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The Somatic Replication of DNA Methylation

Michael Wigler, Daniel Levy, and Manuel Perucho
Cold Spring Harbor Laboratories
Cold Spring Harbor, New York 11724

Summary

We have tested the hypothesis that DNA methylation patterns are replicated in the somatic cells of vertebrates. Using M-Hpa II, the modification enzyme from Haemophilus parainfluenzae which methylates the internal cytosine residues in the sequence $\overset{5'\ CCGG}{3'\ GGCC}$, we methylated bacteriophage ϕX174 RF DNA and the cloned chicken thymidine kinase (tk) gene in vitro and then introduced these DNAs and unmethylated controls into tk$^-$ cultured mouse cells by DNA-mediated transformation. Twenty-five cell generations later, the state of methylation of transferred DNA was examined by restriction endonuclease analysis and blot hybridization. We conclude that methylation at Hpa II sites is replicated by these cultured cells but not with 100% fidelity. We have also noted that methylation of the cloned chicken tk gene decreases its apparent transformation efficiency relative to unmethylated molecules.

Introduction

Methylated bases are found in the genomic DNA of both procaryotes and eucaryotes and arise by postreplicative modification of the elementary bases usually at the 5 position of cytosine or the N-6 of adenine. The function and mechanism of methylation in higher eucaryotes are not understood. In vertebrates the only modified base found in DNA is 5-methylcytosine (Vanyushin et al., 1970) which is found primarily at cytosine residues occurring in the palindromic sequence $\overset{5'\ CG}{3'\ GC}$ (Sinsheimer, 1955; Diskocil and Sorm, 1962). Much has been learned about the distribution of methylated sequences within the vertebrate genome through the use of restriction endonucleases, such as Hpa II, Sma I and Hha I, which will not cleave methylated sequences (Gautier et al., 1977; Bird and Southern, 1978; Waalwijk and Flavell, 1978a). Such studies reveal tissue-specific differences in methylation patterns (Waalwijk and Flavell, 1978b; McGhee and Ginder, 1979; Mandel and Chambon, 1979; van der Ploeg and Flavell, 1980). It has been proposed that methylation of newly replicated DNA strands is mediated by a methylase which acts preferentially on a hemimethylated palindromic sequence (Holiday and Pugh, 1975; Riggs, 1975; Bird, 1978). Evidence in favor of this mechanism has been presented by Bird (1978) who found that the randomly methylated sites of Xenopus ribosomal genes were mostly fully methylated (that is, both strands methylated) rather than hemimethylated (that is, only one strand methylated). This model predicts that DNA methylation patterns are heritable in somatic cells. We describe a series of experiments designed to test this point. A methylase, M-Hpa II, was purified from Haemophilus parainfluenzae which methylates the internal cytosines in the sequence $\overset{5'\ CCGG}{3'\ GGCC}$ (Mann and Smith, 1977). These sequences form a subset of the potentially methylatable sites in vertebrates (Bird and Southern, 1978; Waalwijk and Flavell, 1978a). Using M-Hpa II, we have methylated in vitro either bacteriophage ϕX174 RF DNA or the cloned chicken thymidine kinase (tk) gene (Perucho et al., 1980a). Methylated or unmethylated molecules were then introduced into cultured mouse cells by DNA-mediated transformation (Wigler et al., 1979b), and the methylation state of these sequences was examined in host cells 25 generations later by restriction endonuclease digestion and blot analysis. We conclude from our studies that DNA methylation is heritable.

Results

Inheritance of Methylation within the Chicken tk Gene

In initial experiments we used either the plasmid pchtk-2, containing the complete chicken tk gene as a 2.25 kb Eco RI–Hind III insert in pBR322, or the charon 4A clone λchtk-1, containing additional chicken sequences flanking the gene (Perucho et al., 1980a). This gene contains one Sma I site and at least five asymmetrically clustered Hpa II sites, designated H1 through H5, which are located in essential regions of the gene (see Figure 1a). The Hpa II sites $\left(\overset{5'\ CCGG}{3'\ GGCC}\right)$ and Sma I site $\left(\overset{5'\ CCCGGG}{3'\ GGGCCC}\right)$ were methylated or mock-methylated by M-Hpa II, and the extent of methylation was monitored by resistance to digestion with Hpa II, which will not cleave partially or fully methylated sequences. Methylated (>95% molecules contain no accessible Hpa II sites) and mock-methylated molecules were used to transform a tk$^-$ mouse line, Ltk$^-$ (Kit et al., 1963), to the tk$^+$ phenotype, and transformants were selected in HAT medium. We noted 3–20 times fewer tk$^+$ colonies following transformation with methylated than with mock-methylated DNA (Table 1). Independent transformants were grown to 5×10^7 cells (about 25 cell doublings) and cellular DNA prepared.

