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Phenotypic Variation in a Genetically Identical Population of Mice

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The parental alleles of an imprinted gene acquire their distinctive methylation patterns at different times in development. For the imprinted *RSVIgmyc* transgene, methylation of the maternal allele is established in the oocyte and invariably transmitted to the embryo. In contrast, the methylation of the paternal allele originates during embryogenesis. Here, we show that the paternal methylation pattern among mice with identical genetic backgrounds is subject to extensive variation. In addition to this nongenetic variation, the process underlying *RSVIgmyc* methylation in the embryo is also subject to considerable genetic regulation. The paternal transgene allele is highly methylated in an inbred C57BL/6J strain, whereas it is relatively undermethylated in an inbred FVB/N strain. Individual methylation patterns of paternal alleles, and therefore all of the variation (nongenetic and genetic) in methylation patterns within an *RSVIgmyc* transgenic line, are established in early embryogenesis. For each mouse, the paternal *RSVIgmyc* allele is unmethylated at the day-3.5 blastocyst stage, and the final, adult methylation pattern is found no later than day 8.5 of embryogenesis. Because of the strong relationship between *RSVIgmyc* methylation and expression, the variation in methylation is also manifest as variation in transgene expression. These results identify embryonic de novo methylation as an important source of both genetic and nongenetic contributions to phenotypic variation and, as such, further our understanding of the developmental origin of imprinted genes.

Genomic imprinting is a molecular regulatory process in mammals which distinguishes parental alleles of certain autosomal loci. The main effect of this regulatory process is that the transcriptional activity of the maternal allele is different from that of the paternal allele. A number of embryological and genetic observations indicate that the expression difference between the parental alleles is due to parent-specific modifications (13). These modifications, also called genomic imprints, are established during gametogenesis, transmitted to the offspring, and associated with a difference between maternal- and paternal-allele expression (6). Although the precise molecular nature of these imprints is presently unknown, DNA cytosine methylation has been strongly implicated (6, 7, 21, 33, 34). Imprinted genes, initially identified because of transcriptional differences between the parental alleles, have also been shown to possess parent-specific differences in DNA methylation (4, 28, 31, 32). Moreover, in mouse embryos with targeted disruptions of the DNA cytosine methyltransferase gene *Dnmt* and a marked reduction in genomic methylation, normally imprinted genes lose their imprinted phenotype (20, 21).

The relationship between DNA methylation and genomic imprinting has been further defined in studies on the embryological origin of methylation patterns (3, 5, 7, 16, 31, 33, 36). In the case of the imprinted *RSVIgmyc* transgene, methylation of the maternal allele is established during the late stages of oogenesis, is transmitted to the embryo, and eventually remains in the adult mouse, where it is associated with transcriptional inactivation (6, 7). In contrast, the paternal allele's methylation pattern is established de novo in the early embryo. The extent of this de novo methylation depends on the strain background. The paternal allele is undermethylated in the FVB/N background. In the inbred C57BL/6J strain, however, the paternal allele is as highly methylated as the maternal allele and, as a consequence, is not expressed (8). These results indicate that the regulation of *RSVIgmyc* imprinting is controlled, in part, by the action of strain-specific modifiers that act in the early embryo (1, 2, 8, 14).

Other mouse transgenes besides *RSVIgmyc* are subject to genetic modification of their DNA methylation (14, 27, 35). Even though these transgenes are not themselves imprinted, the mechanisms underlying these modifications may have an effect on the behavior of imprinted genes or be directly associated with the imprinting process. The *TKZ751* transgene is interesting in this regard (1, 35). A modifier gene(s) acts to increase transgene methylation in a BALB/c background, whereas methylation remains low in a DBA/2J background. In F_1 hybrid reciprocal crosses between inbred BALB/c and DBA/2J strains, the BALB/c-specific effect of increasing methylation occurs only when the BALB/c modifier is inherited from the oocyte. Therefore, the BALB/c modifier activity itself may be imprinted (2). Studies on the modification of the *RSVIgmyc* and *TKZ751* transgenes indicate that modifiers are associated with the imprinting process, either by being imprinted themselves or by regulating the behavior of imprinted genes. Thus, further study of transgene modification processes should provide a better understanding of the molecular events in genomic imprinting.

