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Molecular mechanisms of human hemoglobin switching: Selective undermethylation and expression of globin genes in embryonic, fetal, and adult erythroblasts

(control of gene expression/DNA methylation/human ontogeny/erythropoietic differentiation)

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ABSTRACT **The globin chain synthetic pattern and the extent of DNA methylation within embryonic, fetal, and adult β-like globin gene domains were evaluated in ≥90% purified human erythroblasts from yolk sacs and fetal livers in the 6- to 12-wk gestational period as well as from adult marrows. The 6-wk erythroblasts produce essentially embryonic ε chains, whereas the 12-wk erythroblasts synthesize largely fetal gamma globin and the adult marrow erythroblasts synthesize almost exclusively adult β chains. In all phases of ontogenic development, a strong correlation exists between DNA hypomethylation in the close flanking sequences of globin genes and their expression. These results suggest that modulation of the methylation pattern may represent a key mechanism for regulating expression of human globin genes during embryonic → fetal and fetal → adult Hb switches in humans. In ontogenic development this mechanism might in turn correlate with a gradual modification of chromatin structure in the non-α gene cluster, thus leading to a 5′ → 3′ activation of globin genes in a balanced fashion.**

In mammalian DNA ≈2–7% of cytosine residues are converted to 5-methylcytosine shortly after DNA replication (1). More than 90% of 5-methylcytosine residues occur in the dinucleotide CpG with a nonrandom distribution, which is both species and tissue specific (1, 2). The DNA methylation pattern in proliferating cells is preserved by "maintenance" methylase(s), which recognize CpG sequences in the newly synthesized unmethylated DNA strand (3, 4).

Growing evidence indicates that in higher eukaryotes DNA methylation and gene expression are inversely correlated (reviewed in refs. 1, 2, and 5). DNA in germline cells is heavily methylated (1, 2). In differentiated cells, ≈70% of CpG sequences are methylated in the overall DNA vs. only ≈20–30% in chromatin regions containing active gene sequences (6). A variety of protein encoding genes is undermethylated or methylated when expressed or not expressed, respectively (1, 2, 5). Similarly, endogenous proviral genomes are commonly heavily methylated when inactive but unmethylated when expressed (7–9). However, inactive genes may be undermethylated in both cell lines (10, 11) and primary tissues (e.g., placenta and carcinomas) (12, 13). In rare instances, active genes are totally methylated (14).

A cause–effect relationship between DNA undermethylation and gene expression has been suggested by a number of experiments. In particular, (*i*) expression of cloned genes inserted in eukaryotic cells is dependent upon their methylation state prior to insertion (15, 16) and (*ii*) inhibition of DNA methylation by 5-azacytidine leads to hypomethylation and activation of endogenous viral DNAs (7, 17) and cellular genes (18–20).

The methylation pattern of human globin genes also relates to the debated issue of mechanisms underlying the Hb switches in humans. In this regard, the embryonic → fetal (i.e., $\zeta \rightarrow \alpha$, $\varepsilon \rightarrow \gamma$) globin chain switch has been scarcely investigated so far, whereas the perinatal $\gamma \rightarrow \beta$ switch has been extensively studied only at cellular level (reviewed in refs. 21 and 22). Molecular studies suggest that activation of γ and δβ genes might be correlated with undermethylation of their domains in fetal liver (FL) and adult marrow (AM) (12). However, interpretation of these results was hampered by use of hemopoietic tissues *in toto* rather than purified erythropoietic cells.

We have investigated the pattern of DNA methylation within the human ε, γ, δβ globin gene region in ≥90% pure erythroblasts from yolk sacs (YSs), FLs, and AMs. The methylation pattern, evaluated by means of restriction endonucleases differentially inhibited by cytosine methylation within their recognition sequences, was correlated with the level of relative expression of globin chains in the same erythroid population. We demonstrate a strong, direct correlation between hypomethylation in the close neighborhoods of globin genes and their expression in embryonic, fetal, and adult erythroblasts. These results may shed light on the molecular mechanisms underlying both embryonic → fetal and fetal → adult Hb switches.

