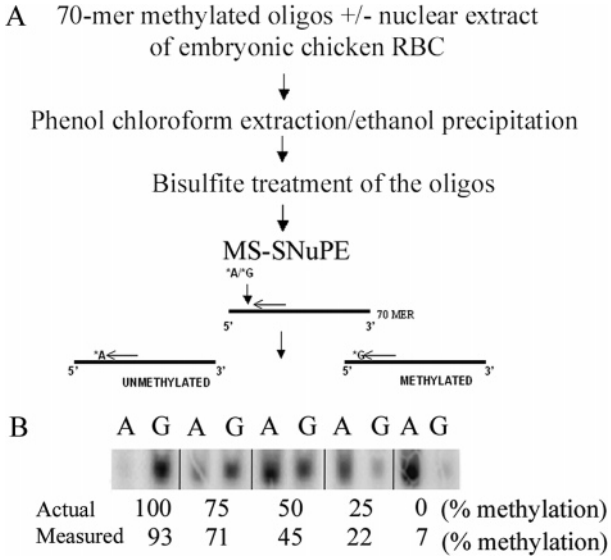

FIGURE 5: Modified MS-SNuPE assay for quantitation of de methylase activity. (A) In this schematic representation, the oligonucleotides after incubation with red cell nuclear extracts were treated with bisulfite to convert unmethylated cytosines to uracil while leaving 5-methylcytosine unchanged. Methylation of specific CpG dinucleotides on the coding and template strand was assayed by incubating the sample with the appropriate primer, buffer, ^{32}P-labeled dATP or dGTP, and Taq polymerase for single-nucleotide primer extension. Radiolabeled products were then electrophoresed on a 12% native polyacrylamide gel and visualized via phosphorimager quantitation. The bands were quantitated using Quantity One (Bio-Rad). (B) MS-SNuPE assay carried out with known ratios of hemimethylated (β_A-MU) and unmethylated (β_A-UU) custom-synthesized 70-mer oligonucleotide sequences that were derived from a CpG rich region of the β_A-globin promoter. β_A-MU and β_A-UU were mixed in different ratios and treated with bisulfite, and methylation of the top strand was assayed by MS-SNuPE. The actual and measured percentage of methylation is given.

Active Demethylation in Red Cell Nuclear Extracts Targets the β_A-Globin Gene Promoter. The results given above show that expression of the β_A-globin gene is correlated to demethylation of the CpG islands. To decipher the mechanism of demethylation and to explore the role of active demethylation in embryonic chicken RBCs, we assayed the nuclear extracts from the erythroid cells for demethylase activity. Earlier assays described in the literature employ methylation-sensitive restriction digestion in assessing the demethylation of methylated oligonucleotide substrates (*9*). The disadvantage of this assay is that individual DNA strands cannot be studied. Further, the lack of sensitivity limits the use of this assay. We developed a novel technique for assaying the demethylation based on principles of methylation-sensitive single-nucleotide primer extension (MS-SNuPE) (*10*). The schematic representation of the assay is shown in Figure 5A. To test the sensitivity of the assay, we used different ratios of methylated to unmethylated substrate. We obtained comparable quantitative values of methylation (Figure 5B). We used a hemimethylated 70-mer oligo derived from the promoter region as a substrate in a demethylase assay with 8-day-old nuclear extracts since the in vivo methylation pattern showed loss of methylation on day 8. This was followed by MS-SNuPE. With this assay, we were able to detect up to 80% of demethylation of the methylated substrate, indicating that the demethylase activity is carried out by an active "demethylase enzyme" present in the chicken red cell nuclear extracts. Demethylase activity is described as percent demethylation calculated as $[A/(A + G)] \times 100$.

Demethylase Activity in Embryonic Chicken Erythroid Cells Is Stage-Specific. As described above, bisulfite genomic sequencing of the β_A-globin gene during the different stages of development of chicken embryo showed that the gene is almost fully methylated on day 5 and subsequent DNA demethylation results in loss of methylation on day 8, and by day 11, the gene becomes almost completely demethylated (Figure 2). To determine if this pattern of demethylation is due to the differential activity of demethylase at the different stages of embryonic development, we carried out demethylase assays followed by MS-SNuPE quantification as described above using nuclear extracts prepared from 5-, 8-, and 11-day-old chicken embryo red cells. We used the hemimethylated oligo (β_A-MU) as the substrate in this assay. We observed maximum demethylase activity (75% demethylation) in 8-day-old nuclear extracts. Nuclear extracts from 5- and 11-day-old chicken embryo erythroid cells exhibited ~6 and ~24% demethylase activity, respectively (Figure 6B). Hence, there seems to be a stage-specific regulation of demethylase activity in embryonic chicken erythroid cells.

