Inheritance of Allelic Blueprints for Methylation Patterns

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Summary

We have developed a strategy to distinguish between the methylation patterns of homologous chromosomes in tissues, and to follow these patterns in human pedigrees. This genetic approach uncovered evidence of variation in the methylation of allelic sites on homologous chromosomes. This variation was tissue-specific and reproducible after transmission through the germ line, demonstrating that homologous chromosomes have distinct blueprints for the tissue-specific regulation of methylation. Furthermore, this approach can be used to study the relationship between parental imprinting and methylation in native mammalian loci.

Introduction

The methylation of deoxycytosines in CG doublets of mammalian DNA is a key developmental process, often invoked in models of cell differentiation (for reviews see Razin and Riggs, 1980; Holliday, 1987), X-dosage compensation (for reviews see Gartler et al., 1985; Lyon, 1988), and parental imprinting (Reick et al., 1987; Sapienza et al., 1987; Swain et al., 1987). Although it is believed that methylation plays a critical role in mammalian development, it is unclear whether methylation is a primary signal for phenomena such as gene expression (Razin and Riggs, 1980) or is a maintenance signal for patterns established by other mechanisms (Bird, 1986; Lock et al., 1987). This dichotomy of ideas arises in part from difficulty in generalizing key observations (e.g., hypomethylated genes are not always expressed; van der Ploeg and Flavell, 1980). Perhaps the resolution of this conflict will come from a better understanding of the phenomenon of methylation itself, especially its regulation during development.

We introduce a novel genetic approach to study mammalian methylation. The strategy has allowed us to distinguish between the methylation status of identical sites in homologous chromosomes (henceforth referred to as allelic sites) and to follow the methylation patterns of these sites in three-generation human pedigrees. We have uncovered evidence that variation in the methylation status of autosomal allelic sites in human tissues is heritable and that allelic sites with identical methylation for one tissue can differ for other tissues, suggesting that variant "blueprints" for the regulation of methylation are transmitted through the germ line unchanged. Furthermore, this genetic approach can be used to study the relationship between methylation and parental imprinting in native human loci, obviating the use of transgenes for similar studies in other mammals (e.g., mice).

Experimental Strategy

A methylation-sensitive enzyme (Bird and Southern, 1978) was used to generate methylation patterns that could be visualized on Southern blots (Southern, 1975) with radio-labeled locus-specific probes. We chose HpaII, which digests CCGG but not C^mCGG (Bird and Southern 1978), because its isoschizomer, MspI, digests both C^mCGG and CCGG. The contribution of each allele to the methylation pattern was defined with the help of restriction fragment length polymorphisms.

Polymorphism in each of the loci we studied reflects variable numbers of small tandem repeats (VNTRs) (Bell et al. 1982; Nakamura et al. 1987); in a heterozygous individual a different number of repeats is present in each of the two alleles of a VNTR locus. Since the methylation sites tested in these experiments surrounded the repeats at each VNTR locus, we were able to distinguish the methylation patterns of maternally derived alleles from those of paternally derived alleles in heterozygous individuals on the basis of allele-size differences.

To optimize resolution of the methylation pattern of each allele, the DNA samples were subjected to further digestion with a methylation-insensitive enzyme(s) whose restriction site(s) flanks closely both the methylation site(s) analyzed and the VNTRs. For example, in Figure 1 the HpaII site is flanked by two BamHI sites. The improved resolution after this second digestion allowed the simultaneous analysis of several methylation sites flanking the repeats.

In this protocol, an allele's methylation pattern is defined by the number of bands present and by the relative intensity of those bands as measured by densitometry. In Figure 1 there are five bands in all heterozygous individuals: four bands with repeats, and a band of constant length that does not include the repeats. The number of bands obtained for each allele reflects the number of sites analyzed. Polymorphic variation permits unequivocal separation of the bands in the methylation pattern that belong to each of the alleles. The father in kindred 1418 in Figure 1 is heterozygous at the 3.4B2 locus: his larger allele (with more repeats) was inherited from his father, his smaller allele from his mother.

Sites methylated in some cells of a given tissue and not methylated in others will be referred to as "partially methylated." For example, heterozygous individuals in Figure 1 have four bands that include the repeats: two bands around 1.2 kb digested by HpaII, and two others around 3.3 kb left undigested by HpaII, presumably because of methylation.
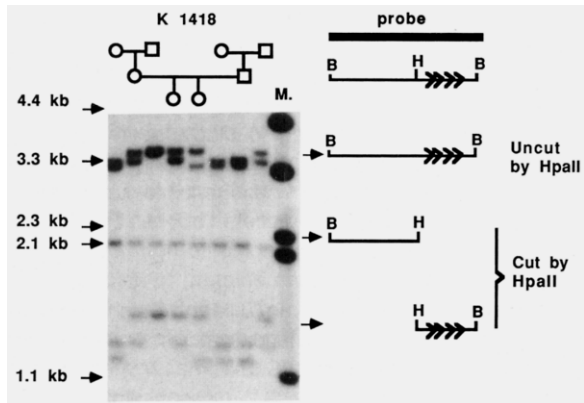
Figure 1. Segregation of Methylation Patterns of the *3.4B2* Locus