MATERIALS AND METHODS

Purification of YS, FL, and AM Erythroblasts. Intact embryos and fetuses, obtained from legal curettage abortions at 6–12 wk of gestational age, were maintained in Iscove's modified Dulbecco's medium (IMD medium). The age was evaluated by both standard ecographic criteria and crown-rump length measurement. A single cell suspension was prepared from YS or FL (or both) as described (23) within 6 hr of abortion. The erythroblastic population was enriched up to ≥90% by removing adherent cells (23). All stages of erythropoietic maturation were similarly represented in both the starting unicellular suspension and the purified erythroblastic population. If necessary, erythroid cells from two to five different specimens were pooled,

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Abbreviations: FL, fetal liver; AM, adult marrow; YS, yolk sac; kbp, kilobase pair(s); kb, kilobase(s); bp, base pair(s); F-WBC, umbilical leukocytes; A-WBC, adult peripheral blood leukocytes.

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but only if their gestational age and globin chain synthesis pattern were strictly equivalent.

AM erythroblasts were enriched up to ≥90% by fluorescence-activated cell sorting with the L 5.1 monoclonal antibody, which specifically recognizes transferrin receptors expressed on the human erythroblastic lineage (24). The positive cell population consisted essentially of pronormoblasts, basophilic, polychromatic, and orthochromatic normoblasts in a normal ratio. The negative cells, utilized as nonerythropoietic controls, contained both myelomonocytes and lymphocytes but no morphologically recognizable erythroid cell. Positive and negative cell populations from 11 normal AMs were pooled.

Control cells included umbilical leukocytes (F-WBC) and adult peripheral blood leukocytes (A-WBC) as well as fibroblasts cultured by standard procedures from both amniotic fluid and adult skin.

Globin Chain Synthesis. Purified erythroblasts were incubated with 100 μCi of [^{3}H]leucine (Amersham, >100 mCi/ml; 1 Ci = 3.7×10^{10} Bq) in leucine-free IMD medium ($0.5–3.0 \times 10^6$ cells per ml) for 2 hr at 37°C. Cells were washed three times in IMD medium, lysed in 0.01 M Tris·HCl, pH 7.4/0.005 M $MgCl_2$. Hb was recovered after high-speed centrifugation, freeze-dried, and resuspended in small volumes of electrophoresis loading buffer. Triton/urea/polyacrylamide gel electrophoresis was carried out as described (25) and 20,000–30,000 incorporated cpm in a 5- to 10-μl volume were loaded on each slot. Globin chain synthetic ratios were determined from densitometric tracings of standard fluorographs.

DNA Methylation Pattern. DNA was obtained from erythroblasts, leukocytes, and fibroblasts by standard techniques. Total DNA (3–5 μg), digested with a methylation-insensitive endonuclease (*Eco*RI, *Hin*dIII, *Bgl* II, and *Pst* I: 2–3 units/μg of DNA), was redigested with a 10- to 20-fold excess of methyl-sensitive endonuclease. The endonucleases include *Msp* I, which cuts C-^{m}C-G-G but not ^{m}C-C-G-G, and *Hpa* II, *Hha* I, *Sal* I, and *Aos* II, which do not cut if the internal C in their recognition sequence [i.e., C-C-G-G, G-C-G-C, G-T-C-G-A-C, and G-R-C-G-Y-C (where R = purine and Y = pyrimidine), respectively] is methylated. Completeness of digestion was assessed as in ref. 12. DNA fragments were separated by electrophoresis on 1.0% agarose gels, transferred to nitrocellulose paper, hybridized, and washed as described (26). Plasmids JW 102 and JW 151 containing human β and γ cDNA sequences (27), and plasmid pε0.7, containing the *Bam*HI 5′ fragment of the human ε gene (28), were ^{32}P-labeled by nick-translation to a specific activity of $3–6 \times 10^8$ dpm/μg and were used as hybridization probes.

