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DNA methylation in chicken α-globin gene expression

(restriction mapping/recombinant DNA/erythrocyte/differentiation)

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ABSTRACT **We have investigated certain specific methylation sites of the chicken α-globin gene cluster in DNA from embryonic and adult erythroid cells as well as from brain and sperm cells. Eight contiguous DNA fragments of the α-globin gene cluster were subcloned from a recombinant λ phage. The subclones were used as probes to map all the *Msp* I/*Hpa* II and *Hha* I sites in the unmethylated cloned DNA and specific sites of methylation in and around the α-globin gene cluster in chromosomal DNA. The data show that sperm DNA is totally methylated at these restriction sites in the globin gene region, as is brain DNA, with some exceptions. Interestingly, the methylation status of specific sites 5′ to the coding sequences is correlated with expression of the embryonic or adult α-globin genes in different stages of erythroid development. Some sites showing partial methylation, however, do not conform to the model that transcribed genes are unmethylated or undermethylated. We also find a well-defined 3.5-kilobase region of DNA 5′ to the α-globin gene cluster in which all C-C-G-G sites are resistant to *Msp* I digestion in all tissues. This "*Msp* block" is presumably caused by 5-MeCpC methylation.**

The phenomenon of enzymatic modification of bases in DNA is a subject of renewed interest as a possible mechanism for controlling gene expression. Several authors have suggested that methylation at specific sites has a role in selective expression of genes in differentiated tissues (1, 2). Recently, McGhee and Ginder (3) have noted an inverse correlation between 5-methylation of specific cytosines in DNA and transcriptional activity of the genes containing those cytosines. The β-globin genes in DNA of chicken erythrocytes were found to be hypomethylated when compared with the β-globin genes in DNA of other chicken somatic tissues (3). Similar results were obtained in the human β-globin gene cluster; Van der Ploeg *et al.* (4) assayed DNA from different tissues by using the restriction isoschizomers *Msp* I and *Hpa* II—the former enzyme can cleave $Cp^{m}CpGpG$ while the latter cannot (5). This protocol has been used by a number of other investigators in correlating undermethylation with actively transcribed genes in eukaryotes (6–12).

In the present study, we have mapped specific sites of methylation in the chicken α-globin gene cluster and correlated the level of methylation at each site with the state of differentiation in chicken erythroid tissues. We have used the restriction enzyme pair *Hpa* II/*Msp* I and the enzyme *Hha* I (G-C-G-C), which is sensitive to methylation of the internal cytosine. The chicken globin system is amenable to such a study because erythrocyte DNA can be prepared from embryos and adult animals in which the α-like globin genes are differentially expressed in development. The gene expressed only in embryos is π/π′-globin while embryos and adults express the α^{A}- and α^{D}-globin genes. The DNA of brain and sperm tissues of adult animals were also compared. We find that the hypomethylation–transcription relationship holds in some, but not all, regions of the gene cluster.

MATERIALS AND METHODS

Tissue DNA Extraction. DNA was prepared by the methods of Blin and Stafford (13) from the following chicken tissues: embryonic erythrocytes from blood of day 4.5 embryos, anemia erythrocytes from a phenylhydrazine-treated adult chicken, adult erythrocytes from whole blood of 3-day-old chicken purchased from Spafas (Norwich, CT), chicken sperm from rooster semen obtained from H. Robinson, Worchester Foundation for Experimental Biology, and adult brain from dissected adult animals. The DNAs were spooled from isopropanol (14) twice to remove RNA and then stored in 10 mM Tris base/0.1 mM EDTA, pH 7.5.

Subcloning. Clone λCαG5 was obtained from J. D. Engel and J. Dodgson (15) and contains the entire chicken α-globin gene cluster. Specific *Eco*RI, *Hin*dIII, and *Eco*RI/*Hin*dIII fragments of λCαG5 were ligated to pBR322 plasmid molecules and transformed into *Escherichia coli* C600 cells as described (16). Methods for growth of bacterial cells and extraction of plasmid DNA were as described (17). Plasmid inserts were prepared by digestion with the appropriate restriction enzymes and were labeled with ^{32}P by nick-translation (18). The plasmid containing the embryonic π′-globin cDNA was isolated in this laboratory by I. Roninson (personal communication).

