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# A physiological delay in human fetal hemoglobin switching is associated with specific globin DNA hypomethylation

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The human fetal-to-adult globin switch normally occurs on a fixed schedule, beginning at 32–34 weeks gestation, and recent studies have suggested an association between this developmental inactivation of the fetal ($\gamma$) globin genes and the appearance of methylation within and around these genes. We have studied a population of infants in whom this switch does not occur before birth (infants of diabetic mothers, IDM) and examined the patterns of methylation surrounding their active $\gamma$-globin genes, in comparison to the $\gamma$-globin genes of age-matched controls who have switched their pattern of globin gene expression on schedule. All genomic DNA samples from infants with delays in the globin switch demonstrated extensive hypomethylation in the region of the $\gamma$-globin genes, comparable to that found in the genomes of fetuses of less than 21 weeks gestation. DNA from the erythroid cells of infants of 32–40 weeks gestation had no detectable hypomethylation in the $\gamma$-globin region. These findings support the concept that hypomethylation is an accurate developmental marker of globin gene switching, and suggest that globin gene expression in IDM may be arrested at an early preswitch stage.

DNA hypomethylation; Gene expression; Globin gene

## 1. INTRODUCTION

Recent studies have suggested an association between human developmental globin gene switching and globin gene expression, and the methylation state of the globin genes [1,2]. To determine whether such a correlation exists, we have compared globin gene methylation in normal infants with age-matched infants of diabetic mothers (IDM), who do not undergo the normally fixed developmental gene switch from $\gamma$-globin to $\beta$-globin synthesis on schedule [3–6]. At term, $\beta$-globin chain synthesis in erythrocytes from cord blood of these infants remains 10–20% of total non-$\alpha$-globin, the range normally found at less than 28 weeks of gestation. We find a consistent difference in genomic globin methylation patterns between IDM and normal infants of the same gestational ages, suggesting that the delayed gene switch may be associated with physical changes at the genomic level.

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## 2. MATERIALS AND METHODS

### 2.1. *Sample collection and preparation*

Cord blood was collected, erythroblasts quantitated, and mononuclear cells separated as described [1].

### 2.2. *Genomic DNA analysis*

High molecular mass DNA was extracted from the nuclei and prepared for restriction endonuclease digestion as described by Bell et al. [7]. 10–15 $\mu$g DNA were digested with *Eco*RI and *Hpa*II or *Msp*I, using reaction conditions suggested by the manufacturer (New England Biolabs, Beverly, MA), electrophoresed on a 1% agarose gel and transferred to nitrocellulose by the method of Southern [8]. The human $\gamma$-globin-specific probe used in the hybridizations was a 0.9 kb *Bam*HI/*Eco*RI fragment of the human $\gamma$-globin gene, containing the large intervening sequence (IVS) of the gene (kindly provided by Dr S. Orkin). The probe was radiolabelled using the oligonucleotide primer method [9]. Hybridizations were carried out in 50% formamide at 42°C, and stringent conditions were

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used in washing the blots. After autoradiography, the intensity of the bands on the film was quantitated by densitometry. The genomic DNA fragments detected by the probe vary in length (7.2, 2.7, 1.5 kb) but all contain the same amount of sequence capable of hybridizing with the probe. Methylation in the $\gamma$-globin gene locus of DNA from IDM and normal term infants was assessed using the restriction enzyme *Hpa*II, which recognizes the sequence CCGG, but will not cleave at this sequence if the internal C residue is methylated. A double digestion of genomic DNA with *Eco*RI and *Hpa*II will yield two fragments of 7.2 and 2.7 kb capable of hybridizing with a $\gamma$ gene IVS probe if the regions upstream of the $\gamma$ genes are methylated and *Hpa*II is unable to cut, but will yield a single band of 1.5 kb if the genes are unmethylated. It was therefore possible to determine semi-quantitatively the fraction of $\gamma$-globin gene DNA that is unmethylated by comparing the intensity of the (unmethylated) 1.5 kb bands to the combined intensities of the (methylated) 7.2 and 2.7 kb bands.

