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Immunoglobulin heavy chain genes: Demethylation accompanies class switching

(gene regulation/complex transcription unit/*Hpa* II restriction sites)

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ABSTRACT **The methylation of immunoglobulin heavy chain genes was examined before and after class switching, by using the *Hpa* II/*Msp* I restriction mapping technique. The μ, δ, and $\gamma 1$ genes all are methylated in cells that do not express them but are demethylated when they are expressed. In particular, the δ gene remains methylated, and thus presumably untranscribed, in a cell line that probably represents an early stage of B-cell differentiation and produces only μ heavy chains. Because μ and δ RNAs are cotranscribed from a single complex transcription unit at a later stage of B-cell differentiation, this finding implies that the μ-plus-δ complex transcription unit is of variable length.**

The immunoglobulin heavy chain genes are generated and activated by a prolific set of somatic mechanisms, including multiple rounds of DNA and RNA rearrangements, and apparently are subject to numerous controls at different stages of the process (see Fig. 1; reviewed in refs. 1 and 2). One potential control point which has not yet been well defined is the onset of transcription of each of the immunoglobulin gene segments. In the light chain gene cluster, studies on chromatin conformation (3) indicated that the unjoined gene for the joining region–constant region of κ light chains (J_κ–C_κ) was in "open" conformation as were complete active genes, but that unjoined genes for the variable region of the κ chain (V_κ) were not, implying that the gene for the constant region (C_κ) becomes available for transcription before V_κ-J_κ joining. Active transcription of unrearranged C_κ genes is seen in cells that also contain a rearranged κ gene (4). In the heavy chain gene cluster, V_H rearrangement first generates an active μ gene, and subsequently other C_H genes become activated in a process known as class switching. It is of interest to ask whether they too may be transcribed before they give rise to stable mRNA species.

The demethylation of methylated cytosines in chromosomal DNA correlates closely with gene expression in many eukaryotic gene systems (for examples, see refs. 5–8). Therefore, demethylation of heavy chain genes might indicate at what stage they become available for transcription.

Most of the methylation in mammalian DNA is 5-methylation of the cytosines in the symmetrical dinucleotide C-G, and most of the C-G sequences contain 5-methylcytosine (C^m) (9–12). The sequence C-C-G-G is cut by restriction enzymes *Hpa* II and *Msp* I, whereas C-C^m-G-G is cut only by *Msp* I (13, 14). Therefore, parallel Southern blots (15) of chromosomal DNA cut with *Hpa* II or *Msp* I show whether these restriction sites are methylated or not. To examine individual C-C-G-G sequences in a gene, *Hpa* II- or *Msp* I-cut chromosomal DNA is also digested with another restriction enzyme (e.g., *Eco*RI) so as to provide a completely cut reference site, and the Southern blots of this DNA are probed with a labeled cloned fragment from close to the reference site in the gene in question. Thus, the *Msp* I/*Eco*RI lane should show a single *Msp* I/*Eco*RI band, corresponding to complete digestion, whereas the *Hpa* II/*Eco*RI lane may show a ladder of *Hpa* II/*Eco*RI bands depending on the percentage methylation of each *Hpa* II site.

We analyzed three of the mouse heavy chain genes (C_μ, C_δ, and $C_{\gamma 1}$) that are activated by three different mechanisms (Fig. 1). The C_μ gene is expressed as a result of the DNA rearrangement which joins the V_H, D_H, and J_H gene segments (V–D–J joining) (16–19). The C_δ gene is expressed by transcription through from the rearranged μ gene, in a VDJ_H–C_μ–C_δ transcription unit (complex transcription unit) (20–22). The $C_{\gamma 1}$ gene is expressed by means of a DNA rearrangement which deletes C_μ and C_δ (and $C_{\delta 3}$) and brings VDJ_H close to the $C_{\gamma 1}$ gene (class switching) (17, 23–27). Because the C_μ, C_δ, and $C_{\gamma 1}$ genes have been cloned and extensively mapped, individual *Hpa* II sites can be located with confidence.

