Cell, Vol. 65, 939–947, June 14, 1991, Copyright © 1991 by Cell Press

A Strain-Specific Modifier on Mouse Chromosome 4 Controls the Methylation of Independent Transgene Loci

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Summary

A transgene, pHRD, is highly methylated in 12 independent mouse lines when in a C57BL/6 strain background, but becomes progressively less methylated when bred into a DBA/2 background. Transgenes inherited from the mother are generally more methylated; however, this parental effect disappears following continued breeding into the nonmethylating strain. Mapping experiments using BXD recombinant inbred mice as well as other inbred strains indicate that a single strain-specific modifier (*Ssm-1*) linked to, but distinct from, *Fv-1* is responsible for the strain effect. In addition to the methylated and unmethylated transgenic phenotypes, certain mice exhibit a partial methylation pattern that is a consequence of an unusual cellular mosaicism. The pHRD transgene, containing target sequences for the V(D)J recombinase, undergoes site-specific recombination only in lymphoid tissues. This V–J joining is restricted primarily to unmethylated transgene copies.

Introduction

DNA modification by methylation plays an important role in regulating gene expression in a wide range of organisms. In mammals the predominant modification is methylation of cytosine, particularly in CpG dinucleotides. In many cases CpG methylation has been inversely correlated with the expression of associated genes (Cedar, 1988), but the mechanism by which methylation regulates gene expression is not understood in most cases. Evidence has been presented demonstrating that cytosine methylation can interfere with the binding of certain transcription factors (Kovesdi et al., 1987; Watt and Molloy, 1988), suggesting the possibility of a direct effect of methylation on expression. In other cases CpG methylation leads to immediate inactivation only if the gene is reconstituted into chromatin prior to microinjection (Buschhausen et al., 1987), which suggests that chromatin structure, not methylation, is the immediate factor affecting gene expression. In light of these and other studies it seems probable that DNA methylation can affect gene expression at several levels and the relationship between the two is complex.

Transgenic mice have proven to be a useful model system for understanding how gene expression is controlled throughout development. A variety of transgenes have been studied with respect to methylation status and the influence of parental heritage and mouse strain. It has been suggested that parental-dependent methylation is the basis for the nonequivalence of the maternal and paternal genomes and so may explain parental imprinting, although the connection between the two phenomena is not certain.

In an early analysis, expression of a metallothionein–thymidine kinase fusion gene was shown in some cases to correlate with hypomethylation (Palmiter et al., 1982), although the inheritance of the methylation and expression patterns was complex. It is possible that some of the complexity could be explained by parental effects as well as strain effects (the transgenic lines were maintained by crossing with (C57BL/6 × SJL)F1 mice). In several cases, a transgene was methylated when inherited from the female parent (Reik et al., 1987; Sapienza et al., 1987; Swain et al., 1987). The methylation was reversible by passage through a male. In two examples, irreversible methylation occurred after one (Hadchouel et al., 1987) or three successive (Allen et al., 1990) passages through a female. In several instances, a strain effect was observed (Sapienza et al., 1989a; Allen et al., 1990). It is not clear whether the mouse strain had an influence in the other transgenic lines, because outbred mice or F2 mice from two different strains were used. In all the transgenic mice in which more than one line with the same transgene had been analyzed, only one or some of the lines showed a parental effect on methylation. Thus, a clear position effect was seen, suggesting that the target for the methylation was the integration site and not the transgene itself.

To study the control of rearrangement of immunoglobulin genes, we previously constructed a rearrangement test gene (pHRD) that upon transfection into pre-B lymphocytes is rearranged in 100% of transfectants (Engler and Storb, 1987; Engler et al., 1991). To analyze the developmental and tissue-specific regulation of rearrangement, pHRD transgenic mice were produced. Contrary to expectations, the pHRD transgene was not rearranged in the lymphoid organs of the first generation offspring of several different transgenic lines, with one exception, a mouse from transgenic line 342. Siblings of this individual and about 40 offspring, however, did not rearrange the transgene. In an attempt to determine the reason for the presumed inaccessibility of the pHRD transgene, the methylation status of the transgene DNA was analyzed in these mice. It was found that all transgene copies were completely methylated in all the mice, except that partial undermethylation was seen in all tissues of the one mouse from line 342 in which spleen, thymus, and bone marrow, but not liver and kidney, showed rearrangement of the pHRD test gene.

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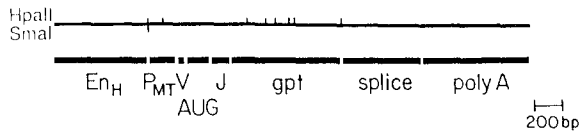

Figure 1. The Structure of the pHRD Transgene

The individual components are mouse immunoglobulin heavy chain enhancer; mouse metallothionein-1 promoter; 7-mer–spacer–9-mer recombinase recognition sequences from an immunoglobulin V_κ region; rat preproinsulin initiation codon and surrounding sequences; 9-mer–spacer–7-mer recognition sequences from a J_κ region; Escherichia coli xanthine-guanine phosphoribosyl transferase coding sequence; mRNA splicing signals from SV40; and polyadenylation signals from SV40. Only those restriction sites used for methylation analysis are indicated.