RESULTS

Synthesis of Embryonic, Fetal, and Adult β-Like Globin Chains in Early Human Ontogeny. In 6-wk YS and FL, erythroblasts produce essentially ε chains, with a minimal amount of gamma globin (synthetic ratios: $\varepsilon/\varepsilon + \gamma$, 0.8–1.0). In 7-wk FL erythroblasts, ε chains are synthesized together with Gγ and Aγ globins ($\varepsilon/\varepsilon + \gamma \approx 0.50$). ε chains represent ≤15% of non-α globin at 8–9 wk and are completely replaced by γ chains starting from ≈11–12 wk of gestation. A minimal but detectable amount of β globin is synthesized at as early as 7–8 wk.

DNA Methylation: The ε-Gene Domain. DNA obtained from purified erythroblasts and control cells was digested with *Hin*dIII to yield a 7.8-kilobase-pair (kbp) fragment, including the entire ε gene and both 5′ and 3′ flanking sequences. It was then redigested with *Hpa* II to test the degree of methylation in two C-C-G-G sequences (Mε1 and Mε3) (28) (Fig. 1). In several instances, *Hpa* II and *Msp* I cleaved at a third site (Mε2) located ≈1.4 kilobases (kb) downstream from Mε1, within the large

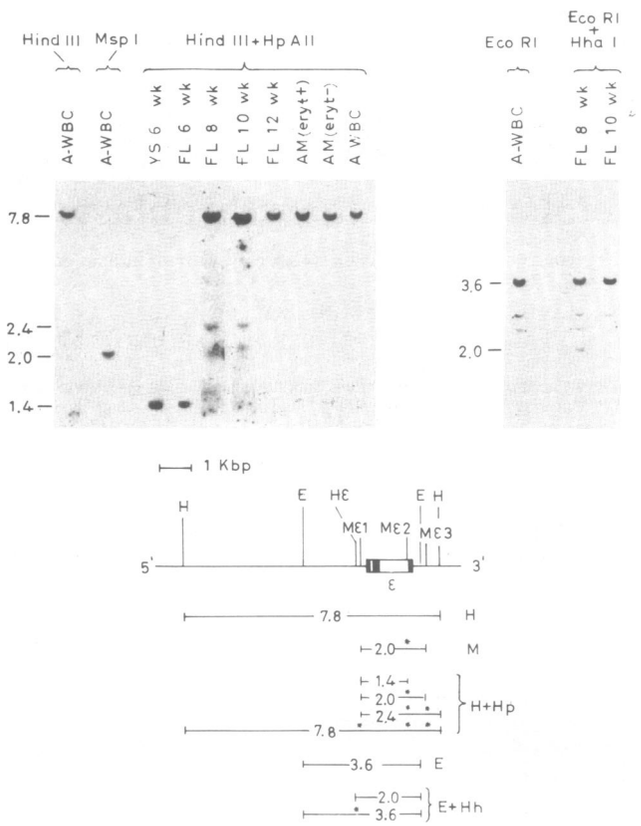

FIG. 1. CpG methylation pattern in human embryonic ε-globin gene region of ≥90% purified erythroblasts from YS, FL, and AM (AM eryt$^+$). The levels of globin chain synthesis in these erythroblastic populations are detailed in the *Results*. Control cells include AM nonerythroblastic cells (AM eryt$^-$) and A-WBC. Data on amniotic fluid and adult skin fibroblasts are not shown (see *Results*). E, *Eco*RI; H, *Hin*dIII; Hh, *Hha* I; Hp, *Hpa* II; M, *Msp* I. Asterisks in double-digestion fragments (H + Hp and E + Hh, *Lower*) indicate methylated cleavage sites (C-^{m}C-G-G and G-^{m}C-G-C, respectively). The asterisk in the *Msp* 2.0-kb fragment indicates ^{m}C-C-G-G methylation (see *Results*).

intron. Although a C-C-G-G sequence is not reported in that position (28), a single base polymorphism at either the C-C-G-T or C-C-T-G sequence located 1,378 or 1,440 bp downstream from the Mε1 site may originate a new *Hpa* II cleavage site.

The G-C-G-C sequence located 414 bp 5′ to the first codon of the ε gene was tested for methylation by *Eco*RI–*Hha* I double-digestion.