The autoradiograph shows a Southern transfer of DNA samples from peripheral blood of members of kindred 1418. DNA was digested with BamHI and HpaII, electrophoresed in a 1% agarose gel, and hybridized with radiolabeled p3.4B2 (Silva et al., 1987). Lane M contains size markers, labeled at left. The pedigree drawn above indicates the sex and kinship of all the samples (circles = females; squares = males). The map of the HpaII and BamHI cleavage sites, the fragments expected from the digestion, and the position of the probe are shown at right. Arrowheads indicate tandem repeats. B = BamHI; H = HpaII.

Results and Discussion

Variation in the Methylation of Allelic Sites in Peripheral Blood and in Colon

The strategy outlined above allowed us to analyze the methylation patterns of each allele in diploid loci. Surprisingly, we found loci in homologous chromosomes that were not identically methylated.

Figure 2A shows an example of variation of methylation between alleles of the *JCZ67* locus. The pJCZ67 probe contains a 4.6 kb MspI fragment with a VNTR marker, cloned in pUC18. When hybridized to MspI-digested DNA from peripheral blood of an individual (2004) heterozygous for *JCZ67*, pJCZ67 revealed two fragments, one from each allele (see lane 1 in Figure 2A), and no other fragments. Since BamHI sites flank the MspI fragment containing the repeats (see diagram in Figure 2A), pJCZ67 also hybridized to two bands (larger than those generated by MspI) in Southern blots of the same DNA digested with BamHI. Double digestions with BamHI and HpaII of the same DNA (Figure 2A, lane 2) generated six bands, three from each allele. Since no pair of bands in Figure 2A, lane 2, was identical in size to the pair in lane 1, we concluded that at least one of the two MspI sites closest to the repeats is highly methylated in the peripheral blood of this individual. However, another site close to the repeats must be only partially methylated in blood, since we can detect two fragments (marked with circles), one from each allele, that are slightly larger than the MspI fragments (Figure 2A, lane 1; marked with crosses). The difference in size between these two fragments is identical to the size difference between the MspI-cut fragments. Two larger bands (marked with squares in Figure 2A, lane 2) with sizes proportional to the allelic pairs just described reveal another partially methylated HpaII site further from the
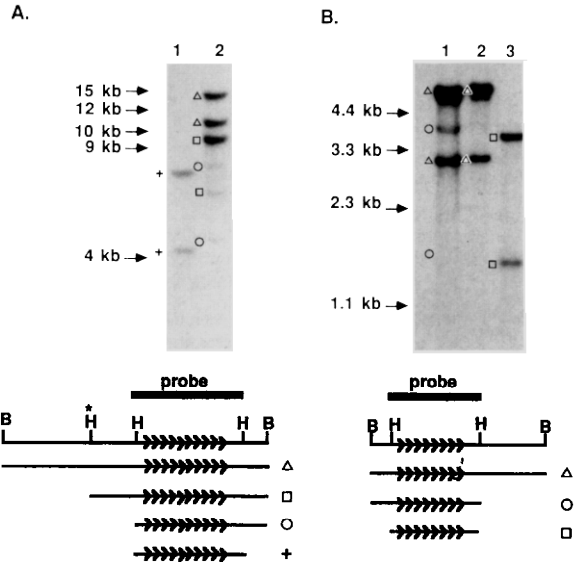

Figure 2. Variation of Allelic Methylation in Two Human Tissues

(A) DNA isolated from peripheral blood of individual 2004 was digested with MspI (lane 1) or with BamHI and HpaII (lane 2). Samples were electrophoresed in a 0.6% agarose gel and transferred to a nylon membrane; the membrane was probed with radiolabeled pJCZ67 and exposed to X-ray film for various times. Here the exposure oversaturated the darker bands to reveal the lighter ones. (B) DNA isolated from normal colon of individual 1795 was digested with BamHI and HpaII (lane 1), BamHI alone (lane 2), or MspI (lane 3); the samples were electrophoresed, transferred to a nylon membrane, and hybridized to radiolabeled pYNH24.