To examine methylation within the chicken tk gene residing in these transformants, DNAs were digested with Xba I and Hpa II or Xba I and Sma I and the digests analyzed by blot hybridization using ^{32}P-labeled chicken tk sequences as probe (Figure 2). In the first series of experiments, we analyzed six transformants obtained using the mock-methylated gene

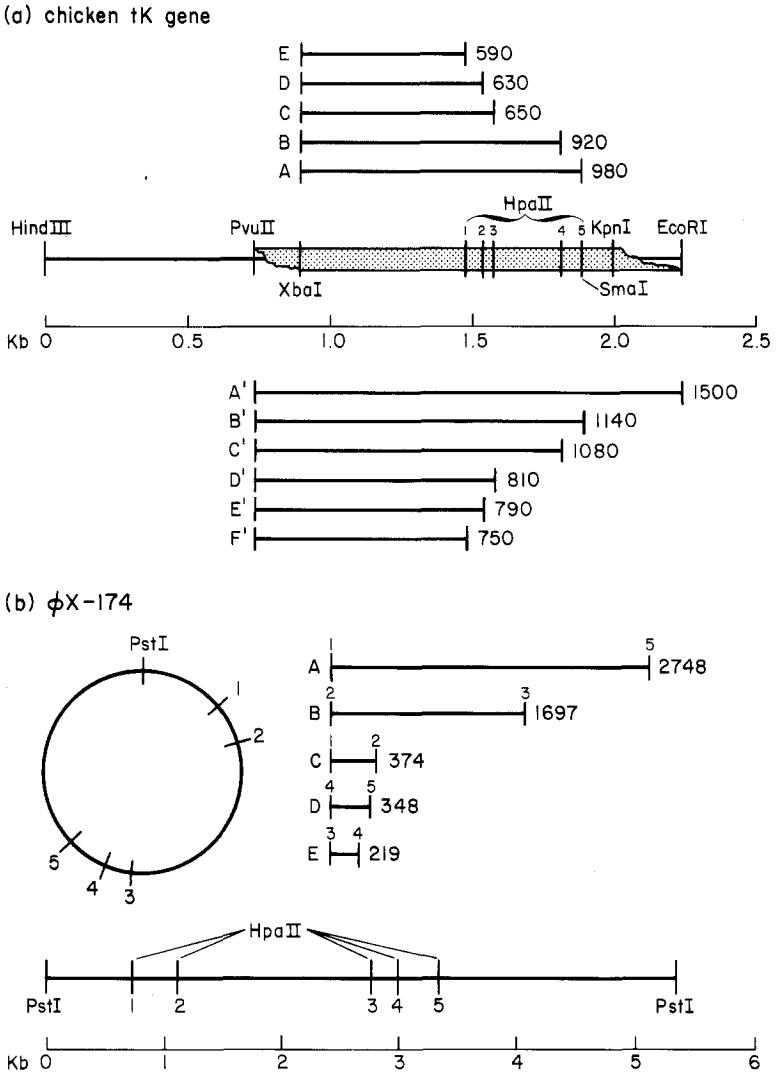

Figure 1. Position of Hpa II Sites in Chicken tk and φX174 DNA

(a) The map of the 2.25 kb Hind III–Eco RI fragment encoding chicken tk is shown with the restriction sites for Pvu II, Xba I, Sma I and Kpn I indicated as reported (Perucho et al., 1980a). These single-cut sites were determined by double digestion. Additional Hpa II sites are indicated and these were mapped as follows: H1 was mapped by double digestion, and others by the method of Smith and Birnsteil (1976). The positions of H1, H2 and H3 have been confirmed by sequencing (J. Kwoh, unpublished results). Above the map are listed the sizes in bp of the five restriction fragments corresponding to Xba I complete, Hpa II partial digestions. Below the map are the Pvu II complete, Hpa II partial digests. The minimum area encoding the chicken tk gene is stippled (Perucho et al., 1980a). (b) A circular and Pst I linearized map of φX174 RF is shown with the positions of the five Hpa II sites indicated. On the right are displayed the lengths in bp of the five Hpa II complete digestion fragments.

and six obtained using methylated tk (Expt. 1, Table 1). The tk genes resident in transformants obtained using mock-methylated tk (Figure 2a) are completely digested by Hpa II. A band of 590 bp is seen in Xba I–Hpa II digests and no traces are seen of partial digestion products. In contrast (Figure 2b), the H1 site of chicken tk is partially protected against Hpa II digestion in each cell line obtained by transformation with the methylated chicken tk gene. The 590 bp Hpa II–Xba I band is present but additional bands of about 630–650 and 920 bp are also present. We estimate that 30–70% of H1 sites are protected from Hpa II digestion in these lines. In separate experiments, DNA digested with Msp I, which cleaves methylated Hpa II sites (Waalwijk and Flavell, 1978a), gave complete digestion patterns indicating that resistance of tk sequences to Hpa II digestion was not due to mutation. We conclude that in cell populations arising by transformation with the methylated chicken gene, a proportion (which we estimate to be 30–70%) of H1 sites are methylated even after 25 cell generations. Moreover, the presence of additional bands of higher molecular weight in Hpa II digests indicate that sites H2 and H3 are also often methylated. On the other hand, populations arising by transforming with unmethylated chicken tk sequences display no evidence of methylation at the H1 site.

We can assess the state of methylation at the unique Sma I site of chicken tk in a similar manner. The Sma I recognition sequence (Endow and Roberts, 1977) is also a recognition sequence for Hpa II and is methylated by M-Hpa II (personal observation). Like Hpa II, Sma I will not cleave its recognition sequence if the central cytosine residues are methylated (personal observation; Gautier et al., 1977). Figure 2 shows that the chicken tk gene from all transformants is extensively cleaved by Sma I yielding a 980 bp Sma I–Xba I fragment. This site is thus not heavily methylated in any line. If, however, a small proportion of sites were methylated, tk gene sequences would be distributed into high molecular weight digestion fragments which might not be readily detected.To examine this ques-

Table 1. Apparent Transformation Efficiencies with Methylated and Mock-Methylated tk Genes

Expt.[a]	DNA[b]	Cleavage	Methylation[c]	Colonies[d] per ng	Colonies[d] per plate	R[d]
1	pchtk-2	Eco RI–Hind III	+	0.21	161/3	0.33
	"	"	–	0.62	618/4	
2	"	Eco RI	+	0.01	1/3	0.05
	"	"	–	0.20	20/3	
	"	Hind III	+	0.04	4/3	0.05
	"	"	–	0.88	88/3	
3	λchtk-1	uncut	+	0.75	12/5	0.02
	"	"	–	~40	~500/5	
4	pchtk-5	"	+	0.025	2/4	0.05
	"	"	–	0.50	41/4	
5	λchtk-1	"	+	0.75	128/3	0.22
	"	"	–	3.4	554/3	

[a] Several transformation experiments were performed over the course of nine months.