DNA from two 6-wk YS and FL erythroblasts synthesizing essentially embryonic globin chains (see above) was found to be totally unmethylated at all three Mε sites, as indicated by the presence of the 1.4-kb ε-gene fragment. In 8- to 10-wk FL erythroblasts most of the ε-gene sequences were methylated, but the presence of a minority of 2.4-, 2.0-, and 1.4-kb fragments indicated that part of the ε-gene sequences was unmethylated at one, two, or three Mε sites. The ε gene was fully methylated in 12-wk FL and AM erythroblasts as well as in control cells.

The *Hha* I site was partially unmethylated in 8-wk FL erythroblasts (2.0-kb band in E+ Hh double-digest in Fig. 1) but was completely methylated in both 10- to 12-wk FL erythroblasts and control cells.

DNA Methylation: The γ-Gene Domain. DNA was first digested with *Bgl* II, which yielded a 14.0-kbp fragment containing both Gγ and Aγ genes, and then was redigested with *Hpa* II to test for methylation at the Mγ1–6 C-C-G-G sequences (12) (Fig. 2).

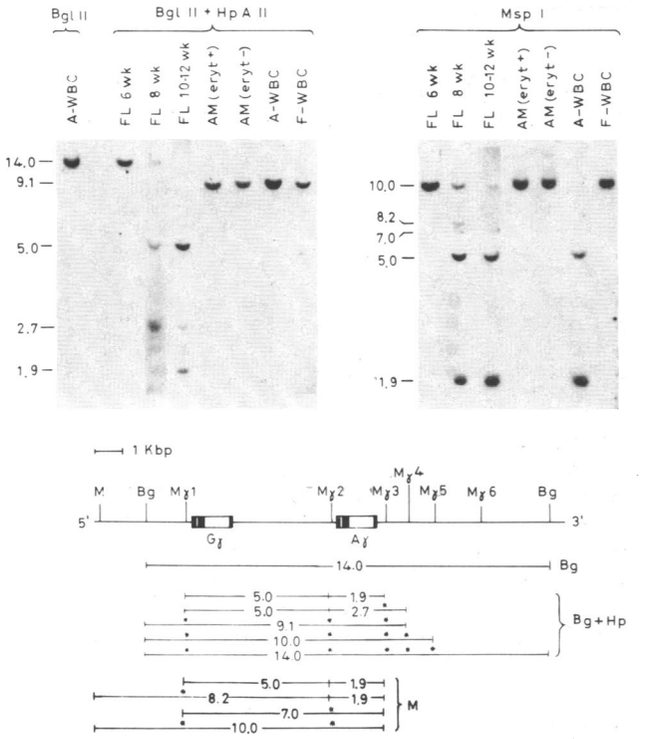

FIG. 2. CpG and CpC methylation pattern in human fetal Gγ- and Aγ-globin gene domain of ≥90% purified erythroblasts from FL and AM (see also legend to Fig. 1). Bg, *Bgl* II; Hp, *Hpa* II; M, Msp I. Asterisks indicate methylated cleavage sites.

In DNA from 6-wk FL erythroblasts all Mγ sites were fully methylated, concomitant with absence of γ-chain synthesis.

DNA from an 8-wk FL, producing ≈85% γ chains, was only slightly methylated at Mγ1, Mγ2, and Mγ4 sites, as indicated by predominance of 5.0- and 2.7-kbp fragments. However, persistence of small amounts of uncut *Bgl* II fragment indicated a low residual degree of methylation at all Mγ sites. The Mγ3 site was still highly methylated, as shown by the relative intensity of the 2.7- vs. the 1.9-kbp fragment.

DNA from 10- to 12-wk FL was completely unmethylated at Mγ1, Mγ2, and Mγ4 sites, and ≈50% methylated at Mγ3. The methylation pattern at Mγ5 and Mγ6 sites was untestable with a γ probe whenever Mγ4 was completely unmethylated. In these cases, demethylation of the Mγ5 site was established by the appearance of a 16.0-kbp instead of a 17.0-kbp fragment in *Hin*dIII–*Hpa* II double-digests hybridized with a β-cDNA probe (see Fig. 3). The Mγ6 was always methylated.

