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Tissue-specific DNA methylation in a cluster of rabbit β-like globin genes

(gene mapping/molecular cloning/gene expression)

CHE-KUN JAMES SHEN AND TOM MANIATIS

Division of Biology, California Institute of Technology, Pasadena, California 91125

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ABSTRACT The relationship between DNA methylation and differential expression of rabbit β-like globin genes was studied by using restriction enzymes that cleave the sequence C-C-G-G but are differentially inhibited by the presence of 5-methylcytosine. The methylation frequency of 13 C-C-G-G sites that flank a set of four closely linked rabbit β-like globin genes was determined. This analysis revealed that certain sites surrounding embryonic and adult globin genes are relatively undermethylated in DNA from embryonic and adult erythroid tissues, respectively. This pattern is most pronounced for three sites that are undermethylated in erythroid cells but are totally methylated in nonerythroid cells. We conclude that the degree of CpG methylation in the rabbit β-like globin gene cluster is correlated with gene activity, but the effect is confined to relatively small regions of DNA.

The genomes of most eukaryotes contain only one major modified base, 5-methylcytosine. In mammalian DNA, this modified base constitutes 2–7% of the total cytosine and is found mainly within the dinucleotide sequence CpG (1, 2). Although the biological function of cytosine methylation has not been established, a number of investigators have proposed that it plays a regulatory role in differential gene expression (3–6).

The distribution of 5-methylcytosine near specific eukaryotic genes has been studied by using restriction enzymes that recognize CpG-containing sequences (7–12). In general, undermethylation is associated with gene activity, but a causal relationship between undermethylation and gene expression has yet to be demonstrated. The restriction enzyme assay is based primarily on the use of enzymes *Hpa* II (13) and *Msp* I (14–16), both of which recognize and cut the unmodified C-C-G-G sequence. If, however, the internal C is methylated, the sequence is resistant to *Hpa* II but not *Msp* I cleavage (14–17). Thus, parallel digestion of a DNA sample with *Hpa* II or *Msp* I makes it possible to differentiate between DNA modification and restriction site polymorphism. We have used this assay to analyze the differential methylation of C-C-G-G sequences within a cluster of rabbit β-like globin genes, using DNA obtained from different rabbit tissues.

The rabbit β-like globin genes provide an interesting system for studying the relationship between DNA methylation and gene activity because these genes are coordinately and sequentially expressed during development. Two types of embryonic β-like globin polypeptides, ε(y) and ε(z), have been detected in the nucleated erythroid cells derived from the yolk sac blood islands of the embryos (18–20). Starting at about 12 days of gestation, these two ε chains are gradually replaced by the adult β-globin chain, and the fetal liver becomes the primary center for erythropoiesis. In the adult, the bone marrow and spleen are the major erythropoietic sites and only the adult β-globin polypeptide is synthesized. The rabbit β-like globin gene cluster has been cloned in a set of overlapping bacteriophage recombinants (21, 22). The cluster contains four genes, which have been designated β1 through β4. The genes β3 and β4 are transcribed in embryonic but not in adult erythroid cells and presumably encode the ε(y) and ε(z) chains. β1, which encodes the adult β-globin polypeptide, is transcribed primarily in adult erythroid cells (Fig. 1). A small amount of the β1 transcript is also detected in embryonic erythroid cells. The β2 gene does not appear to be transcribed in any erythroid tissues (22). Nucleotide sequence analysis indicated that this gene cannot encode a functional globin polypeptide and is a β-globin pseudogene (unpublished data). As shown in Fig. 1, all of the genes are transcribed from the same DNA strand and they are arranged on the chromosome in the order of their expression during development. The same relationship between gene order and developmental expression has been observed in several other mammalian globin gene clusters (see ref. 23 for review).

Although tissue-specific methylation patterns of several individual eukaryotic genes have been reported (7–12), the well-characterized rabbit β-like globin gene cluster provides an opportunity to study the methylation pattern of large regions of chromosomal DNA that contain coordinately as well as sequentially expressed genes. In this paper, we report the results of experiments which indicate that certain C-C-G-G sites surrounding embryonic and adult β-globin genes are relatively undermethylated in the DNA from erythroid tissues, whereas the same sites are methylated to a greater extent in nonerythroid tissues. However, we do not find a uniform pattern of DNA methylation over large regions of the gene cluster.