[b] DNA from pchtk-2 or λchtk-1 (Perucho et al., 1980a), or pchtk-5, a pBR322 derivative containing the 3.0 kb Hind III fragment encoding the chicken tk gene subcloned from λchtk-1, were used in transformation experiments using Ltk$^-$ DNA as carrier as previously described (Wigler et al., 1979a).

[c] tk genes were either methylated (+) or mock methylated (–) in vitro as described in Experimental Procedures.

[d] From 1 to 50 ng per dish of tk gene was used and colonies per ng were calculated from the total number of colonies appearing after growth of cultures for 12–16 days in HAT medium. R is the ratio of colonies per ng obtained for methylated versus unmethylated tk donors within a given experiment. DNA from λchtk-1 give consistently higher transformation efficiencies than DNA from plasmid clones (Perucho et al., 1980a).

tion more closely, we prepared a new set of transformants using methylated and mock-methylated λchtk-1 (Expt. 3, Table 1). Transformant colonies were grown into mass culture (25 cell doublings) and DNA was prepared. DNAs were digested with either Eco RI–Pvu II, or Eco RI–Pvu II–Sma I, and analyzed by blot hybridization using ^{32}P-labeled tk probe (Figure 3). Eco RI–Pvu II digestion of tk yields a 1500 bp fragment. When the Sma I site is not methylated, triple digestion with Eco RI–Pvu II–Sma I yields an 1140 bp fragment. Thus the ratio of 1140 to 1500 bp fragments in triple digests is a measure of the proportion of Sma I sites that are methylated. A limit to the sensitivity of this analysis is that the chicken tk probe hybridizes faintly to sequences in mouse DNA, and one such fragment unfortunately comigrates with the 1500 bp Eco RI–Pvu II chtk fragment (lane N, Figure 3). Within these limits we estimate that not greater than 15% of the Sma I sites are protected in any line whether transformed with methylated or mock-methylated tk genes. In these same lines, we found the H1 site highly protected when derived from the methylated λchtk-1 (data not shown).

Inheritance of Methylation at the Hpa II Sites of φX174 in Cotransformed Cells

Experiments similar to those carried out for the chicken tk gene were performed using the double-stranded replicative form DNA of bacteriophage φX174. φX174 DNA contains five Hpa II sites (Figure 1b). Molecules methylated in vitro were greater than 95% resistant to cleavage with Hpa II. Biochemical analysis indicated that greater than 98% of all Hpa II sites were in fact methylated (see Table 2). Methylated or mock-methylated φX174 DNA was introduced into cultured cells either as the uncleaved circular molecule or the Pst I cleaved linear by the method of unlinked cotransformation using the HSV-1 tk gene as the selectable marker (Wigler et al., 1979b). Several transformants were grown into mass culture (25 cell doublings) and DNA prepared. The φX174 copy number was determined by blot hybridization after digesting host cellular DNA with Hind III (data not shown), which does not cleave within the φX174 genome. The tk$^+$ transformants resulting from this procedure contained as usual multiple copies of φX174 sequences, the exact number varying between individual transformants. We observed no significant difference in either the frequency of cotransformation (7 out of 11 transformants versus 6 out of 8) or the number of φX174 sequences integrated into cotransformed cells (15 versus 12 per cell) when using methylated versus mock-methylated molecules.

To evaluate the extent of methylation at Hpa II sites within the φX174 sequences resident in host cells, we digested cellular DNAs with either Hpa II or Msp I and analyzed digestion products by blot analysis using ^{32}P-labeled φX174 as probe (Figure 4). Cells cotransformed with circular φX174 contain all five internal Msp I specific restriction fragments of sizes 2748, 1697, 374, 348 and 219 bp (lanes A, G, K and M). The reason for this is that although the φX174 sequences are integrated, the points of integration are probably random, thus generating an apparently circular restriction map. Cells transformed with linearized φX174 lack only the 2748 bp fragment (lanes C, E and I). For comparison of the Msp I and Hpa II digestions we must focus on the internal fragments, since

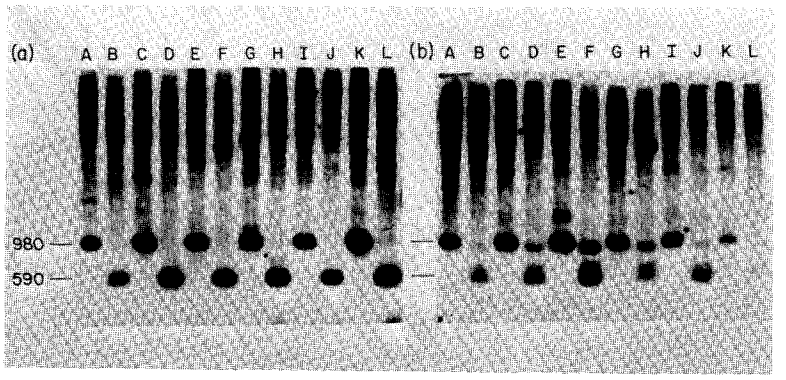

Figure 2. The Blot Hybridization Patterns Are Shown for Sma I–Xba I and Hpa II–Xba I Digests of DNA from Ltk$^-$ Cells Transformed with the Mock Methylated (a) or Methylated (b) R1–Hind III Fragment of Chicken tk