DNA from AM erythroblasts was fully methylated at all sites, except for Mγ4 (9.1-kb band in Fig. 2) and Mγ5 (16.0-kb band in Fig. 3). DNA from control cell was unmethylated only at the Mγ4 site, with the exception of both fetal and adult fibroblasts, in which it was fully methylated (data not shown).

Both *Hpa* II sites 5′ to Gγ and Aγ genes may be methylated at the CpC dinucleotide (12). This was tested by single-digestion with *Msp* I, which in the absence of cleavage at Mγ1 and Mγ2 sites yields a 10.0-kb γ-gene fragment extending from a C-C-G-G sequence upstream from the *Bgl* II site to the Mγ3 site. Mγ1 and Mγ2 were methylated at the first cytosine in DNA from 6-wk FL, partially methylated in 8-wk FL, and completely unmethylated in 10- to 12-wk FL. Conversely, DNAs from AM erythroblasts and control cells (except for A-WBC) were methylated at these sites.

DNA Methylation: The δβ-Gene Domain. DNA was restricted with *Hin*dIII, which yields a 17.0-kbp fragment containing the majority of the $A_{\gamma-\delta}$ intergenic region, the δ gene and its 3′-flanking sequences, and a 7.8-kbp fragment containing the β gene with both 5′- and 3′-flanking sequences (Fig. 3). It was then redigested with *Hpa* II to test for methylation of the Mδ, Mβ1, and Mβ2 sites as well as Mγ5 and Mγ6 in all cases of complete cleavage at Mγ4 (see above).

Methylation at the G-T-C-G-A-C Sδ site, located 67 bp upstream from the first codon of the δ gene (29), was tested by *Eco*RI–*Sal* I double-digestion. The Aβ site, located ≈1.5 kbp 5′ to the β gene (12), was tested by *Pst* I–*Aos* II double-digestion.

DNA from 8- to 12-wk FL and AM erythroblasts was always ≈50% methylated at the Mβ1 site and at least 50% methylated at the Mβ2 site (methylation at Mβ2 cannot be studied by the β-cDNA probe in the portion of DNA cleavage at Mβ1). Both sites were fully methylated in control cells as well as in 6-wk FL synthesizing virtually only embryonic chains (preliminary results not shown here). The Mδ site was found to be methylated in all tested cells. Conversely, the Sδ site was always unmethylated, except in fibroblast DNA (data not shown). The Aβ site was slightly undermethylated in both 12-wk FL and AM erythroblast DNA, whereas it was fully methylated in A-WBC and nonerythroid AM cells.

DISCUSSION

The globin chain synthesis pattern and the extent of DNA methylation within embryonic, fetal, and adult β-like globin gene domains were explored in purified erythroblasts from human YS, FL, or AM. In all ontogenic phases a strong direct correlation exists between hypomethylation in the close flanking sequences of globin genes and their expression (see Fig. 4).

The ε gene, although unmethylated in 6-wk YS and FL erythroblasts (i.e., at the time of its peak expression), was both methylated and fully suppressed starting from 10 wk of gestation. Conversely, the γ gene region (Mγ1–3 sites) is meth-

