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A dominant goal of research focused on the nonclassical human leukocyte antigen G (HLA-G) gene is to understand the molecular mechanism involved in its limited expression. In the present report, we examined DNA methylation as a potential regulatory mechanism of HLA-G transcription in two cell types of the adult lymphomyeloid lineage: CD2$^+$ lymphocytes express several mRNA isoforms while transcripts are undetectable in CD34$^+$ hematopoietic cells. The methylation status of 63 CpG sites in the promoter and in the 5′ CpG island was established using bisulfite-treated genomic DNA sequencing. Methylation was first analyzed by the direct sequencing of bisulfite-treated and amplified products. The general patterns of CpG methylation in the 5′ part of the gene were found to be similar for CD34$^+$ cells and CD2$^+$ lymphocytes: the distribution of methylation was not uniform across the 63 CpG sites. In the promoter region, both CpG dinucleotides were partially or fully methylated whereas in the CpG island, several CpG sites were totally demethylated. Unexpectedly, in HLA-G positive CD2$^+$ lymphocytes, a great number of CpG dinucleotides displayed a higher frequency of methylation relative to that found in CD34$^+$ cells. However, the sequence analysis of cloned products revealed that the molecules have different methylation patterns which suggests that the HLA-G gene is differentially expressed in CD2$^+$ cells. Our results suggest that methylation is not the sole mechanism that achieves the repression of HLA-G transcription in immature CD34$^+$ cells.

Key words: HLA-G gene – transcriptional regulation – methylation – hematopoietic cell
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The human leukocyte antigen G (HLA-G) gene is a nonclassical class I gene which codes for the only detected major histocompatibility complex antigen in fetal placental tissues: HLA-G is expressed at high levels in early gestation tissues as cytotrophoblast populations, and transcripts and proteins decline as the pregnancy progresses to term (1, 2). Several alternative HLA-G transcripts including soluble isoforms are expressed in placenta (3–5). The corresponding proteins were identified as an array of 37 to 39 kDa proteins and a secreted form can be found in supernatants of cultured cytotrophoblasts and cell lines such as JEG-3 (1, 3). HLA-G proteins may play a critical role in maternal-placental immune response. Most likely, this role is to protect fetal cells from lysis by natural killer cells and large granular lymphocytes (6, 7), and possibly to present viral antigens in virally infected placenta cells (8). HLA-G transcriptional isoforms have been detected in several human tissues and in peripheral blood leukocytes (9). However, expression of these different isoforms varies according the cell type and tissue. For example, several HLA-G mRNA isoforms are expressed in lymphocytes (5, 10, 11) and in monocytes (12) whereas the immature hematopoietic cells and polynuclear cells have an HLA-G negative phenotype. The functions of HLA-G products in leukocytes have not been established yet.

Both mechanistically and functionally, the limited expression of the HLA-G gene poses interesting

problems. HLA class I gene regulation is generally mediated by a multitude of *trans*-acting factors which interact with *cis*-regulating elements. However, several regulatory elements have been found to be lacking or altered in the HLA-G promoter: i) the palindromic sequence related to enhancer A, which interacts with the proteins of the KBF/NFkB/c-rel family, is lacking. ii) The interferon (IFN) consensus sequence (ICS) is modified, which suggests that either this gene cannot be IFN inducible or that it possesses other IFN regulatory elements located outside the promoter region (12). iii) Site α, related to the cAMP-responsive element, and the enhancer B motif are also altered in HLA-G (13). Moreover, a recent report has demonstrated the presence of a positive regulatory element 1.1 kb from the beginning of the first exon. This element may be important for efficient transcription of HLA-G gene in extraembryonic tissues in vivo (14).

It appears that the transcriptional regulation of class I genes is influenced by DNA methylation. In mammalian cells, methylation occurs at the cytosine residues on the CpG dinucleotide sequences. Many genes contain DNA segments of 0.5 to 3 kb with a high G+C content. These DNA segments, designated as CpG islands, cover the transcription initiation in all housekeeping genes and widely expressed genes. Forty percent of the genes with a tissue or limited expression are also associated with CpG islands. But in the latter cases, their position is not biased toward the 5′ end of the transcription unit. Identified CpG islands are also associated with the 5′ end of HLA class I genes (15, 16). Several studies have tried to establish a relationship between the level of CpG island methylation in the HLA-G gene and its transcriptional activity. Heretofore, methylation changes in the HLA-G gene were mainly detected and localized by using Southern blot analyses with methylation-sensitive restriction enzymes. The trophoblast-derived human cell line Jar, which expresses no any HLA class I genes except HLA-E, exhibited methylated CpG islands in all HLA class I loci other than HLA-E (17). After a 5′ azacytidine demethylating agent treatment followed by cell cloning, these cells recover the HLA-G gene and other HLA class I genes transcription, and cell surface expression. The methylation status and transcriptional expression of HLA-G gene were also analyzed in human trophoblast cells from term placenta. Whereas HLA-G is undermethylated in both cytotrophoblast and syncytiotrophoblast, it is clearly transcribed in the former but is minimally transcribed in the latter subpopulation (18). The authors suggest that the downregulation of HLA-G gene expression in the syncytiotrophoblast is unlikely to be caused by DNA methylation.