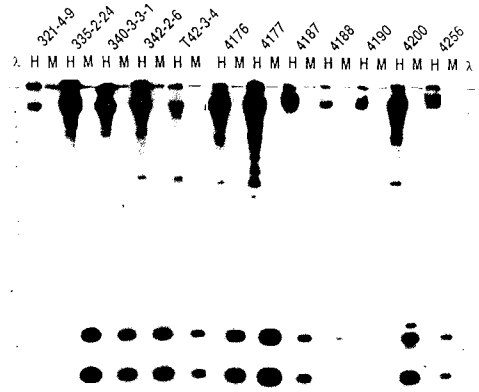

Figure 2. Methylation Analysis of 12 Independent pHRD Transgenic Lines

Spleen DNA was cut with either HpaII (H) or MspI (M), and Southern blots were hybridized with an E. coli gpt probe identical to the gpt fragment in pHRD. All mice were of mixed C57BL/6 and SJL background (see Experimental Procedures). The first five mice are offspring of the founder mice, while the last seven DNA samples were prepared directly from the spleens of the founder animals. Mouse 4177 is actually a mosaic; offspring of both integration sites have methylated pHRD in a C57BL/6 background. The two panels of this figure were hybridized and exposed separately so that copy numbers may not be directly comparable. Marker lanes (λ) contain bacteriophage λ DNA cut with HindIII: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb.

Since the original founder mouse was a (C57BL/6 × SJL)F2 and since the first generation had been backcrossed to F1 mice, but later generations to C57BL/6, we assumed that the C57BL/6 strain may be responsible for the hypermethylation of the transgene. This was confirmed by backcrossing the mice to the SJL strain; the transgene became undermethylated after two generations of backcrossing. Undermethylation was also achieved by crossing with another inbred strain, DBA/2. We have studied the inheritance of methylation pattern in these mice and have mapped a gene that plays a major role in its control. This will be a first step toward understanding how methylation patterns are established, maintained, and altered and how this affects gene expression during development.

Results

The pHRD Transgene Is Highly Methylated in a C57BL/6 Strain Background

The pHRD transgene contains about 100 CpG dinucleotides, 8 of which correspond to HpaII restriction sites (Figure 1). Transgene methylation was assessed by HpaII cleavage of transgenic mouse DNA, Southern transfer, and probing with guanine phosphoribosyl transferase (gpt) sequences identical to those in the pHRD plasmid. A methylated transgene array remains uncut by HpaII and appears at the top of the gel, while an unmethylated array is cut into hybridizing fragments of 0.6 and 0.4 kb (the smaller gpt-containing fragments are run off the gel). The methylation-insensitive enzyme MspI cuts the transgene into these small fragments regardless of methylation. When assayed in this manner, 12 of 12 independent pHRD transgenic mouse lines showed a high level of transgene methylation despite a wide range of copy number and despite different integration sites (Figure 2). All of the DNA samples were cut to completion with the methylation-insensitive isoschizomer MspI, indicating that the restriction sites were indeed present in the transgenes. The first five transgenic lines shown in Figure 2 were derived by injecting pHRD into the male pronucleus of (C57BL/6 × SJL)F2 zygotes, and the mice shown in Figure 2 are offspring of these founders. The last seven transgenic lines were made by injecting the plasmid into the male pronucleus of (C57BL/6 × SJL)F1 eggs fertilized with C57BL/6 sperm; the DNA analyzed in Figure 2 is from the founder animals. Evidence will be presented suggesting that the C57BL/6 strain background is responsible for this observed transgene methylation.

Inheritance of Methylation Pattern

Two independent lines (termed 342 and 335) were chosen for a detailed analysis of methylation pattern. Both lines contain approximately six copies of pHRD mostly in head-to-tail orientation but integrated at different sites (data not shown). Mice in Figure 3 are identified by a generation number, with I being the founder mouse, and by an individual number (small arabic numerals). The 342 line (Figure 3a) was maintained by backcrossing into C57BL/6 for four generations after an initial cross with (C57BL/6 × SJL)F1. With a single exception (II.2) all of these mice had highly methylated transgenes. This single mouse, with a mixed C57BL/6 and SJL background, showed definite evidence of partial undermethylation even though its littermates and offspring had completely methylated transgenes.

