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Immunology

Methylation patterns of immunoglobulin genes in lymphoid cells: Correlation of expression and differentiation with undermethylation

(immunoglobulin μ, δ, $\gamma 2b$, α, κ, and λ gene expression)

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ABSTRACT **Different states of eukaryotic gene expression are often correlated with different levels of methylation of DNA sequences containing structural genes and their flanking regions. To assess the potential role of DNA methylation in the expression of immunoglobulin genes, which require complex rearrangements prior to expression, methylation patterns were examined in cell lines representing different stages of lymphocyte maturation. Methylation of the second cytosine in the sequence 5' C-C-G-G 3' was determined by using *Hpa* II/*Msp* I endonuclease digestion. Four C_H genes (C_μ, C_δ, $C_\gamma 2b$, and C_α), C_κ, V_κ, C_λ, and V_λ genes were analyzed. The results lead to the following conclusions: (*i*) transcribed immunoglobulin genes are undermethylated; (*ii*) the *C* gene allelic to an expressed *C* gene is always also undermethylated; and (*iii*) all immunoglobulin loci tend to become increasingly undermethylated as B cells mature.**

The details of the control of Ig gene expression have yet to be determined. Functional immunoglobulins are only produced after rearrangement of variable (V) and constant (C) genes (1, 2). It is not known what triggers rearrangement. The observation was made that C_κ genes in germline (unrearranged) configuration are DNase I sensitive in myeloma cells that express a rearranged κ gene (3). DNase I sensitivity is paralleled by transcription of the germline genes (4, 5). Thus, perhaps alterations in chromatin structure precede rearrangement. Because alterations in chromatin structure are often correlated with changes in DNA methylation (6, 7), the present analysis was undertaken to determine the methylation patterns of Ig genes in lymphoid cells that express different Ig genes.

METHODS

Hybridization probes for C_μ (8), C_δ (9), $C_\gamma 2b$ (10), C_α (11), C_κ (12), $V_\kappa 167$ (12), V_κ-*MOPC-21* (13), $V_\lambda 1$ (14), and $C_\lambda 1$ (14) were described as referenced and are illustrated in Fig. 2. $J_\kappa 3,4$ was subcloned from a germline JC_κ clone. It extends from a *Sau*3A site just 5' of $J_\kappa 3$ to a *Sau*3A site just 3' of $J_\kappa 4$ (unpublished data; see Fig. 2*E*). The lymphoid cell lines analyzed in this study are listed in Table 1. DNA was prepared from cell nuclei (3) (except for whole MOPC-21 and L cells; ref. 12). Restriction enzyme digestion and Southern blotting were done as described (12, 25).

RESULTS

Representative Southern blots are shown in Fig. 1. The methylation patterns are summarized in Fig. 2. The analyses were performed by digesting the DNA with a restriction enzyme recognizing a hexamer sequence (e.g., *Eco*RI). This enzyme was used alone, in combination with *Msp* I to determine all potential restriction sites, or with *Hpa* II to determine which of the sites were methylated and therefore insensitive to *Hpa* II digestion (Fig. 1). In some cases several other restriction enzyme combinations were also used to facilitate the mapping of *Hpa* II/*Msp* I sites (not shown).

The C_μ Gene. The lymphoid cell lines studied for C_μ methylation all produce μ protein, except MOPC-167, which produces α (see Table 1). Three *Msp* I sites are present in the vicinity of the $C_\mu 1$–$C_\mu 2$ exons (Fig. 2*A* and ref. 18). Restriction digestion at these sites results in two restriction fragments of 0.9 and 0.5 kb (Fig. 1). As shown in Fig. 1, the majority of the C_μ DNA of liver is not digested with *Hpa* II. However, in about 20% of the liver DNA, site B is unmethylated—i.e., is susceptible to *Hpa* II digestion—whereas sites A and C are methylated (see *Discussion*).