We examined the methylation of these genes both in DNA from liver, which does not express any of them, and in DNA derived from three cultured cell lines which express each of the three genes in turn.

MATERIALS AND METHODS

DNA from cell nuclei (28) was restriction-digested to completion, and samples (15–20 μg) were electrophoresed in 0.7% agarose in Tris acetate/EDTA and blotted onto nitrocellulose paper (15). The blots were hybridized overnight (24) with fragments of DNA labeled by nick-translation with [α-^{32}P]dCTP. Sources of DNA were: pSpμA1, containing the C_μ gene from ChSpμ7 (30); p(C_δH), a gift of K. Moore, containing part of the C_δ gene from ChSp37 (21); and pγ1.13, containing the $C_{\gamma 1}$ gene from λIgCγ1.1 (31). All procedures were in accordance with the current National Institutes of Health guidelines on recombinant DNA.

RESULTS

The C_μ Gene. Southern blots with the μ probe are shown in Fig. 2*B*. Each panel contains three lanes, digested with *Eco*RI, *Hpa* II plus *Eco* RI, and *Msp* I plus *Eco*RI, respectively. The proportion of larger fragments visualized in the middle (*Hpa* II/*Eco*RI) lane reflects the degree of methylation of the *Hpa* II sites. The restriction pattern should not be altered by V–D–J joining because these rearrangements all take place 5′ to the C_μ *Eco*RI fragment.

Abbreviations: V, variable region; D, diversity region; J, joining region; C, constant region; M, membrane-binding region; κ, κ light chains; μ, δ, $\gamma 1$, etc., specific classes of heavy chains; H, heavy chains in general (these symbols denote regions of the immunoglobulin chain and of the DNA that encodes it; in the DNA, these elements should be termed "gene segments," but the traditional terminology of V and C "genes" is used here for simplicity); kb, kilobase(s).

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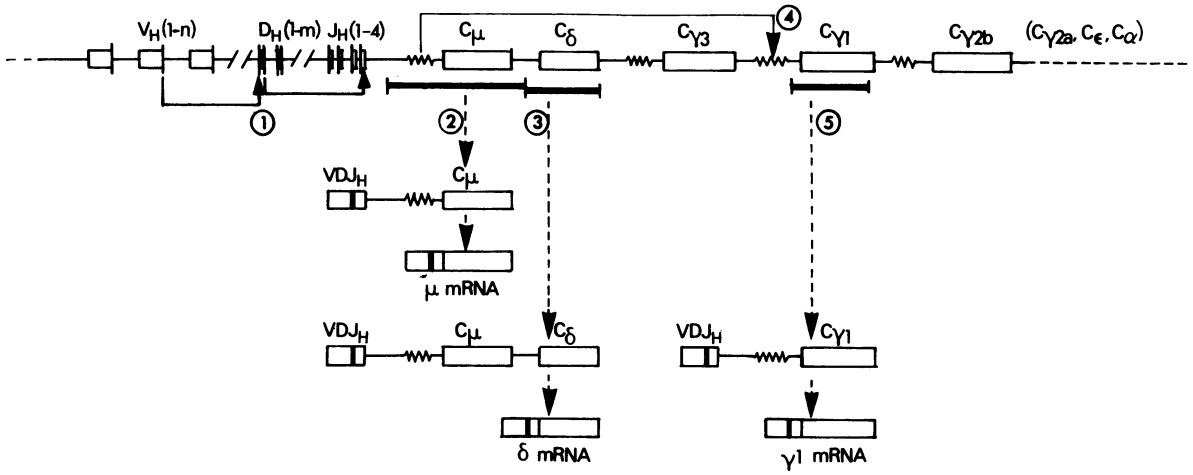

FIG. 1. Simplified scheme of immunoglobulin heavy chain gene expression. Processes indicated are: 1, V–D–J joining by DNA recombination; 2, μ gene transcription and RNA splicing; 3, μ-plus-δ gene transcription and RNA splicing; 4, C_H class switching by DNA recombination; 5, γ1 gene transcription and RNA splicing. Small introns within genes are omitted. Bars represent *Eco*RI fragments shown in more detail in Figs. 2–4.