Beneath both autoradiographs are diagrams for the interpretation of the restriction fragments. Symbols identify each fragment class in the autoradiographs. B = BamHI; H = HpaII. The asterisk in (A) marks a site of the smaller allele that is methylated to a greater extent in peripheral blood than the same site in the larger allele.

repeats. Unlike the other pairs of bands just described, the difference between the intensities here is striking: the larger band is markedly more intense than the smaller one. This intensity difference is not caused by the greater number of repeats in the larger allelic band, because the MspI allelic bands (Figure 2A, lane 1), which also contain the VNTR marker, have similar intensities. Densitometry readings of the intensities of the bands in the methylation pattern of the small allele (from the largest to the smallest) are 78%, 16%, and 6% (where the numbers represent the percentage of the total intensity of all the bands contributed by the allele). Similar densitometry for the large allele revealed substantial differences: 33%, 57%, and 10%, respectively. The smaller allele has a site (marked with an asterisk in the diagram in Figure 2A) methylated in more cells of peripheral blood than is the large allele, demonstrating that allelic sites can be methylated to different extents in the same tissue. A key implication of this analysis is that some cells in peripheral blood of individual 2004 must have one of the homologs methylated and the other unmethylated at that site within the same nucleus.

Figure 2B shows another example of variation in the methylation of allelic sites within the same tissue. In this
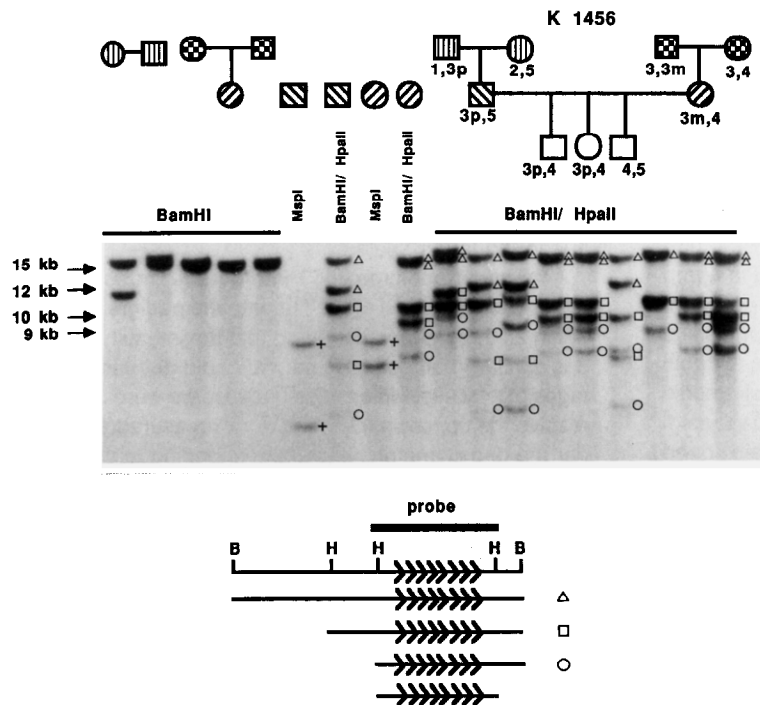
Figure 3. Inheritance of Variant Methylation Patterns of the *JCZ67* Locus

DNA samples isolated from peripheral blood of members of kindred 1456 were digested with BamHI and HpaII (see diagram of kindred above autoradiograph at right); DNA samples from the grandparents and the mother were also digested with BamHI alone; in addition, DNA samples from both parents were again digested with BamHI and HpaII and with MspI side by side. All samples were electrophoresed in a 0.6% gel, transferred to a nylon membrane, and probed with pJCZ67. Below is a diagram of the restriction fragments observed; each class of fragments in the diagram has a symbol that identifies it in the autoradiograph. B = BamHI; H = HpaII.

case the variation is in normal colon cells, and the locus is *YNH24*. Even though the larger allele is clearly visible on the allelic pair digested by HpaII (bands with circles), the smaller one is almost invisible. The intensity ratio between the two alleles digested with MspI (Figure 2B, lane 3) is 2.2/.99; the intensity values were measured as the areas under the densitometric tracings. However, the intensity ratio between the variable allelic bands in the BamHI–HpaII double digest is 3.7/(<0.3) (bands with circles in lane 1). This differs strikingly from the ratio expected for these alleles if they were equally methylated (3.7/1.7).

Transmission of Methylation Patterns in Human Pedigrees

In the last section we demonstrated that the methylation patterns of allelic loci may vary. In this section we establish that the blueprints for the variant methylation patterns are transmitted through the germ line unchanged.

Since individual 2004 (Figure 2A) is part of Utah kindred 1456 (Figure 3, the father), we followed the segregation of his two differentially methylated alleles of the *JCZ67* locus (alleles 3p and 5). Comparisons of methylation patterns at this locus in three generations of the pedigree demonstrated that the two allelic variants were stably inherited. Densitometry measurements of the small allele (allele 5) in the paternal grandmother, father, and one of the siblings yielded the following relative intensities: 71% ± 8%, 21% ± 6%, and 8% ± 2% for the larger, the middle, and the smaller bands, respectively. (The numbers are presented with one standard deviation.) Similar measurements on the other allele in individual 2004 (allele 3p) yielded 34% ± 7%, 55% ± 2%, and 11% ± 6%. Statistical analysis