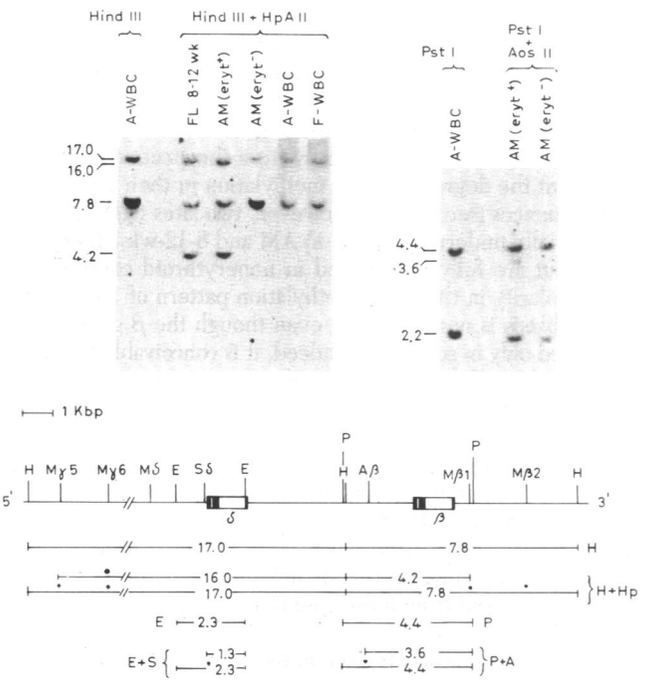

FIG. 3. CpG methylation pattern in human adult δ- and β-globin gene domain in ≥90% purified erythroblasts from FL and AM (see also legend to Fig. 1). A, *Aos* II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa* II; P, *Pst* I; S, *Sal* I. Asterisks indicate methylated cleavage sites.

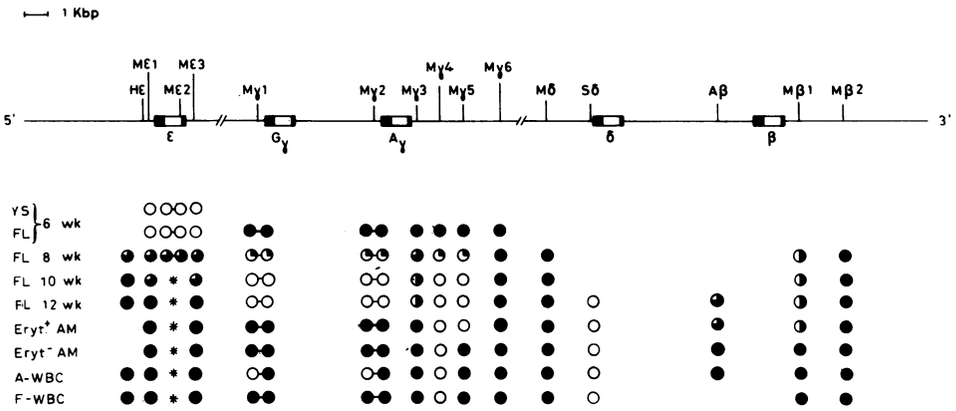

FIG. 4. DNA methylation pattern in human ε-, γ-, δβ-globin gene regions in ≥90% purified erythroblasts from YS, FL, and AM (see also legend to Fig. 1). The corresponding pattern of β-like globin chains synthesis (see also *Results*): 6-wk FL and YS erythroblasts produced virtually only ε globin; those from 8- to 10-wk FL produced mostly γ chains with residual ε- and initial β-globin synthesis; those from 12-wk FL produced largely γ chains with a minor aliquot of β globin; and AM erythroblasts (AM eryt+) produced almost exclusively β chains. M, *Msp* I/*Hpa* II restriction endonuclease recognition site; H, *Hha* I site; S, *Sal* I site; A, *Aos* II site. ●, Fully methylated; ◕, 70–90% methylated; ◑, 40–60% methylated; ◔, 10–20% methylated; ○, unmethylated. ○○ represents the 1st and 2nd cytosine in single C-C-G-G sites, whereby methylation of both ^{m}C-C-G-G and C-^{m}C-G-G types may concomitantly occur. The Mε2 site is probably polymorphic, because it has not been reported in the ε-gene sequence (28). This site is hence marked by an asterisk when lack of cleavage may have been caused by either its methylation or its absence.

ylated immediately prior to the ε → γ switch and becomes unmethylated in >10-wk FL erythroblasts (i.e., at peak gene expression), but it is fully methylated in AM erythroblasts when γ genes are virtually not expressed. In the ε → γ mid-switch period, the relative degree of methylation in both ε- and γ-gene domains appears to parallel the relative synthetic level of the corresponding globin. In DNA from 8- to 10-wk FL erythroblasts synthesizing ≈10% ε chains, residual undermethylation was still detectable 5′ and 3′ to the ε gene, whereas γ genes were only slightly methylated at all explored sites. This partial demethylation pattern may reflect asynchronous switching of different erythroblastic clones or heterogeneity in DNA methylation between homologous chromosomes within the same cells (or both).