Southern Hybridization. Enzyme-restricted DNA was analyzed by gel electrophoresis and transferred to nitrocellulose filters according to established procedures (19). Hybridization and washing procedures were as described by Jeffreys and Flavell (20). Some filters were successively hybridized with different probes. For this purpose, the filters were washed for 24 hr in sterile water at 65°C and then prehybridized and hybridized as above.

RESULTS

Subclones of the Chicken α-Globin Cluster. All *Hha* I and *Msp* I (*Hpa* II) sites in λCαG5 were mapped (Fig. 1). In this map, the subcloned restriction fragments used as probes are shown above the map as plasmid inserts, along with the positions of the adult α-globin genes α^{A} and α^{D}. The position of the embryonic α-like globin gene π was mapped by using the embryonic π′ cDNA clone. It appears from these results that π′, which differs from π by one amino acid, is an allele of π rather than a separate gene, since mapping data with this probe did not show any other π-globin gene in the chicken genome (results not shown).

Tissue-Specific Methylation of the α-Globin Cluster. The same set of subcloned probes was used to evaluate the level of methylation of specific restriction endonuclease sites in DNA from different tissues. For example, Fig. 1 illustrates a Southern

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Abbreviation: kb, kilobase(s).

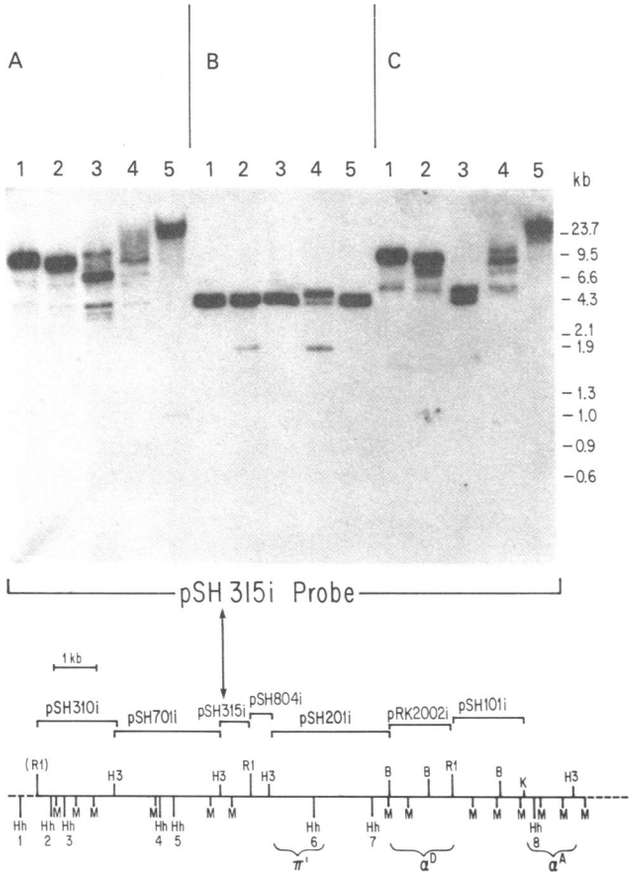

FIG. 1. *Hha* I (*A*), *Msp* I (*B*), and *Hpa* II (*C*) fragments of the α-globin cluster from tissue DNA. Lanes: 1–3, adult, anemia, and embryonic erythrocytes, respectively; 4, brain; 5, sperm. Southern blots of restricted DNA were hybridized with a radiolabeled *Eco*RI/*Hin*dIII fragment of the subclone pSH315. A map of the restriction sites of cloned DNA containing the α-globin cluster is shown below. Subcloned fragments are shown as bracketed sequences above the map. Positions of the embryonic α-like gene, π, and the adult genes α^A and α^D are also shown. The cloned DNA shown is Engel and Dodgson's λCαG5 (15), containing their *Eco*RI (R1), *Hin*dIII (H3), BamHI (B), and *Kpn* I (K) restriction sites; R1 is an artificial site introduced during construction of the recombinant phage. We determined the *Msp* I (M) and *Hha* I (H) sites (data not shown). *Hha* I sites are numbered for convenience; Hh-1 is a site in tissue DNA (data not shown).