CpG methylation and HLA-G gene transcription

The same authors showed that male germ cells differed from somatic cells in the methylation of the HLA-G gene and suggested that the absence of HLA-G transcriptional activity in peripheral blood leukocytes might be due to de novo methylation occurring in the course of development (19). The transcriptional activity of the HLA-G gene has been demonstrated recently in lymphocytes (10, 11) and in monocytes (12). Using methylation-sensitive endonucleases, only the 3′ extremity of CpG islands is analyzed, and detecting variable levels of methylation at different CpG sites is difficult. In this report, we questioned whether primary sites of demethylation are indeed critical for the transcriptional activity of the HLA-G gene in $CD2^+$ lymphocytes. To analyze the occurrence of methylation at both CpG sites in the 5′ part of the HLA-G gene, we applied PCR amplification of bisulfite-treated genomic DNA (20). We established and compared the methylation status of CpG dinucleotides close to the initiation transcription site in HLA-G negative hematopoietic $CD34^+$ cells and in HLA-G positive $CD2^+$ lymphocytes.

Material and methods

Hematopoietic cell isolation

The peripheral blood $CD34^+$ cells were purified from a mobilized apheresis product using a Ceprate LC CD34 kit (Cellpro, Merignac, France). The patient was mobilized with chemotherapy and a granulocyte-macrophage-colony stimulating factor to yield progenitor cells in the circulation. Mononuclear cells obtained by Ficoll gradient centrifugation (d=1.077) (Pharmacia Biotech, Uppsala, Sweden) were incubated with a biotin-labeled anti-CD34 antibody and the biotin-labeled target cells, retained on avidin-coated polyacrylamide beads, were released by gentle agitation. The enrichment rate of $CD34^+$ cells, estimated by flow cytometry, was at 86%.

Blood $CD2^+$ lymphocytes were obtained from a healthy donor. Mononuclear cells were separated from polymorphonuclear cells and erythrocytes by Ficoll gradient density centrifugation. After the removal of monocytes from mononuclear cells by an overnight plastic adherence, the fraction enriched with $CD2^+$ lymphocytes was obtained through immunoadsorption using magnetic Dynabeads coated with an anti-CD2 antibody (Dynal, Compiegne, France).The rate of lymphocytes enrichment, evaluated by flow cytometry, was greater than 90%.

Reverse transcription and amplification (RT-PCR)

Poly A^+ m-RNAs were purified using Dynabeads oligo (dT) 25 (Dynal). cDNAs were prepared by ran-

dom hexamer priming of 0.5 μg of poly A^+ mRNA. The first strand cDNA reaction was performed using Superscript II RNase H- reverse transcriptase (Life Technologies, Eragny, France). A hot-start PCR was carried out with half of the cDNA reaction mixture in a total volume of 50 μl using HLA-G-specific primers (9). The PCR was run at 94°C for 1 min, at 65°C for 1 min, and at 72°C for 1 min for 35 cycles, with a final extension of 10 min. In the same way, amplification of actin cDNA was performed to check RNA quality. 10 μl of each PCR reaction were analyzed following electrophoresis on a 1.2% agarose gel and staining with ethidium bromide.

To confirm the specificity of the RT-PCR, the amplified fragments were transferred onto positively charged Nylon membranes (Boehringer Mannheim, Meylan, France) in a 0.4 N NaOH solution using a vacuum blotter and fixed by backing at 120°C for 30 min. An HLA-G specific probe was labelled by PCR amplification using a nonradioactive nucleotide, digoxigenin-dUTP (Boehringer Mannheim) and an HLA-G genomic clone. Membranes were hybridized in 5 × SSC, 0.5% blocking reagent, 0.1% N lauroyl sarkosine, 0.02% SDS containing 0.5 ng/ml of digoxigenin-labelled DNA probe. The membranes were incubated overnight at 65°C. After stringent washing, the blots were incubated with antidigoxigenin antibodies conjugated to alkaline phosphatase. The digoxigenin-labelled nucleic acids were detected using a chemiluminescent substrate, CSPD (Boehringer Mannheim). The membranes were then exposed to X-ray film to record the chemiluminescent signal.