of the intensities of the large and middle bands in the methylation patterns of alleles 5 and 3p yielded a significant difference at the 98% confidence level (T distribution). The intensities of the smaller bands in the two alleles are similar. Allele 4 is the only other allele segregating in all three generations that is represented in the autoradiograph in Figure 3; its pattern is 36% ± 11%, 51% ± 9%, and 14% ± 6%, respectively, for the larger, middle, and smaller bands. Since the band intensities in alleles 4 and 3p are within one standard deviation of each other, we cannot argue that they represent different degrees of methylation. Allele 3m (from the maternal side) has a different pattern, again consistent in all three generations (data for the third generation not shown): 64% ± 8% and 56% ± 8%, respectively, for the larger and medium bands. Absence of the smaller band could be due to a high degree of methylation at one of the HpaII sites close to the repeats (see diagram in Figure 3) or to a base pair mutation of that HpaII site. Our Southern data on the MspI restriction digest of the locus exclude the latter possibility (see the MspI digest of the mother in kindred 1456 and the diagram of the restriction sites in Figure 3).

Figure 4 illustrates the segregation of variant methylation patterns at the *YNH24* locus. In the last section we demonstrated that the alleles of the *YNH24* locus in colon cells of individual 1795 were methylated differently. Since we were not likely to obtain colon samples from complete kindreds, we searched for allelic variation at this locus in peripheral blood. However, the sites shown to be variant in colon were completely methylated in the peripheral blood of 21 unrelated individuals tested. Hence, we looked further from the repeats by choosing a methylation-insensitive enzyme (BglII) that yielded larger fragments than
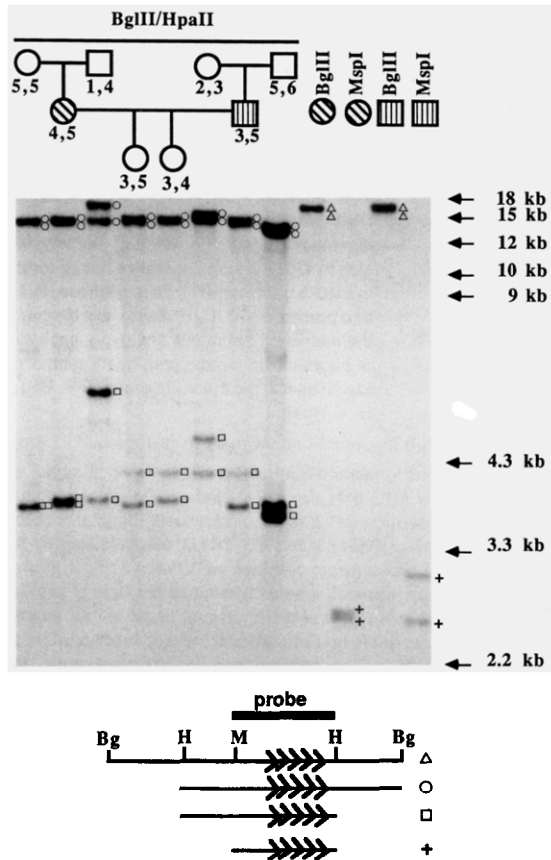
Figure 4. Inheritance of Variant Methylation Patterns of the YNH24 Locus

DNA samples isolated from peripheral blood of members of kindred 1459 were digested with BglII and HpaII; DNA samples from the parents were also independently digested with BglII and MspI. All samples were electrophoresed in a 0.6% agarose gel and probed with radiolabeled pYNH24. Fragments in the autoradiograph are represented in the diagram below; symbols identify each fragment class.

BamHI, with more methylation sites. The probe used was pYNH24, a 2 kb MspI fragment containing the entire VNTR region, cloned in pUC18; an MspI digest of DNA from peripheral blood of the father in kindred 1459 (Figure 4) shows that he is heterozygous for YNH24. The intensities of the two MspI bands were similar (51% of the total inten-

sity for the larger band and 49% for the smaller band; bands marked by crosses in Figure 4). However, an allelic pair of HpaII-digested bands (marked by squares in Figure 4) from the same individual shows a striking difference in intensity: densitometry readings were 4.4 times more intense in the smaller band. Because the intensities of the MspI bands were nearly identical, we concluded that a site in the smaller allele is methylated in fewer cells than it is in the larger allele.

Analysis of pedigree 1459 in Figure 4 shows that the larger allele (allele 3) of the father in the pedigree is present in all three generations and that the intensity of allele 3 is similar in all generations. We could not use percentages of total allele intensities because we were unable to resolve the upper bands. Hence, we measured areas under the densitometric peaks corresponding to the bands of interest. The area for allele 3 was 36 ± 13 (all three generations), while the area for the maternal allele 5 was 85 ± 07. Unlike previous measurements of percentage of total allele intensity, these do not account for differences in the amount of total DNA loaded in each lane, so standard deviations were larger. T-distribution analysis gave us 90% confidence that alleles 3 and 5 were of different intensities but that the same allele in different generations had the same intensity. This is another example of allelic variants in methylation inherited in a codominant Mendelian fashion.