In liver DNA, both copies of the C_μ gene were in germ-line configuration [12.5-kilobase (kb) *Eco*RI fragment] and substantially methylated.

In W279 (μ^+) DNA, two *Eco*RI fragments were seen; one of germ-line size (≈12.5 kb) and a rearranged one (10.2 kb). W279 was the only cell type in this experiment whose mouse genotype is not entirely BALB/c (Table 1), and the larger fragment could be either the BALB/c allele (12.5 kb) or the NZB allele (12.2

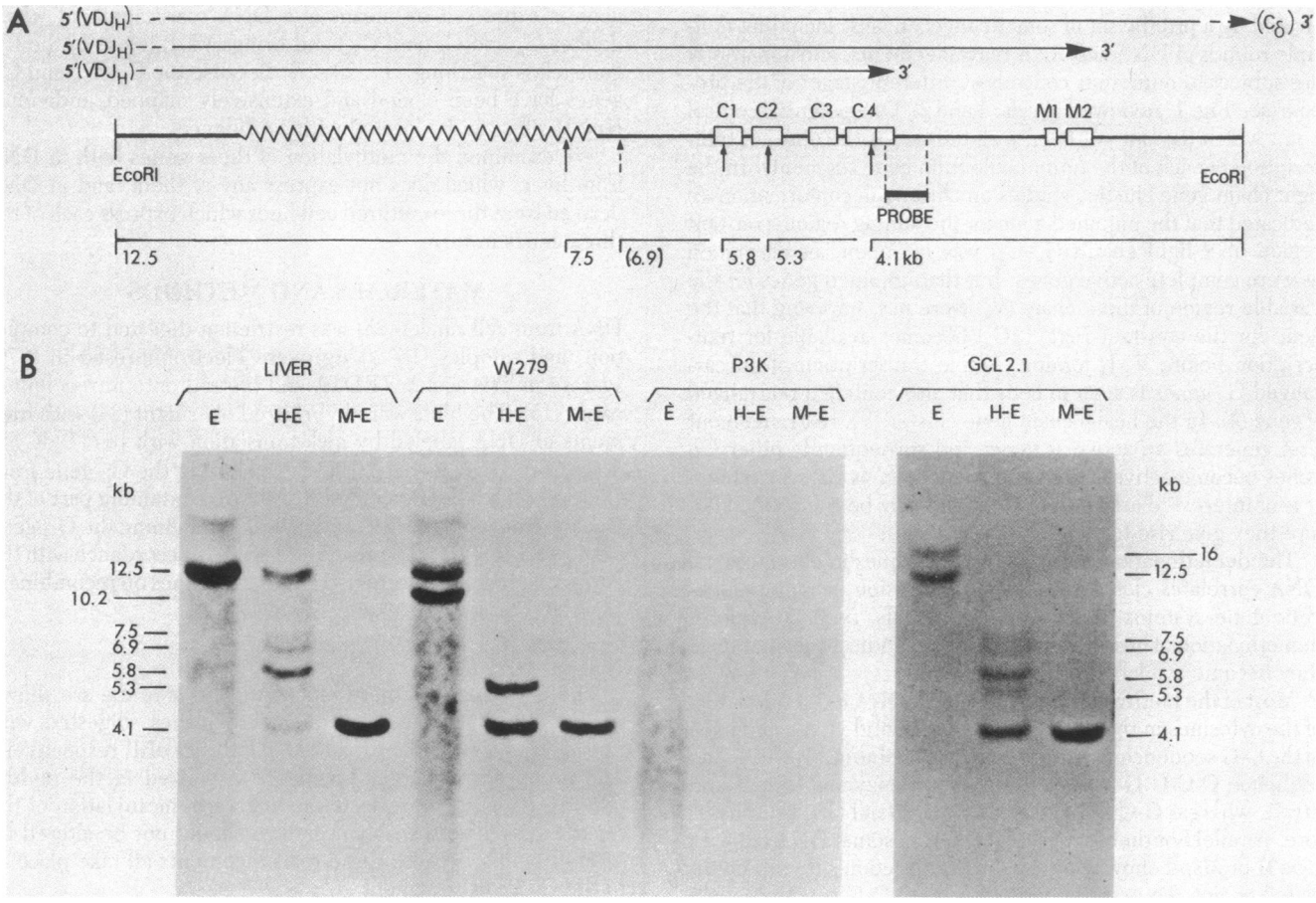

FIG. 2. (*A*) Scale map of the C_μ gene. Upward-pointing arrows are *Hpa* II sites. Restriction sites and exons in the right half are from refs. 35 and 37. An *Hpa* II site in the left half (dotted arrow) was inferred from the *Hpa* II/*Eco*RI digestion of liver DNA shown here. The zigzag segment indicates the region of repetitive sequences involved in class switching (17, 25). Deletions occur frequently in this region. Such a deletion has probably occurred in one of the chromosomes of W279, giving rise to the 10.2-kb *Eco*RI band. The arrows above the map indicate the extents of polyadenylylated primary transcripts for μ_s (bottom), μ_m (middle), and δ (top) mRNAs (21, 22, 32, 33). Lines below the map indicate fragments that might be seen in an *Hpa* II/*Eco*RI digest hybridized with the indicated probe, which is a 475-base-pair *Hha* I fragment. (*B*) Southern blots of the C_μ gene. Each panel displays three digests of chromosomal DNA: *Eco*RI (E), *Hpa* II plus *Eco*RI (H-E), and *Msp* I plus *Eco*RI (M-E). The faint bands at >12.5 kb in the *Eco*RI lanes are plasmid contaminants.