High molecular weight DNAs were extracted from clones MMchtk-1, -2, -3, -4, -5 and -6 (a) and Mchtk-1, -2, -3, -4, -5 and -6 (b) 25 generations after transformation, and 10 μg of each was digested with Xba I and Hpa II (lanes B, D, F, H, J and L, respectively) or with Xba I and Sma I (lanes A, C, E, G, I and K, respectively). DNAs were electrophoresed in 1.5% agarose gels, transferred to nitrocellulose filters and probed with the gel-purified 1.3 kb Xba I–Eco RI chicken tk fragment ^{32}P-labeled by nick translation to a specific activity 2×10^8 cpm/μg. Lines on the left indicate the position and size (in bp) of the Xba I–Sma I and the Xba I–Hpa II fragments of chicken tk. Controls for the completeness of digestion are described in Experimental Procedures.

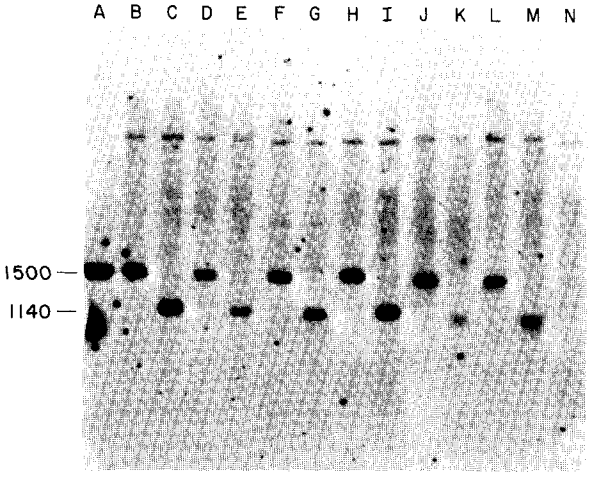

Figure 3. The Blot Patterns Are Shown for Eco RI–Pvu II and Eco RI–Sma I–Pvu II Digests of DNA from Ltk$^-$ Cells Transformed with Methylated or Mock-Methylated λchtk-1

DNA from two cell lines derived following transformation with mock-methylated λchtk-1 were digested either with Eco RI–Pvu II (lanes B and D, respectively) or with Eco RI–Sma I–Pvu II (lanes C and E, respectively). DNA from four cell lines derived following transformation with methylated λchtk-1 were digested either with Eco RI–Pvu II (lanes F, H, J and L, respectively) or with Eco RI–Sma I–Pvu II (lanes G, I, K and M, respectively). (Lane A) 20 pg of pchtk-2 digested with Eco RI–Pvu II, with 5 μg of uncleaved Ltk$^-$ DNA as carrier. (Lane N) 10 μg of Ltk$^-$ DNA cleaved with Eco RI–Sma I–Pvu II. Blots were hybridized with the 1140 bp Sma I–Pvu II fragment of chicken tk ^{32}P-labeled by nick translation to 5×10^6 cpm/μg. The numbers on the left indicate the position and size (bp) of the Eco RI–Pvu II and Pvu II–Sma I fragments of the chicken tk gene (see Figure 1a).

the variation in the intensity of other bands will reflect methylation of cellular sequences flanking ϕX174 specific sequences. Figure 4 shows that Hpa II digestion of DNA from lines cotransformed with mock-methylated ϕX174 DNA generates internal fragments of intensities equal to those generated by digestion with Msp I (lanes A–H). Thus in these lines, the Hpa II sites of ϕX174 are not significantly methylated. By the same criterion, however, the Hpa II sites of the lines derived by cotransformation with methylated ϕX174 DNA are heavily methylated (lanes I–N). We estimate that the internal fragments generated by Msp I digestion are about 4-fold more intense than those generated by Hpa II digestion. Hence at least 50% of each Hpa II site within the ϕX174 sequences is still protected. We conclude that there must be replication of methylation at these sites.

Quantitative Assessment of Inheritance

While our studies indicate that cells do replicate methylation patterns it seems that they do not do so with complete fidelity. After 25 cell generations, all sites in the sequences we introduced are accessible in degrees to Hpa II cleavage. From estimates of the proportion of methylated sites we can make estimates of the fidelity of inheritance. We shall assume in the following calculations that the frequency of transmission of methylation is a constant per generation and that there is no selective pressure in favor of loss of methylation. In the following we may therefore be underestimating fidelity.

Let $P_{1,n}$ be the proportion of sites on double-stranded DNA that is hemimethylated just prior to DNA synthesis after n cell generations and $P_{2,n}$ be the proportion that is fully methylated. Then $P_n = P_{1,n} + P_{2,n}$ is the proportion of Hpa II protected sites. Let q be the probability that when a site on the template strand is already methylated then the daughter strand will become similarly methylated. Finally, let r be the probability of de novo methylation. For the following we assume that r is small in comparison to q and that r will be ignored. This assumption is justified by our inability to observe any spontaneous methylation of exogenous sequences in transformed cells. We also assume that the absence of methylation in progeny strands can arise only by the failure to methylate. This assumption is based on the lack of demethylating activity in animal cells (Burdon and Adams, 1968). Nevertheless, even if there is a low probability of demethylation, it would not distort our mathematical model. If s is the rate of demethylation, its main