Table 1 summarizes results on the number and frequency of variant methylation patterns found in peripheral blood for ten loci studied. Each locus was analyzed in at least two pedigrees with heterozygous parents and grandparents. DNA from peripheral blood of each individual in the pedigrees selected was digested with HpaII and then with a methylation-insensitive enzyme optimized for band resolution on Southern blots. As controls, all pedigrees were also independently digested with MspI and the methylation-insensitive enzyme. In all cases the methylation status (partially methylated, fully methylated, or completely unmethylated) of the sites studied remained unchanged (see Figures 1, 3, and 4 for examples).

There are differences in the frequency of variants among the loci studied (Table 1). For example, YNZ22 revealed highly polymorphic methylation patterns in 21 unrelated heterozygous individuals tested, while YNH37 did not show any variation among 16 unrelated heterozygous

Table 1. Summary of Methylation Data for Ten Loci in Peripheral Blood

Locus	Chromosome Localization	Pedigrees Tested	Sites Partially Methylated	Variant Sites and Frequency[a]
YNZ22	17p	6	2	1, 21/22
YNH37	17p	3	2	0, 0/16
YNH24	2q	3	2	1, 3/21
CMM65	16p	2	2	0, 0/9
JCZ67	7q	3	3	1, 3/9
RMU3	17q	2	3	0, 0/5
MCOC12	14	2	1	0, 0/5
c-Ha-ras-1	11p	3	4	0, 0/12
3.4B2	14q	3	1	0, 0/15
CMM101	14q	4	2	0, 0/8

[a] Ratios of unrelated heterozygous individuals with variant methylation patterns to total number of heterozygous individuals tested.

BamHI/MspI

BamHI

BamHI/HpaII

N.C N.C C.O. C.C N.C

4.3 kb→

△ allele 1
△ allele 2

3.1 kb→

non-VNTR band "b"

2.3 kb→

non-VNTR band "a"

□ allele 1

□ allele 2

1.1 kb→

○ allele 1

○ allele 2

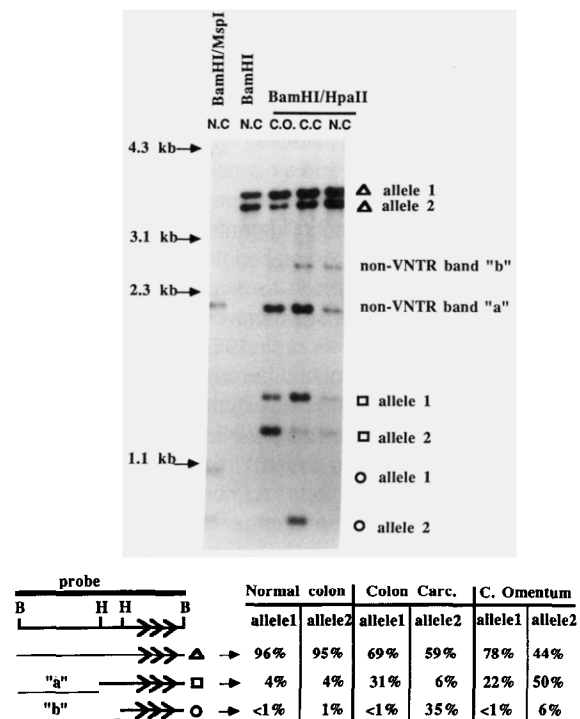| probe			Normal colon		Colon Carc.		C. Omentum	
B H H B			allele1	allele2	allele1	allele2	allele1	allele2
→→→ △ →			96%	95%	69%	59%	78%	44%
"a" →→→ □ →			4%	4%	31%	6%	22%	50%
"b" →→→ ○ →			<1%	1%	<1%	35%	<1%	6%

Figure 5. Comparison of Methylation Patterns of the Locus 3.4B2 in Three Different Tissues from Individual 1795

DNA was extracted from normal colon (N.C.), a colon carcinoma (C.C.), and a metastatic carcinoma in the omentum (C.O.) from individual 1795. The samples were digested as indicated with BamHI alone, BamHI and MspI, or BamHI and HpaII, electrophoresed, transferred to a nylon membrane, and probed with radiolabeled p3.4B2. Below the autoradiograph is a diagram of the fragment classes obtained, and a table with the densitometry measurements of the methylation patterns of each of the alleles. Each horizontal column in the table corresponds to a fragment size class in the diagram. B = BamHI; H = HpaII.

individuals. Curiously, the methylation patterns of the same loci revealed extreme variation in cell lines of B lymphocytes transformed with Epstein-Barr virus. These variations were not inherited in these cell lines derived from members of pedigrees in which heritable allelic methylation for peripheral blood had been demonstrated (data not shown). This observation was consistent with evidence suggesting that the methylation patterns of cells tend to drift in culture (Reis and Goldstein, 1982; Wigler et al., 1981; Pollack, 1980). All the variants detected were in sites partially methylated.