Bisulfite genomic modification

Genomic DNA from hematopoietic cells was prepared using a QiaAmp blood kit (Qiagen, Hilden, Germany). The bisulfite reaction was carried out on 10 μg of DNA under conditions whereby cytosine was converted stoichiometrically to uracil, while 5-methyl-cytosine remains nonreactive (21). Genomic DNA was linearized with the restriction enzyme *Hin*dIII, then phenol-extracted, alkali-denatured, neutralized, and precipitated. Denatured DNA was incubated in a total volume of 1.2 ml with freshly prepared 3.1 M sodium bisulfite/0.5 M hydroquinone (Sigma, St Quentin-Fallavier, France) pH 5.0 at 50°C for 20 h under mineral oil. The sample was removed from underneath the oil and the DNA was extracted from the solution with 20 μl of Geneclean glass milk (Bio 101, La Jolla, CA). After 3 washes, DNA was eluted with 100 μl of 10 mM Tris-HCL, 0.1 M EDTA, pH 7.5 at 50°C. Desulfonation was performed by adding NaOH to a final concentration of 0.3 M at room temperature for 10 min followed by the addition of ammonium acetate (pH 7.0) to a final concentration of 3 M and precipitated with 4 volumes of ethanol for 30 min at –20° C. The DNA pellet was resuspended in 100 μl of TE buffer and stored at –20°C.

PCR amplification

PCR amplifications were carried out to yield a fragment in which all uracyl and thymine residues were amplified as thymine and only 5-methylcytosine residues were amplified as cytosine. The two strands of bisulfite-treated duplex DNA are rendered noncomplementary by bisulfite treatment. We designed oligonucleotides primers to amplify the upper strand of the HLA-G gene on the assumption that the priming sequences do not contain CpG dinucleotides. Furthermore, to amplify HLA-G specific sequences, these primers were compared to all HLA class I gene and pseudogene sequences after theoretical cytosine conversion. Locations of primers specific for the HLA-G promoter and the HLA-G 5′ CpG island in bisulfite-reacted human DNA are shown in Figure 1. Primers GP328 (5′ AAG TGT ATA GGA GGA TAG GTA AGG 3′) and GP 776 (5′ AAC ACC ATA ACC ACC ATC CTT AAC 3′) were used to produce a 472-bp fragment from the HLA-G promoter. Primers GC 776 (5′ GTT AAG GAT GGT GGT TAT GGT GTT 3′) and GC 1496 (5′ CCA AAT CAC AAC CAA TCA TCC ACT 3′) were used to amplify a 744-bp fragment from the CpG island. Because of the "three-letter code", PCR amplification was performed by using a hot-start technique to reduce the number of nonspecific amplimers. Each amplification reaction was normally carried out on 5–10 μl of treated DNA for 35 cycles under standard conditions, with denaturation at 94°C, annealing at 55-60°C, and extension at 72°C. The amplified fragments were analyzed on an ethidium-bromide stained agarose gel to deter-

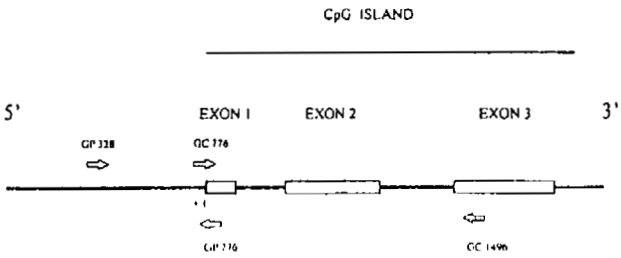

Figure 1. Schematic representation of the HLA-G gene 5' region according to ref 33. Exon 1 (position 783 to 855), exon 2 (position 985 to 1254) and exon 3 (position 1481-1756) are shown (box). Primers GP328 and GP776 are specific for the upper strand and amplify a 472 bp fragment from the promoter. Primers GC 776 and GC 1496 are specific for the upper strand and amplify a 744 bp fragment from the CpG island. +1: transcription start.