The DNA of the pre-B cell line 18-81M contains two differently rearranged C_μ genes (Fig. 1). The rearrangements are located 5' of the *Msp* I sites, which are considered here and do not complicate the analysis. All C_μ genes of 18-81M are undermethylated, but only about two-thirds of the DNA molecules are *Hpa* II restricted at all three sites (Fig. 1). The rest are cut only at site B, not at site A or C. This is probably not due to insufficient enzyme, because twice the amount of *Hpa* II led to exactly the same pattern (not shown).

The results for other cell types are summarized in Fig. 2*A*. In the case of the B lymphoma WEHI 279, 80% of the DNA molecules were *Hpa* II sensitive at all three sites. The C_μ genes of the myelomas MOPC-104E and PC3741 are completely unmethylated (Fig. 2), supporting previous observations with myelomas (33).

The C_δ Gene. The δ genes are generally methylated in liver and 18-81M, except for about 20% of the molecules of liver DNA, which are unmethylated at site A. As with the C_μ gene, the C_δ gene shows increasing undermethylation with lymphoid cell differentiation (compare Fig. 2 and Table 1).

The $C_\gamma 2b$ Genes. All *Hpa* II sites in the $C_\gamma 2b$ gene are completely methylated in liver, 18-18M, and WEHI 279 (Figs. 1 and 2). In the myelomas MOPC-104E, MOPC-21, and PC3741, which do not synthesize $\gamma 2b$, the $\gamma 2b$ gene is partially undermethylated (see Figs. 1 and 2*C*). Restriction fragments containing the $\gamma 2a$ gene are also seen in Fig. 1, due to cross-hybridization with the $\gamma 2b$ probe (34). The $\gamma 2a$ gene appears to be completely methylated in liver, 18-81M, and WEHI 279, but again in MOPC-104E it is partially undermethylated.

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Abbreviations: C, constant; H, heavy; D, diversity; J, joining; V, variable; L, light; kb, kilobase(s).

Table 1. Cells and tissues used in this study

Name	Cell type*	Ig produced†	Ig genes rearranged‡
BALB/c	Liver	—	—
NZB	Kidney	—	—
L cells	Fibroblast	—	—
18-81M	Pre-B	μ (16)	μ (16)
4-9-12-7-7	Pre-B hybridoma	μ (15)	μ (5)
15-58-6	Pre-B hybridoma	μ (15)	?§
15-23-3	Pre-B hybridoma	μ (15)	μ (5)
WEHI 279	B	μ, κ (17)	μ, κ (18)
MOPC-167	Myeloma	α, κ (19)	α, κ (12) $\gamma 2b$ deleted¶
MOPC-21	Myeloma	$\gamma 1$, κ (21)	$\gamma 1$, κ, λ (12, 13) μ deleted
MOPC-104E	Myeloma	μ, λ (21) κmRNA (22)	μ, λ, κ α deleted
PC3741	Myeloma	μ, κ (23)	μ, κ (24)
MOPC-41	Myeloma	κ (21)	κ (12)

References are shown in parentheses.

* The pre-hybridomas were derived by fusion of fetal liver cells with the myeloma Ag8653 (15); 4-9-12-7-7 is derived from C57BL mice. WEHI 279 arose in an inbred strain derived from (BALB/c × NZB)F_1 hybrids; the BALB/c gene codes for the C_μ allotype. PC3741 arose in an NZB mouse. All other cell lines are BALB/c derived.

† Protein synthesized as referenced.

‡ Rearrangement of Ig genes determined in this study, unpublished, and referenced. Ig genes that are not listed were found in germline context in the particular cells and genes shown in Fig. 2.

§ Presumably there is a variable-diversity-joining (*VDJ*) rearrangement 5′ of C_μ (nonanalyzed).

¶ The remaining μ gene of MOPC-167 does not seem to have undergone a joining-heavy (*JH*) gene rearrangement: a *Kpn* I site found just 5′ of JH1 in unrearranged DNA (20) is present in MOPC-167 (not shown).

The C_α Gene. Both *Msp* I sites in the C_α gene are methylated in liver, 18-81M, and WEHI 279, but they are partially unmethylated in the myelomas MOPC-21 and PC3741, which do not produce α (Figs. 1 and 2), and are completely unmethylated in MOPC-167, which produces α chains. Thus, the different cell types behave similarly with respect to the C_α and the $C_\gamma 2b$ genes.