Allelic Differences in Methylation in Other Tissue Samples

It is well established that the extent of methylation of certain genes can vary from one tissue to another (for review see Razin and Riggs, 1980). In the previous section we described a site in the YNH24 locus that is completely methylated in blood and is methylated to varying extents for different alleles in colon. As part of the present study, we compared heterozygous methylation patterns in three tissue samples from the same individual: normal colon, a colon carcinoma, and a carcinoma in the omentum. The

DNA isolated from these tissues was digested with the combinations of enzymes indicated above the autoradiograph in Figure 5. Densitometric analysis of the autoradiograph (3.4B2 locus) shows that one of the MspI/HpaII sites closest to the repeat (marked with circles in the diagram and in the autoradiograph in Figure 5) is consistently more methylated in the larger allele in all the tissue samples. However, densitometry readings of the two bands (one from each allele) marked with squares in the autoradiograph of Figure 5 show that the second site, further from the repeats, is equally methylated in both alleles in normal colon, more methylated in the smaller allele in a colon carcinoma, and less methylated in the smaller allele in a carcinoma in the omentum. As predicted, these changes in intensity are accompanied by proportional changes in the bands digested by BamHI but not HpaII (marked by triangles). This feature of the strategy can be a control for other phenomena that could affect band intensity, such as the extent of DNA transfer to the nylon membrane, unequal hybridization to the radiolabeled probe, or aberrant X-ray film exposures. Curiously, the 3.4B2 locus had revealed no allelic methylation differences in peripheral blood (Table 1 and Figure 1). Furthermore, one of the sites that was partially methylated in colon was completely methylated in the peripheral blood of 15 unrelated individuals tested.

Three other loci (YNH24, YNZ22, and c-Ha-ras-1) were tested in the same DNA samples described above. The methylation of the YNZ22 alleles was different in the three tissues sampled. However, we did not detect any variation in the methylation of the YNH24 and the c-Ha-ras-1 loci in the three tissue samples. The tissue samples we analyzed were polyclonal; even the carcinoma samples included cells from surrounding tissue. Thus, it is possible that the differences in methylation observed between the tissues analyzed reflect their varied cell composition.

Methylation in Sperm versus Peripheral Blood

The results described in previous sections suggest that cell types have specific methylation patterns. The only homogeneous cell type that we studied was sperm (which are resistant to lysis in the absence of dithiothreitol while lymphocytes in semen are not; Giusti et al., 1986). We compared methylation in sperm and peripheral blood for three unrelated individuals; Figure 6 illustrates the results. All eight loci studied had homogeneous methylation patterns in sperm but not in peripheral blood: some sites were fully methylated, others were fully unmethylated, but none were partially methylated in sperm.

A band in the larger YNZ22 allele of the peripheral blood of individual S001 (Figure 6A, top triangle in blood B/H lane) has half the intensity of its counterpart in the smaller allele (second triangle from the top), even though the intensity of the two allelic bands is similar in a MspI digest (Figure 6A, blood M lane); the opposite is true for the allelic bands marked with squares (Figure 6A). However, both alleles are fully unmethylated in sperm (the MspI pattern was similar to the BamHI–HpaII pattern in Figure 6A).

In peripheral blood, locus YNZ22 was partially methylated at two sites flanked by two BamHI sites. The same methylation sites in sperm were apparently completely un-
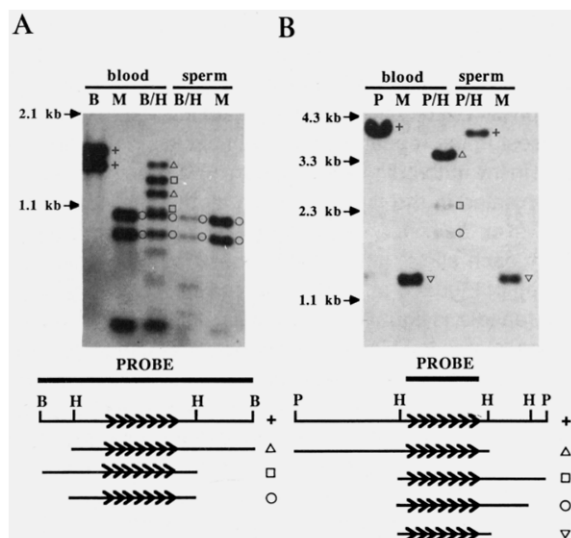
Figure 6. Comparison of Methylation Patterns of Blood and Sperm for Two Loci, *YNZ22* and *MCOC12*

DNA was isolated from blood and semen of individuals S001 (A) and R001 (B). DNA extracted from semen was depleted of cells other than sperm; a small degree of contaminating lymphocytes might have escaped the purification steps. The samples were digested with the combinations of enzymes shown (B = BamHI; H = HpaII; M = MspI; P = PvuII), electrophoresed, and transferred to nylon membranes. Membranes were probed with pYNZ22 (A) or pMCOC12 (B). Below the autoradiographs are diagrams of the fragments obtained.