MATERIALS AND METHODS

DNA Preparation. Rabbit DNA was purified from different tissues by using published procedures (24). DNA from bone marrow and blood islands was isolated from a single female New Zealand White rabbit (rabbit I). This bone marrow DNA will be referred to as bone marrow I DNA. DNA of the bone marrow, kidney, liver, and spleen of a second rabbit (rabbit II) was kindly provided by V. Parker. The bone marrow DNA of this rabbit is designated as bone marrow II DNA. The brain DNA of a third rabbit was a gift from A. Efstratiadis.

Hybridization Probes. The subclones containing mainly the large introns of the four rabbit globin genes were provided by R. Hardison (β4, β3), E. Lacy (β2), and C. O'Connell (β1). The intron fragments were purified by polyacrylamide gel electrophoresis and labeled with ^{32}P by nick translation (25).

Blot Hybridization. Genomic rabbit DNA was digested to completion with *Hpa* II or *Msp* I, alone or in combination with other enzymes (New England BioLabs, Beverly, MA, and Be-

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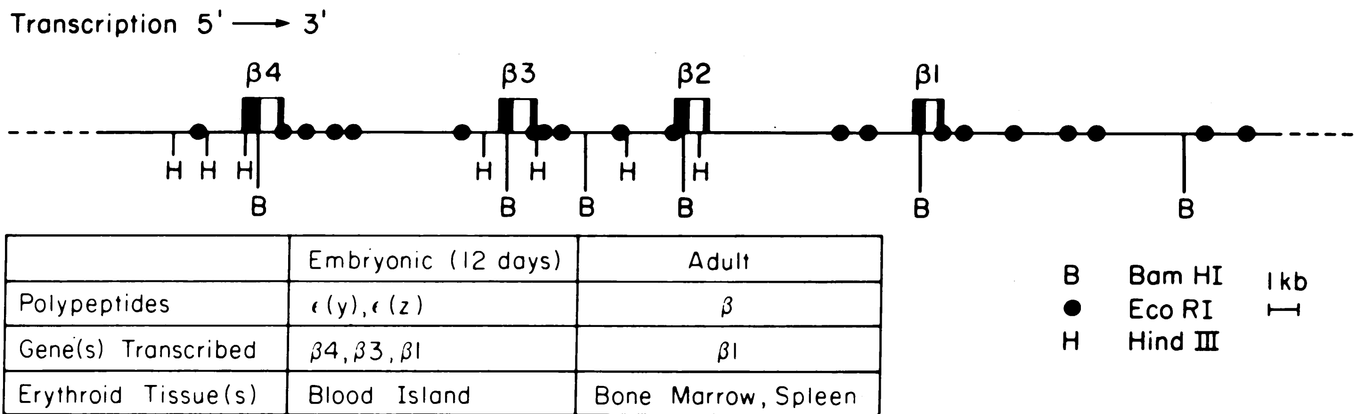
Abbreviation: kb, kilobase.



	Embryonic (12 days)	Adult
Polypeptides	ε(y), ε(z)	β
Gene(s) Transcribed	β4, β3, β1	β1
Erythroid Tissue(s)	Blood Island	Bone Marrow, Spleen

FIG. 1. The linkage arrangement of rabbit β-like globin genes. The maps of the *Eco*RI, *Bam*HI, and *Hin*dIII sites have been determined previously (21, 22). The gene locations are indicated by black (exon sequences) and white (intron sequences) boxes. The small intron present in each of the genes is not shown. The *Bam*HI/*Eco*RI and *Bam*HI/*Hin*dIII restriction fragments containing mainly the large introns of the four genes were subcloned and used as hybridization probes. The ontogeny of rabbit β-like globin gene expression (18–22) is summarized in the table below the map. kb, kilobase.