In addition to strain-specific modifier (genetic) effects on methylation of the paternal *RSVIgmyc* allele, we show here that there is a significant nongenetic contribution to the methylation of the paternal *RSVIgmyc* allele. The paternal allele's behavior can be examined independently of that of the maternal allele in transgenic mice that carry a hemizygous transgenic locus inherited from the male parent. In particular, we observe significant differences in the degree of methylation of the paternal allele among genetically identical F_1 hybrid mice. These F_1 hybrids are generated from matings between inbred FVB/N

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and C57BL/6J mice. The F_1 hybrid variation arises in early embryogenesis, coincident with the major increase in genome-wide DNA methylation (7, 18, 25). Furthermore, the variation in methylation persists, being closely associated with significant differences in expression of the transgene in the adult heart. The extent of *RSVIgmyc* expression is known to have a profound phenotypic effect, producing myocyte hyperplasia and an increase in cardiac size (17). As such, these findings identify a source of significant nongenetic contribution to phenotypic variation.

MATERIALS AND METHODS

Mouse strains. The C57BL/6J and FVB/N inbred strains of mice were used in the experiments. *RSVIgmyc* transgenic lines of mice were produced either in inbred FVB/N (TG.AAJ line) or in inbred C57BL/6J (TG.NL and TG.NN lines) mice. The TG.AAJ line exhibits imprinting characteristics (allele-specific differences in DNA methylation and transgene expression) identical to those of other *RSVIgmyc* lines produced in the inbred FVB/N background (8) (data not shown). This result is consistent with the imprinting characteristics of the *RSVIgmyc* transgene construct being independent of its genomic integration site (7, 8). The inbred nature of the C57BL/6J and FVB/N mice and the hybrid nature of the transgenic F_1 offspring utilized in the present studies were confirmed by using a series of microsatellite markers that distinguish C57BL/6J and FVB/N alleles (D1Mit108, D3Mit124, D4Mit58, D5Mit23, D8Mit120, D9Mit43, D10Mit117, D13Mit139, D14Mit101, and D15Mit16). Specifically, for each microsatellite marker, the size of the PCR product is different for FVB/N and C57BL/6J genomic DNA; F_1 hybrid genomic DNA contains both PCR products (data not shown).

Transgene methylation studies. Transgene methylation was analyzed by Southern blotting of DNA from individual inbred or hybrid mice. Both DNA and RNA were isolated from individual hearts (9). Because the *RSVIgmyc* transgene is expressed only in the heart, the association between transgene methylation and expression could be analyzed. Individual day-8.5 embryos were dissected free of attached extraembryonic membranes, and the DNA was isolated following proteinase K (Boehringer Mannheim, Indianapolis, Ind.) digestion of the whole embryo. A portion of each DNA sample was used in a PCR-based assay to determine the sex of the embryo (26). At day 3.5 of embryogenesis, blastocysts were isolated from natural matings and pooled together, and the DNA was isolated following proteinase K digestion. All isolated DNA samples were digested with the appropriate restriction endonuclease and electrophoresed on 1% agarose gels. Controlled digestion with the methylation-insensitive enzymes *Msp*I and *Bgl*II was used to demonstrate the complete digestability of the DNA samples. Following transfer of the DNA to nylon membranes (GeneScreen; NEN Research Products), the Southern blots were hybridized in 40% formamide with the Cα fragment of the transgene (7) and washed at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate plus 0.1% sodium dodecyl sulfate). The labeled Southern blots were then analyzed by autoradiography and, quantitatively, with a PhosphorImager, as described below.

Transgene expression analysis. The level of heart-specific transgene expression was determined by a previously described RNase protection assay (8, 32). RNA was isolated from individual hearts (9), and the level of transgene-specific c-*myc* expression was determined with a mouse c-*myc* RNase protection probe. The ribosomal L32 gene transcript was used as an internal control to normalize for the amount of RNA assayed (12, 24, 30).