methylated. The opposite was true for the locus *MCOC12* (Figure 6B): sites partially methylated in peripheral blood were completely methylated in sperm (the PvuII pattern was identical to the PvuII–HpaII pattern in Figure 6B). The results were consistent in the three individuals tested. Previous studies have reported that human satellite DNA (Gamma-Sosa et al., 1983) and low-copy sequences (Ehrlich et al., 1985) are hypomethylated in sperm relative to other adult tissues. Here we provide evidence that single-copy sequences can also be hypomethylated in sperm and that specific sites unmethylated in sperm can become partially methylated in adult tissues, confirming claims that de novo methylation events occur during development and that tissue-specific patterns are not solely the result of selective demetylation events. However, only two of eight loci studied in sperm were unmethylated at the sites observed, corroborating other reports on the hypermethylation of single-copy sequences in sperm (Rahe et al., 1983) and on the abundance of demethylation events in development.

Conclusions

We have developed the foundation for a genetic approach to the study of methylation in DNA. One of the key features of our strategy is the ability to distinguish between the methylation status of sites in homologous chromosomes and then to follow the methylation of these allelic sites through human pedigrees. Since VNTRs are ubiquitous in the human genome (Nakamura et al., 1987) and in the ge-

nomes of other mammals (Jeffreys et al., 1987; Jeffreys and Morton, 1987), this methodology will be applicable in the study of biological phenomena involving methylation, such as parental imprinting.

Since the methylation patterns of the ten loci we studied were inherited unchanged from parents of both sexes, the methylation of these loci was not parentally imprinted. In contrast, the methylation of "imprinted" transgenic loci in mice changes with the parental origin of the allele: maternally derived alleles tend to be hypermethylated, while paternally derived ones are more often hypomethylated (Sapienza et al., 1987; Reik et al., 1987; Swain et al., 1987). The combined frequency of differentially methylated "imprinted" loci in mice deduced from transgenic experiments (Sapienza et al., 1987; Reik et al., 1987; Swain et al., 1987), and assuming random integration of the transgenes, is 6/21, in strong contrast to our results (0/10). This suggests either that the phenomenon is less frequent in humans or that the disparity between the two ratios arises from differences between the methods. The experiments in mice measured the methylation of hemizygous transgenic loci, while our experiments analyzed undisturbed diploid loci in humans.

Studies on X-chromosome inactivation in females revealed differences in methylation between homologous loci in the active versus the inactive X-chromosome (for review see Lyon, 1988). Even though these chromosome-specific modifications are somatically inherited, they are apparently erased in the germ line. While the studies described here were in progress, Chandler et al. (1987) suggested that allelic versions of the c-Ha-*ras*-1 gene in human cell lines are not equally methylated. However, the instability of methylation in cultured cells demonstrated by these authors and by others (Reis and Goldstein, 1982; Wigler et al., 1981; Pollack et al., 1980) complicates the interpretation of their results. The allelic differences found by Chandler et al. (1987) could be accounted for by random drift in the methylation of the two alleles due to this instability in cells in culture. To avoid this problem, we focused our analysis on uncultured human tissues (colon, blood, carcinomas).

We demonstrated that homologous chromosomes can have different methylation patterns in uncultured tissue samples. In peripheral blood these patterns are always reproducible after transmission through the germ line; since methylation changes throughout development, the entity responsible for the Mendelian inheritance of methylation must not be the methylation status of the gametes, but the elements segregating with chromosomes that regulate methylation. Changes in these elements could be responsible for the variations between allelic sites. These *cis*-acting elements could either be sequences surrounding the methylation sites, proteins that are associated with the site, or DNA structures (Cedar, 1988) (or a combination of these). Weissbach et al. (1985) and Bolden et al. (1986) showed that synthetic oligonucleotides with high GC content are preferentially methylated de novo by HeLa DNA methylases, and Bird (1986) has suggested that the hypomethylation of "HTF islands" is caused by a permanent association with transcription factors that obstruct

the activity of the methylases. Furthermore, Wang et al. (1986) isolated a methylation- and sequence-specific human protein. We did not detect gross sequence differences between the alleles with variant methylations.

The loci studied all contain VNTRs, which could result in structural variations around the methylation sites tested. Since chromosome structure has been implicated in the regulation of methylation (Cedar, 1988; Groudine and Conkin, 1985; Razin and Riggs, 1980), we compared the methylation status of sites within alleles of different sizes for the loci that showed allelic variation in methylation. However, the number of tandem repeats does not appear to affect the regulation of methylation at VNTR loci, since we found numerous examples of alleles with the same number of repeats that were methylated differently, and alleles of different sizes that were identically methylated. Furthermore, the methylation sites studied were not within the repeats.