thesda Research Laboratories, Rockville, MD). The digestions were performed by using the buffer conditions suggested by the commercial sources, with a 10-fold excess of enzyme and a 4- to 8-fold excess of digestion time to ensure complete digestion. The digested DNA was fractionated on 1% agarose gels along with suitable size markers, blotted onto nitrocellulose filters (26), and hybridized with the ^{32}P-labeled intron probes (27). The blots were washed stepwise under stringent conditions as described (28) and autoradiographed on Kodak XR-5 x-ray film with Du Pont intensifier screens. The autoradiographs were traced with a microdensitometer (Quick Scan, Jr., Helena Laboratories, Beaumont, TX). When necessary, several autoradiographs were examined to avoid the problem of nonlinearity in film response due to under- or overexposure.

Although there is a difference in the overall efficiency of transfer and binding of large and small DNA fragments, in practice these two effects are compensatory, because we find a linear relationship between the amount of hybridizing sequence and band intensity for a wide range of DNA molecular weights. For example, the 6.9-, 2.7-, and 1.8-kb bands shown in Fig. 2*a* are of equal intensity.

RESULTS

Specific Probes. To survey the methylation pattern of all C-C-G-G sites within the rabbit β-globin gene cluster, we used the large intron of each β-globin gene as a hybridization probe. These probes were chosen because they are single copy in the genome and do not cross-hybridize with each other in genomic blotting experiments (see *Materials and Methods* for a description of the probes).

Methylation of C-C-G-G Sites Surrounding the Embryonic β3 Globin Gene. The locations of *Msp* I sites surrounding the embryonic β3 are shown in Fig. 2*b*. The extent of methylation of these sites in the blood island and bone marrow I DNA was analyzed as shown in Fig. 2*a*. When the β3 intron is used to probe *Hpa* II-digested blood island DNA, a minimum of four bands is observed (Fig. 2*a*, lane 2). These bands correspond to DNA fragments of 2.7, 5.8, and 6.4 kb and fragments larger than 14 kb. The locations of these fragments (Fig. 2*b*) were determined by carrying out double digestions with the restriction enzymes *Hpa* I and *Hpa* II (Fig. 2*a*, lane 4; also see Fig. 2*b* for location of *Hpa* I sites). The *Hpa* II (Fig. 2*a*, lane 7) and (*Hpa* I + *Hpa* II) (Fig. 2*a*, lane 9) patterns observed with bone marrow I DNA are distinctly different from the corresponding patterns of blood island DNA. In particular, the 2.7-kb (*Hpa* II) and 1.8-kb (*Hpa* I + *Hpa* II) fragments that are present in blood island DNA (Fig. 2*a*, lanes 2 and 4) are absent in bone marrow I DNA (Fig. 2*a*, lanes 7 and 9). We conclude from this result that the C-C-G-G site (site 6) near the 5′ end of the β3 gene is totally methylated in bone marrow I DNA and partially methylated in blood island DNA.

Both the blood island and bone marrow tissues contain erythroid as well as nonerythroid cells. This heterogeneity in cell type may contribute to the observed heterogeneity in methylation at different C-C-G-G sites. To obtain an approximate estimate of the frequency at which a given C-C-G-G site is methylated, we scanned autoradiographs with a densitometer and measured the area under individual peaks. From the areas we calculated the weight proportion P of each band within a lane. Because every band contains all of the sequence represented in our intron probes, the weight proportion of each band equals the number proportion. P of each band is thus related to the methylation frequencies f of the C-C-G-G sites contained within the fragment as indicated by the expression presented in Fig. 2*b*. For example, in order to detect the 4.9-kb band in the (*Hpa* I + *Hpa* II) digest, site 6 must be methylated and site 5 must be unmethylated. Thus, the probability of detecting the 4.9-kb band, which is equivalent to the weight proportion of this band ($P_{4.9}$), is equal to the product of $(1 - f_5)$ and f_6, in which f_5 and f_6 are the methylation frequencies of sites 5 and 6, respectively.

