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Methylation patterns of testis-specific genes

(*de novo* methylation/CpG islands/spermatogenesis)

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ABSTRACT **The methylation patterns of genes expressed in the mouse male germ line have been examined. *Int-1*, *Hox-2.1*, and *Prm-1*, all of which contain 5′ CpG islands, were found to be completely unmethylated at many sites in these domains, both in somatic tissues and in sperm DNA. Many other testis-specific genes have a similar structure and are probably also constitutively unmethylated. *Pgk-2*, a non-CpG-island gene, is similar to somatic tissue-specific genes in that it is highly methylated in nonexpressing cell types but undermethylated in pachytene spermatocytes and round spermatids, where it is actively transcribed. At later stages of spermatogenesis, however, the gene becomes remethylated and thus acquires the full modification pattern in sperm DNA. In all these cases, the sperm DNA that emerges from the testis does not contain any germ-line-specific unmethylated sites and thus carries the methylation pattern typical of that in somatic tissues.**

DNA methylation is thought to play a role in the regulation of tissue-specific genes in animal cells (1). Not only is there a clear-cut correlation between the capacity of a gene to undergo transcription and its level of undermethylation, but transfection experiments have demonstrated that modification indeed inhibits gene expression, probably by interfering with protein–DNA interactions required for activity (2, 3). For this mechanism to be effective, the DNA modification pattern of these genes must go through dynamic changes in methylation during development. Most tissue-specific genes are fully methylated in sperm and in almost all somatic tissues of the adult organism (4). In the tissue of expression, however, these same genes undergo a striking "demethylation" that could either be necessary for gene transcription or represent a secondary response to induction which serves to maintain the new active state.

In a parallel fashion, genes that are expressed specifically in the male germ line during the process of spermatogenesis should be fully methylated in all somatic tissues but undergo demethylation in the proper germ-line cell types. As opposed to somatic-cell demethylation, where the undermethylated DNA is not passed on to future generations, demethylation in germ-line lineages might require an additional mechanism for regenerating the fully methylated state, and this could be accomplished either in the later stages of germ-line development or at some stage of embryogenesis. Alternatively, gene activation in the germ line may not be coupled with any changes in methylation. To investigate a possible role of DNA methylation in the regulation of germ-line genes, we have undertaken a study of selected genes that are expressed at various stages of spermatogenesis. The results suggest that many testis-specific genes have a fixed methylation pattern which does not vary during development. At least one gene, *Pgk-2*, does undergo demethylation in an expression-specific manner but is restored to its native methylation state before sperm maturation.

MATERIAL AND METHODS

Mouse sperm were taken from the ductus deferens and genomic DNA was isolated by lysis in 10 mM Tris·HCl, pH 7.5/10 mM EDTA/140 mM 2-mercaptoethanol/2% SDS with proteinase K at 200 μg/ml, incubation for 1 hr at 50°C, purification by phenol and chloroform extractions, and precipitation with ethanol. DNA from mouse liver, kidney, or spleen was extracted from frozen tissues by the same procedure but without the mercaptoethanol. Pachytene spermatocytes and round spermatids were isolated as described (5).

Southern blot analysis was carried out using restriction fragments labeled to high specific activity (multiprime system, Amersham) as hybridization probes. These probes were isolated from plasmids kindly provided by E. Keshet (Hebrew University) (*Int-1*), M. Groudine (Fred Hutchinson Cancer Research Center, Seattle) (protamine 1), A. Fainsod (Hebrew University) (*Hox-2.1*), N. Cowan (New York University) (Mα3), and C. Adra (University of Ottawa) (*Pgk-2*).

RESULTS

Numerous male gonad-specific genes have been cloned and characterized, but their methylation patterns have not been studied. One example that is amenable to study is the *Int-1* gene, which is expressed in postmeiotic spermatids as well as in the early embryo but is not transcribed in any somatic tissues (6). An examination of this gene sequence revealed the presence of a CpG island covering its entire length with peaks of CpG dinucleotide residues located within the exon portions of the sequence domain (7). This observation immediately suggested that *Int-1* may be perpetually unmethylated in this island, as are all known housekeeping genes that have been characterized (8). A careful analysis of the *Int-1* methylation pattern with methyl-sensitive restriction enzymes revealed that this was indeed the case. Using a cDNA probe, we could detect a 3400-base-pair (bp) *Stu* I fragment that contained the entire gene. This region was completely digested by all methyl-sensitive enzymes tested, including *Hpa* II and *Hha* I (Fig. 1). Most informative were cleavages with *Xho* I and *Sma* I, which detected single identifiable sites, and in this case also, the CpG residues were found to be completely unmethylated in both liver and sperm DNA. Additional experiments confirmed that a similar modification pattern is also present in DNA from other somatic tissues, including spleen and kidney, suggesting that the same is most likely true of all cell types in the organism.