Table 1. Sources of DNA

Cells	Strain	Heavy chains	Ref.
Liver	BALB/c	—	—
WEHI 279 (B lymphoma)	NZC*	μ	34, 35
GCL2.1 (hybridoma)	BALB/c†	μ,δ	36, 37
P3K (myeloma)	BALB/c	$\gamma 1$	38

* WEHI 279 (W279) originated in an inbred strain derived from a (BALB/c × NZB)F_1 mouse. The W279 μ chains bear the BALB/c allotype.

† GCL2.1 is a subclone of GCL2, a hybridoma between mouse lipopolysaccharide-stimulated spleen cells and a Syrian hamster B-cell lymphoma. It has only a single copy of mouse chromosome 12, which carries the heavy chain genes (ref. 37). The Syrian hamster genes apparently do not cross-hybridize with the mouse probes used here.

kb) (39). The rearrangement in the other chromosome is probably a deletion in the repetitive sequences of the "switch region" 5′ to the C_μ gene, because V–D–J joining would take place 5′ to this *Eco*RI fragment, and the 3′ portion of this fragment is not rearranged (see the *Msp* I/*Eco*RI lanes in Fig. 2). Deletions in the switch region are found between different mouse strains (39, 40) and in some individual μ-producing cell lines (32, 41), as well as in almost all cloned isolates of the μ gene (17, 24, 39).

The *Hpa* II/*Eco*RI digest of W279 DNA displayed the definitive 4.1-kb fragment prominently, indicating demethylation in conjunction with μ transcription. Because both the 12.5-kb and 10.2-kb fragments were missing from this digest, both C_μ genes must be demethylated, even though only one [the BALB/c allele (34, 35)] is expressed in IgM protein.