Demethylase Activity in Embryonic Chicken Erythroid Cells Targets the Fully Methylated β_A-Globin Gene Promoter. Earlier reports indicated that active demethylation occurs only after one round of passive demethylation via DNA replication. These results were based on methylation-sensitive restriction digestion assays that fail to distinguish between individual DNA strands. Also, the lack of sensitivity is a limitation for this assay. Recent reports show that demethylation of a fully methylated substrate can occur in cells in which replication has been blocked, evidence of the presence of an active demethylation process regulating gene expression (*16*, *17*). To check if the demethylase activity in developing chicken embryos can target a fully methylated

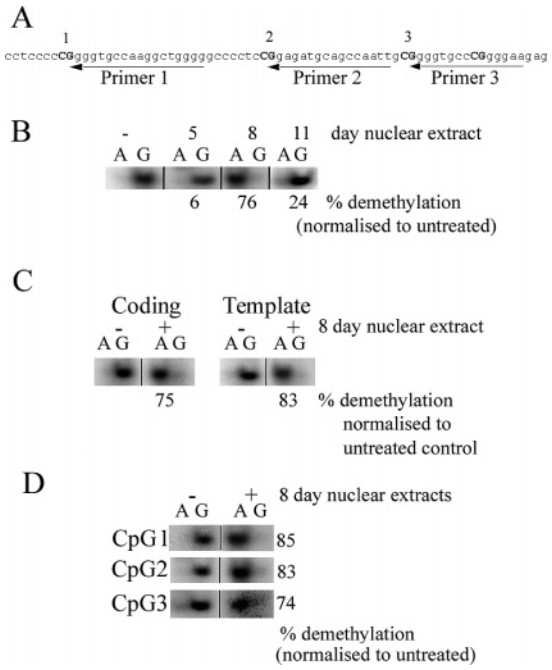

FIGURE 6: Demethylase activity in primary chicken erythroid cell nuclear extracts. (A) The 70-mer oligonucleotide sequence used as a substrate for the demethylase assay. The CpG dinucleotides are indicated by numbers, and the primers used in the MS-SNuPE assay are also indicated. (B) Stage-specific demethylase activity. Hemimethylated (β_A-MU) substrates for the demethylase assay were custom-synthesized as 70-mer oligonucleotide sequences that were derived from a CpG rich region of the β_A-globin promoter. Nuclear extracts (240 μg of protein) from 5-, 8-, and 11-day-old chicken embryonic RBCs were incubated with 10 μg of the substrate oligonucleotides in a total reaction volume of 100 μL containing 1× demethylase buffer for 18 h at 37 °C. The oligos were then extracted and assayed by MS-SNuPE quantitation. The oligos after demethylase treatment were treated with sodium bisulfite, and demethylation of the top strand was assayed using the appropriate primer. Untreated oligos were used as a control in the assay. (C) Demethylase activity on the fully methylated substrate. Nuclear extracts from 8-day-old chicken embryonic RBCs were incubated with 10 μg of the fully methylated 70-mer oligonucleotides, and demethylase activity on the same CpG site was assayed for the coding and template strand. Untreated oligos were used as controls in this assay. (D) Site-specific analysis of demethylase activity on the fully methylated oligonucleotide substrate. Nuclear extracts from 8-day-old chicken embryonic RBCs were incubated with 10 μg of the fully methylated 70-mer oligonucleotide, and demethylase activity on the individual CpG site was assayed for the coding strand. Untreated oligos were used as controls in this assay.

substrate, we used symmetrically methylated 70-mer oligo as the substrate in the demethylase assay. We studied the same CpG on both strands to obtain a comparative demethylase activity on both strands. As seen in Figure 6C, there was similar demethylase activity on the template strand (83%) and the coding strand (75%). Activity on fully methylated 70-mer oligos was similar to that on the hemimethylated substrate. Further, similar levels of demethylase activity were observed on all three CpG dinucleotides that were analyzed (Figure 6A,D). These results conflict with earlier reports (*9*, *18*, *19*) that show that demethylase activity can target only a hemimethylated substrate.

DISCUSSION

The main findings in this study are as follows. The expression of the β_A-globin gene is concomitant with DNA demethylation. Using a novel quantitative assay, we found that demethylase activity in the chicken red cell nuclear extracts can target both fully methylated and hemimethylated oligonucleotide substrates equally. Further, the demethylase activity appears to be stage-specific. There is maximum demethylase activity on day 8 in comparison to activity of nuclear extracts derived from 5- and 11-day-old embryonic primary erythroid cells. These results conform to the bisulfite genomic sequencing data showing the methylation pattern of the β_A-globin gene at different stages of development of chicken embryos.