A systematic breeding program was initiated in an attempt to determine the genetic basis for this difference. When male or female transgenic mice (C57BL/6 background) were bred with either DBA/2 or SJL mice, all offspring were uniformly methylated regardless of the sex of the parent or the strain (VII). However, when these offspring were backcrossed to either DBA/2 or SJL mice, significant undermethylation was observed (VIII). In some individuals the transgenes were completely unmethylated, in others they were totally methylated, while some gave an intermediate pattern (similar to that seen in offspring

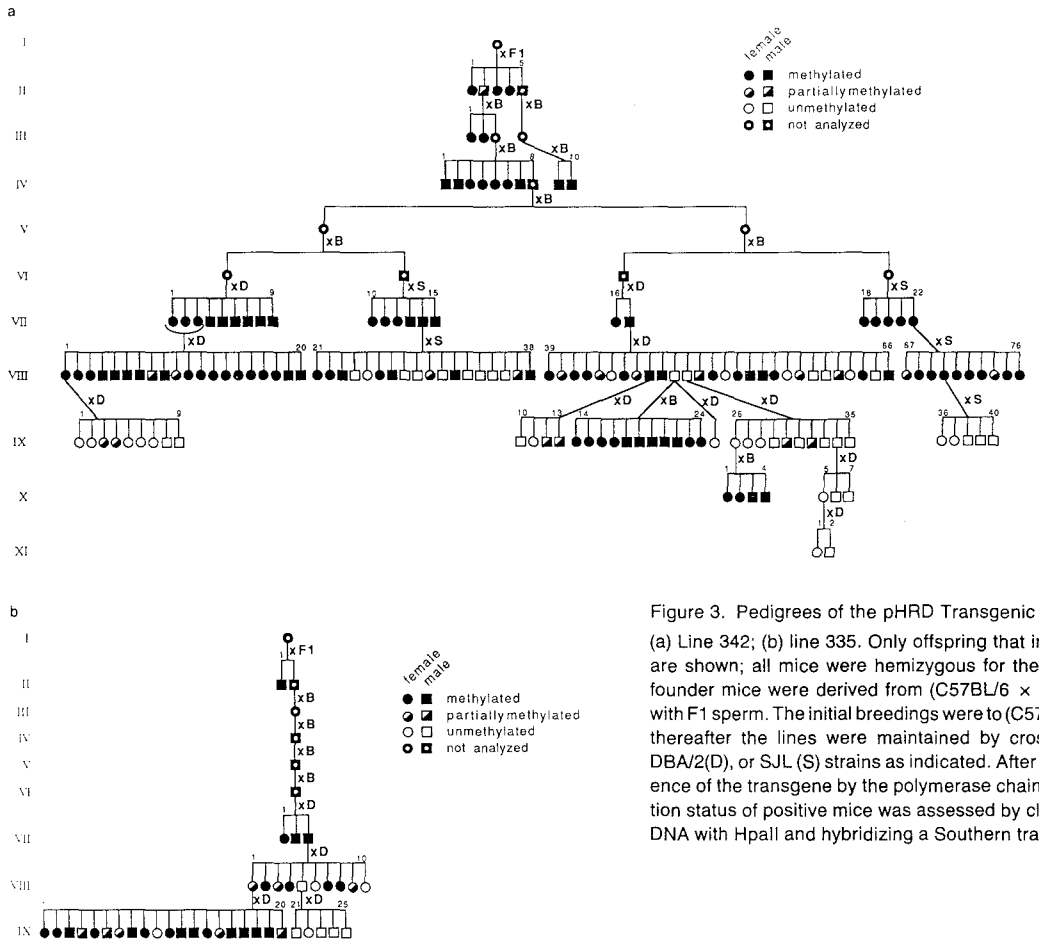

Figure 3. Pedigrees of the pHRD Transgenic Lines 342 and 335
(a) Line 342; (b) line 335. Only offspring that inherited the transgene are shown; all mice were hemizygous for the transgene locus. The founder mice were derived from (C57BL/6 × SJL)F1 eggs fertilized with F1 sperm. The initial breedings were to (C57BL/6 × SJL)F1 males; thereafter the lines were maintained by crossing to C57BL/6 (B), DBA/2(D), or SJL (S) strains as indicated. After screening for the presence of the transgene by the polymerase chain reaction, the methylation status of positive mice was assessed by cleaving a sample of tail DNA with HpaII and hybridizing a Southern transfer with a gpt probe.

from BXD-31 in Figure 4 or in offspring from the A strain in Figure 6). In this generation a parental effect was apparent in both the DBA/2 and the SJL background sublines: the transgene array was, on average, more methylated when inherited from the female (compare VIII.1–20 with VIII.39–66 and VIII.21–38 with VIII.67–76). However, after further backcrossing into a nonmethylating strain this parental effect disappears. For example, mice IX.36–40 are unmethylated even though they inherited the transgene from a methylated female. A similar lack of parental effect is seen with different strain and sex combinations (see IX.26–35, X.5–7, and XI.1–2).

Significantly, when an unmethylated transgenic male is crossed with a C57BL/6 female, complete methylation is observed in the progeny (IX.14–24), while the same mouse bred with a DBA/2 female gives unmethylated transgenic progeny (IX.25). This immediate and complete methylation is also seen in the reciprocal cross (X.1–4), in which an unmethyated transgenic female is bred with a C57BL/6 male. These observations form the basis for the mapping experiment described in the next section.