In GCL2.1 ($\mu^+\delta^+$) DNA, there is only a single copy of mouse chromosome 12, which contains the heavy chain gene cluster, and the C_μ *Eco*RI fragment is of germ-line size (12.5 kb). (The origin of the larger *Eco*RI fragment in that lane is unknown; it could represent cross-hybridization with Syrian hamster sequences in this hybridoma, but no nonmouse bands were seen in the C_μ *Hpa* II/*Eco*RI lanes or in the C_δ lanes in Fig. 3.) As with W279, the C_μ gene is largely demethylated in these cells.

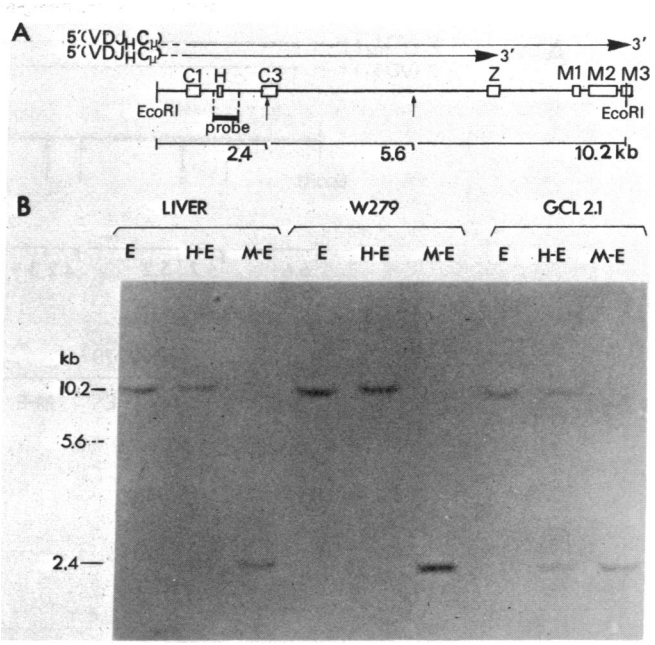

FIG. 3. (*A*) Scale map of the C_δ gene. Upward-pointing arrows are *Hpa* II sites (ref. 17; unpublished data). Positions of exons are taken from refs. 22 and 42; Z denotes the 3′-terminal exon for δ_s mRNA. The exact postion of the *Eco*RI site relative to the M exons is uncertain. The arrows above the map indicate the extents of polyadenylylated primary transcripts postulated for δ_s mRNA (lower) (21, 42) and δ_m mRNA (upper) (21, 22). The probe is an ≈500-base-pair *Sau*3AI fragment. Hybridization to Southern blots of the cloned δ gene (data not shown) and of chromosomal DNA (this figure) showed that it contains no repeated sequences. (*B*) Southern blots of the C_δ gene. Lanes are as in Fig. 2.

Table 2. Percentage of *Hpa* II sites methylated

Site, kb	Liver	W279	GCL2.1	P3K
μ gene				
7.5	90	—	0	
6.9	75	—	40	
5.8	60	—	35	
5.3	95	0	80	
4.1	90	40	50	
δ gene				
2.4	75	100	≈40	
5.6	100	100	100	
$\gamma 1$ gene				
5.7	80	100		—
5.2	80	100		—
4.2	85	100		—
3.9	85	100		—
3.2	90	100		—
2.5	95	100		0

For each gene, *Hpa* II sites are identified by their distance from the reference *Eco*RI site (see Figs. 2–4). The percentage methylation at each site was derived from the relative intensities of the bands in the *Hpa* II/*Eco*RI lane, which were measured by scanning the autoradiograph with a Beckman-25 spectrophotometer. If the bands are numbered from 1 (the smallest *Hpa* II/*Eco*RI band) to N (the full-length *Eco*RI band), and I_i is the intensity of band i, the percentage methylation of the site corresponding to band n is given by

$$100 \sum_{i=n+1}^{N} I_i \Big/ \sum_{i=n}^{N} I_i$$

—, site could not be observed because of complete demethylation closer to the probe fragment.