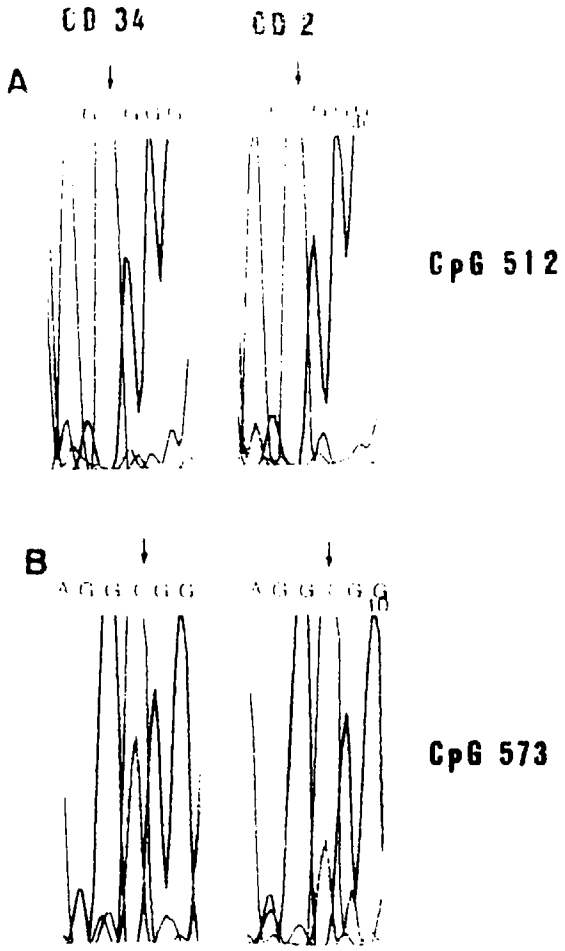

Figure 2. Direct sequencing of the amplified promoter region after bisulfite treatment. CD34⁺ cells (CD34) and CD2⁺ lymphocytes (CD2) DNAs were treated with sodium bisulfite, amplified and directly sequenced with a primer reading the upper strand from the 5′ to 3′ direction. On the electropherograms, unmethylated C appear as T while methylated C remain as C. A) CpG 512 is fully methylated in the two hematopoietic cells. B) A mixture of C and T is seen at the CpG 573: the T peak is more intense in CD34⁺ cells which indicates that the level of methylation at this CpG site is lower in CD34⁺ cells than in CD2⁺ lymphocytes. The CpG sites are numbered according to ref 33.

mine the quality and quantity of the desired PCR products.

Cloning

To clone the PCR fragments, blunt ends were produced by utilizing the 3′–5′ exonuclease activity of the Klenow fragment of DNA polymerase (Pharmacia Biotech). PCR fragments are then concomitantly phosphorylated by T4 polynucleotide kinase (Pharmacia Biotech), purified using glassmilk, then ligated to a blunt-ended PUC18 vector.

Sequencing

Recombinant plasmid DNAs and double-stranded PCR products were purified on Qiagen columns. Sequencing reactions were then carried out with M13 universal and reverse sequencing primers (for individual clones) and internal specific primers (for individual clones and PCR products) by using Taq FS DNA polymerase (Applied Biosystem, Foster City, CA) and dye terminator chemistry. The reaction products were then separated and analyzed on a Model 373DNA sequencer (Applied Biosystem).

Analysis of methylated CpG sites in genomic DNA

Bisulfite treatment converts preferentially unmethylated cytosine (C) residues to uracyl so that C residues are amplified as thymine (T) and only methylated cytosine (m^5c) residues appear as C in the PCR product. Direct sequencing of the PCR products provides the means of identifying DNA methylation in genomic DNA. Moreover, the format of the DNA sequences produced by automated DNA sequencers makes the determination of partially methylated cytosines possible in a certain target sequence (22). In our study, the position of each m^5C residue in the original DNA was identified by the presence of a C peak (or a G peak for the complementary strand) in the electropherogram. Double C/T (G/A) peaks consistent with a mixture of non-reactive m^5C and reactive C dem-

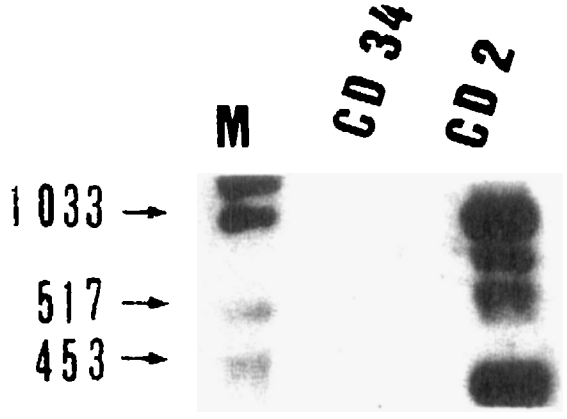

Figure 3. RT-PCR amplification results obtained with HLA-G specific primers on hematopoietic CD34⁺ cells (CD34) and CD2⁺ lymphocytes (CD2) analyzed by hybridization with the HLA-G locus specific probe. The bands detected in CD2⁺ lymphocytes are assigned to full length mRNA copy and alternatively spliced transcripts. M: DNA molecular weight markers VI, digoxigenin labeled (Boehringer Mannheim).