A second gene specifically expressed during spermatogenesis is *Prm-1*, encoding protamine 1, a protein that replaces the normal histone species and is involved in the formation of the chromatin that is packaged into the mature

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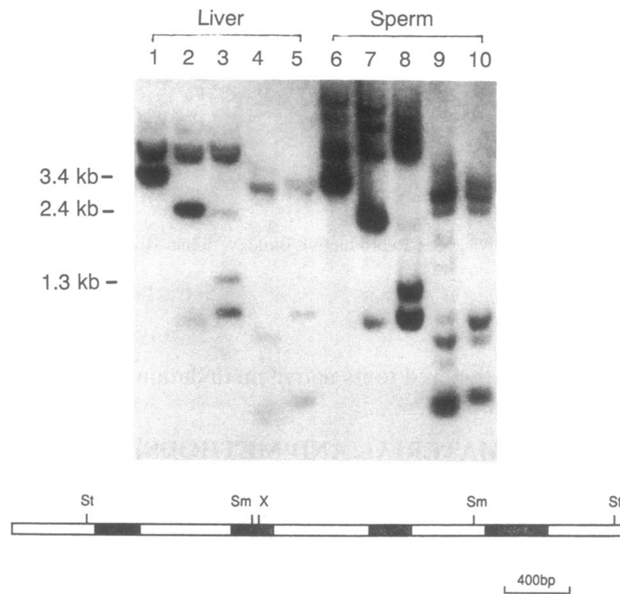

FIG. 1. Methylation pattern of the mouse *Int-1* gene. Genomic DNA (20 μg) from mouse liver (lanes 1–5) or sperm (lanes 6–10) was digested with *Stu* I (lanes 1 and 6), *Stu* I/*Xho* I (lanes 2 and 7), *Stu* I/*Sma* I (lanes 3 and 8), *Stu* I/*Hha* I (lanes 4 and 9), or *Stu* I/*Hpa* II (lanes 5 and 10). Samples were electrophoresed in 1% agarose gels, blotted, and hybridized with a 2.1-kilobase (kb) *Eco*RI fragment containing the *Int-1* cDNA (9). In the schematic of the mouse *Int-1* gene (7), dark boxes represent exons and light boxes represent introns or flanking sequences. Sites for *Xho* I (X), *Stu* I (St), and *Sma* I (Sm) are indicated. In addition, there are >60 *Hpa* II and *Hha* I sites distributed over the length of the gene. This entire region has been sequenced and has an average CpG content of 6%, with peaks in some exon regions as high as 12%.

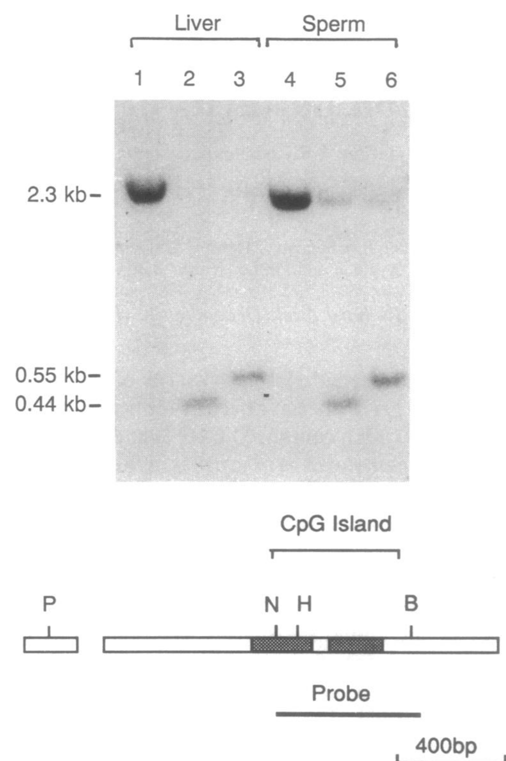

FIG. 2. Methylation pattern of the mouse protamine 1 gene (*Prm-1*). Genomic DNA from liver (lanes 1–3) or sperm (lanes 4–6) was digested with *Pst* I/*Bgl* II (lanes 1 and 4), *Pst* I/*Bgl* II/*Hpa* II (lanes 2 and 5), or *Pst* I/*Bgl* II/*Nci* I (lanes 3 and 6). Samples were electrophoresed in 1% agarose gels, blotted, and hybridized to the 575-bp *Nco* I–*Bgl* II fragment. In the map is a schematic of the mouse *Prm-1* gene (11). Dark boxes represent exons and light boxes represent introns. All samples were cut with *Pst* I (P) and *Bgl* II (B) to yield a 2.3-kb fragment. The sites for *Nci* I (N) and *Hpa* II (H) are indicated, as well as the island-containing region.