The percentage methylation at each *Hpa* II site was calculated from the relative intensities of the autoradiographic bands (Table 2). These values should be interpreted with caution because sites far from the probe fragment can only be observed on chromosomes in which the sites closer to the probe fragment are methylated and also because large and small fragments may not be visualized with equal efficiency by Southern blotting. However, they clearly show that neither methylation nor demethylation is uniform across the μ gene. In particular, the *Hpa* II site in the $C_\mu 2$ exon (corresponding to the 5.3-kb *Hpa* II/*Eco*RI band) is still methylated in GCL2.1 almost as much as in liver but totally demethylated in W279; most of the other sites are methylated in GCL2.1 to only about half the extent that they are in liver. Because there is only one copy of chromosome 12 in GCL2.1, these values must reflect unequal demethylation of this one chromosome in different cells.

In P3K ($\gamma 1^+$) DNA, the C_μ genes are absent, as expected from the deletion mechanism for heavy chain class switching.

The C_δ Gene. Southern blots with the δ probe are shown in Fig. 3. In liver DNA and in W279 (μ^+) DNA, both copies of the C_δ gene are in germ-line configuration (10.2-kb *Eco*RI band) and mostly methylated. In GCL2.1 ($\mu^+\delta^+$) DNA, there is no C_δ gene DNA rearrangement, which confirms previous reports (21, 22), but the *Hpa* II site in the $C_\delta 3$ exon is mostly demethylated. The other *Hpa* II site, which should correspond to a 5.6-kb *Hpa* II/*Eco*RI band, is fully methylated in all three DNAs. Although this site is not detectable in any of the blots