A less thorough breeding analysis was performed with the 335 transgenic line, but the results are compatible with the conclusions drawn from the 342 pedigree. Specifically, the pHRD transgene is highly methylated in a C57BL/6 background (Figure 3b; generations II to VII) and this methylation is reversible upon breeding the transgene into a DBA/2 background (VIII.1–10 and IX.1–25). Besides the strain effect in this second transgenic line, the inheritance of the transgene is also consistent with a parental effect. Ongoing breeding experiments, so far with three additional founder mice (numbers 4187, 4190, and 4200 in Figure 2), have yielded unmethylated transgenes following breeding to DBA/2 mice.

Mapping the Strain-Specifc Modifier

As described in the previous section, an unmethylated pHRD transgene bred into a C57BL/6 (B) background becomes methylated but remains unmethylated if crossed into DBA/2 (D). To map the gene responsible for this strain-specific modification, unmethylated transgenic mice were bred with a set of previously created and characterized recombinant inbred mice derived from C57BL/6J and DBA/2J progenitor strains (BXD; Taylor, 1989), and the methylation patterns of the offspring were determined. The strategy was to correlate methylation of the transgenic offspring with the B allele of previously characterized polymorphic loci.

Male mice from the 342 lineage that had been derived by crossing for two generations with DBA/2 and whose

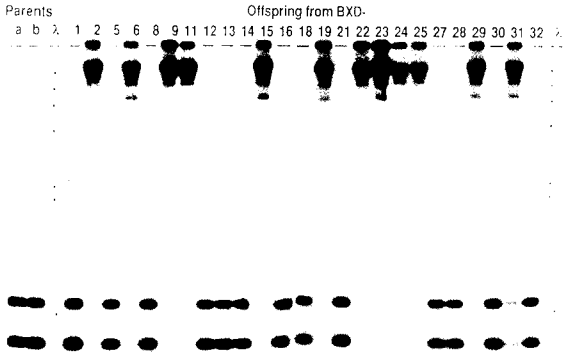

Figure 4. Methylation Analysis of Offspring of Unmethylated pHRD Transgenic Male Mice (342 Line) Bred to Female BXD Recombinant Inbred Mice

Whole-body DNA from the offspring (or tail DNA from the parents) was cut with HpaII, and a Southern transfer was hybridized with a gpt probe. Two male parents (a and b) were used for breeding to the entire panel of BXDs. In each case the entire litter was screened, although only a single representative individual offspring is shown in this figure. Marker lanes (λ) contain bacteriophage λ DNA cut with HindIII: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb.

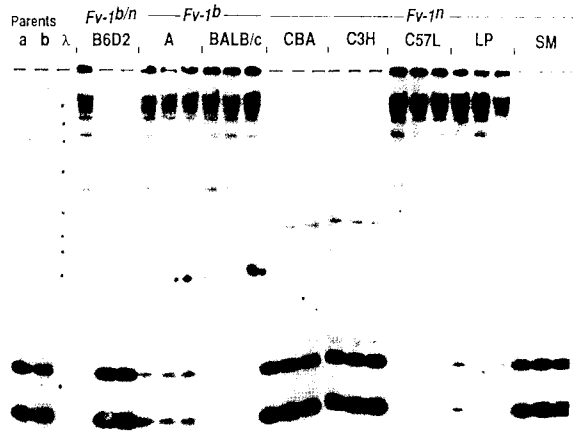

Figure 6. Methylation Analysis of Offspring of Unmethylated pHRD Transgenic Male Mice (342 Line) Bred to Female Mice of Various Inbred Strains

B6D2 is a (C57BL/6 × DBA/2)F1. Whole-body DNA from the offspring (or tail DNA from the parents) was cut with HpaII, and a Southern transfer was hybridized with a gpt probe. Results from groups of three offspring are shown. The strains are grouped according to *Fv-1* type. Marker lanes (λ) contain bacteriophage λ DNA cut with HindIII: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb.

transgenes were therefore unmethylated were mated with 25 different BXD females, and whole-body DNA of newborn offspring was analyzed by HpaII Southern blots (Figure 4). The offspring gave one of two patterns: either unmethylated (like the parents) or highly methylated (as if bred to C57BL/6). Results from only a single offspring of each cross with BXD are shown in Figure 4, but an average of 3.4 offspring that had inherited the transgene were analyzed from each breeding with identical results. Comparison of the methylation pattern with the known strain distribution patterns of polymorphic loci in BXD strains revealed an exact concordance between transgene methylation in the offspring and the presence of the B allele of *Fv-1*. This result localizes the strain-specific modifier, *Ssm-1*, to chromosome 4, probably between *Ly-31* and *Gpd-1* (Figure 5; strain distribution patterns and order of *Ly-31*, *Fv-1*, and *Gpd-1* are from Taylor, 1989). A similar analysis was performed with transgenic mice from the 335 lineage. Again complete concordance (15 of 15; Figure 5) was found between pHRD transgene methylation and the B allele of *Fv-1*, suggesting that the same modifier controls the methylation of a different transgene locus.