Table 2. Completeness of in Vitro Methylation of ϕX174

Fragment(s)	(kb)	Methylated[a]	cpm in Mononucleotides[b] dAMP	dCMP	dmeCMP	dGMP	TMP		R[c]
A	2748	+	NS	16	915	NS	NS	<	0.02
B	1697	+	27	75	2643	20	18		0.02
C & D	374 & 348	+	20	48	3209	19	17	<	0.02
E	219	+	27	31	986	NS	NS		0.02
B	1697	−	NS	3380	NS	NS	NS		—
C & D	374 & 348	−	NS	3207	NS	NS	NS		—

[a] M-Hpa II methylated (+) or mock-methylated (−) ϕX174 was digested with Msp I, the 5′ ends were labeled with ^{32}P and single-stranded restriction fragments were resolved in denaturing gels. Fragments were eluted from gel slices, hydrolized with DNAase I and snake venom phosphodiesterase, and 1000–3000 cpm of mononucleotide digestion products were fractionated by high-pressure, reverse-phase liquid chromatography (see Experimental Procedures for details).

[b] Fractions were assayed by Cherenkov counting for 10 min, and cpm in mononucleotides are the summation of peak fractions after subtraction for background radiation. NS indicates that counts were less than two standard deviations from the mean background radiation.

[c] R is the ratio of dCMP to dmeCMP calculated after correction for end-labeled randomly nicked molecules which comigrated with specific restriction fragments. (—) Not applicable.

contribution could be subsumed by a new rate factor $q' = (q - s)$. There would be a very small contribution from the loss of both methyl groups from a fully methylated site. This factor would be of the order s^2 and could therefore be ignored. The following recursive equations describe the state of methylation of Hpa II sites for an exponentially expanding clonal population:

$$P_{1,n+1} = (1 - q)P_{2,n} + \tfrac{1}{2}(1 - q)P_{1,n}.$$

$$P_{2,n+1} = qP_{2,n} + \tfrac{1}{2}qP_{1,n}.$$

We take our initial conditions to be $P_{1,0} = 0$ and $P_{2,0} = 1$; that is, the initial transforming molecule is fully methylated. This is justified by our biochemical analysis of molecules methylated in vitro (see Experimental Procedures). From these equations we can compute the degree of protection against Hpa II digestion after n cell generations, as a function of transmission, q. These computations are presented in Table 3 for $q = 0.80$ to $q = 0.99$ at $n = 25$ and $n = 50$ cell doublings.

From our estimate of 30–70% protection at the H1 site of chicken tk, we estimate that the fidelity of inheritance is 94 ± 3% per generation at this site. The values for the H2 and H3 sites are probably similar. On the other hand, inheritance of methylation at the Sma I site is probably less than 85% per generation. Similarly, we estimate that the fidelity of inheritance of methylation at Hpa II sites within ϕX174 is about 95%.

Discussion

Our studies show that cultured cells will replicate the DNA methylation patterns established in vitro at Hpa II sites and that mechanisms must exist whereby template strands direct the methylation of daughter strands at complementary sites. In our transformation experiments we have found no evidence for de novo methylation, although others have (Desrosiers et al., 1979; Sutter and Doerfler, 1980; Pollack et al., 1980). We must in any event consider the possibility that most somatic methylation is inherited and that differences in the methylation patterns observed in different tissues of the same organism (McGhee and Ginder, 1979; Mandel and Chambon, 1979; van der Ploeg and Flavell, 1980; Waalwijk and Flavell, 1978b) arise by specific interference with replication. In support of this idea, essentially all the Hpa I and Hpa II sites that are found even partially protected against digestion in the DNA of somatic tissues are found fully protected in sperm DNA (Mandel and Chambon, 1979; van der Ploeg and Flavell, 1980). (The level of methylation is actually lower in sperm DNA than in somatic tissues [Vanyushin et al., 1970]. It is possible, therefore, that DNA from sperm is predominantly hemimethylated). If this model is correct, either a complete methylation pattern is repeatedly reestablished during gametogenesis, or a methylation pattern is its own genetic determinant, transmitted through the germ line lineage of both male and female from generation to generation.

While the molecules we used in transformation were highly methylated at the Hpa II sites (greater than 98% of all the CCGG sequences in ϕX174, see Table 2), we observed less extensive methylation in progeny of the transforming molecules. Thus, either cells select against the incorporation of fully methylated molecules during the transformation event or methylation is incompletely inherited. Two considerations indicate the latter is the correct inference. First of all, no significant difference was observed in the frequency of cotransformation with ϕX174 DNA or in the number of copies of ϕX174 DNA incorporated when using either methylated or unmethylated molecules. Secondly, unpublished data indicate that one cell line (Mchtk-4 of Figure 2b) was transformed initially by a