RESULTS

Variation in *RSVIgmyc* methylation. To understand the effect of different strain backgrounds on the extent of methylation of the paternal *RSVIgmyc* allele, we studied three groups of mice: those in which an *RSVIgmyc* transgene was in an inbred FVB/N background (TG.AAJ line), those in which this transgene was in an inbred C57BL/6J background (TG.NL and TG.NN lines), and those in which it resided in an F_1 hybrid background produced from the parental FVB/N and C57BL/6J strains. Specifically, the F_1 hybrid mice were produced from a mating between an FVB/N inbred female and a C57BL/6J inbred male containing the *RSVIgmyc* transgene (TG.NL or TG.NN line). In this mating scheme, the female parent always contributed a haploid FVB/N genome to the F_1 offspring and the C57BL/6J male parent always contributes a haploid C57BL/6J genome. With the exception of sex chromosome differences between male and female members of the group, each of the three groups of mice [inbred FVB/N, inbred C57BL/6J, and the F_1(FVB/N × C57BL/6J) hybrids] was genetically identical, including having an integrated *RSVIgmyc* transgene allele. For each group, Southern blot analysis was performed on mouse genomic DNA digested with the methylation-sensitive restriction endonuclease *Hpa*II. Because the transgene had integrated as a tandem array of unit copies in the lines used, the methylation of the entire array was evaluated with the Cα probe from the transgene construct (Fig. 1A).

Methylation patterns of individual mice from the three genetic groups are shown in Fig. 1B. The inbred FVB/N mice are from the transgenic line TG.AAJ, produced directly in the FVB/N background. The other mice are derived from the TG.NL line, produced in the inbred C57BL/6J strain. The F_1 hybrid mice were produced by mating FVB/N females to TG.NL males. The paternal *RSVIgmyc* alleles of three inbred FVB/N mice shown have essentially identical methylation patterns (lanes 1 to 3). More specifically, the number of bands, their relative intensities, and their sizes are very similar. Likewise, the paternal *RSVIgmyc* alleles of the four inbred C57BL/6J mice shown also have essentially identical patterns (lanes 13 to 16), although this pattern differs from that of FVB/N. In contrast to the uniformity of *RSVIgmyc* methylation patterns in the inbred strains, methylation patterns from the nine F_1 hybrid transgenic mice (Fig. 1B, lanes 4 to 12) are very different from one another. The patterns range from one that is similar to the pattern in C57BL/6J mice (lane 4) to one that approximates the FVB/N pattern (lane 11). The strong strain-specific effects on *RSVIgmyc* methylation seen in the inbred FVB/N and C57BL/6J backgrounds and the large degree of variation in the methylation patterns in the group of genetically identical F_1 mice indicate that methylation is governed by a combination of genetic and nongenetic influences.

To define the degree of variation in *RSVIgmyc* methylation in a group of genetically identical mice, each individual methylation pattern was analyzed. The methylation pattern of a paternal allele in an FVB/N background is composed of prominent bands of characteristic molecular size (1.6, 2.7, 3.2, and 4.3 kb), whereas an *RSVIgmyc* allele in an inbred C57BL/6J background has a prominent 16-kb band (Fig. 1B). (Both methylation patterns have additional bands that contribute slightly to the transgene hybridization signal and are not included in this analysis.) A methylation pattern of an F_1 hybrid mouse can be described as a composite of five bands of characteristic molecular size (1.6, 2.7, 3.2, 4.3, and 16 kb) (Fig. 1A and B). The relative intensities of these bands are a measure of the extent of restriction endonuclease digestion of *Hpa*II sites in the *RSVIgmyc* transgene construct (7, 32). The largest band (16 kb) represents a highly methylated allele, whereas the four smaller-molecular-size bands, taken together, define the undermethylated pattern of the paternal allele in an FVB/N background (8) (Fig. 1B). Because the integrated *RSVIgmyc* transgene allele is a tandem array of *RSVIgmyc* unit copies, an individual methylation pattern can be described as a composite of highly methylated and undermethylated unit copies. In this formulation, the ratio of the intensity of the largest band to the sum of the intensities of all five bands is a quantitative measure of methylation of the transgene allele. The stability of the sizes of the observed hybridization bands in the group of mice indicates that major internal structural rearrangements of the transgene insertion could not account for the variation in Southern hybridization patterns. Furthermore, the characteristic heart methylation pattern of each mouse is also found in DNA isolated from multiple adult somatic tissues (data not shown), indicating that the pattern is probably established uniformly in all cells at an early embryonic stage.