The methylation frequencies of the four C-C-G-G sites near the β3 gene as calculated in Fig. 2 are listed in Fig. 6. Both sites 5 and 6 are extensively methylated in bone marrow I DNA ($f_5 = 0.8, f_6 = 1.0$) and relatively undermethylated in blood island DNA ($f_5 = 0.3, f_6 = 0.7$), while no significant difference is detected for sites 4 or 7. Although this analysis is only semiquantitative, it does provide a meaningful indication of the relative degree of methylation in DNA samples from different tissues that are analyzed on the same agarose gel.

Methylation of C-C-G-G Sites Surrounding the Embryonic β4 Globin Gene. An analysis similar to that described in Fig. 2 was carried out by using the β4 intron as a hybridization probe. The mapping results of this analysis are summarized in Fig. 3. Differential methylation at the 5 C-C-G-G sites (sites 1–5) within 12 kb of the β4 gene will produce a number of DNA fragments when rabbit DNA is digested with *Hpa* II and hybridized with the β4 intron probe. All of the fragments shown in Fig. 3 can be detected in blood island DNA, including a 3.3-kb fragment that is produced when sites 2 and 3 are un-

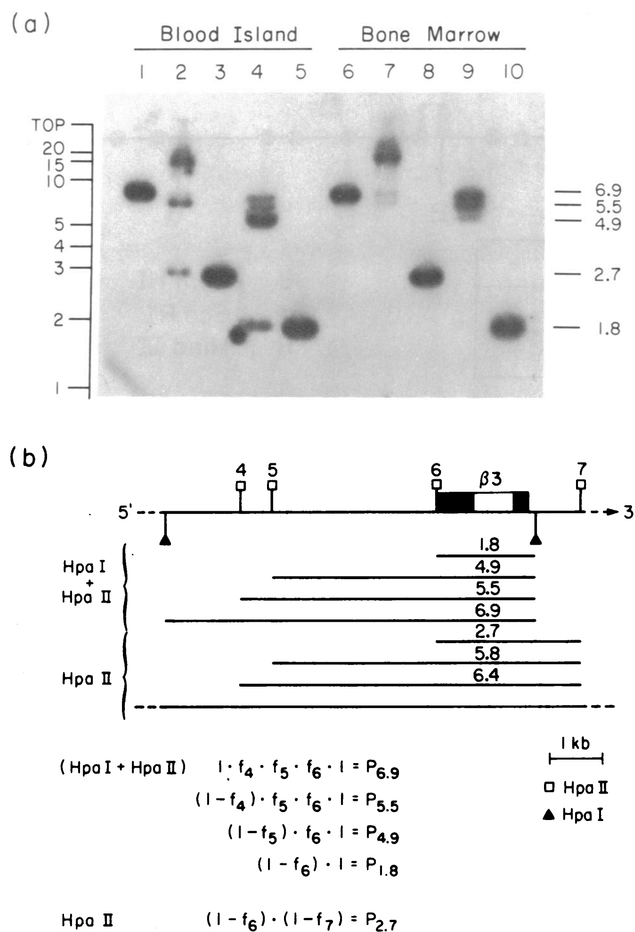


FIG. 2. Methylation of C-C-G-G sites surrounding the embryonic β3 globin gene. Blood island and bone marrow I DNA were digested with *Hpa* I, *Hpa* II, or *Msp* I as indicated below and hybridized to a ^{32}P-labeled β3 intron probe. (*a*) Autoradiograph showing the hybridization pattern of blood island (lanes 1–5) and bone marrow I (lanes 6–10) DNA. Lanes 1 and 6, *Hpa* I alone; lanes 2 and 7, *Hpa* II alone; lanes 3 and 8, *Msp* I alone; lanes 4 and 9, *Hpa* I + *Hpa* II; lanes 5 and 10, *Hpa* I + *Msp* I. Scales on sides are in kb. (*b*) Map of C-C-G-G sites surrounding the β3 globin gene and an expression for estimating methylation frequencies. The C-C-G-G sites are indicated by small white boxes which are numbered 4–7. The lines below the map represent the fragments detected by β3 intron probe. The fragment lengths are indicated in kb. The methylation frequencies f_4 through f_7 could be calculated from the five equations in which the weight proportion (*P*) values are known from the autoradiographs.

methylated, or when the DNA is digested with *Msp* I. In contrast, the 3.3-kb fragment is not detected in *Hpa* II digests of bone marrow I DNA. Additional mapping experiments using *Xba* I and *Hpa* II show that site 3 is partially methylated in all tissues (data not shown). Thus, site 2 is totally methylated in bone marrow I DNA. The methylation frequencies of sites 1–5 are listed in Fig. 6.