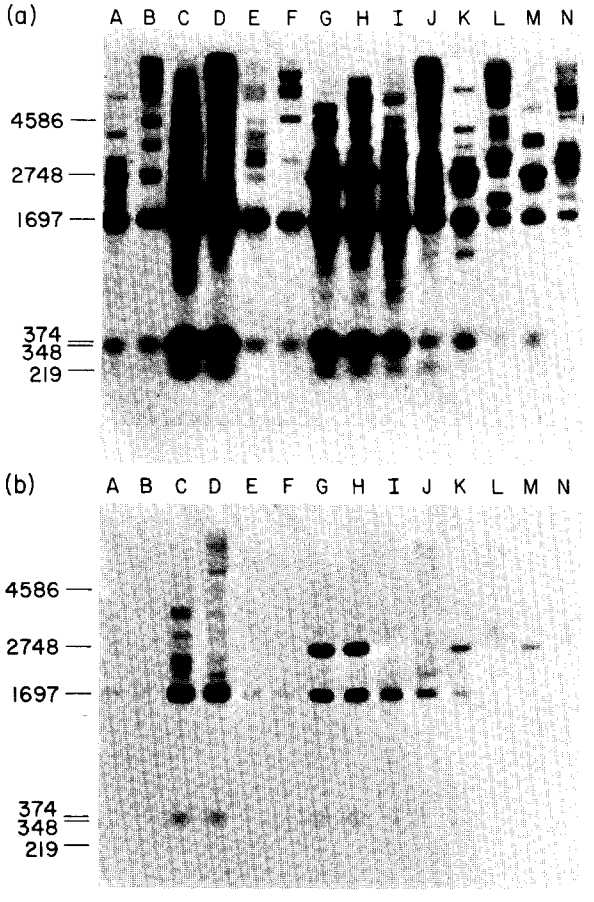

Figure 4. The Blot Patterns Are Shown for Hpa II and Msp I Digests of DNA from Lines Cotransformed with Methylated and Mock-Methylated ϕX174 RF DNA

DNAs from MMϕX-1 and MMϕX-4, lines cotransformed with mock-methylated, circular ϕX174 DNA, were digested with Msp I (lanes A and G, respectively) and Hpa II (lanes B and H, respectively). DNAs from MMϕX-2 and MMϕX-3, lines cotransformed with mock-methylated, Pst I linearized ϕX174 DNA, were digested with Msp I (lanes C and E, respectively) and Hpa II (lanes D and F, respectively). DNA from MϕX-1, a line cotransformed with methylated, Pst I linearized ϕX174 DNA, was digested with Msp I (lane I) and Hpa II (lane J). DNAs from MϕX-2 and MϕX-3, lines cotransformed with methylated, circular ϕX174 DNA, were digested with Msp I (lanes K and M, respectively) and Hpa II (lanes L and N, respectively). Digested DNAs were electrophoresed in 1.5% agarose gels, and transferred to a nitrocellulose filter which was hybridized with ^{32}P–ϕX174 DNA labeled by nick translation to 3 × 10^8 cpm/μg. (a) is a 72 hr exposure and (b) an 8 hr exposure of the autoradiograms. Figures on the left are sizes in bp of full-length linear ϕX174 and Hpa II digestion products.

single copy of the methylated chicken tk gene, and this gene was more highly methylated in cell populations 25 generations after transformation than in cell populations derived after yet another 25 generations. We conclude, therefore, that the incomplete methylation observed in progeny strands is due at least in part to the incomplete inheritance of methylation within transformant populations.

The fidelity of inheritance we observe, which we estimate to be about 95% per site per generation, is not sufficient to account for the stability of the methylation pattern seen in somatic tissues. There are several reasons why we may be underestimating the fidelity of somatic inheritance of methylation. First, the recipient Ltk$^-$ cells may be deficient in this function. Second, there may be a transient period early in the transformation process when replication of methylation does not occur. Third, as we have previously demonstrated (Perucho et al., 1980b), exogenous sequences incorporated during transformation undergo intermolecular ligation into large concatamers composed mainly of exogenous DNA. As a consequence, the sequence environment of foreign DNA in transformants may be fundamentally disorganized and may lack critical features that facilitate the inheritance of methylation. Fourth, there may be selective pressure for the loss of methylation from transformed sequences. Until these questions are resolved we may say only that the somatic cell does have the potential to mediate the faithful inheritance of methylation.

In one exception to the otherwise general observation of the persistence of methylation, the Sma I site of chicken tk did not remain methylated in vivo. Two possibilities can be considered. Cellular methylases may not recognize this particular site even though Sma I sites are found methylated in mammalian DNA (Gautier et al., 1977; Desrosier et al., 1979). Alternatively, methylation at the Sma I site might inhibit the expression of chicken tk providing a selection pressure against its maintenance. Consonant with this, we have observed a 3- to 20-fold lower transformation efficiency with the chicken tk gene when it is methyl-

Table 3. Inheritance of Methylation after N Generations

q	P_{25}	P_{50}
1.00	1.00	1.00
0.99	0.89	0.79
0.98	0.78	0.61
0.97	0.70	0.47
0.96	0.62	0.37
0.95	0.55	0.28
0.94	0.49	0.22
0.93	0.43	0.17
0.92	0.38	0.13
0.91	0.33	0.10
0.90	0.29	0.08
—	—	—
0.85	0.14	0.01
—	—	—
0.80	0.06	0.00

Proportion of Hpa II sites protected, P_N, after N generations as a function of q, the probability of inheritance of methylation per site per cell generation. Values have been computed according to the recursive equations described in the text. N = 0 corresponds to the initial condition in which all sites within the population are completely methylated.

ated (Table 1). Whatever the reasons, failure to observe the inheritance of methylation at other sites in other sequences has been reported recently (Pollack et al., 1980).

It has been proposed that DNA methylation might control gene expression in vertebrates, that the tissue-specific differences in methylation pattern account in some way for tissue-specific gene expression and that the somatic inheritance of methylation could therefore provide a molecular basis for such phenomena as stable differentiation and chromosome inactivation (Holiday and Pugh, 1975; Riggs, 1975). Consistent with this idea is an inverse correlation observed between expression and methylation of integrated or latent viral genomes (Desrosiers et al., 1979; Cohen, 1980; Sutter and Doerfler, 1980). These observations, however, do not distinguish a causal role for methylation from a correlative one. One way to resolve this question is to follow the expression of methylated and unmethylated genes when they are introduced into living cells. In this regard, we have observed a decreased transformation efficiency using the methylated chicken tk gene (Table 1) as well as the methylated HSV-1 tk gene (unpublished observations). Further work in progress (D.L. and M.W.) suggests that DNA methylation can directly inhibit gene expression in mammalian cells.