onstrated that there was a heterogeneity of methylation at the CpG sites in the DNAs. Typical sequencing electropherograms are shown in Figure 2. The comparison of double peaks in the two hematopoietic cells allowed us to evaluate the variation of methylation at each CpG site. Cloning and sequencing provided methylation maps of single DNA molecules. In every modification reaction performed during our study, all non-CpG cytosines appeared as thymine in the final sequence. This indicates that under our conditions, bisulfite modification was complete.

Results

HLA-G m RNA transcription

RT-PCR was performed on polyA$^+$ mRNA using primers derived from exon 2 and the 3′ untranslated region respectively (9). The products were analyzed by electrophoresis on agarose gel and hybridized with an HLA-G locus-specific probe. The hybridized Southern blot is shown on Figure 3. The RT-PCR of mRNA from CD34$^+$ cells did not show any hybridization signals. In CD2 lymphocytes, several bands corresponding to alternative HLA-G mRNA isoforms were observed.

Frequency of methylation at the CpG sites in PCR products

We analyzed and compared the methylation pattern of the HLA-G gene from hematopoietic CD34$^+$ cells and CD2$^+$ lymphocytes to determine whether there were cellular-specific differences in the extent of methylation. Direct sequencing of the PCR products after bisulfite treatment was used to evaluate the level of methylation at each CpG site in the genomic DNA.

Analysis in the HLA-G promoter region. The methylation status of 17 CpG sites was determined at first in a 424-pb region located just downstream from the transcription start site (Figure 1). The electropherograms obtained for the CpGs 512 and 573 are shown as example in Figure 2. In the 3′ part, this promoter region contains the regulatory elements of the class I genes which are altered in the HLA-G gene except for the CCAAT and TATA motifs. The results have been summarized in Table 1, panel A. No CpG site of the promoter region turned out to be unmethylated in the DNAs of the two hematopoietic cells. In CD34$^+$ cells, CpG 512 and CpG 523 dinucleotides were fully methylated and the other CpGs were par-

Table 1

General patterns of methylation in the HLA-G gene 5′ region. The level of methylation at CpG sites in CD2$^+$ lymphocytes is compared to that assessed in CD34$^+$ cells. The CpG sites are numbered according to ref 33. M: fully methylated CpG, P: partially methylated CpG, 0: unmethylated CpG, P↗: indicates a higher level of methylation, P↑: indicates a much higher level of methylation, P↘: indicates a lower level of methylation. A) Promoter: Enh A: enhancer A, Enh B: enhancer B, CAAT: CAAT box, TATA: TATA box, ATG: transcription start codon. B) Exon 1 and intron 1. Because of a nucleotide variation, CpG 915 do not exist in CD34$^+$ cells. C) Exon 2