The putative cis-acting elements must be close to the sites to explain the complete cosegregation observed in three generations for all loci with allelic variations in methylation. Since we found variation of methylation patterns in three out of ten loci, it is likely that most pairs of homologous chromosomes will prove to have polymorphic methylation patterns at numerous places along the chromosome in any given tissue. In addition, meiotic recombination might increase the diversity in methylation among homologs by reshuffling variant segments between them. The heritable allelic variation in methylation demonstrated here implies that each chromosome carries a unique blueprint for methylation. The apparent abundance of differences in methylation between homologs suggests that most are not likely to affect gene expression. Perhaps these variant methylation sites are reference points for phenomena that require the distinction between homologs.

Allelic sites equally methylated in one tissue were shown to be methylated to different extents in other tissues (sperm versus blood, colon versus blood, and colon versus carcinomas). These differences are unlikely to be caused by somatic mutations at CG sites because the methylation patterns showed Mendelian inheritance; furthermore, several tissues within the same individual revealed "partially methylated" patterns, which would require unusually high rates of somatic mutations. Since we have shown that the methylation patterns of peripheral blood cells are inherited, it is unlikely that the differences observed between tissues are random. This suggests that the postulated cis elements interact with cell-specific factors in the regulation of methylation and that the differences documented between the tissue samples reflect the various cell types of the samples tested. This interpretation is consistent with the results of others (Razin et al., 1985) and with our results for the analysis of sperm, the only homogeneous cell population that we studied. Unlike other samples, which contained a mixture of cell types, sperm cells contained sites that were either completely methylated or unmethylated.

The trans-acting factors could be proteins that either modify the methylases or interfere with them. For example, rabbit liver contains methinin, an inhibitor of DNA methylation (Lyon et al., 1987).

It is unlikely that every cell type possesses a unique methylase complex and a specific cis-acting element for each site. However, permutations of a small number of these elements could account for the complexity and the specificity documented here and in other reports.

We have demonstrated that methylation is a dynamic process because we saw differences in the methylation of independent sites, of the same site in different alleles, and of allelic sites in different tissues. However, we have also shown that this diversity is tightly regulated since the blueprints for these complex patterns are stably inherited in pedigrees. The results presented here suggest that homologous autosomes might carry different developmental programs for methylation.

Experimental Procedures

DNA Samples and Isolation
Blood samples were collected in tubes of acid–citrate–dextrose solution. High molecular weight DNA was isolated from white blood cell nuclei and from tissues as described by Wyman and White (1980).

Enzyme Digestion, Electrophoresis, and Hybridizations
Restriction enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, and Molecular Biology Resources and were used under the conditions specified by the supplier. Human DNA samples were digested with a 5- to 10-fold excess of enzyme; reactions were monitored for completion by mixing an aliquot of the digestion mixture containing 0.5 μg of human DNA with 0.5 μg of an appropriate marker DNA for parallel digestion. HpaII (Molecular Biology Resources enzyme) digestions were done as above except that each sample was digested twice, and each time the controls reported full digestion. Electrophoresis in agarose was as described elsewhere (Wyman and White, 1980). Nylon-66 charged Biotrace membrane was used in Southern blot transfers (Southern, 1975). The membranes were hybridized with 8 × 10⁶ cpm of probe per ml of hybridization solution (50% formamide, 5× SSC, 0.02 M NaPO₄ [pH 6.7], 100 μg/ml of total human carrier DNA, and 10% dextran) at 42°C. Labeling of DNA with [³²P]dCTP was done by the random primer method (Feinberg and Vogelstein, 1984). Routinely, hybridization membranes were washed at 65°C for up to 1 hr with 0.1× SSC, 0.1% SDS.

Analysis of Methylation Patterns
The analysis of the methylation patterns proceeded in three stages. First, we identified the bands in the pattern belonging to each of the alleles, and compared the sizes of the bands obtained with those resulting from single digestion with MspI and with the methylation-insensitive enzyme used. Using this information we constructed a map accounting for the pattern seen; with this map we determined the number, the status (methylated, partially methylated, or unmethylated), and the order of the methylation sites assayed. The diagrams of the restriction sites in all the figures illustrate these results. Second, we measured the intensities of the bands with a densitometer, using exposures that maximized the linear response of the film. Finally, we calculated the sum of all band intensities for each allele and the percentage of the total allele intensity present in each band. When we could not resolve all the allelic bands (as in Figure 4), we compared intensities from sample to sample without normalizing them for total intensity in each allele. The intensity was measured as the area under the densitometric tracing corresponding to the respective band in the methylation pattern. Bands that did not include the repeats (e.g., the band around 2.2 kb in Figure 1) were not included in the calculations. Since the fragments that do not include the repeats are not polymorphic, we could not separate the contributions of each of the alleles to the total band intensity.

Densitometry

Measurements were done in a Beckman DU-8 spectrophotometer with the Scan II software package. Background was subtracted with reference to the "defined lowest valley," and the areas under the peaks were automatically calculated by the machine.

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