Experimental Procedures

Cells, DNA and Transformation

Ltk$^-$, a tk$^-$ murine cell (Kit et al., 1963), was maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% calf serum (Flow Laboratories), penicillin (50 U/ml) and streptomycin (50 μg/ml). Transformations, cotransformations and selection in HAT medium were performed as described previously (Wigler et al., 1979a, 1979b). Genomic DNA extracted from Ltk$^-$ cells was used as carrier. For cotransformation experiments, 1 μg of φX174 RF DNA (Bethesda Research Labs) was added with 20 μg of carrier per plate. Plasmid DNAs were grown in DH1, an r$^-$m$^+$ recA Escherichia coli K12 strain, using a modified high salt/SDS procedure of Tanaka and Weisblum (1975). λchtk-1 DNA was prepared from phage grown in DP50supF. Cellular DNAs were prepared as previously described (Wigler, 1979a).

Restriction Endonuclease Digestion and Blot Hybridization

DNAs were cleaved with restriction endonucleases in buffers recommended by suppliers (New England Biolabs and Bethesda Research Labs). When DNAs were to be cleaved with more than one enzyme, digestions were performed either simultaneously (if buffers were compatible) or sequentially in adjusted buffers. To prepare restricted DNAs for blot hybridization, additional DNA markers were added for purposes of control as described below. DNAs, 10 μg in 30–50 μl, were loaded per slot and electrophoresed in horizontal agarose slab gels in 40 mM Tris, 4 mM sodium acetate, 1 mM EDTA (pH 7.9). Blot hybridization (Southern, 1975) was performed using double nitrocellulose sheets (Jeffreys and Flavell, 1977) as described previously (Wigler et al., 1979b). The first sheet was probed with ^{32}P-DNA labeled by nick translation as described previously (Maniatis et al., 1975; Wigler et al., 1979b). In some cases, the second nitrocellulose sheet was hybridized with another probe (see below).

Two sorts of controls were performed in blot hybridizations. To monitor the completeness of digestion, cold-marker DNAs (either SV40, Ad2, or φX174 RF, where appropriate) were added to the digestion reactions (from 10 to 100 ng per 10 μg cellular DNA), and aliquots were electrophoresed separately for a second filter hybridization with viral-specific probes. Only those digestion reactions judged at least 95% complete have been reported. This type of control was performed for each experiment. A second type of control was included in all experiments where Hpa II restriction digests were to be compared to Msp I digests. In those experiments, unlabeled marker viral DNA was mixed thoroughly with cellular DNAs and two equal aliquots were taken, one for digestion with Msp I and one for digestion with Hpa II. After electrophoresis and blotting, the second nitrocellulose filter was hybridized with ^{32}P-labeled viral probe, and the intensity of hybridization in paired companion lanes was compared. We report only those experiments judged to have equal amounts of DNA present in companion lanes.

Preparation of Hpa II Methylase

Four liter cultures of Haemophilus parainfluenzae were harvested by centrifugation (5,000 rpm for 25 min) from well aerated, early stationary phase cultures grown in brain–heart infusion and 2 μg/ml NAD. Cell pellets were frozen at −70°C. Unless otherwise specified, enzyme isolation was performed at 4°C. Cell pellets (15 ml) were thawed and resuspended in 2.5 times volume of 10 mM Tris (pH 7.5), 10 mM β-mercaptoethanol (βME) and the cells disrupted by sonication. (Branson sonifier; ½ inch probe; 10 min total sonication at setting 0.5 of full power; done in 1 min bursts with 1 min rest intervals between bursts.) The sonicate was then centrifuged for 2.5 hr at 100,000 g and the resulting supernatant was decanted, made 2% streptomycin sulfate, and allowed to stand at 0°C for 15 min. Precipitated material was removed by centrifugation at 9,000 g for 20 min. To the clarified supernatant ammonium sulfate was slowly added to 50% saturation and the mixture was stirred overnight. The precipitated proteins were collected by centrifugation at 9,000 g for 30 min and resuspended in a small volume (4–5 ml) of Biogel column buffer (1 M NaCl, 10 mM Tris, 1 mM EDTA). This solution was dialyzed for 3 hr against 1 l of column buffer to remove ammonium sulfate and then chromatographed on a 2.5 cm × 90 cm Biogel column. One hundred twenty 5 ml fractions were collected and assayed for methylase activity by the procedures outlined below. A single peak of activity was found that protected against Hpa II digestion. Fractions comprising this peak were pooled, dialyzed overnight against 50 mM Tris (pH 7.5), 10 mM EDTA, 5 mM βME, concentrated 10-fold by placing dialysis tubes of extract in contact with a hydrophilic gel (Aquacide II, Calbiochem) and further concentrated another 4-fold by exhaustive dialysis against 50% glycerol, 50 mM Tris (pH 7.5), 10 mM EDTA, and 5 mM βME. The final product, approximately 1 U/μl (see below), was then stored at −20°C.