The JC_κ Gene. Because there is only one *Msp* I site present in the JC_κ region, site A in Fig. 2*E*, located 5 kb 3′ of C_κ, we have also determined the methylation pattern of three *Hha* I sites (A′–C′) (28) located in the J_κ region. *Hha* I recognizes the sequence 5′ G-C-G-C 3′ and does not cut DNA where the 5′ C is methylated (35). All *Hha* I or *Msp* I sites are methylated in liver and 18-81M DNA (Figs. 1 and 2), except in about 10% of the liver DNA, which is unmethylated at sites B′ and A (Figs. 1 and 2). L cells show some undermethylation at site B′. In all of the other cell lines sites A′, B′, and C′ are completely unmethylated. Various methylation patterns were seen at site A (Figs. 1 and 2). Variable behavior of site A was also found by Mather and Perry (36) in several κ-producing cell lines; therefore, methylation at this site seems not tightly controlled.

The $V_\kappa 167$ Gene. The $V_\kappa 167$ gene is rearranged and expressed in the myeloma MOPC-167, where it is undermethylated at site A, 11.8 kb upstream of *V*, and site C, within the *V* gene. MOPC-167 seems to be haploid for κ and does not have a germline $V_\kappa 167$ gene (29). The $V_\kappa 167$ gene is not rearranged in the other lymphoid cells considered here. Nevertheless, it shows a somewhat varied methylation pattern at three of the four *Msp* I sites (Fig. 2*F*). The Southern blot with a $V_\kappa 167$ cDNA probe shows a weak band of 1.7 kb after *Eco*RI/*Hpa* II and *Eco*RI/*Msp* I digestion (Fig. 1*F*). This represents another V_κ gene that has partial homology with $V_\kappa 167$. Apparently this particular *Hpa* II site is constitutively unmethylated.

The $JC_\lambda 1$ and $JC_\lambda 4$ Genes. The *Msp* I sites in the vicinity of the $C_\lambda 1$ and $C_\lambda 4$ genes are completely methylated in liver, 18-