To examine methylation patterns surrounding the insulin gene, genomic DNA was digested with *Pvu*II and *Hpa*II or *Msp*I. Following electrophoresis and transfer, the immobilized DNA was hybridized to an insulin-specific probe, pHI-1 (a 0.23 kb long, *Ava*II to *Rsa*I fragment of a cDNA clone, generously provided by Lydia Villa-Kamaroff).

### 2.3. *Globin synthesis*

Globin synthetic ratios were determined by [$^3$H]leucine labelling and column chromatography as in [3].

## 3. RESULTS

Erythroid precursors from these IDM synthesized a significantly higher percentage of $\gamma$-globin (mean 88 ± 5.8% of total non-$\alpha$-globin) than did cells from normal infants (mean 58 ± 5.2% of total non-$\alpha$-globin) (table 1), consistent with previous data [3,5,6].

The mononuclear cell fraction from cord blood consisted of a mixture of erythroblasts and lymphocytes. Centrifugation density techniques which have been effective for enrichment of erythroblasts from bone marrow mononuclear cell populations were not successful in enriching erythroblasts from cord blood. We therefore compared cord blood from IDM and normal infants with varying numbers of erythroblasts to determine whether any cells within the mononuclear cell population had hypomethylation of the $\gamma$-globin genes, and to evaluate whether a correlation existed with the proportion of erythroblasts in the samples.

Fig.1 shows autoradiograms of genomic DNA from cord blood of normal term infants and IDM, and from K562 cells (which express no $\beta$-globin and have an unmethylated pattern in this region). Mononuclear cell populations from normal infants with percentages of erythroblasts ranging from 5 to 49% (lanes A2,A3,C3) displayed no evidence of a (unmethylated) 1.5 kb band (limits of detectability ~5%). In contrast, mononuclear cell populations from all IDM studied, with erythroblast counts ranging from 5 to 60%, demonstrated hypomethylation in the $\gamma$-globin gene region. The degree of hypomethylation correlated well with the proportion of erythroblasts in the mononuclear population (coefficient determinant = 0.928, table 1). For example, in the IDM with the lowest erythroblast fraction (5%, lane A5), the percentage of unmethylated $\gamma$-globin genes was 7%; an IDM with 40% erythroblasts had 32% of its $\gamma$-globin genes unmethylated (lane C4); whereas an IDM with 60% erythroblasts had approx. 50%

Table 1

Correlation of degree of methylation with percent erythroblasts

| Gestation | % $\beta$-globin[a] | % erythroblasts[b] | % methylation[c] | Lane[d] |
|---|---|---|---|---|
| Term | 49 | 12 | <5 | A2 |
| | 61 | 40 | <5 | A3 |
| | 60 | 18 | <5 | – |
| | 56 | 10 | <5 | C3 |
| | 58 | 20 | <5 | – |
| 32–34 weeks | 64 | 28 | <5 | B2 |
| 33 weeks | 56 | 25 | <5 | D4 |
| Fetal liver | 79 | 90 | 18 | A9 |
| Term IDM | 89 | 20 | 14 | A4 |
| | 88 | 5 | 7 | A5 |
| | 95 | 32 | 35 | A6 |
| | 79 | 60 | 50 | A8 |
| | 93 | 18 | 15 | A10 |
| | 96 | 28 | 24 | B3 |
| | 88 | 20 | 21 | B4 |
| | 82 | 30 | – | B5 |
| | 85 | 40 | 32 | C4 |
| | 91 | 42 | 39 | C5 |
| | 91 | 30 | 22 | D2 |
| | 79 | 25 | 21 | D3 |
| 31 weeks IDM | 94 | 10 | 12 | A7 |

[a] $\gamma$-Globin synthesized as a fraction of total non-$\alpha$-globin
[b] Reticulocytes as a fraction of the mononuclear cells in the sample
[c] Percentage of $\gamma$-globin genes with methylation, calculated as described in text
[d] Corresponding lane of gels shown in fig.1