Methylation Analysis in Other Strains

To determine if *Fv-1* and *Ssm-1* are allelic, line 342 male mice with unmethylated transgenes were mated with normal females of eight different strains of known *Fv-1* type (Figure 6). The *Fv-1* locus controls susceptibility to Friend leukemia virus (Jolicoeur, 1979), and most mice can be classified as either *Fv-1*^{b} (resistant to N tropic virus as is C57BL/6) or *Fv-1*^{n} (susceptible to N tropic virus as is DBA/2). Both of the *Fv-1*^{b} strains tested, A and BALB/c, caused an increase in transgene methylation, although the A strain was somewhat unusual in that this genetic background was associated with an incompletely methylated phenotype. The five *Fv-1*^{n} strains, however, were split between methylators and nonmethylators. Clearly, there is no absolute correlation between pHRD methylation and *Fv-1*, suggesting that *Ssm-1* and *Fv-1* are different genes.

In addition, the unmethylated 342 transgenic males were bred with (C57BL/6 × DBA/2)F1 females (B6D2; Figure 6). The offspring were either methylated or nonmethylated in approximately equal numbers. This result indicates that maternal cytoplasmic factors do not contribute significantly to the observed methylation during embryonic development.

Results similar to those in Figure 6 were obtained when unmethylated 335 transgenic males were bred with B6D2, BALB/c, CBA, and LP females (data not shown).

	BXD- 1	2	5	6	8	9	11	12	13	14	15	16	18	19	20	21	22	23	24	25	27	28	29	30	31	32
Ly-31	D	B	B x	B	D	B	B	D	D	D	D x	D	D	B		D	B	D x	B	B	B x	D	B	D	B	D
Fv-1	D	B	D	B	D	B	B	D	D	D	B	D	D	B	B	D	B	B	B	B	D x	D	B	D		
Gpd-1	D	B	D	B	D	B	B	D	D	D	B	D	D	B	B	D	B	B	B	B	B	D	B	D	B	D
Meth																										
342	-	+	-	+	-	+	+	-	-	-	+	-	-	+		-	+	+	+	+	-	-	+	-	+	-
335		+	-		-	+	+	-	-		+		-			-				+	-	-		-	+	

Figure 5. Strain Distribution Patterns of *Fv-1* and Its Flanking Markers *Ly-31* and *Gpd-1* in the BXD Recombinant Inbred Series

The upper section data are from Taylor (1989). The last two lines summarize the pHRD methylation pattern seen in the offspring from an unmethylated pHRD 342 male (data from Figure 4) or from an unmethylated pHRD 335 male (original data not shown). "x" represents crossover between the two strains.

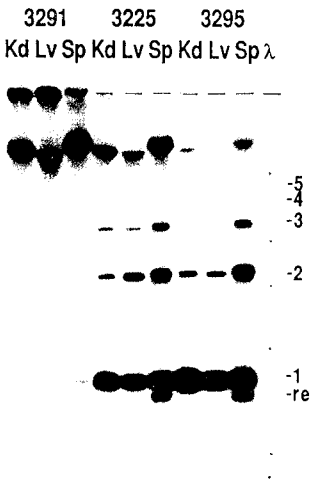

Figure 7. Analysis of Transgene Methylation Mosaicism and Rearrangement in Three Mice of the 342 pHRD Transgenic Line That Display the Partial Methylation Pattern

Kidney, liver, and spleen DNAs were digested with SmaI, and a Southern transfer was hybridized with a gpt probe. The dots in the marker lane (λ) represent sizes of 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb. Multiples of the transgene monomer (3.6 kb) are indicated, as is the rearranged fragment ("re") resulting from site-specific V–J recombination.

Transgene Methylation Mosaicism and Rearrangement

In addition to the fully methylated and unmethylated transgenic phenotypes, some mice show evidence of partial methylation of the transgene array (e.g., offspring of BXD-31 in Figure 4 and offspring of the A strain in Figure 6). Since mice of the 342 lineage contain about six head-to-tail copies of the pHRD plasmid, a number of possibilities are compatible with the observed patterns seen on HpaII Southern blots. Several general patterns could be envisioned: either all cells constituting a particular organ could have an identical partial methylation pattern or there could be cellular mosaicism with respect to methylation. Cellular mosaicism could be further subdivided into two types: a mixture of cells with fully methylated and fully unmethylated transgenes (all-or-none mosaicism) or mixtures of cells with intermediate patterns (mixed mosaicism). Furthermore, it is of interest to determine if the pattern is identical in different organs of mice with a partial methylation phenotype.