When transgene methylation patterns of DNA derived from

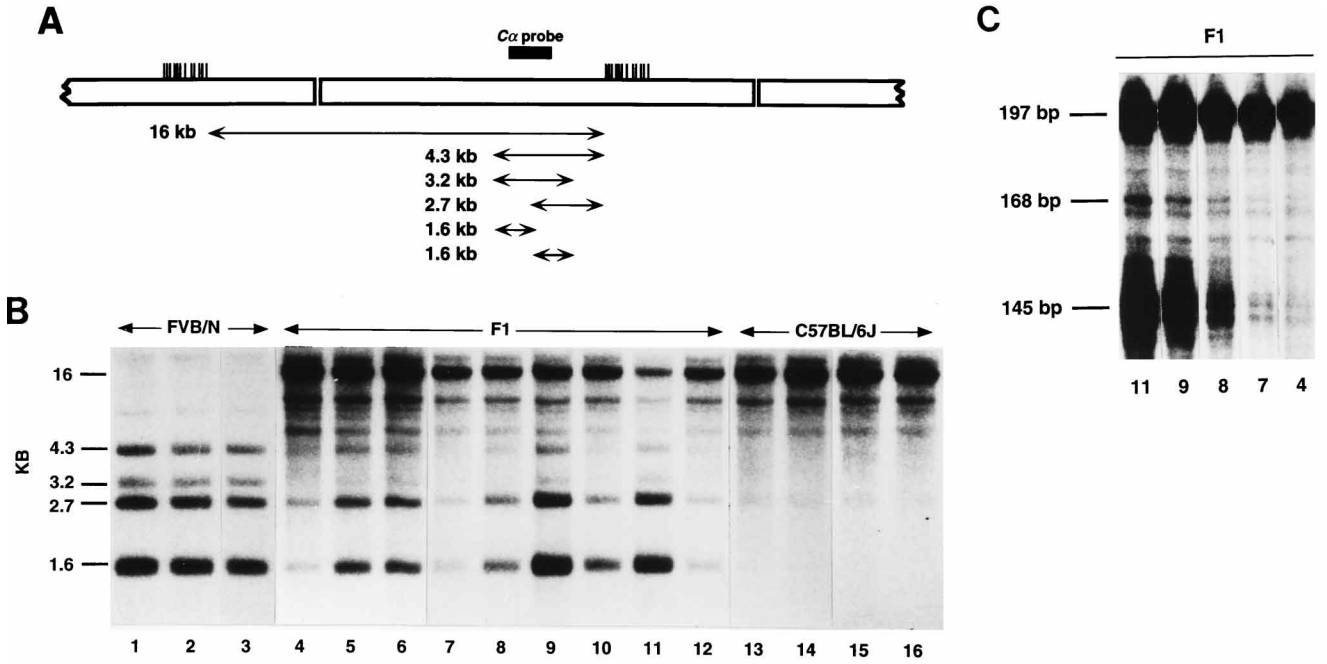

FIG. 1. Variation in methylation and expression of the paternal allele of the *RSVIgmyc* transgene. (A) Schematic of an *RSVIgmyc* allele, shown as a tandem array of unit copies. One complete and two partial copies are depicted as joined head-to-tail-oriented copies. The locations of the prominent *Hpa*II restriction digestion fragments identified on Southern blots hybridized with the Cα probe are indicated by bidirectional arrows. The 16-kb fragment is formed from two adjacent unit copies and results from the methylation of all *Hpa*II sites between two CpG islands in adjacent unit copies (shown as tight clusters of vertical lines). Refer to reference 8 for details on the construct. (B) Variation in methylation of DNA samples derived from hearts of F_1(FVB/N × TG.NL) hybrid mice. For comparison, paternal-allele methylation patterns of *RSVIgmyc* in inbred-strain backgrounds FVB/N and C57BL/6J are shown. The FVB/N *RSVIgmyc* line is TG.AAJ, and the C57BL/6J line is TG.NL. DNA was isolated from whole hearts of adult transgenic mice, and Southern blots were prepared as previously described and hybridized with the Cα probe (6). (C) Variation in transgene expression in hearts of F_1(FVB/N × TG.NL) hybrid mice. For ease in comparison, numbers at the bottom of the gel indicate individual mice that were analyzed in panel B. Fifteen micrograms of RNA isolated from the whole heart was assayed for transgene-specific c-*myc* expression by a previously described RNase protection assay (8, 32). The 197-bp band protects the L32 ribosomal protein gene internal loading control, and the 168-bp band protects transgene-specific c-*myc* transcripts. Nonspecific protection (transgene and endogenous c-*myc*) of exon 2 by the c-*myc* cDNA probe is also shown as a 145-bp protected fragment.