hybridization in which DNAs from five chicken tissues—adult, anemia, and embryonic erythrocytes, brain, and sperm—were digested with restriction enzymes sensitive to cytosine methylation (*Hha* I, *Msp* I, and *Hpa* II). The probe used in this example was the 0.6-kilobase (kb) *Hin*dIII/*Eco*RI insert of plasmid pSH315, which is a fragment 5′ to the π-globin gene (Fig. 1). This probe hybridizes to those restriction enzyme fragments of tissue DNA that contain the probe sequence, but the sizes of the fragments will depend on the availability to digestion of the enzyme sites. Methylation blocks cleavage and larger fragments result. The detailed map of cloned unmethylated DNA shows a number of C-C-G-G (*Msp* I/*Hpa* II) and G-C-G-C (*Hha* I) sequences near the pSH315 region and throughout the α-globin cluster. Those sites that are present and cut in the unmethylated cloned DNA but are not cleaved in a particular tissue DNA are interpreted as being methylated in the DNA of that tissue. Thus, if the two *Hha* I sites (Hh-5 and Hh-6) flanking the pSH315 probe were unmethylated, a 3.5-kb fragment would be seen on the autoradiograph of Southern hybridization. However, none of the tissue DNAs show a 3.5-kb fragment. Adult and anemia erythrocyte DNAs have a very large hybridizing fragment, indicating blockage by methylation at several neighboring *Hha* I sites (i.e., G-^{m}C-G-C). Embryonic erythrocytes DNA produces two fragments, smaller than in the adult but larger than the expected 3.5-kb fragment, and hence must have more sites free of methylation than does the adult tissue DNA. Moreover, finding two fragments implies the presence of an *Hha* I site at which only some of the molecules in the population are methylated—i.e., partial methylation. Sperm DNA, which shows a single very large fragment, probably has fully methylated sites extending for a long distance on either side of the probe.

In contrast, the patterns of *Msp* I digestion are almost the same in all five tissues examined, as shown in Fig. 1*B*. Here, a prominent doublet of approximately 4.5 kb appears in each DNA sample, along with a faint 1.9-kb band. *Hpa* II recognizes the same sequence, C-C-G-G as *Msp* I but is inhibited by methylation of the *inner* cytosine. Tissue DNAs cut with *Hpa* II and then probed with pSH315i demonstrate very different restriction patterns than those cut with *Msp* I (Fig. 1*C*) and correspond in extent of methylation to those seen with *Hha* I digestion. Adult and anemia erythrocyte DNAs show intense large fragments, and the embryonic erythrocyte DNA, which is less methylated in the π-globin gene region, shows a doublet of 4.2- and 4.4-kb restriction fragments. The sperm DNA shows very large DNA fragments, indicating a high level of methylation near the globin genes.

A fine mapping experiment with the pSH315i probe is shown in Fig. 2. By carrying out a series of double digestions with

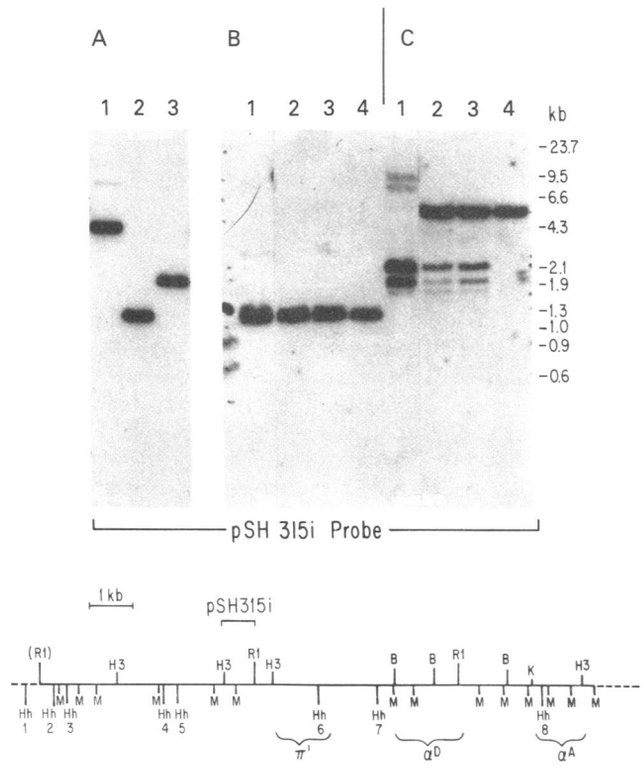

FIG. 2. Comparison of restriction sites in cloned (λCαG5) and tissue DNA. Southern blots of restricted DNA were hybridized to radiolabeled pSH315 insert fragments (5′ to the π-globin gene). (*A*) Autoradiograph of cloned DNA. Lanes: 1, *Hha* I; 2, *Hin*dIII; 3, *Hha* I/*Eco*RI. (*B* and *C*) Autoradiographs of *Hin*dIII- and *Hha* I/*Eco*RI-cleaved genomic chicken DNAs, respectively. Lanes: 1 and 2, adult and embryonic erythrocytes, respectively; 3, brain; 4, sperm. The *Hha* I/*Eco*RI pattern of the cloned DNA is not seen in the tissue DNA. Instead, genomic DNA has a tissue-specific pattern corresponding to partially open and closed *Hha* I recognition sequences.