To address these questions, we made use of a methylation-sensitive restriction enzyme (SmaI) that has only a single site in each transgene monomer (see Figure 1). Since the transgene locus in the 342 line consists of about six copies mostly in head-to-tail orientation, it is possible to distinguish between the various patterns by examining the array of fragments generated by cleavage with this single cutter. Analysis of DNA from kidney, liver, and spleen from three individual mice (Figure 7) indicates that the overall level of transgene methylation is similar, though not identical, in different organs of the same individual and that in many cases a mixture of transgene multimers can be clearly identified. This ladder of transgene multimers is incompatible both with nonmosaic and all-or-none mosaic methylation patterns. Therefore the partial methylation phenotype results from a distinctive cellular mosaicism in which the population of cells expresses a multitude of individual methylation patterns.

The pHRD transgene was designed as an immunoglobulin gene rearrangement test construct. When transfected into a pre-B cell line, virtually every cell that incorporated the construct showed evidence of site-specific rearrangement (Engler and Storb, 1987; Engler et al., 1991). In contrast, the initial results in transgenic mice were uniformly negative. However, the one unusual mouse (II.2 in Figure 3a) that showed evidence of partial undermethylation of the transgene also showed evidence of V–J recombination. Southern blot analysis of spleen and other organ DNAs from over 20 individual mice with methylated pHRD transgenes provided no evidence of transgene recombination, yet every mouse with partial or complete transgene undermethylation has also had readily detectable V–J joining. This V–J rearrangement results in a deletion of 0.3 kb and occurs only in lymphoid tissues (spleens in Figure 7 and data not shown).

In mice with a partial methylation phenotype most of the rearrangements can be shown to be associated with the unmethylated copies (note the recombination products below the monomer bands in the spleen lanes of Figure 7; see Discussion). After longer exposure of this blot without an intensifying screen (data not shown) rearranged bands are also seen below the dimer band in the spleen DNAs, indicating that rearrangement does occur in a cell where some transgene copies have methylated CpGs. It is not possible to discern rearranged bands at the higher multimers, because of the low resolution of the small size differences. The exact relationship between transgene methylation and V–J recombination in these mice is currently being investigated.

Discussion

Methylation as a Consequence of Chromatin Structure

We have identified a strain-specific modifier on mouse chromosome 4 that controls the methylation of a particular transgene independent of integration site. Such a modifier could act by directly affecting the specificity of the methylase itself or through other less direct means. The available evidence suggests that all CpG methylation activities, both de novo and maintenance, are carried out by very similar or identical methyltransferases (Bestor and Ingram, 1983, 1985) whose cDNA has been cloned (Bestor et al., 1988). The C-terminal portion is probably the catalytic domain, whereas the N-terminal portion may be a regulatory region. It is possible that this regulatory domain differs between mouse strains and may target the methyltransferase in a strain-dependent way.

Since we have not observed any major global methylation differences between various mouse strains, a more reasonable possibility is that the normal DNA methyltransferase is equally present in all mouse strains and that the

differential transgene methylation we have described is a secondary phenomenon. One attractive possibility to explain how the modifier might act to regulate methylation of chromosomal sequences is that it controls chromatin structure and that methylation is a consequence of this altered structure. This could be similar to what has been observed in mammalian X chromosome inactivation during development, where inactivation of *hprt* precedes methylation by several days (Lock et al., 1987). Thus it may be that the primary event leading to strain-specific transgene methylation is analogous to the initiation of heterochromatin formation.

Position Effect Variegation in Drosophila

A number of parallels between transgene methylation in mice and position effect variegation in Drosophila have been pointed out (Sapienza et al., 1989b; Allen et al., 1990), and the body of Drosophila work serves as a useful framework within which to interpret our results. Despite some obvious differences (particularly the fact that cytosine methylation is not known to be a major DNA modification in Drosophila) there are some striking similarities (such as control by modifying loci, parental effect, and cellular mosaicism), and it will be interesting to see how far the analogy can be extended. In a particularly well-studied case in Drosophila, *In(1)w^{m4}*, an inversion of the X chromosome has relocated the normally euchromatic *white* gene near a block of centromeric heterochromatin (Eissenberg, 1989; Spradling and Karpen, 1990; Henikoff, 1990). Cytogenetic characterization has shown that the boundary of heterochromatin has spread differentially through the *white* gene, inactivating it in some cells but not in others. This differential inactivation results in variegated expression of the pigment gene, causing an eye with a mottled appearance.

The degree of variegation due to position effect is known to be modified by various factors, both environmental and genetic (Spofford, 1976). A number of modifier genes, perhaps 30, have been identified. Some of these modifiers, such as histone genes, are undoubtedly involved in other processes as well, but some may be directly involved in heterochromatin formation. For example, *Suvar205*, which suppresses position effect variegation, has been shown to inactivate a heterochromatin-specific protein, HP-1 (Eissenberg et al., 1990). Similarly, another suppressor of position effect variegation, *Suvar(3)7*, disrupts a protein with an unusual zinc finger structure (Reuter et al., 1990). The protein, predicted to have five widely spaced zinc fingers, has been postulated to be involved in heterochromatin formation by binding to distant sites on DNA. It is tempting to speculate that *Ssm-1* might be similar in some way to previously identified modifiers of position effect variegation in Drosophila.