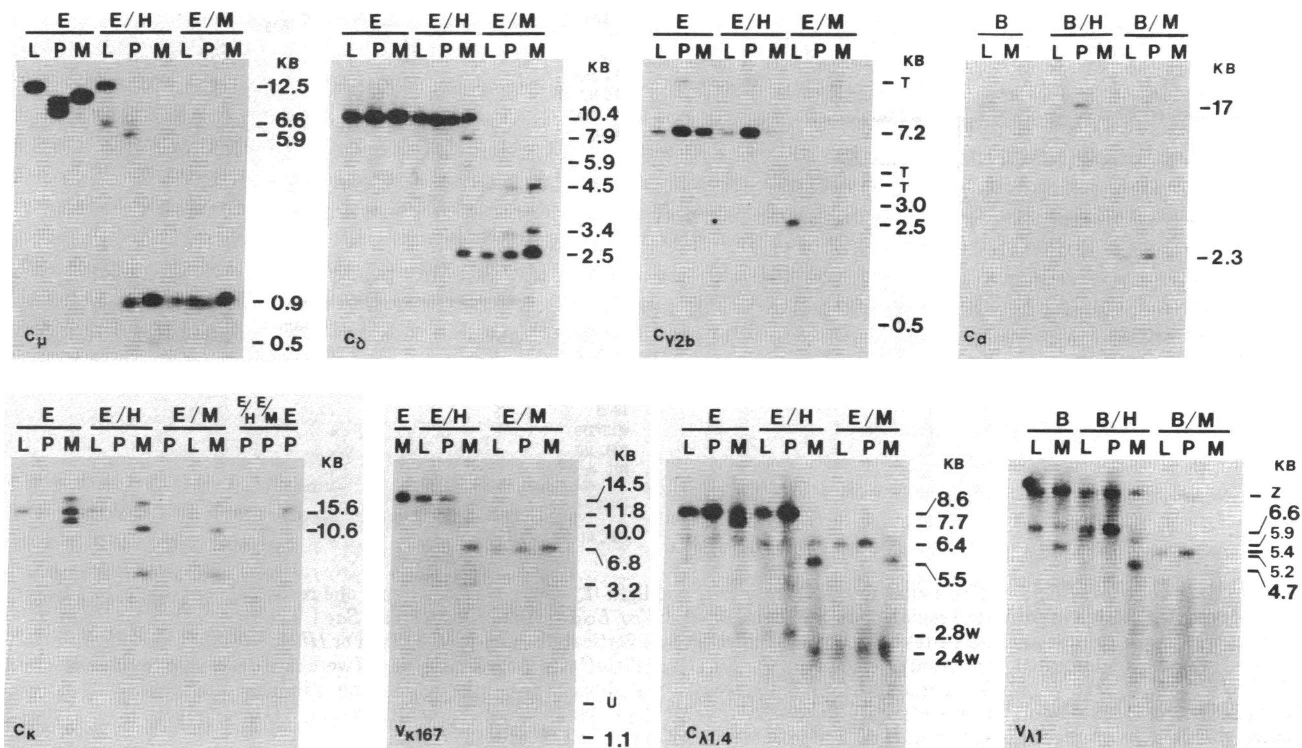

FIG. 1. Southern blots of *Hpa* II/*Msp* I analysis of Ig genes. B, *Bam*HI; E, *Eco*RI; E/H (B/H), *Eco*RI(*Bam*HI)/*Hpa* II; E/M (B/M), *Eco*RI(*Bam*HI)/*Msp* I. L, liver; P, pre-B cell 18-81; M, myeloma MOPC-104E. T, $\gamma 2a$ gene: 23.5-kilobase (kb) *Eco*RI band, 4.3-kb *Eco*RI/Hpa II band, 3.6-kb *Eco*RI/*Msp* I band; U, $V_\kappa 167$-related gene, 1.7 kb; W, $C_\lambda 4$ gene; Z, $V_\lambda 2$ gene.