methylation-insensitive and methylation-sensitive restriction enzymes, we can identify which particular site in tissue DNA is unavailable for cleavage. Hybridization with pSH315i of the restriction fragments of cloned DNA λCαG5, is shown in Fig. 2A. The *Hha* I fragment of λCαG5 that hybridizes to the pSH315i probe is 3.5 kb long and digestion with *Hin*dIII yields the 1.2-kb *Hin*dIII/*Hin*dIII fragment containing the probed region; the *Hha* I/*Eco*RI digestion results in a 1.9-kb fragment of DNA. These results were then compared with the restricted tissue DNA. The *Hin*dIII digest of genomic DNA shows the same 1.2-kb *Hin*dIII/*Hin*dIII fragment in all tissues. This result is expected, since *Hin*dIII is insensitive to inhibition by methylation. The *Eco*RI site defines the 3′ end of the probed region, so that the methylation state of the two *Hha* I sites (Hh-4 and Hh-5) in the 5′ direction can be examined. In adult erythrocyte DNA, intense restriction bands at 1.9 and 2.2 kb are seen. The 1.9-kb band corresponds to the 1.9-kb Hh-5–RI fragment of the *Hha* I/*Eco*RI digest of cloned DNA, and the 2.2-kb band is interpreted to mean that *Hha* I site Hh-5 is partially methylated. The complex digestion pattern remains unchanged when greater amounts of enzyme (10-fold excess) and longer incubation times (4 hr) were used (data not shown). Methylation is more extensive in embryonic erythrocyte DNA, as evidenced by the predominance of larger fragments. The 1.9 and 2.2-kb bands are present at much lower intensity, as is a 5.2-kb fragment, which cannot be due to the artificial *Eco*RI site at the 5′ end of the cloned DNA, but a *Hha* I site (Hh-1) in tissue DNA just beyond the insert of λCαG5 is present (results not shown). The four nearest *Hha* I sites (Hh-2, Hh-3, Hh-4, Hh-5) 5′ to the pSH315i probe must therefore be fully methylated in embryonic erythrocyte DNA. The pattern of brain DNA is similar to that of embryonic erythrocyte DNA in this particular experiment. Other preparations of brain DNA showed lesser amounts of the 2.2- and 1.9-kb bands. There may be some contamination with blood cells in brain preparations. Sperm DNA also shows a fully methylated pattern, and a single large restriction fragment results. Interestingly, the unmethylated Hh-1 site in sperm DNA is so far the only potentially sensitive site found around the globin genes in this tissue.

The mapping of methylated *Hha* I sites 5′ to the pSH315i DNA region (Hh-2, Hh-3, Hh-4, Hh-5) is confirmed when another probe, pSH701i, is used (Fig. 3). The pSH701i insert is the 2.55-kb *Hin*dIII fragment immediately 5′ to pSH315. Again, the 2.2- and 1.9-kb *Hha* I/*Eco*RI fragments (Hh-4–RI, Hh-5–RI) are seen in adult and anemia erythrocyte DNAs, in addition to two other fragments that hybridize to the pSH701i probe. The 0.3-kb *Hha* I fragment (Hh-4, Hh-5) (faint because of its small size) is seen in the adult erythrocyte DNA digest. In addition, a 3.0-kb *Hha* I fragment extends in the 5′ direction from Hh-4 to Hh-1. The faint 2.7-kb fragment (Hh-2–Hh-4) might have arisen from incomplete methylation at the Hh-2 site. By contrast, the most prominent *Hha* I/*Eco*RI band in the other tissue DNAs (embryonic erythrocyte, brain, and sperm) is the same 5.2-kb *Hha* I/*Eco*RI fragment seen with the pSH315i probe, which extends from Hh-1 to the *Eco*RI site in pSH315.