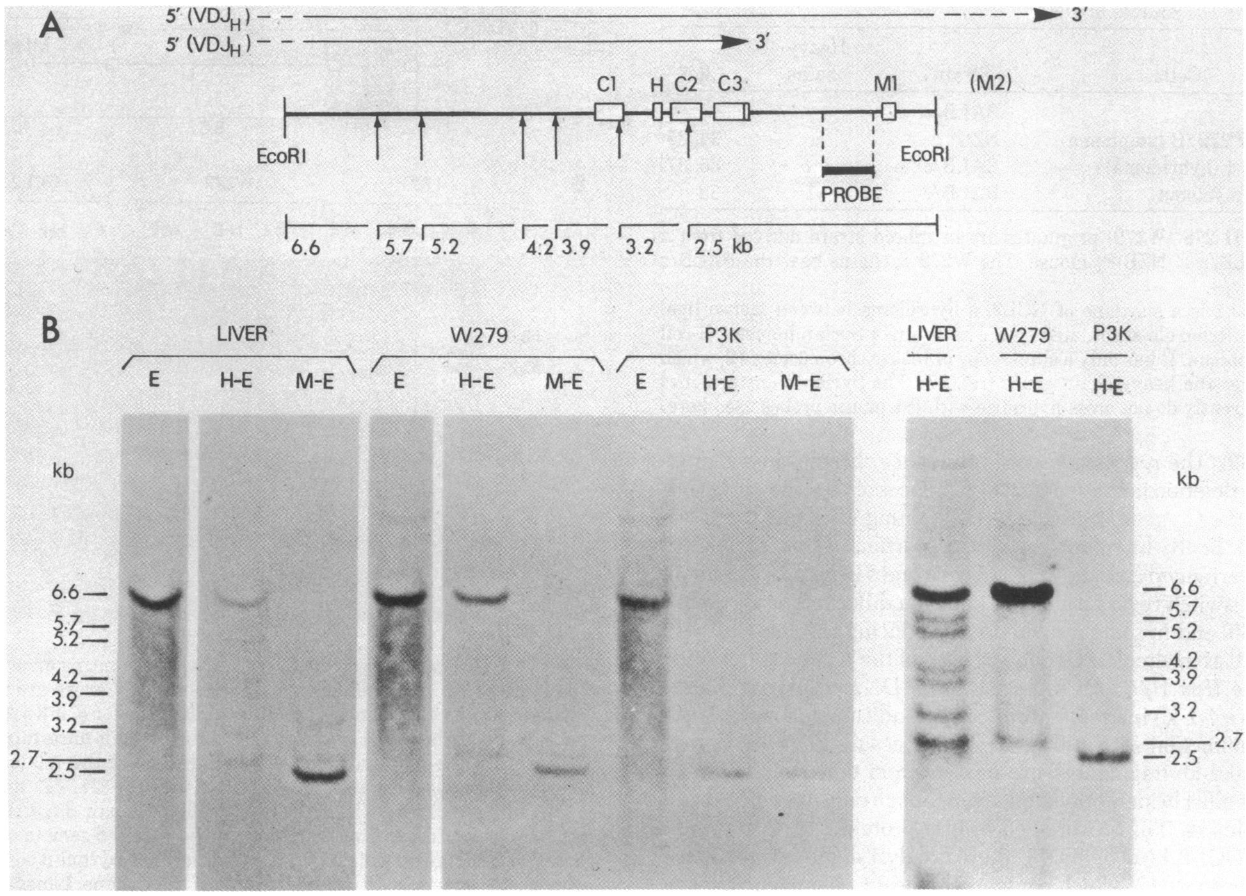

FIG. 4. (*A*) Scale map of the $C_{\gamma 1}$ gene. *Hpa* II sites (upward-pointing arrows) and exons are from refs. 31, 43, and 44 and unpublished data. The arrows above the map indicate the predicted extents of polyadenylylated primary transcripts for $\gamma 1_s$ (lower) and $\gamma 1_m$ (upper) mRNAs (44). The probe is a 510-base-pair *Kpn* I/*Pst* I fragment. (*B*) Southern blots of the $C_{\gamma 1}$ gene. Lanes are as in Fig. 2. Hybridization of the same γ1 probe to the cloned γ2b and γ3 genes showed that it cross-hybridizes partially with both, and cross-hybridization with these and with the γ2a gene probably explains the faint bands seen in the *Msp* I/*Eco*RI lanes (2.7 and 3.2 kb) and in the *Eco*RI lanes (≥15 kb) (unpublished data). Because of a background smear in the *Eco*RI lanes, shorter exposures of these lanes are presented. The right-hand panel shows a repeat of the *Hpa* II/*Eco*RI lanes.

shown, its existence in chromosomal DNA was confirmed by rehybridizing *Msp* I/*Eco*RI lanes to a probe from the δ M exons, which showed the predicted 4.6-kb band.

In P3K ($\gamma 1^+$) DNA, the C_δ genes are deleted.

The $C_{\gamma 1}$ Gene. Southern blots with the γ1 probe are shown in Fig. 4. There is partial cross-hybridization with the γ2a, γ2b, and γ3 genes, but these bands are faint, and the γ1 bands can be identified unambiguously from the known restriction map.

In liver DNA and in W279 (μ^+) DNA, both copies of the $C_{\gamma 1}$ gene are in germ-line configuration (6.6-kb *Eco*RI fragment) and substantially methylated. All *Hpa* II sites are 80–95% methylated in liver and 100% methylated in W279 (Table 2).

In P3K ($\gamma 1^+$) DNA, the size of the transcribed $C_{\gamma 1}$ *Eco*RI fragment is unchanged, presumably because the DNA rearrangement responsible for the γ1 class switch occurred 5′ to this *Eco*RI fragment (31). The active $C_{\gamma 1}$ gene, together with any other $C_{\gamma 1}$ genes which may be present in this myeloma, is now fully demethylated, at least at the *Hpa* II site in the $C_\gamma 2$ exon, as shown by the 2.5-kb band in the *Hpa* II/*Eco*RI lane.