a group of mice are analyzed in this manner, the variation in methylation pattern is reflected in the standard deviation of the mean value of methylation. As shown in Table 1, the variation in *RSVIgmyc* methylation in F_1 hybrids of the TG.NL line is much greater than that in the inbred TG.AAJ and TG.NL lines. These results are in complete accord with the difference in variation observed in Fig. 1B.

Variation in *RSVIgmyc* expression. There is a strong association between the extent of DNA methylation and the level of gene expression, particularly with imprinted genes (29, 32). Because of this, we explored the possibility that the variation in *RSVIgmyc* methylation among the genetically identical F_1 hybrid mice is manifest as variation in transgene expression. To address this, transgene expression in the F_1 animals was measured in an RNase protection assay using total RNA isolated from individual hearts of F_1 hybrid mice (8, 32) (Fig. 1C). The ribosomal L32 gene transcript was used as an internal control to normalize for the amount of RNA assayed (12, 24, 30).

The variation in the extent of transgene expression among individual F_1 hybrid mice was large (a 10-fold range of expression), and there was a strong inverse correlation between transgene methylation and expression; when the degree of methylation was high, the level of expression was reduced (Fig. 2). This was evident when the methylation pattern and the level of expression for the same animal were compared (identical lane numbers in Fig. 1B and C correspond to the same animal). Moreover, there was a small degree of variation in the expression (but no apparent variation in methylation) of paternal TG.NL and TG.NN alleles in the C57BL/6J inbred background. For example, two of nine TG.NL mice carrying a paternal *RSVIgmyc* allele in a C57BL/6J background exhibited a low but detectable level of transgene expression (8) (data not shown). We conclude from this that there is a large difference in transgene expression among genetically identical F_1 hybrid individuals. These findings are consistent with previous observations on the relationship between the highly methylated maternal allele and *RSVIgmyc* silencing and that between the undermethylated paternal allele and *RSVIgmyc* expression (8, 32).

Embryonic origin of F_1 hybrid variation. Analysis of the TG.AAJ and TG.NL *RSVIgmyc* paternal alleles (TG.AAJ in the FVB/N strain and TG.NL in the C57BL/6J strain) at different stages of embryogenesis indicated that the transgene was unmethylated in the blastocyst and that the final adult pattern was acquired by embryonic day 7.5 (7, 8). In the F_1 hybrid animals, the TG.NL transgene was also unmethylated in blastocysts (Fig. 3). By day 8.5 of embryogenesis, transgene methylation was present in F_1 individuals of both the TG.NL and TG.NN lines and the variation in methylation among the TG.NL F_1 individuals was the same as that observed in adults (Table 1 and Fig. 3). We examined transgene methylation in individual mice at embryonic day 8.5 because at earlier times the quantity of DNA isolated from an individual embryo was insufficient to accurately determine transgene methylation. These results indicate that the variation in *RSVIgmyc* methylation arises during the interval in which methylation of the paternal allele is normally established in the inbred FVB/N and C57BL/6J backgrounds (7, 8). This is roughly coincident with