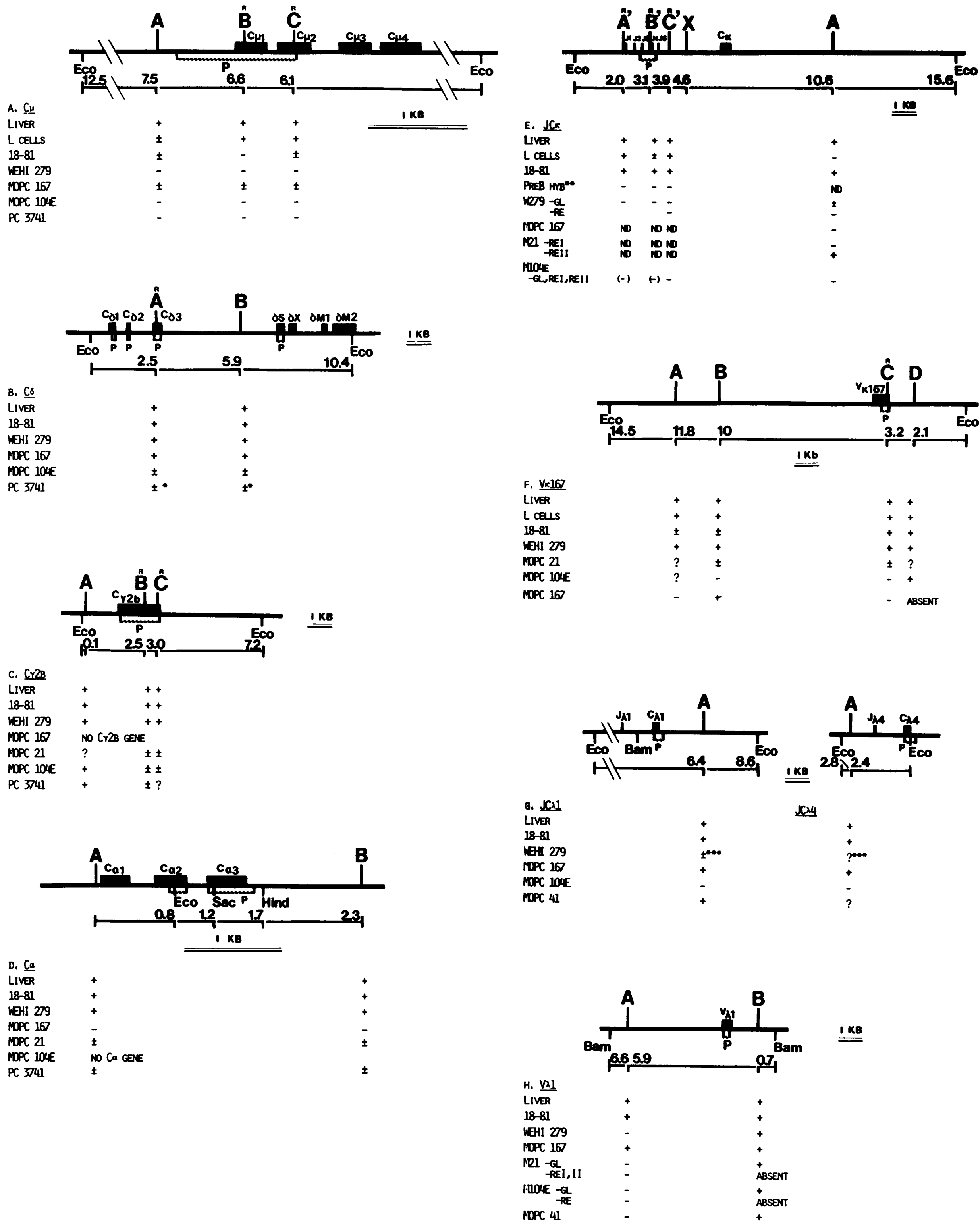

FIG. 2. Maps of *Hpa* II/*Msp* I or *Hha* I sites in the vicinity of *H* and light (*L*) genes. Generally only one restriction enzyme with a hexamer recognition site is indicated to define the region of a gene. Bam, *Bam*HI; *Eco*, *Eco*RI; Hind, *Hin*dIII; Sac, *Sac* I. Restriction sites for *Hpa* II/*Msp* I are indicated by a vertical line and A–D; sites for *Hha* I are indicated by a vertical line and A′–C′. *Msp* I or *Hha* I sites that have been subjected to sequence analysis are indicated by reference (R) [C_μ (26), C_δ (27), $C_\gamma 2b$ (10), JC_κ (28), $V_\kappa 167$ (29)]; maps were also derived from these references and C_δ (30), C_α (31), C_λ, and V_λ (32). The extent of methylation was estimated from the Southern blots and is indicated in the legends as + (80–100% methylation), ± (30–70% methylation), and − (0–20% methylation). gl, Gene in germline configuration; ND, not done; P, DNA probe used in Southern blot; re, rearranged gene; X, new *Hpa* II site in rearranged gene of MOPC-104E.

* PC-3741 shows the NZB C_δ germline restriction map, which differs from that of BALB/c mice.

** Three pre-B hybridomas were analyzed (see Table 1).

*** WEHI 279 shows a composite of BALB/c and NZB gl C_λ restriction fragments. Apparently, no rearrangement of $C_\lambda 1$-related genes has taken place in WEHI 279 compared with BALB/c and NZB kidney DNA.

81M, MOPC-41, and MOPC-167. However, the germline $C_\lambda 1$-like genes of WEHI 279 are partly unmethylated. As expected, the rearranged $C_\lambda 1$ gene is unmethylated in MOPC-104E but so are the germline $C_\lambda 1$ and surprisingly the $C_\lambda 4$ genes (Fig. 1).