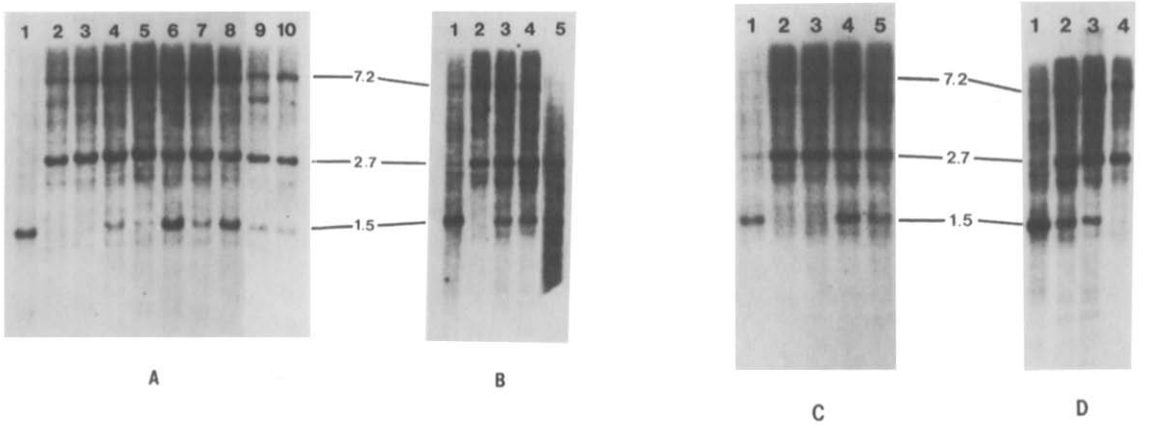


Fig.1. Southern blot analysis of cellular DNA methylation in the region of the $\gamma$-globin genes. The positions of the $\gamma$-globin restriction fragments of 7.2, 2.7 and 1.5 kb are marked. K562: lanes A1, B2, C1 and D1. Adult lymphocytes: lane C2. Normal term infants: lanes C3, A2, A3. Premature infants of 32–34 weeks gestation: lane B2; of 33 weeks gestation: lane D4. Fetal liver (21 weeks gestation): lane A9. Infants of diabetic mothers: lanes A4–A8, A10, B3–B5, C4, C5, D2, D3.

hypomethylation of DNA in the same region (lane A8).

DNA methylation patterns surrounding a gene which is not expressed in erythroid cells, the insulin gene, were examined in genomic DNA from normal infants and from IDM. Digestion of human cellular DNA with *Pvu*II and *Hpa*II and hybridization with a probe specific for the 5′ end of the insulin gene produces a 1.6 kb band if fully methylated, but yields two bands of 0.59 and 0.54 kb when unmethylated [10,11]. The results in fig.2 demonstrate that this inactive gene remains fully methylated in DNA from both normal infants and IDM.

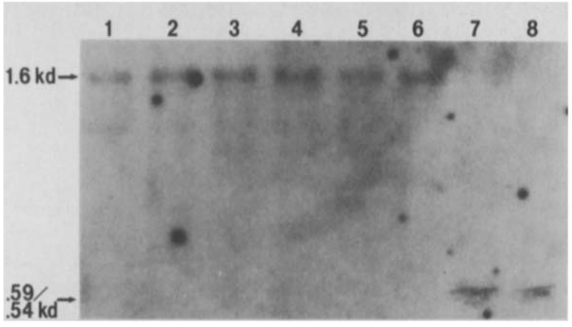


Fig.2. Southern blot analysis of cellular DNA methylation in the region of the insulin gene. The positions of insulin-specific restriction fragments of 1.6, 0.59 and 0.54 kb are indicated. The DNA samples analyzed in this autoradiogram correspond to those used in fig.1, as follows: Normal term infants: lanes 1 (fig.1, A2) and 2 (fig.1, A3). Infants of diabetic mothers: lanes 3 (fig.1, A5), 4 (fig.1, A8), 5 (fig.1, A7) and 6 (fig.1, C4). Lanes 7,8 are the same DNA preparations as used for lanes 1,6, respectively, but were digested with *Pvu*II and *Msp*I (methylation insensitive) to show the restriction pattern expected if methylation were not present in the DNA digested with *Pvu*II and *Hpa*II.