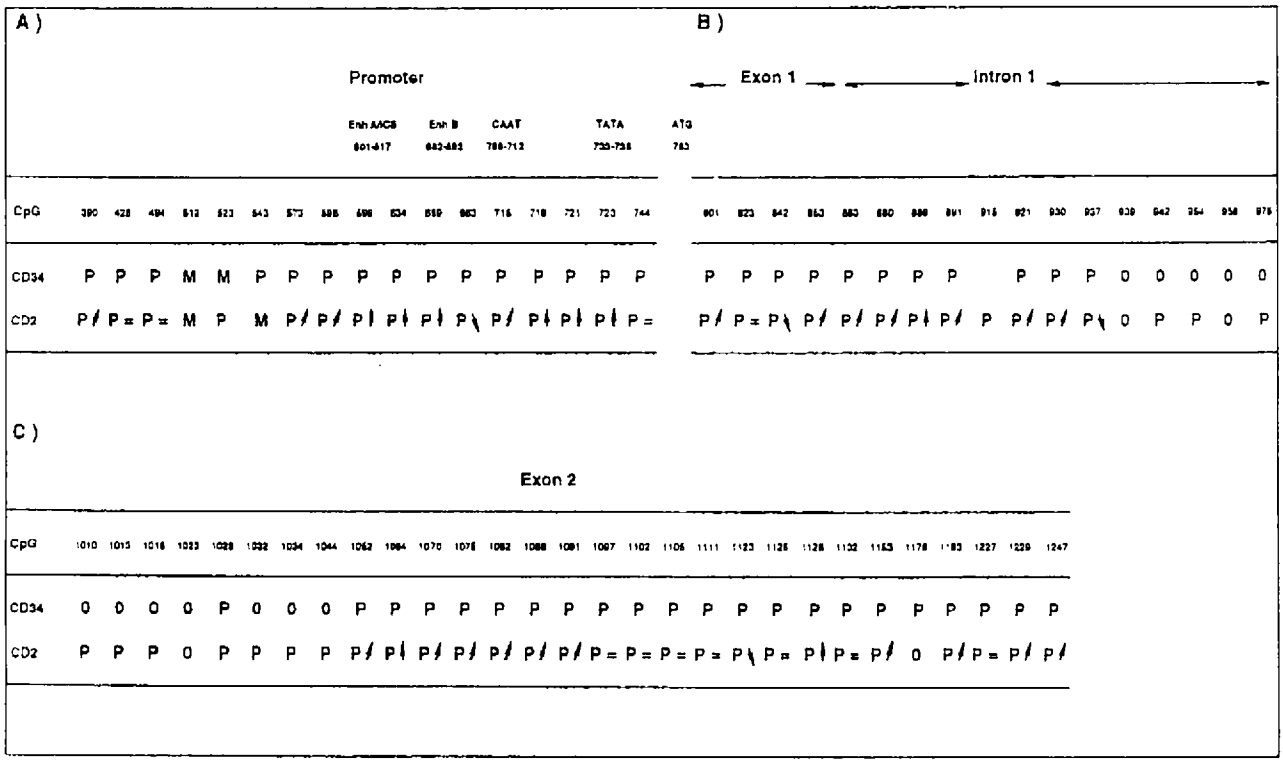

Table 2.
Distribution of methylated CpG sites from the HLA-G promoter in cloned PCR products derived from CD34⁺ cells (CD34) and CD2⁺ lymphocytes (CD2). The CpG sites are numbered according to ref 33. M: methylated CpG.

									Enh A/CS 601-617		Enh B 682-692		CAAT 708-712			TATA 733-738		
CpG	390	428	494	512	523	543	573	596	598	634	659	663	715	718	721	723	744	Total
Clones																		
CD34-1			M	M	M	M	M	M					M	M	M	M	M	11
CD34-2	M	M	M	M	M	M	M			M		M				M		10
CD34-3	M	M		M	M	M	M				M	M	M	M				10
CD34-4	M	M	M	M	M	M						M						7
CD34-5	M	M	M	M	M	M	M											7
CD34-6		M	M	M	M		M											5
CD34-7	M			M	M	M												4
Clones																		
CD2-1	M	M	M	M	M	M	M	M	M		M		M	M		M	M	14
CD2-2		M		M		M	M			M	M		M	M		M	M	10
CD2-3	M	M	M	M	M	M	M		M				M	M		M		11
CD2-4	M	M		M	M	M	M		M	M				M		M		10
CD2-5	M		M	M	M	M	M	M	M		M		M	M	M			12
CD2-6	M		M	M	M	M	M		M		M	M		M	M			11
CD2-7	M	M	M	M	M	M	M			M					M			9

tially methylated. In CD2⁺ lymphocytes, most CpG sites displayed a similar or increased amount of methylation relative to those of CD34⁺ cells. Only two CpG sites (CpG 523 and CpG 663) were less methylated in T lymphocyte than in CD34⁺ cells. The increase in methylation was particularly significant in the region containing the altered regulatory elements. CpG 512 and CpG 543 were fully methylated in CD2⁺ lymphocytes.

Analysis in the HLA-G CpG island. The HLA-G gene contains a 1070 bp CpG island which covers the first three exons, including the transcription start (15) We analyzed the methylation pattern of a 457 bp region in the 5′ CpG island upstream from the transcription initiation site. This region includes exon 1, intron 1 and exon 2 sequences and contains 46 CpG sites. The comparison of CpG methylation in the DNA taken from the two hematopoietic cells are presented in Table 1, panels B and C. No CpG site appeared to be fully methylated in the two DNAs. Unlike the promoter region, several CpG sites were not methylated: 12 and 4 CpGs were found to be unmethylated in CD34⁺ cells and CD2⁺ lymphocytes respectively. Several CpG dinucleotides, such as CpGs 939, 958, 1023, were demethylated in both DNAs. In CD34⁺ cells, unmethylated CpG dinucleotides are concentrated in the 3′ part of intron 1 and in the 5′ part of exon 2. In CD2⁺ lymphocytes, like the promoter region, most CpG sites displayed a similar or higher amount of methylation than those in CD34⁺ cells. Four CpG sites, scattered all over the CpG island (CpG 842, CpG 937, CpG 1123, CpG 1176), were found to be less methylated than those in CD34⁺ cells. CpG 1176 was the only CpG site which was methylated in CD34⁺ cells and totally demethylated in CD2⁺ lymphocytes.