Methylation of C-C-G-G Sites Surrounding the Embryonic Globin Genes in the DNA from Tissues Not Expressing These Genes. The frequency of methylation of C-C-G-G sites surrounding the embryonic genes in DNA from nonexpressing tissues was estimated by hybridizing *Hpa* II- or *Msp* I-digested kidney, adult liver, spleen, and brain DNA with the β3 or β4 intron probe. The results of the β4 experiment are presented in Fig. 4. The hybridization pattern of *Msp* I-digested DNA is the same in all the tissues examined (lanes 2, 5, 6, 8, 10, 12). The 3.3-kb DNA fragment produced by cutting sites 2 and 3 (see Fig. 3) is present in erythroid as well as nonerythroid tissues.

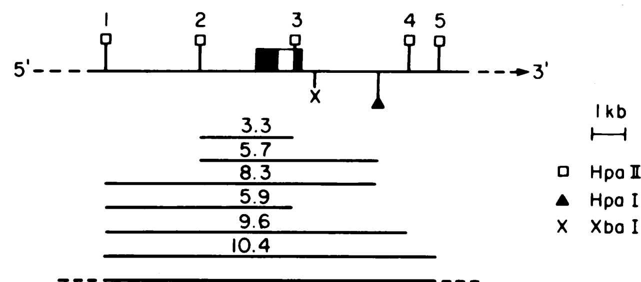


FIG. 3. Methylation of C-C-G-G sites surrounding the embryonic β4 globin gene. A summary of the results of an analysis similar to the one described in Fig. 2 is shown. The C-C-G-G sites are indicated by small white boxes which are numbered 1–5. The methylation frequencies of these sites in different tissues are listed in Fig. 6. The lines below the map represent the fragments detected by the β4 intron probe. The fragment lengths are indicated in kb.

The minor DNA band that corresponds to a 5.7-kb fragment has not been identified. The fact that the β4 probe detects a minor band in DNA digested with several other enzymes suggests that it corresponds to a rabbit DNA fragment located outside the β-globin gene cluster (data not shown). Alternatively, the 5.7-kb fragment could result from the methylation of the C-C-G-G sequence at site 2. Methylation of the first C in the C-C-G-G sequence has been shown to block *Msp* I digestion (12).

The *Hpa* II digestion pattern of blood island DNA is different (Fig. 4, lane 1) from that of DNA from other tissues (Fig. 4, lanes 3, 5, 7, 9, 11). In particular, a 3.3-kb DNA fragment is detected only in blood island DNA (arrow, Fig. 4). Site 2 (Fig. 3) is therefore totally methylated in the DNA of all tissues except the embryonic blood island. A similar analysis revealed that the C-C-G-G site 6, which is located in the 5′ portion of the β3 gene, is totally methylated in all tissues except the blood islands (see Fig. 6).

Methylation of C-C-G-G Sites Surrounding the Adult β1 Globin Gene. A total of 6 bands is detected when blood island and bone marrow I DNA are digested with *Hpa* I + *Hpa* II and hybridized to the β1 intron probe (Fig. 5*a*). The locations of the DNA fragments corresponding to these bands are indicated below the map in Fig. 5*c*. The methylation patterns of sites 10, 11, and 12 are similar for the blood island and bone marrow I DNA (Fig. 5*a*).