DISCUSSION

These data demonstrate a close correlation between demethylation and expression of immunoglobulin C_H genes, in agreement with results in other eukaryotic gene systems (5–8). Although our criterion for gene expression is the production of stable mRNAs, it is likely that demethylation reflects active transcription of chromosomal DNA regardless of the fate of the transcripts. In support of this interpretation, we find that *Hpa* II sites are demethylated in introns as well as exons of an active μ gene, just as demethylation occurs in introns and transcribed 3′ flanking sequences of active globin genes (6–8). It is possible that demethylation may even precede transcription as a mechanism by which gene expression is initiated (45, 46), but this would not affect the following discussion.

Two points of particular relevance to immunoglobulin gene regulation emerge from the results with the B-cell lymphoma W279. First, although the lymphoma makes only a single allotype of μ chain (34, 35), both alleles of the C_μ gene are demethylated. This may indicate that the second allele is transcribed. Although W279 does not contain the μ RNAs characteristic of nonproductively rearranged C_μ genes (47, 48), other nonfunctional rearrangements of the J_H–C_μ locus are possible which would give rise to mRNAs identical in size to the functional μ_m and μ_s mRNAs observed in W279 (33). The second J_H–C_μ allele is commonly rearranged in normal B cells and in B-cell lymphomas, so its rearrangement and transcription in W279 would not be unusual.

Second, the results in W279 suggest a novel mechanism for regulating the expression of the complex transcription unit (VDJ_H–C_μ–C_δ) for μ and δ mRNAs. The W279 cells, which are presumed to represent an early stage in B-cell maturation, make only μ_s and μ_m mRNAs, but no δ mRNAs (ref. 33; unpublished data). In contrast the GCL2.1 cells, which are presumed to correspond to a more developed B cell, make μ_s, μ_m, δ_s, and δ_m

mRNAs from the VDJ_H–C_μ–C_δ transcription unit. We have proposed that the differential RNA splicing which generates these different μ and δ mRNAs is the consequence of differential polyadenylylation at the four alternative sites in this transcription unit (20–22, 32). This model was based on the findings in transcription units for simian virus 40, adenovirus late genes, and β-globin, in which RNA splicing follows polyadenylylation (49–52). In these cases, polyadenylylation apparently occurs via the cleavage of a nascent transcript as an event separate from the termination of transcription. For the VDJ_H–C_μ–C_δ complex transcription unit we left open the question of whether the transition for μ to μ-plus-δ mRNA production in B-cell development was regulated through the cleavage of transcripts at specific poly(A) sites (with the transcripts continuing along the entire transcription unit) or through the termination of transcription prior to the δ gene in cells making only μ mRNAs. Comparison of the methylation patterns in GCL2.1 and W279 can shed light on this question. In GCL2.1, in which the whole VDJ_H–C_μ–C_δ transcription unit is active, we find that the C_μ and C_δ genes are largely demethylated. In contrast, in W279, in which only μ mRNAs are produced, the C_μ genes are demethylated but the C_δ genes remain methylated. This suggests that the production of μ_s and μ_m mRNAs in W279 cells is caused by a regulatory factor affecting the termination of transcription at or 3′ to the μ_m poly(A) site. The subsequent production of μ_s alone in antigen-stimulated B cells, which is a dominant property in hybridomas, may be caused by a similar factor which halts transcription at or 3′ to the μ_s poly(A) site.

It has recently been reported that the first mRNA family contained in the major "late" complex transcription unit of adenovirus is selectively expressed early in infection, through termination of transcription after the first poly(A) site in this large transcription unit (53, 54). This is another case in which the choice of RNA splicing patterns is dependent on the choice of poly(A) site, which in turn is dependent on regulated termination of transcription. Therefore, this postulated mechanism for controlling gene expression in complex transcription units is not unique to immunoglobulin genes.

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