TABLE 1. Quantitative analysis of methylation in *RSVIgmyc* lines

Transgenic line[a]	Genetic background	Tissue	Methylation[b]	n[c]
TG.AAJ	FVB/N	Heart	0.01 ± 0.00	6
TG.NL	C57BL/6J	Heart	0.94 ± 0.03	7
TG.NL	F_1(FVB/N × C57BL/6J)	Heart		
Total			0.37 ± 0.21	30
Females			0.35 ± 0.20	15
Males			0.37 ± 0.23	15
TG.NL	F_1(FVB/N × C57BL/6J)	Embryo		
Total			0.31 ± 0.18	17
Females			0.30 ± 0.20	4
Males			0.35 ± 0.17	9
TG.NN	F_1(FVB/N × C57BL/6J)	Embryo		
Total			0.39 ± 0.23	21
Females			0.48 ± 0.24	13
Males			0.25 ± 0.10	8
TG.AAJ	F_1(C57BL/6J × FVB/N)	Embryo	0.20 ± 0.12	6
TG.NN	F_2(C57BL/6J × F_1(C57BL/6J × FVB/N))	Embryo	0.47 ± 0.19	38

[a] TG.AAJ, TG.NL, and TG.NN are *RSVIgmyc* transgenic lines, produced independently in either an FVB/N (TG.AAJ) or a C57BL/6J (TG.NL and TG.NN) inbred background. All three lines have similarly sized insertions (approximately 10 to 15 copies in tandem arrays) (data not shown). Alleles were always evaluated in hemizygously transmitted states and in whole day-8.5 embryos or whole hearts of adult mice. The sex of each day-8.5 embryo was determined by PCR analysis with oligonucleotide primers to the Y-linked *zyf* gene (26). The sex of four TG.NL F_1 hybrid embryos was not determined.

[b] Transgene methylation was analyzed from Southern blots of paternal *RSVIgmyc* alleles (Fig. 1), with a PhosphorImager being used to determine the signal from ^{32}P-labeled hybridization probes. The methylation for each individual was calculated as the ratio of the intensity of the signal from the 16-kb band to the sum of the signal intensities of all five discrete transgene-specific hybridization bands seen on Southern blots (see text) (Fig. 1A and B). The data from all individuals of the same background genotype are displayed as means ± standard deviations.

[c] n = the number of mice analyzed per group.

the establishment of de novo methylation of the embryonic genome and with the determination of early embryonic cell lineages (7, 18, 25). Variation in methylation of the paternal *RSVIgmyc* allele is also present in a group of reciprocal F_1 hybrid mice obtained from a mating between a C57BL/6J female and a TG.AAJ male (Table 1). Therefore, the variation in a genetically identical F_1 population is not specific to the parental origin of either inbred background, indicating an absence of strain-specific parental effects (such as a strain-specific cytoplasmic component or an imprinted strain-specific modifier).

The variation in *RSVIgmyc* methylation in genetically identical populations of F_1 hybrid mice provides an estimate of the degree of nongenetic variation. How significant is this nongenetic variation? To address this, we measured the variation in the paternal *RSVIgmyc* methylation in an F_2 backcross population of embryos produced by mating wild-type C57BL/6J females to F_1 hybrid male TG.NN transgenics. The variation measured in the F_2 group was the sum of nongenetic and genetic variation; the segregation of FVB/N and C57BL/6J modifier alleles in the germ line of an F_1 male parent produced genetic variation among the F_2 mice. The mean (± standard deviation) *RSVIgmyc* methylation ratio for the F_2 backcross group was 0.47 ± 0.19 (Table 1). The mean methylation ratio

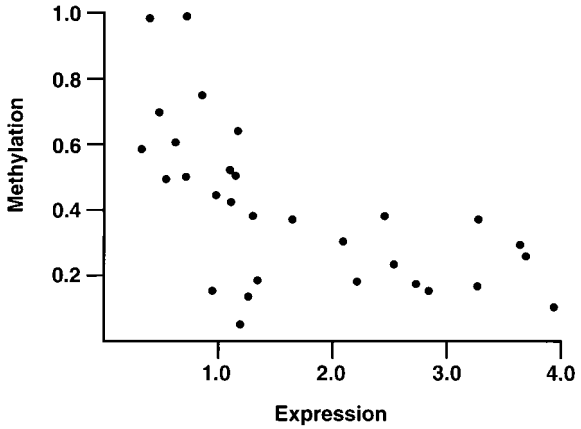

FIG. 2. Relationship between DNA methylation of the paternal allele of the *RSVIgmyc* transgene and its level of expression in F_1 hybrids of the TG.NL line. These data are from the TG.NL mice described in Table 1. The data are plotted as the level of methylation (the intensity of the 16-kb band divided by the sum of the intensities of all five *Hpa*II bands) versus expression (percent intensity of the 168-bp band relative to the intensity of the 197-bp L32 normalization band).