A comparison of the methylation pattern of C-C-G-G sites surrounding the β1 gene in DNA from bone marrow I, kidney, liver, and brain is presented in Fig. 5*b*. A 3.8-kb fragment is detected in bone marrow I DNA (Fig. 5*b*, lane 1), liver DNA (Fig. 5*b*, lanes 4 and 5), and spleen DNA (data not shown), but is absent in all other tissues. The absence of the 3.8-kb fragment

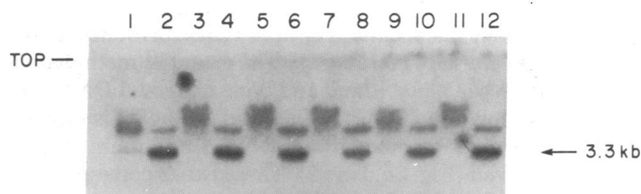


FIG. 4. Methylation of C-C-G-G sites surrounding the β4 globin gene in different tissues. DNA isolated from different tissues was digested with *Hpa* II (odd-numbered lanes) or *Msp* I (even-numbered lanes) and hybridized with the β4 intron probe. Lanes 1 and 2, blood island; lanes 3 and 4, bone marrow I; lanes 5 and 6, bone marrow II; lanes 7 and 8, kidney; lanes 9 and 10, liver; lanes 11 and 12, spleen. The arrow points to the position of the *Hpa* II-generated 3.3-kb band that is present only in the blood island DNA (lane 1).

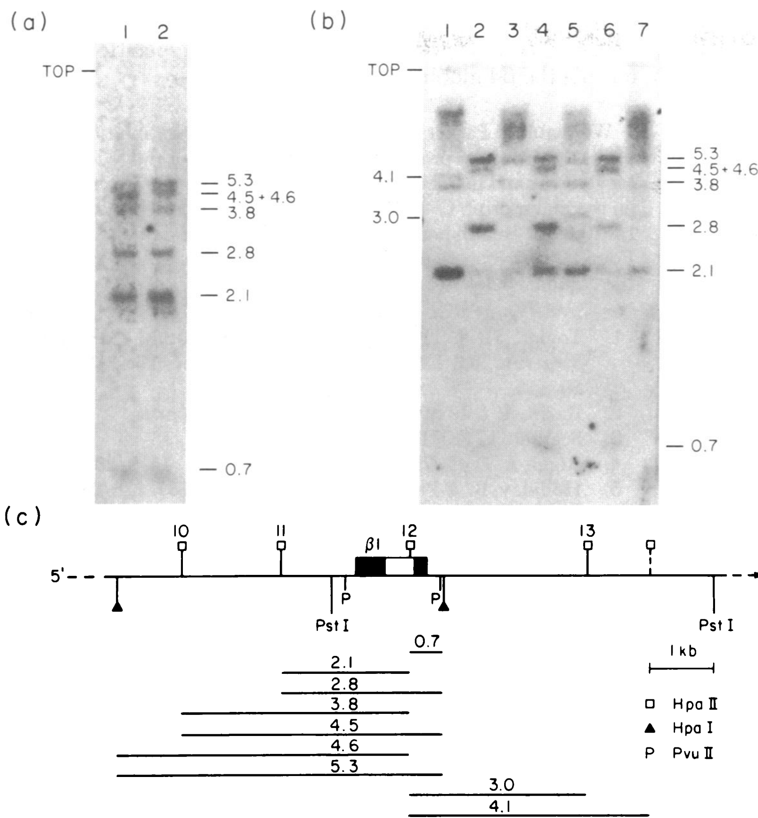
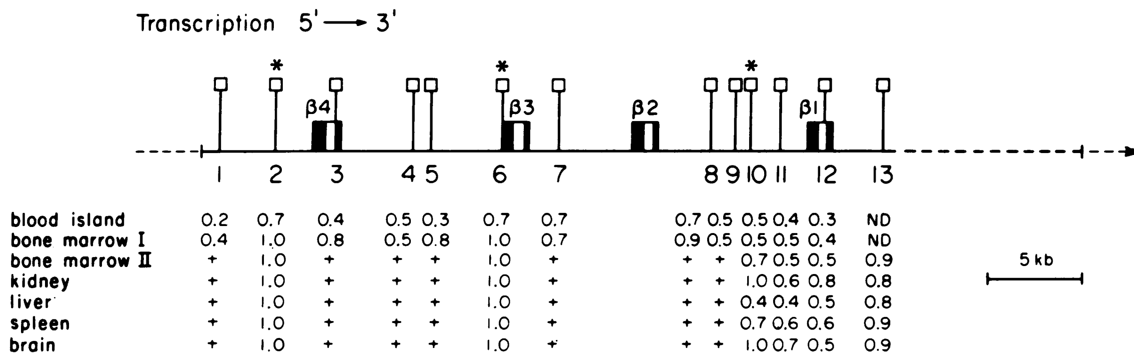