The $V_\lambda 1$ Gene. There are two *Msp* I sites, one upstream and one downstream of the $V_\lambda 1$ gene (Figs. 1 and 2*H*). [The $V_\lambda 2$ gene has no *Msp* I site in its vicinity (Fig. 1) and was not further studied.] Both *Msp* I sites of $V_\lambda 1$ are completely methylated in liver, 18-81M, and MOPC-167 in accordance with the absence of $\lambda 1$ synthesis in these cells. In the $\lambda 1$ producer MOPC-104, site B is missing in one $V_\lambda 1$ gene because of the rearrangement to $JC_\lambda 1$. Site A is completely unmethylated in the rearranged gene and also in the germline $V_\lambda 1$ gene of MOPC-104E. However, site B is completely methylated in the germline gene.

The myeloma MOPC-21 has two rearranged $V_\lambda 1$ genes (not shown). They are both unmethylated. Furthermore, site A, but not site B, of the germline $V_\lambda 1$ gene of MOPC-21 is also unmethylated. Undermethylation in germline $V_\lambda 1$ genes of cells that also contain a rearranged $V_\lambda 1$ gene was unexpected, but, more surprisingly, site A was also found to be unmethylated in two cell lines, the B lymphoma WEHI 279 and the myeloma MOPC-41, which show no rearrangement of $V_\lambda 1$.

DISCUSSION

Liver and L Cells. In liver DNA generally all Ig genes are *Hpa* II insensitive (Fig. 2), suggesting that these genes, when not expressed, are fully methylated and that undermethylation of Ig genes in lymphoid cells may be an indicator of gene activity. However, in about 10–20% of the DNA from liver some *Msp* I sites are unmethylated in C_μ, C_δ, and JC_κ genes but not in other Ig genes (Fig. 1). These unmethylated genes may derive from lymphocytes present in the highly vascularized liver tissue that express mainly μ, δ, and κ. We have also determined the methylation status of C_μ, JC_κ, and V_κ genes of fibroblastoid L cells as additional control for the lymphoid cell lines because L cells are also a rapidly dividing cell population but do not produce Igs. The C_μ and JC_κ genes were found to be partially undermethylated (Figs. 1 and 2). Therefore, the Ig genes concerned may be partially undermethylated in some cell types, regardless of expression.

Correlation of Undermethylation with Expression of Ig Genes. Without exception, Ig genes that are expressed are unmethylated or at least partly undermethylated (compare Fig. 2 with Table 1). This confirms previous work (18, 33) and extends it to the consideration of λ genes of pre-B cells and several myelomas not previously studied. The methylation patterns are also in agreement with our previous findings regarding DNase I sensitivity of Ig genes (ref. 3; unpublished data).

In the C_μ gene, the undermethylation seems to be well confined to the region of the expressed locus. The C_μ genes are undermethylated in all of the lymphoid cells that we studied, which synthesize μ chains. However, the C_δ gene, which is only 2.3 kb 3′ of C_μ (37), is completely methylated in the pre-B and B-cell lines and at least partially in the myelomas. δ protein is only produced at a certain stage of B-lymphocyte development. Our study did not include a B-lymphocyte line that produces both μ and δ, but Rogers and Wall (18) have studied such a cell line and found undermethylation of both C_μ and C_δ genes. In addition, they also found that, in the B-lymphoma WEHI 279, the C_δ genes of the BALB/c chromosome, which code for the μ chains of WEHI 279, are methylated. Their results and ours together suggest that transcription of the C_μ locus in μ-only-producing cells may be terminated after the last C_μ exon.

Undermethylation of Germline Ig Genes. The germline counterpart in a cell that has a rearranged C_κ, C_λ, or C_μ gene is both undermethylated (this study) and DNase I sensitive (ref. 3; unpublished data). We interpret this finding as a coordinated activation of a particular Ig locus that may precede rearrangement. The observation that transcription of germline C_κ and C_μ genes occurs in myeloma and pre-B hybridoma cells is compatible with this conclusion (4, 5).