Results described by others (see Introduction) in which only a fraction of transgenic lines show strain- or parental-dependent methylation seem to indicate that position effect is the most reasonable explanation. However, it seems unlikely that the strain-specific methylation we have described is due to position effect. Since transgene methylation was observed in all 12 independently derived lines, it is probable that the pHRD transgene itself contains the signals required for targeting the methylation. These signals may be analogous to the postulated heterochromatin initiator sites (Tartof et al., 1984). It will be interesting to determine if the pHRD transgene influences chromatin structure and methylation of its flanking sequences in a strain-dependent manner.

A Model for Parental- and Strain-Dependent Transgene Methylation

We have documented both parental- and strain-dependent methylation of a particular transgene and have mapped a modifier locus that plays a major role in the strain-dependent methylation of the transgene. Is the same modifier also responsible for the parental effect? Our model, which is speculative in most aspects, assumes that *Ssm-1* is involved in the regulation of chromatin structure and that this structural change allows subsequent methylation. The model predicts that a similar alteration is responsible for both the parental effect (gametic modification) and the strain effect (zygotic modification). Results presented here, particularly the breeding analysis with BXD recombinant inbred mice, indicate that a single dominant gene is responsible for the methylation observed following the breeding of an unmethylated transgene into a methylating strain. This alteration of transgene methylation is clearly a zygotic effect since the same parent can produce unmethylated transgenic offspring if bred with a nonmethylating strain. We suggest that at some postfertilization stage the *Ssm-1* product recognizes sequences associated with the pHRD transgene and marks them for later methylation.

A similar recognition and marking could also explain the parental effect. In the pHRD transgenic mice as well as in most other lines that show a parental effect (see Introduction), transgenes are more methylated when inherited from a female. This is in contrast to the overall methylation level in gametes, where sperm DNA is considerably more methylated than is oocyte DNA (Sanford et al., 1987). We postulate that the transgene becomes unmethylated and demodified early during gametogenesis and that modification of the transgene occurs preferentially during oogenesis. This gametic modification could involve chromatin structure, methylation, or both. *Ssm-1* may be expressed only during oogenesis or during gametogenesis in both sexes. If the latter is true, erasure of the imprinted chromatin structure could be a consequence of the special events in spermatogenesis. During spermatogenesis the normal nucleosome structure is replaced by a characteristic DNA–protamine complex, while no such drastic alteration of chromatin structure is known to take place during oogenesis. This difference was pointed out by Spofford (1976) as being important for understanding parental effects in both Drosophila and mice.

Partial Methylation of Transgenes and Cellular Mosaicism

When the pHRD transgene is in a C57BL/6 background it is completely methylated, but when carried in a DBA/2 background it is totally unmethylated. However, in mixed

C57BL/6 and DBA/2 strain backgrounds a partial methylation phenotype is often observed (see, for example, Figure 3a, generation VIII). A degree of partial transgene methylation is also seen in the BXD-31 cross (Figure 4; though clearly there is a very great shift toward methylation in this example) and in the cross with the A strain (Figure 6). It is significant that when we examined various organs from mice with partially methylated transgene arrays, they all had a similar if not identical overall degree of methylation in different organs (Figure 7 and data not shown). Furthermore, in mice with partially methylated transgene arrays it appears that a given organ is composed of a mosaic population of cells with a diversity of individual methylation patterns. Several hypotheses to explain these partial methylation patterns may be offered. First, it is possible that other genes, differentially expressed in various strain backgrounds, alter the effect of *Ssm-1*, resulting in altered methylation of the transgene. Second, multiple alleles of *Ssm-1* may exist in strains other than C57BL/6 and DBA/2 that have slightly different modifier effects. Finally, it is possible that the resetting to the unmethylated state during gametogenesis is incomplete, allowing partially modified transgenes to be transmitted to the next generation.

Effect of Methylation on Transgene Recombination

The finding that pHRD transgenic mice do not rearrange the transgene when it is hypermethylated may be due to either chromatin condensation or a direct effect of DNA methylation on the rearrangement process or both. Immunoglobulin genes must be in an accessible state to be targeted for rearrangement (Blackwell and Alt, 1989). While DNA methylation as a control of gene expression has generally been linked to the state of chromatin, some data exist that suggest that methylation may directly inhibit the binding of transcription factors. Methylation of certain CpG dinucleotides at the adenovirus major late promoter inhibits binding of a promoter-specific protein and transcription (Watt and Molloy, 1988). It has been suggested that methylation at the sensitive sites may prevent the molecular contacts between the DNA and the regulatory proteins (Dynan, 1989).