FIG. 5. Methylation of C-C-G-G sites surrounding the β1 globin gene. (*a*) Hybridization of (*Hpa* I + *Hpa* II)-digested blood island DNA (lane 1) or bone marrow I DNA (lane 2) with the β1 intron probe. (*b*) Hybridization of various restriction enzyme digests with the β1 intron probe. Lane 1, *Hpa* II-digested bone marrow I DNA; lane 2, (*Hpa* I + *Hpa* II)-digested kidney DNA; lane 3, *Hpa* II-digested kidney DNA; lane 4, (*Hpa* I + *Hpa* II)-digested liver DNA; lane 5, *Hpa* II-digested liver DNA; lane 6 (*Hpa* I + *Hpa* II)-digested brain DNA; lane 7, *Hpa* II-digested brain DNA.(*c*) Map of C-C-G-G sites surrounding the β1 gene. The *Hpa* I sites were mapped by E. Lacy (E. Lacy and T. Maniatis, unpublished results). The 4.1-kb band is a result of sequence polymorphism in the rabbit I DNA.

in kidney and brain DNA could be the result of complete methylation of site 10 or 12. However, in studies of (*Pvu* II + *Hpa* II) digests of DNA from different tissues (data not shown), the methylation of site 12 varies from 0.3 to 0.8 (Fig. 6). Thus, the absence of the 3.8-kb fragment in kidney and brain DNA is explained by the total methylation at site 10. The only adult nonerythroid tissue in which site 10 is partially methylated is the adult liver. The significance of this observation is not known because the liver has not been examined for the β-globin transcript.

Methylation of C-C-G-G Sites Surrounding the β2 Globin Gene. When the β2 intron was used to probe various restriction digests of DNA from erythroid and nonerythroid tissues, methylation at sites 8 and 9 (Fig. 6) was detected. However, these two sites are methylated to approximately the same extent in all tissues examined.

Methylation of C-C-G-G Sites of the Total Genome in Different Rabbit Tissues. To demonstrate that the extent of methylation of C-C-G-G sites within the globin gene cluster is not simply a reflection of the state of methylation of the genome as a whole, we have compared the fluorescent patterns of gel-fractionated *Msp* I and *Hpa* II digests of DNA from the rabbit blood islands, bone marrow (I and II), kidney, spleen, adult liver, and brain. In all cases, approximately 55% of the C-C-G-G sites in the total genome were found to be resistant to *Hpa* II digestion. This observation is consistent with the chemical analysis of DNA from different rabbit tissues (29).



	1	2	3	4	5	6	7	8	9	10	11	12	13
blood island	0.2	0.7	0.4	0.5	0.3	0.7	0.7	0.7	0.5	0.5	0.4	0.3	ND
bone marrow I	0.4	1.0	0.8	0.5	0.8	1.0	0.7	0.9	0.5	0.5	0.5	0.4	ND
bone marrow II	+	1.0	+	+	+	1.0	+	+	+	0.7	0.5	0.5	0.9
kidney	+	1.0	+	+	+	1.0	+	+	+	1.0	0.6	0.8	0.8
liver	+	1.0	+	+	+	1.0	+	+	+	0.4	0.4	0.5	0.8
spleen	+	1.0	+	+	+	1.0	+	+	+	0.7	0.6	0.6	0.9
brain	+	1.0	+	+	+	1.0	+	+	+	1.0	0.7	0.5	0.9