Several criteria indicated that this final preparation contained M–Hpa II methylase activity and no other detectable methylases. First, incubation of DNA with this extract under the reaction conditions outlined below protected greater than 95% of all sites from digestion by Hpa II assayed by the conversion of form I and II circular molecules to form III linears. Second, the extract methylated greater than 98% of all available Hpa II sites as determined by the high-pressure liquid chromatography method outlined below. Third, methylated DNA was fully sensitive to digestion by the enzyme Hpa I and Mbo I, which will not cleave methylated GATC, a site found methylated in H. parainfluenzae DNA (Lacks and Greenberg, 1977), even when methylations were done in vast enzyme excess. Last, Hpa II cleaved SV40 DNA failed to incorporate radioactivity in a standard methylase assay containing ^{3}H-S-adenysylmethionine. Incorporation of radioactivity was identical to that seen when no DNA was added and less than 5% of that incorporated by uncleaved DNA.

Methylation and Mock-Methylation of DNA

Standard reaction conditions for methylation of DNAs were 3 units of methylase to 1 μg DNA in a 10λ reaction containing 50 mM Tris–HCl (pH 7.5), 10 mM EDTA, 80 μM S-adenosylmethionine (SAM) and 5 mM βME for 3 hr at 37°C. We define one unit of M–Hpa II as the lowest amount of methylase that will render pBR322 DNA greater than 95% resistant to Hpa II digestion in a 1 hr incubation at 37°C.

Mock-methylated DNAs were prepared by incubation of the DNA under the same conditions as above but the SAM was omitted. All DNAs were then brought to 150 mM NaCl and extracted once with phenol and once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated from the aqueous phase by the addition of EtOH to 70%. Incubation of form I circular molecules under methylation or mock methylation conditions converted not greater than 50% of DNA to form II and negligible amounts to the linear form.

Assays for Methylation

Three assays for methylation were used. For purification, methylase activity was assayed as described by Mann and Smith (1977). Eight micrograms of salmon sperm DNA were incubated for 1 hr in a 20 μl reaction mixture containing 8 μl of enzyme, 50 mM Tris (pH 7.5), 10 mM EDTA, 5 mM βME and 1 μCi of ^{3}H-methyl SAM (approximately 2 μM). Twelve microliters of this mixture were then spotted on polyethyleneimine thin-layer chromatography sheets. These were dried in vacuo for 10 min at 60°C and developed by ascending chromatography in 1 N HCl. The ratio of radioactivity remaining at the origin of the chromatogram to that migrating with the solvent was measured by scintillation counting.

Alternatively, circular DNA was incubated with our enzyme preparations and the degree of protection against Hpa II digestion monitored by the conversion of form I or II circular molecules to form III linears, as assayed by gel electrophoresis. All DNAs used in our experiments were checked in this fashion, and all methylated preparations were greater than 95% resistant to digestion by Hpa II, while mock-methylated preparations were completely sensitive.

Because resistance to Hpa II digestion indicates only the proportion of Hpa II sites that are at least hemimethylated, we analyzed the proportion of CCGG sequences methylated by biochemical means for one methylated sample, the φX174 RF DNA used in cotransformation experiments. To this end, methylated and mock-methylated DNAs were digested with Msp I, treated with calf intestinal alkaline phosphatase at 0.1 U to 10 μg DNA in 40 μl containing 10 mM Tris–HCl (pH 7.9) and 0.1 mM EDTA for 30 min at 60°C (enzyme was a gift from R. J. Roberts). The resulting 5′OH groups were then labeled with γ-^{32}P–ATP using T4 polynucleotide kinase (Bethesda Research Labs) in a reaction containing 2 U kinase, 10 μg DNA, 50 mM Tris–HCl (pH 9.5), 10 mM $MgCl_2$, 5 mM DTT and 1 mCi γ-^{32}P–ATP (300 Ci/mmole), incubated at 37°C for 30 min. The reaction was stopped by the addition of EDTA to 20 mM, SDS to 0.5% and NaCl to 150 mM, and extracted once with an equal volume of phenol. Kinased DNA was separated from unreacted ATP by passage through a G-50 Sephadex column. DNA was then precipitated by the addition of EtOH to 70% and resuspended in 100 μl of 1 mM Tris–HCl (pH 7.9), 1 mM EDTA. Msp I generated fragments were then separated in alkaline agarose gels in order to decrease the contribution of incorporation of γ-^{32}P into randomly nicked molecules: DNA was loaded in 100 mM NaOH into a 1.0% agarose horizontal gel containing 30 mM NaOH and 1 mM EDTA, with the same running buffer. After electrophoresis, the gel was stained and neutralized by incubation in 1 M Tris–HCl (pH 7.9) containing 1 μg/ml ethidium bromide. Gel pieces containing φX174 fragments C and D (which could not be resolved), A, B, and E (see Figure 1b) were separately cut out, and DNAs were electroeluted into dialysis bags containing 40 mM Tris–HCl (pH 7.9), 4 mM sodium acetate and 1 mM EDTA. Forty micrograms sheared, denatured salmon testes DNA were added to each eluate. NaCl was added to 150 mM and DNA was precipitated by the addition of EtOH to 70%. Precipitated DNA was resuspended in 40 μl of 0.01 M sodium acetate pH 5.0. One microgram of pancreatic DNAase was added and reaction incubated for 60 min at 37°C. The reaction was then brought to 100 mM Tris–HCl (pH 8.9) and 10 mM $MgCl_2$ and snake venom phosphodiesterase was added to 100 μg/ml. The reaction was continued for an additional 60 min at 37°C. The resulting mononucleotide digestion products were separated by reverse phase chromatography on a Waters HPLC using modifications of an unpublished procedure of G. Acs and J. Christman. This method yields excellent resolution of all five mononucleotides: dCMP, dmeCMP, dTMP, dGMP, and dAMP. By this assay, greater than 98% of all Hpa II sites were methylated.

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