By analogy, methylation of DNA at CpG dinucleotides may interfere with recombinase activity, for example by inhibiting its progression. There is some evidence that the V(D)J recombinase may operate in a one-dimensional tracking mode (Yancopoulos et al., 1988; Storb et al., 1989). In this case, methylation at sites between the rearrangement signal sequences may, but methylation outside may not, interfere with rearrangement. Other schemes can be envisaged where the recombinase would be inhibited by methylation at any site within a certain distance from the sites of rearrangement. It is quite striking that in transgenic mice with a partial methylation pattern of pHRD unmethylated transgene copies can be rearranged, while methylated copies presumably in the same array are not. This was confirmed by digesting spleen DNA with an enzyme (such as PstI; see Engler and Storb, 1987; Engler et al., 1991) that isolates a DNA fragment containing both rearrangement recognition sequences and the gpt gene and gives a band containing all unrearranged copies and a different one containing all the rearranged genes. In mice with the partial methylation phenotype the rearranged band is completely eliminated by HpaII digestion (data not shown).

Thus, V–J rearrangement can apparently only take place in transgene copies with at least one unmethylated HpaII site. The distance between the rearrangement sites in adjacent pHRD transgenes is only 3.6 kb. It will be interesting to determine the role of methylation in this very localized effect.

Functions of *Ssm-1* and Other Mammalian Modifier Genes

Although methylation of transgene sequences clearly is an artificial situation, the modifier(s) that controls this phenomenon may also play a role in controlling gene expression in nontransgenic animals. Perhaps endogenous target sequences, analogous to those present in the pHRD transgene, play a role in regulation of methylation or chromatin structure. It is possible that a family of modifiers exists, each with distinct but overlapping target sequences. Such a degree of functional redundancy could explain why the overall levels of methylation do not vary dramatically between different mouse strains. Some of these modifiers might be involved directly as structural chromatin proteins, as regulators of chromatin assembly, or as regulators of the methylase itself. This type of organization would allow coordinate regulation of entire subsets of genes during development. Alternatively it has been proposed (Solter, 1988) that such modifiers only recognize foreign or transposed DNA and that endogenous genes in their normal environment would not be altered by these modifiers. If this is the case then linkage of *Ssm-1* and *Fv-1*, which controls viral susceptibility, may not be fortuitous. It will be of great interest to clone *Ssm-1* and other mammalian modifier genes and to determine their functions in the overall regulation of gene expression.

Experimental Procedures

Transgenic Mice

The plasmid pHRD, used to generate the transgenic mice, has been described in detail (Engler and Storb, 1987) and its components are summarized in Figure 1. A 3.6 kb EcoRI–HindIII fragment, lacking pUC vector sequences, was isolated from an agarose gel using hydroxylapatite (Tabak and Flavell, 1978) and used for microinjection after phenol extraction and ethanol precipitation. Two sets of transgenic mice were made, both using (C57BL/6J × SJL/J)F1 eggs; the first set of five mice was derived from eggs fertilized with F1 sperm, while the second set of seven was from eggs fertilized with C57BL/6J sperm. All procedures related to the transgenic mice have been described (Brinster et al., 1985; Hogan et al., 1986). The transgenic lines were maintained by breeding to either (C57BL/6J × SJL/J)F1, C57BL/6J, SJL/J, or DBA/2J mice as noted in Figure 3. The mapping experiments described in Figures 4 and 6 were performed by breeding selected (see next section) male mice with unmethylated transgenes to female BXD/Ty recombinant inbred mice or to females of other inbred strains as described in the Results. All mice were obtained from The Jackson Laboratory or bred in our colonies.

Methylation Analysis

Methylation status was assessed by cleaving DNA with HpaII (sensitive to 5meC modification), MspI (an isoschizomer of HpaII but insensitive to 5meC modification), or SmaI (which cuts a single site in the pHRD transgene and is sensitive to 5meC modification) and probing a South-

ern transfer with ^{32}P-labeled gpt sequences (identical to those contained in pHRD and completely transgene specific). The DNA used for analysis was prepared from tail clippings of young mice, from whole bodies of newborn mice, or from various organs of adults as stated in the Results. To confirm cleavage by HpaII, Southern transfers were rehybridized with a probe derived from the 5′ end of a mouse thymidine kinase cDNA. This 0.1 kb PstI–ApaI fragment from pMtk4 (Lin et al., 1985) hybridizes to a 1.0 kb HpaII genomic DNA fragment that is constitutively unmethylated in all tissues (data not shown).

Acknowledgments

We are especially grateful to W. Reik for several key suggestions during the course of this work. We would also like to express our gratitude to D. Lo for help with the initial analysis of the transgenic mice, to D. Gottschling, C. Laird, R. Braun, J. Spofford, and B. Taylor for helpful comments on this work, and to D. Solter, M. Gellert, and A. Weng for suggestions about the manuscript. We also acknowledge the contributions of G. Bozek for her assistance with the maintenance of the mouse colony. This work has been supported by NIH grants AI24780 and HD23089. P. E. was supported by NIH postdoctoral training grant HD07136 and L. D. by a fellowship from the National Multiple Sclerosis Society.

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Received February 19, 1991; revised April 4, 1991.

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