## 4. DISCUSSION

Infants of diabetic mothers experience a delay in the normal developmental switch from $\gamma$- to $\beta$-globin synthesis in utero, but the molecular mechanisms underlying this delay are not yet completely established. The amount of $\gamma$-globin in IDM erythrocytes remains high at term and in vitro globin synthetic studies in mature progenitors show continued production of $\gamma$-globin as the predominant non-$\alpha$-globin species [3–6]. These synthesis data imply that the proportion of mRNA coding for $\gamma$-globin is higher in erythroid cells from IDM than from normal infants. Differential instability of $\gamma$ and $\beta$ mRNA in IDM erythroid cells should manifest itself as changing ratios of $\gamma$- to $\beta$-globin synthesized with time. In a previous study, we found no such change in $\gamma$ to $\beta$ chain synthesis in IDM or normal infants, once balanced $\alpha$ to non-$\alpha$ chain synthesis was achieved [5]. This was consistent with the work of other investigators, who have found stable and equivalent half-lives of $\gamma$ and $\beta$ mRNAs in cord blood reticulocytes [12,13]. The persistence of higher relative transcriptional activity from the $\gamma$-globin

genes in IDM would explain our findings; however, we have not been able to study directly the transcriptional activity of the globin genes in cord blood samples due to the limited number of nucleated erythroid cells available. We therefore examined methylation patterns surrounding the $\gamma$-globin genes in IDM for evidence at that level of a developmental delay.

Methylation of the fetal globin genes in erythroid cells occurs during the course of development from fetus to adult. Van der Ploeg and Flavell have shown that erythroid tissues from fetuses early in gestation display areas of hypomethylation around the $\gamma$-globin genes, while non-erythroid tissues, with presumably inactive globin genes, appear to have these genes fully methylated. Erythroid tissues from adults (no longer expressing $\gamma$-globin mRNA) are also fully methylated [1,2]. Ley and colleagues postulated that demethylation of the $\gamma$-globin genes in the adult is necessary, although not solely sufficient, for reactivation of these genes, as hypomethylation has been observed in association with increased $\gamma$-globin expression under conditions of erythropoietic stress [11,14,15]. However, an obligatory relationship has not been definitively established between hypomethylation at this particular site and $\gamma$-globin gene expression [11,16–19]. Indeed, it is noteworthy that the $\gamma$-globin genes of the normal term infants remain active (as shown by their synthesis of $\gamma$-globin as a mean 56% of non-$\alpha$-globin), yet their genomes display full methylation of identifiable sites surrounding their $\gamma$-globin genes.

Irrespective of whether it is directly associated with gene activation, the methylation state of the $\gamma$-globin genes may still be a marker for their developmental stage. Our findings that the genomes of mononuclear cells from cord blood of IDM, a group of infants who continue at term to synthesize $\gamma$-globin at early fetal levels, contain a population of $\gamma$-globin genes which remain unmethylated suggests that these $\gamma$-globin genes from term IDM may have been arrested at an earlier developmental stage. The fraction of unmethylated $\gamma$-globin genes correlated with the proportion of erythroblasts in the IDM cord blood samples. This unmethylated fraction was not found in cord blood from normal term infants or even in cord blood from premature infants immediately before the globin switch normally occurs, but was observed in erythroid cells from fetal liver. Because cord blood samples from IDM containing predominantly lymphocytes showed substantially less hypomethylation of the $\gamma$ genes, the presence of unmethylated $\gamma$-globin genes appeared specific for erythroid cells in the IDM, and was not a general feature of non-erythroid cells. Analysis of methylation patterns around another gene, the insulin gene, which is not expressed in erythroid cells or lymphoid cells, provides further evidence for the selectivity of the hypomethylation of $\gamma$-globin genes observed in erythroid cells from IDM. The insulin gene, which maps to the same chromosome as the $\gamma$-globin gene, remains fully methylated in DNA from both normal infants and from IDM.

This distinct difference at the genomic level between the erythroid $\gamma$-globin genes of IDM and normal infants may provide a clue to the cause of the persistence of $\gamma$-globin synthesis in this population. The $\gamma$-globin genes from these IDM resemble those observed in fetal liver at 21 weeks gestation, suggesting that globin gene expression in IDM may be arrested at the pre-switch stage. Elucidation of those factors in the maternal diabetic intrauterine milieu responsible for this developmental arrest could theoretically prove useful in determining the cause of the many delayed developmental processes identified in the IDM population [20], and, paradoxically, might have beneficial therapeutic implications for the $\beta$ chain hemoglobinopathies and thalassemias.

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