The filter probed with pSH701i was washed and then hybridized to a cDNA probe of embryonic π-globin cDNA. The *Hha* I/*Eco*RI digest of the tissue DNA confines the DNA region probed to the *Hha* I sites 3′ to the *Eco*RI site. There are two such *Hha* I recognition sites before the next *Eco*RI site (4.8 kb), one at the 3′ border of the π-globin gene (Hh-6) and one just 5′ to the adult α^D-globin gene (Hh-7). The results with all five tissue DNAs demonstrate the 4.8-kb *Eco*RI restriction band. Embryonic erythrocyte DNA in addition shows an *Eco*RI/*Hha* I fragment of 1.2 kb; hence, the *Hha* I site (Hh-6) at the 3′ end of the π-globin gene is only partially methylated. Interestingly,

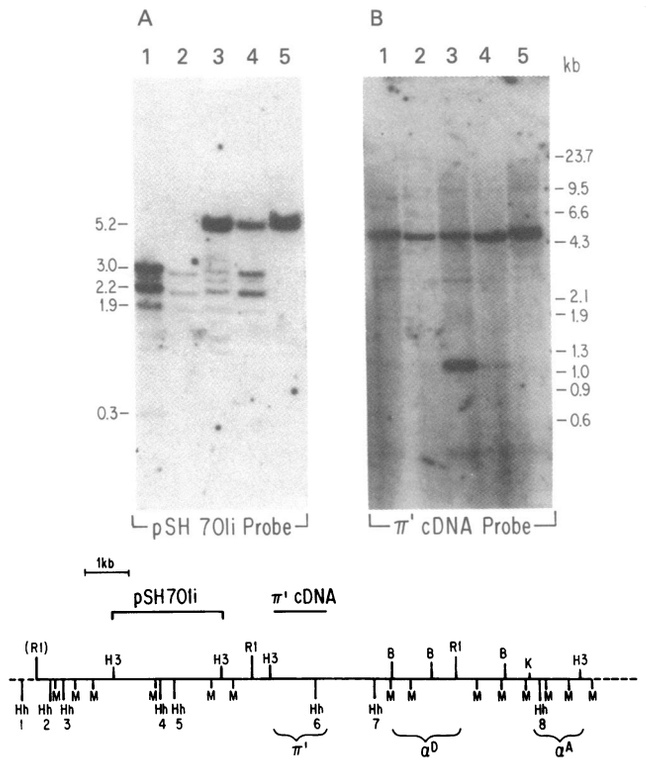

FIG. 3. Restriction mapping of *Hha* I methylation in tissue DNA. Double digestions with *Hha* I/*Eco*RI were carried out on tissue DNAs. (A) Southern blots were hybridized to the nick-translated pSH701i insert fragment. Adult erythrocyte DNA shows partially methylated *Hha* I sites spanned by this probe. (B) The same Southern blot was filter hybridized to a cloned π cDNA probe. Here, the *Hha* I site at the end of the π-globin gene is available only in embryonic erythrocyte DNA. Lanes: 1–3, adult, anemia, and embryonic erythrocytes, respectively; 4, brain; 5, sperm.

this site is fully methylated in adult and anemia erythrocyte DNAs as well as in brain and sperm DNAs.

Similar experiments using *Hha* I and the probes indicated in Figs. 1, 2, and 3 led to results summarized in Fig. 4. Mapping of *Msp* I/*Hpa* II sites was also carried out in a similar manner (data not shown). Parallel experiments with *Hpa* II and *Msp* I directly show the presence or absence of Cp^mCpGpG methylation, since the former enzyme is inhibited while the latter is not. The results of these experiments and those with *Hha* I are summarized in Figs. 4 and 5.

CpC Methylation in Tissue DNA. We have mapped several *Msp* I recognition sequences (C-C-G-G) in cloned DNA containing the α-globin gene cluster. In the region under study, seven sites are available to *Msp* I cleavage in all tissue DNAs. At the 5′ end of the α-globin cluster, however, there is a 3.5-kb region of DNA in which four *Msp* I sites are blocked in all tissues examined. Since all other restriction sites are identical, this phenomenon is probably not a result of rearrangement of the cloned DNA versus the tissue DNA. We therefore interpret these results as ^{m}CpC type methylation of tissue DNA, since *Msp* I will not cleave where this sequence is methylated (4).