The $V_\kappa 167$ and $V_\lambda 1$ genes were found to be completely methylated in L cells or liver (or both). However, in several of the lymphoid cells, V genes in germline context were undermethylated. In two myeloma cells that have rearranged $V_\lambda 1$ genes (MOPC-21 and MOPC-104E), not only the rearranged but also the germline $V_\lambda 1$ gene is unmethylated at site A (Fig. 2). In several cases V_κ and $V_\lambda 1$ genes were found to be undermethylated in cells that are not known to express these genes at all. Thus, site A of $V_\lambda 1$ is unmethylated in MOPC-41 and WEHI 279 (Fig. 2*H*) and several sites in the vicinity of $V_\kappa 167$ are partially or completely unmethylated in 18-81M, MOPC-21, and MOPC-104E (Fig. 2*F*). On the other hand, in another study, it was found that in the case of $V_\kappa 19$ and $V_\kappa 21$ genes, only the expressed allele is undermethylated and not other $V_\kappa 19$ or $V_\kappa 21$ genes (36). Possibly there may be a rather tight regulation of undermethylated domains to delineate expressed V genes, but unexpressed V genes may not always be fully methylated.

In all cases in which $V_\kappa 167$ and $V_\lambda 1$ germline genes were unmethylated at *Msp* I sites upstream of the V gene, a downstream site was methylated (Fig. 2 *F* and *H*). The downstream region that is removed by functional *VJ* recombination may be involved in the transcriptional down-regulation (38) from germline V genes.

Although κ genes appear to have the potential to be expressed much more often than λ genes (39), this does not correlate with the observed methylation patterns. At least in the sample of cell lines studied here, λ genes were almost as frequently undermethylated as κ genes.

Correlation of Undermethylation with Differentiation. The methylation patterns analyzed in this study show a trend towards a decreased methylation of all Ig genes with increasing differentiation of the lymphoid cell type. In the pre-B cell 18-81M only the C_μ and $V_\kappa 167$ genes are partially undermethylated. Because in another Abelson virus-transformed pre-B-lymphocytic cell line, PD, the JC_κ region is undermethylated (R.-J. Sen and D. Baltimore, personal communication), it may depend on the differentiation status of pre-B cells whether the JC_κ locus is activated. In three pre-B cell hybridomas, JC_κ genes were unmethylated (Fig. 2*E*). In these pre-B hybridomas the germline JC_κ genes are also DNase I sensitive (unpublished data). Furthermore, germline JC_κ genes were transcribed in all pre-B hybridomas tested by Perry *et al.* (5). It remains to be determined if in pre-B cell hybridomas the C_κ genes may have become activated by some *trans*-acting mechanism that operated in the fusing myeloma cell.

A higher degree of differentiation is represented by the B-lymphocyte line WEHI 279. In these cells C_μ genes are more undermethylated than in 18-81M, though not completely. C_δ genes are methylated, except for 20% of the NZB genes. The other C_H genes are completely methylated, but κ and λ genes are at least partially undermethylated.

Finally, in most myelomas C_μ is completely and the three other C_H genes studied are at least partially undermethylated. Also, C_κ and C_λ genes and often V_κ and V_λ genes are at least partially undermethylated. Myelomas have about 100 times more immunoglobulin mRNA per cell than B-lymphoid cell lines (22), presumably due to a higher transcription rate. The higher rate of transcription may be correlated with increased undermethyl-

ation of the expressed genes. However, Ig genes not known to be expressed in myelomas are also undermethylated. For example, the μ-producing myeloma PC3741 does not show rearrangement (24) or detectable transcription (40) of $C_\gamma 2b$ or C_α genes; nevertheless, both of these genes are partially undermethylated. Furthermore, myelomas with germline context of C_δ genes (MOPC-104 and PC3741), of $C_\gamma 2b$ genes (MOPC-104E and MOPC-21), and of C_α genes (MOPC-21) show partial undermethylation of these genes. The C_γ and C_α genes are >100 kb (41) away from the C_μ gene expressed in PC3741 and MOPC-104E and 20 and 65 kb (41), respectively, from the $C_\gamma 1$ gene expressed in MOPC-21.

In conclusion, the analysis of the methylation state of Ig genes seems useful as an indication for a potentially expressed gene: all expressed Ig genes were found unmethylated at least at some sites within or in the vicinity of the gene. Therefore an Ig gene found to be methylated is generally not expressed. However, a gene found to be undermethylated is not necessarily expressed. The significance of transcriptionally silent undermethylation of Ig genes remains to be determined.

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