is greater than that of the F_1 populations examined, a finding expected from the increase in the proportion of individuals of the C57BL/6J genotype in the F_2 backcross animals compared to that in the F_1 animals. The similarity in variation (shown as similar standard deviations of the mean) between the F_1 and F_2 populations indicates that the genetic variation present in the F_2 population is quite low compared to the nongenetic variation. We conclude from these measurements that the nongenetic variation is indeed a very significant contributor to the total observed variation.

A possible explanation for the nongenetic variation in *RSVIgmyc* methylation is random X chromosome inactivation, a known source of epigenetic variation in female placental mammals. Random X chromosome inactivation is known to arise in early embryogenesis, in the interval between the blastocyst stage and the early postimplantation embryo (22, 23). In principle, random inactivation of one of the two different X chromosomes in female F_1 hybrids could indirectly influence the methylation and expression of an autosomal transgene. If the variation in the methylation and/or expression of the autosomal *RSVIgmyc* transgene in the TG.NL and TG.NN lines is a consequence of random X chromosome inactivation, then

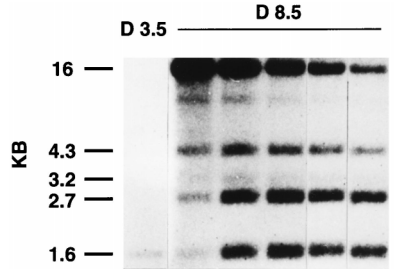

FIG. 3. Variation in the level of DNA methylation of the paternal allele of the *RSVIgmyc* transgene in F_1(FVB/N × TG.NL) hybrid embryos. Approximately 60 day-3.5 blastocysts (D 3.5) were collected and pooled, and the DNA isolated from them was digested with *Hpa*II. Day-8.5 embryos (D 8.5) were carefully dissected away from maternal decidual tissue and extraembryonic membranes. DNA from individual embryos was prepared, digested with *Hpa*II, and subjected to Southern blotting as described in Materials and Methods. The filters were hybridized with the probe Cα, a 1.7-kb *Eco*RI-*Xba*I fragment of the *RSVIgmyc* transgene (8). The positions of molecular size markers are shown on the left.

the variation should be present only in female transgenic carriers. Male mice, which do not undergo X chromosome inactivation, would not show differences in transgene methylation and/or expression. However, to the contrary, both male and female F_1 hybrid mice showed similar degrees of variation in *RSVIgmyc* methylation (Table 1), indicating that the variation was not a consequence of random X chromosome inactivation.

DISCUSSION

Allele-specific methylation of the imprinted *RSVIgmyc* transgene. In a feasible model of the *RSVIgmyc* imprinting process, each unit copy of a transgene allele acquires one of two possible methylation patterns, a highly methylated one or an undermethylated one. In the oocyte, all unit *RSVIgmyc* copies of the inserted transgene allele acquire the highly methylated pattern. In contrast, each paternal *RSVIgmyc* allele acquires a methylation pattern during early embryogenesis, soon after blastocyst formation and roughly coincident with genome-wide de novo methylation (7, 18, 25). Each unit copy of the paternal allele acquires either a highly methylated or an undermethylated pattern. The extent of embryonic de novo methylation of paternal *RSVIgmyc* alleles can be different in a group of mice, producing variation in the methylation and expression of the paternal allele, even in a genetically identical population. Because *RSVIgmyc* expression in the heart is associated with myocyte hyperplasia and cardiac enlargement (17), the variation in the molecular properties of the transgene is in correlation with variation in cardiovascular phenotype.