Modification of gene activity has been largely attributed to DNA methylation, the pattern of which is stably inherited in somatic cells (*2*, *20*). Methylation of CpG dinucleotides carried out by DNA methyltransferases (Dnmts) in the promoter as well as transcribed regions has been reported to cause inactivation of the gene in vitro as well as in vivo. It has been shown that transient depletion of xDnmt1 leads to premature activation of *Xbra*, *Cerebrus*, and *Otx2* in developing *Xenopus* embryos and that DNA methylation at the promoter regions has a role in regulating the timing of gene activation in these embryos (*20*, *21*). Our results with transient transfection of a luciferase reporter construct driven by the β_A-globin promoter support this theory since the methylation of the whole plasmid represses expression of the reporter gene. It is more likely that the gene expression was repressed by methylation of the promoter rather that of the enhancer because of the dense distribution of CpGs in the promoter sequence. Thus, silencing of the β_A-globin gene seems to occur via methylation-mediated repression of transcription. Demethylation of the regulatory regions of genes alleviates repression, leading to transcriptional activation of the gene. Our data on the in vivo methylation pattern show almost complete methylation of the promoter and transcribed regions of the β_A-globin gene during early stages of embryonic development and nearly complete loss of methylation during the later stages. Thus, loss of methylation seems to correlate with activation of gene expression.

Previous reports have shown the occurrence of active DNA demethylation in chicken embryonic nuclear extracts using the methylation-sensitive restriction digestion assay (*9*). However, this assay is not sensitive and requires large amounts of DNA template. The assay also fails to distinguish between individual strands and is also limited to the assay at restriction sites which may not represent the target DNA sequence. The MS-SNuPE assay, on the other hand, is highly sensitive, enables quantitation of demethylation activity, and allows the assay of individual strands of the target sequence (*10*). This is important since demethylase enzyme is believed to target one strand at a time, although sufficient evidence for this is lacking. MS-SNuPE quantitation also permits the assay of all the CpG dinucleotides of the substrate DNA sequence. Moreover, in vitro demethylase assays that have been reported in the literature so far have been designed with an artificial substrate. The substrate used in our assay is a target of demethylation in vivo. The in vitro demethylation activity correlates with the progressive demethylation observed in chicken erythoid cells during development. Hence, this is the first report of an in vitro stage-specific demethylase activity on a fully methylated substrate that mimics demethylation of a native developmentally regulated gene.

DNA demethylation has been proposed to occur via two mechanisms: a passive replication-mediated process and an enzyme-mediated active process (*22*). It was initially believed that DNA demethylation occurs by passive mechanisms involving the exclusion of DNA methyltransferases following DNA replication (*23*) and that an initial passive process may be followed by enzymatic demethylation (*15*). Active demethylation was proposed to occur through 5-methylcytosine DNA glycosylase activity, which removes the methylated cytosine leaving the deoxyribose intact. Local DNA excision repair then adds back cytosine in the nucleotide form (*9*). However, this enzyme targets only the hemimethylated DNA, and action on fully methylated DNA results in the formation of double-stranded nicks that results in the breakdown of DNA and no demethylation (*9*). Another proposed mechanism of demethylation is via MBD2b which has specific demethylase activity for CpG dinucleotides and converts 5-methylcytosine to cytosine and methanol (*24*). 5-Methylcytosine glycosylase activity was also observed in the human MBD4 (G/T mismatch glycosylase) and its chicken homologue (*18*, *19*). This protein bound to symmetrically methylated, hemimethylated, and unmethylated DNA even when the methyl binding domain was deleted. MBD4 exhibited the strongest 5-MeC DNA glycosylase activity only on hemimethylated DNA with weak substrate affinity toward fully methylated DNA. These findings suggested that replication-mediated demethylation is required as a priming step for enzyme-mediated active demethylation to take place. However, recent reports show that demethylation of a fully methylated substrate can occur in cells in which replication has been blocked, evidence of the presence of an active demethylation process regulating gene expression (*16*, *17*). Our study on stage-specific demethylation of individual DNA molecules yielded similar results. The presence of an intermediate methylation pattern suggested that demethylation occurred by a replication-independent pathway. Moreover, we found that the demethylase activity present in the chicken primary erythroid cells could target the fully methylated and hemimethylated DNA substrate equally.

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