DISCUSSION

We have examined the occurrence of DNA methylation, specifically 5-MeCpG and 5-MeCpC, in chicken tissues that show developmental regulation of expression of the α-like globin genes π/π', α^D, and α^A. A region of approximately 12 kb of DNA is involved. Erythroid differentiation in the chicken em-

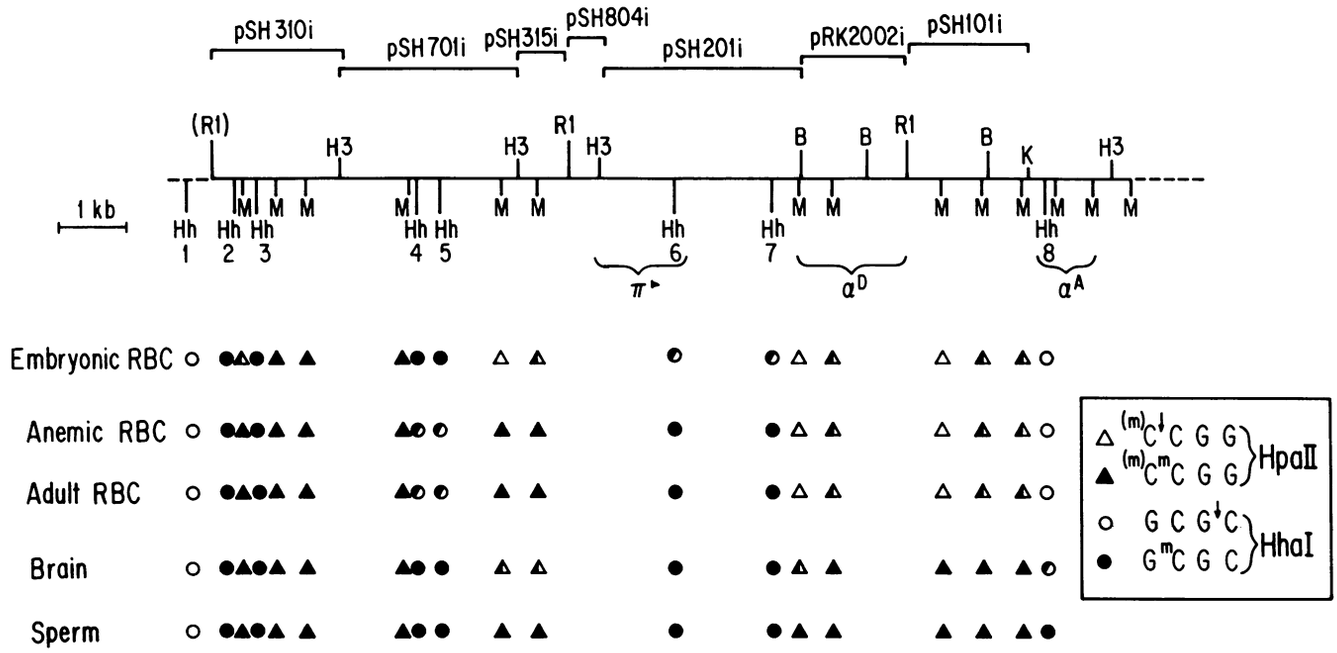

FIG. 4. Distribution of ^{m}CpG type methylation of the α-globin gene cluster in tissue DNA. Restriction analysis was carried out on cloned DNA to map all possible *Hha* I and *Msp* I (*Hpa* II) sites. This restriction map was then compared with restriction digestions of tissue DNA. Single and double digests were made for each tissue DNA and the mixtures were then hybridized to the subcloned probe inserts. △ and ○, Presence of an *Hpa* II or *Hha* I site available for enzymatic digestion; ▲ and ●, absence of such sites; ◭ and ◐, partial availability of enzyme sites. RBC, erythrocyte.

bryo consists of a change from an embryonic (primitive) to an adult (definitive) erythrocyte population and a switch in globin gene expression. Embryonic cells have as α-like globins mainly π-globin or its allele π' and smaller amounts of α^{D}- and α^{A}-globins. After 5 days of embryonic development, adult (definitive) cells begin to appear, a group of cells persisting into adult life. Adult cells express only α^{D}- and α^{A}-globin genes.

The role of tissue-specific DNA methylation in regulating gene activity is not clear, but it has been proposed by Ginder and McGhee (3) and by others (6–12) that the DNA of genes that are expressed in a tissue is unmethylated or undermethylated at specific sites within or near the genes concerned. Conversely, these sites are predicted to be methylated in DNA from tissues that do not express these genes. This model is supported by experimental evidence from gene transfer (21, 22) and drug inhibition studies (23); it is unknown, however, which specific methylated sites might be important in gene expression and which are not. Moreover, the techniques available can examine only those CpG or CpC sites that happen to be part of the recognition sequence of a small number of specific restriction enzymes.