The highly methylated and transcriptionally silent phenotype of the maternal *RSVIgmyc* allele is remarkably invariant compared to the very different methylation patterns of the paternal *RSVIgmyc* allele. This difference between the two parental alleles suggests that once certain methylation patterns of *RSVIgmyc* are established (during either gametogenesis or embryogenesis, or thereafter), they are faithfully perpetuated. (Primordial germ cells, which have poorly methylated genomes, are exceptions to this maintenance of methylation [6, 11, 25]). A heritable, highly methylated pattern, established in the single-cell oocyte, would be uniformly maintained in all cells of the embryo. Likewise, for each paternal allele, the composite de novo methylation pattern of the entire tandem array of unit copies (transgene allele) is stably maintained for the duration of the individual animal's life. A prediction of this clonal effect is that alleles of imprinted genes that inherit methylation from the germ line should be uniformly methylated in all somatic cells of the offspring. This appears to be the case for *RSVIgmyc* (32) and is also likely to be true for endogenous imprinted genes (4, 15, 31).

Embryonic methylation and phenotypic variation. Studies of targeted mutations in the mouse *Dnmt* gene demonstrated the essential role for DNA methylation in cellular differentiation and embryonic development (19, 20, 21, 34). In view of this, variation in embryonic de novo methylation of endogenous genes would be expected to exhibit significant effects on embryogenesis, manifested as phenotypic variation. Given the extent of embryonic de novo methylation, it is feasible that many endogenous genes are affected. This effect occurs on the paternal allele of *RSVIgmyc*, where methylation is regulated both genetically and nongenetically.

The genetic contribution to variation in *RSVIgmyc* methylation is well demonstrated by comparing the characteristics of the paternal *RSVIgmyc* alleles in the inbred FVB/N and C57BL/6J strains (8). The allele is highly methylated and generally silent in the C57BL/6J background and is undermethylated and active in the FVB/N background. When paternal alleles in F_1 hybrids are examined, intermediate F_1 methylation patterns result, suggesting that the methylation phenotype is a quantitative trait, governed by the inheritance of alleles of multiple modifier genes. The low level of genetic variation compared to the nongenetic variation suggests that many strain-specific modifier loci contribute to the methylation phenotype, each significantly (10). As a consequence, the total variation in an F_2 backcross population of transgenic mice is mostly due to the substantial nongenetic variation, which is apparent as the sole source of variation in the genetically identical F_1 hybrid mice (Fig. 1B and Table 1).

Even though the nongenetic variation is most evident in the F_1 hybrid group of mice, it is probably also present in one or both of the inbred parental strains. For example, given the strong relationship between methylation and expression, the slight variation in paternal *RSVIgmyc* allele expression among inbred C57BL/6J transgenics suggests that there is also a slight variation in methylation. Why was the nongenetic variation in methylation evident in the F_1 hybrid population yet imperceptible in the inbred FVB/N and C57BL/6J populations? A likely explanation is that the genetic modification of *RSVIgmyc* methylation in the C57BL/6J background is sufficiently potent to ensure a maximization of de novo methylation on *RSVIgmyc*. This saturation effect would preclude recognition of any nongenetic variability. Similarly, a strong modification effect in the FVB/N background would preclude methylation above a minimal methylation pattern. The strength of this genetic modification effect would also prevent the appearance of nongenetic variability. In contrast, in an F_1 hybrid background, variation in methylation would be apparent against the balance of the FVB/N and C57BL/6J background effects.

What is the mechanism underlying the nongenetic variation in *RSVIgmyc* methylation? Because the genetic and nongenetic contributions to *RSVIgmyc* methylation both arise near the time of embryonic de novo methylation, the most likely mechanism for the origin of the nongenetic variation is one which affects the molecular mechanism of *RSVIgmyc* methylation. Components of this machinery include a de novo methylase and factors that modulate its activity, possibly encoded by genetic modifiers of *RSVIgmyc* methylation. Thus, we can postulate that nongenetic variation arises by variation in the level of a de novo methylase enzyme or in the level of a modifier of its activity. This variation, in turn, might arise by stochastic fluctuations in levels among a group of genetically identical individuals or, alternatively, might result from environmental fluctuations outside of the embryo (for example, in the uterus). These environmental fluctuations, in turn, would have an impact on the mechanism of de novo methylation. Further details on the molecular source of this nongenetic variation await a better understanding of the mechanism of de novo methylation, including the identification of the de novo methylase enzyme (*Dnmt* or some other methylase gene), and the identification of factors that regulate its activity.

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