Distribution of methylated CpG sites in the cloned PCR products

A sequence analysis of the cloned PCR products was used to test whether the C/T or G/A (complementary strand) patterns observed in DNAs from CD34⁺ cells and CD2⁺ lymphocytes represented a aggregate of methylated and non-methylated sequences.

Analysis in the HLA-G promoter. The detailed methylation status of the 17 CpG sites from the promoter region was established by sequence analysis of 7 randomly clones. The results, which are described in Table 2, revealed several features: i) no particular clone appeared exempt from methylation or was to-

tally methylated: the number of methylated CpG sites ranged from 4 to 11 and 9 to 14 out of 17 CpGs in CD34$^+$ cells and in CD2$^+$ lymphocytes respectively ii) the methylation pattern of individual clones was heterogeneous within each hematopoietic population, but the domain downstream from the enhancer A motif maintained a higher density of methylation: the seven CpG dinucleotides (CpG 390 to CpG 573) were methylated in two molecules from CD34$^+$ cells (clones CD34-2, CD34-5) and in three molecules from CD2$^+$ lymphocytes (clones CD2-1, CD2-3, CD2-7) iii) on average, the clones derived from CD2$^+$ lymphocytes were more methylated than the CD34$^+$ cells clones: there were 45.4% methylated sites in CD34$^+$ cells and 64.7% methylated sites in CD2$^+$ lymphocytes. The CpG sites in the 200 bp region close to the initiation site were unmethylated in three clones from CD34$^+$ cells (CD34-5, CD34-6, CD34-7) suggesting the presence of two different populations of molecules.

Analysis in the HLA-G CpG island. Methylation of 46 CpG sites was also analyzed in 6 randomly cloned PCR products from the CpG island. The methylation patterns obtained in exon 2 are shown on Table 3. The number of sites methylated in each clone derived from the CpG island was less than that observed in the promoter clones: on average, 2.9% of the sites in CD34$^+$ cells were methylated as compared to 9.8% in CD2$^+$ lymphocytes. In addition, the distribution of methylation was not uniform across the 46 CpG sites. The CD34$^+$ cells clones displayed no methylation at any CpG site in exon 1, intron 1 (data not shown). In one clone (CD34-1), we detected 6 methylated CpG sites (CpG 1082 to CpG 1102 and CpG 1193) localized in the 3′ part of exon 2. A single CpG (CpG 1247) located in the 3′ part of exon 2 was methylated in 2 clones (CD34-2, CD34-3). The three other clones were completely demethylated. In CD2$^+$ lymphocytes, the number of methylated CpG sites per clone ranged from 1 to 13 of 46 CpGs and a single molecule (CD2-1) was methylated in the domain close to the transcription initiation site at the CpGs 823 and 842 in exon 1 and CpGs 889, 891 and 958 in intron 1 (data not shown).

Discussion

Hematopoiesis is a highly regulated process by which a small population of self-renewing primitive progenitors generates distinct lineages of increasingly differentiated cells characterized by specific functional activities. Programmed active demethylation of CpG islands represents an essential step in the activation of tissue-specific genes during differentiation. The importance of DNA methylation is becoming apparent with the demonstration that mouse DNA methyltransferase knockout mutations are lethal during early development (23). Changes in genome methylation have been demonstrated during the in vitro development of MEL cells, a model system for blood cell differentiation (24). The demethylation of tissue specific immunoglobulin Kappa genes is probably also developmentally regulated, because these genes remain methylated in pre-B cells, even when transcribed and become demethylated only in B cells (25).

In this report, we raised the question whether methylation might influence HLA-G transcription during lymphoid differentiation. For this purpose, we have established and compared the methylation status of 63 CpG sites in the 5′ part of the gene in two distinct cells of the adult lymphomyeloid lineage

Table 3.
Distribution of methylated CpG sites from the HLA-G CpG island in cloned PCR products derived from CD34$^+$ cells (CD34) and CD2$^+$ lymphocytes (CD2). The CpG sites are numbered according to ref 33. M: methylated CpG.