Examination of the methylation status of *Hpa* II sites near the π/π'-, α^{D}-, and α^{A}-globin genes (Fig. 4) shows that the model is supported quite well by the data in this study. In par-

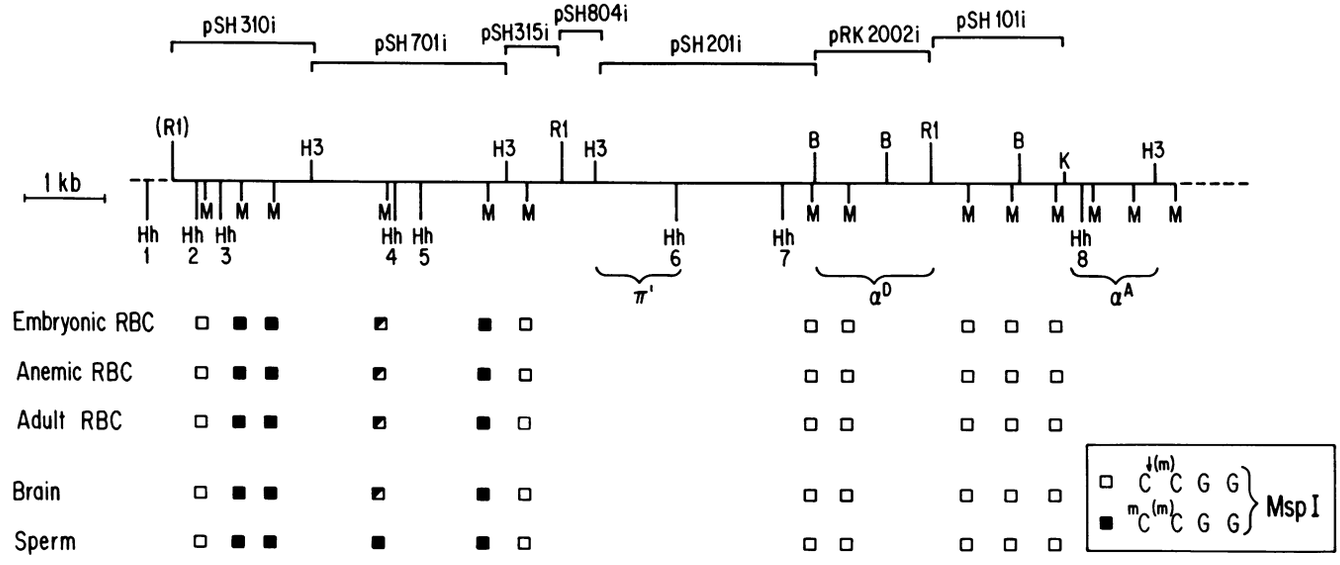

FIG. 5. Distribution of ^{m}CpC type methylation of the α-globin cluster in tissue DNA. *Msp* I is not affected by C^{m}CGG methylation. Single and double digestions of each tissue were carried out and these Southern blots were probed with the subcloned sequences. □ and ■, Presence and absence, respectively, of an *Msp* I site available for enzymatic digestion; ◩, partial availability of an *Msp* I site. RBC, erythrocyte.

ticular, an *Hpa* II site at the 5′ end of the α^D-globin gene and another just 3′ to the α^D-globin gene are unmethylated in embryonic as well as in adult erythrocyte DNA but are completely or partially methylated in brain and sperm DNA; both embryonic and adult erythrocytes express α^D-globin, while brain and sperm do not. Another *Hpa* II site inside the α^D-globin gene and two others just 5′ to the α^A-globin gene are only partially methylated in the DNA from cells expressing these genes but are fully methylated in brain and sperm DNA. There is also a pair of *Hpa* II sites 5′ to the π/π'-globin gene that are completely unmethylated or partially methylated, respectively, in DNA from embryonic erythrocytes, which express π/π'-globin, but are fully methylated in DNA from adult cells, and which do not make π/π'-globin. Brain DNA is partially methylated in these positions and therefore only partly supports the model; sperm DNA as expected is fully methylated here. Other *Hpa* II sites are fully methylated in all tissues and are perhaps too far away from the genes to be involved.