FIG. 6. Summary of the methylation study of rabbit β-like globin gene cluster. The four genes (β4 through β1) are represented by the black (exon sequences) and white (intron sequences) boxes in the top map. The positions of the C-C-G-G sites mapped are indicated by the small white boxes and labeled as sites 1–13. The C-C-G-G sites to the right (or 3′ end) of site 13 have not been mapped due to the lack of appropriate probes. Below the map is the list of the methylation frequency at each one of the 13 C-C-G-G sites sites in different tissues. The + symbol means that the methylation frequency of a specific C-C-G-G site in some tissues is approximately the same as that in the bone marrow I DNA. However, a calculation similar to that described in Fig. 2 has not been carried out. The methylation frequency of the site 13 in the blood island and bone marrow I DNA was not estimated (ND) because of the presence of sequence polymorphism at this position (see *Results*). The * on top of sites 2, 6, and 10 indicates that the three sites are totally methylated in the nonexpressing tissues (see text for details).

Mapping of C-C-G-G Sites Within Cloned Fragments of the Rabbit β-Like Globin Gene Cluster. We have performed *Msp* I digestion of the recombinant phage DNA containing the rabbit β-like globin genes as well as the subcloned *Eco*RI fragments isolated from the gene cluster. All 13 C-C-G-G sites derived from the genomic blotting experiments are confirmed. In particular, site 6 has been mapped within the small intron of the β3 globin gene. The existence of sites 3 and 12 is also consistent with the DNA sequence data (22, 30). There is one extra C-C-G-G site found within the 0.85-kb *Eco*RI fragment located at the 3′ end of β4 gene. It is most likely to be a result of restriction site polymorphism in the liver DNA from which the recombinant clones were derived.

DISCUSSION

The tissue-specific pattern of DNA methylation of 13 C-C-G-G sites within the rabbit β-like globin gene cluster is summarized in Fig. 6. The following conclusions can be made on the basis of this information:

(*i*) In general, C-C-G-G sites flanking the β3 and β4 globin genes that are transcribed in the embryo (sites 1, 2, 3, 5, and 6) are undermethylated in the DNA from embryonic erythroid tissue relative to the bone marrow cells and adult nonerythroid tissues.

(*ii*) With the exception of site 10, the pattern of methylation of C-C-G-G sites surrounding the adult β1 globin gene is not significantly different in expressing and nonexpressing tissues.

(*iii*) C-C-G-G restriction sites 2 and 6, which are located adjacent to or within the β4 and β3 gene, respectively, are totally methylated in all tissues except embryonic blood islands, where they are relatively undermethylated. Site 10, which is located near the β1 gene, is undermethylated in both embryonic and adult erythroid tissues. This site is also undermethylated in adult liver; the liver is an erythroid tissue in fetus but not in the adult. In the two nonerythroid tissues examined, the brain and kidney, site 10 is totally methylated.

A study of tissue-specific DNA methylation in a cluster of four differentially expressed human β-like globin genes was reported (12). It is interesting to note that an analogous pattern of differential DNA methylation is found in the human and rabbit β-like globin gene clusters. In particular, C-C-G-G sites 2, 6, and 10 in the rabbit cluster and sites 1, 2, 4, 6, 12, and 15 in the human cluster (12) are undermethylated in the tissues in which they are expressed and totally methylated in all other tissues.

The interpretation of the methylation pattern reported here is complicated by the fact that only a small fraction of CpG sites is detected in the restriction enzyme assay and by the fact that expressing tissues are heterogenous in cell type. However, the undermethylation of certain C-C-G-G sites can be correlated with gene activity in both the rabbit and human β-like globin gene clusters. It is interesting to note that the adjacent sites within any given region of a gene cluster do not display similar patterns of tissue-specific methylation. Thus, if methylation plays a role in differential globin gene expression, the sites of action may be only a few specific sequences within the gene cluster. These sequences may or may not be located within the transcribed globin genes. For example, tissue-specific methylation of site 6 within the β3 gene can be correlated with gene activity, but site 12 within the β1 gene is methylated to approximately the same extent in erythroid and nonerythroid tissues. Site 2, which also shows the tissue specificity, maps outside the β4 globin gene.

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