CpG	1010	1013	1018	1023	1028	1032	1034	1044	1052	1064	1070	1075	1082	1088	1091	1097	1102	1105	1111	1123	1125	1128	1132	1153	1176	1193	1227	1229	1247	Total
Clones																														
CD34-1													M	M	M	M	M									M				6
CD34-2																													M	1
CD34-3																													M	1
CD34-4																														0
CD34-5																														0
CD34-6																														0
Clones																														
CD2-1			M					M		M	M			M										M			M	M		8
CD2-2	M	M														M	M					M	M			M			M	8
CD2-3																											M	M		2
CD2-4																						M							M	2
CD2-5																								M						1
CD2-6								M																						1

which differ in their HLA-G transcription level. The $CD2^+$ lymphocytes expressed several mRNA isoforms while no transcript was detectable in the immature hematopoietic $CD34^+$ cells. Analysis of PCR products showed that most CpG sites from $CD2^+$ lymphocytes tended to maintain or increase methylation frequency relative to those of the $CD34^+$ cells. In the same way, most clones derived from $CD2^+$ lymphocytes displayed a higher density of methylated CpG sites than the $CD34^+$ clones. These data suggest that developmentally regulated demethylation of HLA-G gene does not take place during hematopoiesis. However, we found that the analyzed clones have considerably different methylation patterns. Certain subsets of unmethylated CpG dinucleotides might be critical for transcriptional reactivation in $CD2^+$ subpopulations. It is correspondingly important that we consider to what extent methylation is a clonal property of the target population and to what extent it is an unregulated event. If we were able to measure weakly surface HLA-G expression using monoclonal antibodies on $CD2^+$ lymphocytes, it would be possible to separate them by cell sorting into categories of different expression levels and to analyze methylation patterns independently.

We have shown that the HLA-G gene is transcriptionally inactive in immature $CD34^+$ cells in spite of an hypomethylation of the 5′ part of the gene. As in the syncytiotrophoblast, the transcriptional regulation of the HLA-G gene is unlikely to be cause by DNA methylation. The question of the mechanism by which DNA methylation prevents the transcription of methylation sensitive genes remains to be overcome (26, 27). One proposed mechanism suggests that methylation of cytosines within the binding sites of transcription factors interferes directly with DNA-Protein interactions. A second proposed indirect mechanism is thought to involve protein mediators, such as MeCP, which bind in a non-sequence specific manner to methylated DNA, prevent transcription factors access, and thereby maintain the chromatin in a transcriptionally inactive state. A crucial determinant of the repression seems to be the density of methylated CpGs located near the promoter (27, 28). This system resembles a switch in which full activity or complete repression depends on the balance between methyl-CpG density and promoter strength. Christophe and Pichon (1995) have revived the debate about the relationship between DNA methylation and gene activity: they demonstrate that the repression of the bovine thyroglobulin gene is maintained in non-differentiating thyrocytes or in case an enhancer element is lacking (29). The authors suggest that the negative effect of DNA methylation is maintained only under conditions where the complete set of transcription factors are activated in fully differentiated thyrocytes. Such a reversal of the negative effect of DNA methylation in conditions where transcription is optimally stimulated had been also observed in the transcription of the rat α-actin gene (30) and of the *Xenopus* estrogen-inducible vitellogenin genes (31). In the same way, our data suggest that in fully differentiated $CD2^+$ lymphocytes, transcription may be activated by a complete maximally activated set of transcription factors which are not affected by CpG methylation in the promoter. Repression of the HLA-G gene in $CD34^+$ cells may be maintained by specific subsets of CpG sites which sustain high methylation frequencies in the promoter and/or by the absence of specific activating factors.

In mammalian cells, genes do not respond in an uniform manner to DNA methylation. An inverse relationship between the level of DNA methylation and gene activity is clearly apparent when considering typical housekeeping genes. In the event that methylation of CpG islands occurs, as in the case of one of the two X chromosomes in mammalian females, genes turn out to be inactive (32). No such clear pictures emerge from the data collected on genes that present a limited pattern of expression. In contrast to typical housekeeping genes, these genes exhibit individual patterns of methylation in the promoter. Most of the experiments aiming at the determination of a causative link between the methylation state of these genes and the fact that they show limited expression have produced conflicting results. Due to their overall CpG deficiency, especially in the 5′ end, these genes become only weakly methylated. CpG island sequences are found only in a minority of these genes and rarely in the 5′ extremity (16). One could speculate that classical class I genes are regulated by establishing a specific methylation pattern in the CpG island allowing for their continuous expression in somatic cells. The HLA-G gene that appears to be expressed irregularly (12, unpublished observations) can hardly be controlled by changes in DNA methylation, since this would involve replication of the DNA at each time. In hematopoietic cells, methylation does not appear to be the sole mechanism that achieves HLA-G transcriptional regulation but has to be interpreted in a general functional context and after a detailed structural and functional analysis of its transcriptional unit.

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