On the other hand, the methylation status of the *Hha* I sites does not always agree with the model. Sites Hh-6 and Hh-8, contained within the π/π'- and α^A-globin genes, respectively, are undermethylated in tissues that express those genes. There is considerable tissue-specific variation in the degree of methylation of *Hha* I sites Hh-4, Hh-5, and Hh-7, which is apparently unrelated to the status of expression of known genes.

Interestingly, there is a region of fully methylated *Hpa* II and *Hha* I sites between *Hha* I sites Hh-1 and Hh-4; methylation at these sites is not tissue specific. The region is bounded at its 5′ end by *Hha* I site Hh-1, which is unmethylated in all tissues including sperm DNA. In that same region is a long stretch of DNA containing four *Msp* I sites (Fig. 5) that are also blocked in all tissues. This is an unusual type of blockage that probably involves methylation of the outer cytosine of the C-C-G-G recognition sequence and thus must be the unusual 5-MeCpC type of methylation. Since most of these sites are also blocked to *Hpa* II, we conclude that there is a cluster of $^mCp^mCpGpG$ modification in many, and perhaps all, tissues. The region is sharply defined. Perhaps this feature is a part of the chromatin structure in that region.

The basis for finding partial methylation—on the order of 50%—at certain sites is not clear. Presumably, some DNA molecules are methylated while others are not. We do not know whether this represents heterogeneity in the cell population or whether the two homologous chromosomes within a cell differ. The possibility cannot be ruled out that only one strand of the DNA is methylated, instead of both strands. Yet another possibility is the presence of heterozygosity at certain sites where partial methylation is found, such that one allele has lost the enzyme recognition site while the other is either methylated or unmethylated in different tissues. Such a situation might exist at sites Hh-6 and Hh-7 or at three of the *Hpa* II sites between Hh-7 and Hh-8. Moreover, in the case of heterozygosity, we would expect some sites to be present in tissue DNA but absent in clone λCαG5.

Sperm DNA yields the same data as any other tissue DNA examined here when cut with methylation-insensitive enzymes such as *Eco*RI, *Bam*HI, *Kpn* I, and *Hin*dIII (data not shown). To a first approximation, no gross structural DNA rearrangement in the α-globin gene cluster has occurred during the developmental stages that we have examined.

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1. Riggs, A. O. (1975) *Cytogen. Cell Gen.* **14**, 9–25.
2. Holliday, R. & Pugh, J. E. (1975) *Science* **187**, 226–232.
3. McGhee, J. D. & Ginder, G. D. (1979) *Nature (London)* **280**, 419–420.
4. Van der Ploeg, L. H. T. & Flavell, R. A. (1980) *Cell* **19**, 947–958.
5. Bird, A. P. & Southern, E. M. (1980) *J. Mol. Biol.* **118**, 27–47.
6. Desrosier, R. C., Mulder, C. & Fleckenstein, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3839–3843.
7. Mandel, J. L. & Chambon, P. (1979) *Nucleic Acids Res.* **1**, 2081–2103.
8. Cohen, J. C. (1980) *Cell* **19**, 653–662.
9. Shen, C. K. & Maniatis, T. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6634–6638.
10. Weintraub, H., Larsen, A. & Groudine, M. (1981) *Cell* **24**, 333–344.
11. Sutter, D. & Doerfler, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 253–256.
12. Bird, A., Taggert, M. & Macleod, D. (1981) *Cell* **26**, 381–390.
13. Blin, H. & Stafford, T. (1976) *Nucleic Acids. Res.* **3**, 2303–2309.
14. Marmur, J. (1976) *J. Mol. Biol.* **3**, 208–212.
15. Engel, J. D. & Dodgson, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2596–2600.
16. Kaufman, R., Kretschmer, P., Adams, J., Coon, H., Anderson, W. F. & Nienhuis, A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4229–4233.
17. Benz, E., Kretschmer, P., Geist, C., Kantor, J., Turner, P. & Nienhuis, A. (1979) *J. Biol. Chem.* **254**, 6880–6888.
18. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
19. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–506.
20. Jeffreys, A. & Flavell, R. (1977) *Cell* **12**, 1097–1108.
21. Wigler, M., Levy, D. & Perucho, M. (1981) *Cell* **24**, 33–40.
22. Stein, R., Gruenbaum, Y., Pollack, Y., Razin, A. & Cedar, H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 61–65.
23. Sager, R. & Kovac, P. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 480–484.