Admittedly, results obtained in the δβ domain are less clear-cut than in the ε and γ regions. Position of the restriction endonuclease recognition sequences containing CpG dinucleotides is highly unfavorable in the β-gene region. Indeed, they are generally located too far away from the structural genes to represent the degree of DNA methylation in their close flanking sequences (see Fig. 4). However, two sites (Aβ and Mβ1) are partially undermethylated in AM and 8-12-wk FL erythroblasts but are fully methylated in nonerythroid control cells. The similarity in the DNA methylation pattern of FL and AM erythroblasts is not surprising even though the β gene is fully expressed only in adult cells. Indeed, it is conceivable that DNA methylation reflects an active conformation of a transcriptional unit but is not involved in the quantitative regulation of gene expression.

At the level of the δ-gene region, in hemopoietic cells no correlation apparently exists between DNA methylation and transcriptional activity. However, the δ gene is the only globin gene that, even when fully active, is transcribed at very low levels (30). This suggests that its activity is controlled via mechanism(s) different from those operating at the level of the other β-like globin genes.

These results clearly reflect on the role of DNA methylation in the control of gene expression in higher eukaryotes. In this regard, our studies show that DNA undermethylation may be a prerequisite to allow activation of a gene cluster coordinately expressed during human development. In particular, they demonstrate a three-step methylation process at the level of γ genes, which entails embryonic methylation and repression, fetal undermethylation and activation, and adult methylation and suppression. A developmental correlation between DNA methylation and gene activation was previously reported for the globin gene system in chicken embryos (31). In this case, undermethylation of the active globin gene domains correlated with specific rearrangement in their chromatin configuration, as indicated by both DNase I hypersensitivity and selective binding to the nucleosomes of proteins of "high-mobility group" type. Both undermethylation and DNase I hypersensitivity apparently precede and do not follow globin gene transcription (11). In humans, preliminary evidence by Groudine and coworkers (32) indicates that both γ- and β-gene domains are DNase I hypersensitive in FL, whereas only the β gene shows an active chromatin configuration in AM. These findings are strictly in line with our data, showing undermethylation of both γ and β domains in FL, but only of the latter domain in the AM. By extrapolation from the avian model, it may be inferred that both DNase I hypersensitivity and undermethylation are prerequisites for, rather than effects of, globin gene activation in human ontogenic development. This concept may lead to a model whereby the chromatin configuration in human ontogeny is gradually modified at the level of the non-α gene cluster, thus leading to a 5′ → 3′ globin gene activation in a balanced fashion (see ref. 22).

Our results may also shed light on the expression of the Hb synthesis program in the erythropoietic differentiation pathway. Indeed, the undermethylation of γ and β domains in fetal and adult life is largely restricted to the erythroblastic lineage, whereas it is not expressed in other hemopoietic lineages (compare results on erythroblast-deprived AM cells, F-WBC, and A-WBC). This suggests that the globin gene undermethylation pattern, and possibly the concomitant alterations in chromatin configuration, is not present in hemopoietic stem cells. Conceivably, it is acquired at the level of erythroid progenitors (BFU-E and CFU-E) or early erythroblasts. The exact cytogenetic step associated with these changes might be elucidated by analysis of purified populations of erythroid progenitors or early erythroblasts. This approach might even elucidate whether in cytogeny undermethylation precedes rearrangements in chromatin configuration or vice versa.

Cellular studies on human Hb switching (21, 33–35) suggest

that a single population of erythroid progenitors is gradually reprogramed from an embryonic to a fetal and finally an adult Hb synthesis program. In molecular terms, "reprograming" may imply that, in the early erythropoietic pathway, a different "choice" is made for an alternative chromatin configuration or pattern of undermethylation (or both), which in turn allows transcription of selected globin genes. The intrinsic or extrinsic mechanism(s) (or both) underlying the time-dependent reprograming of erythroid progenitors is still unknown.

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