sperm (10). This gene also harbors a small CpG island (11) near its 5′ end, and restriction analysis confirmed that the detectable sites in this region are indeed unmethylated. Our assay picked up one *Hpa* II and one *Nci* I site within a small portion of the coding region, which contains 12% CpG, and both were completely unmethylated in several somatic tissues and in sperm DNA (Fig. 2). The same *Hpa* II site has been studied by Trasler *et al.* (12), who found that this CpG residue is undermethylated in testis from mice at different stages of maturity.

This same sequence pattern was also observed for the mouse *Hox-2.1* homeobox gene, which has been shown to be transcribed in the male germ line and in early embryos (13, 14). In this case, the CpG island covers an 800-bp domain at the 5′ end of the transcribed region, which contains numerous sites for *Hpa* II, *Hha* I, and other methyl-sensitive enzymes. Digestion of this gene with any of these restriction enzymes yielded the limit digestion pattern (Fig. 3), indicating that many of these sites are indeed unmethylated in various somatic tissues and in sperm. Although this gene is preferentially expressed in the testis, it may also be transcribed in other, but not all, adult tissues (14).

In the course of searching for additional testis-specific sequences in various organisms, it soon became obvious that the large majority of these genes contain 5′ CpG islands (Table 1). It seemed likely, in light of the above studies, that these would probably behave like all other known CpG island genes and thus remain unmethylated in all tissues and at all stages of development. In some cases islands are built into tissue-specific genes as well, including such examples as the human α-globin (31) and mouse Thy-1 (32) genes, which are probably subject to a system of regulation that does not involve DNA methylation.

Despite the abundance of CpG islands in testis-specific genes, a small number do not contain CpG islands and have a sequence structure that is more typical of other known tissue-specific genes and thus may undergo changes in their methylation pattern. One such example is *Pgk-2*, which is an autosomal processed gene that is homologous to the somatic *Pgk-1* gene located on the X chromosome. Using the published sequence for the murine form of this gene, we identified four CpG sites amenable to analysis (33). Two of these, an *Aha* II and an *Hha* I site, were located near the 5′ end of the coding sequence of this gene, while another two *Hha* I sites were present at the 3′ end, downstream to the polyadenylation site. Unlike the previously examined island-containing genes, all of these CpG residues were methylated in somatic cells and in sperm DNA (Fig. 4). Since *Pgk-2* is expressed specifically during the meiotic and postmeiotic stages of spermatogenesis (34), we felt the necessity to determine whether this gene is transiently undermethylated in these cell types. Indeed, analysis of two transcriptionally active stages of germ-line cell types, pachytene spermatocytes and round spermatids, revealed a clear-cut total demethylation at both sites at the 5′ end of the gene, but not at the 3′ loci (Fig. 4). This change in the modification pattern is probably specific for the *Pgk-2* gene, since other, non-testis-tissue-specific genes are known to remain fully methylated at these stages (35). These data indicate that demethylation accompanies the activation of *Pgk-2* but that the full state of methylation is then restored before the final maturation of the sperm DNA, thus ensuring that the next generation will receive the correct paternally derived methylation pattern typical of a tissue-specific gene.

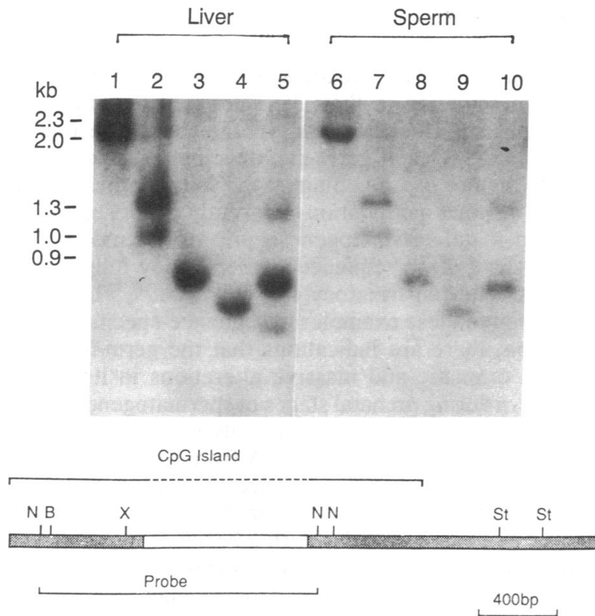

FIG. 3. Methylation pattern of the mouse homeobox *Hox-2.1* gene. Genomic DNA (20 μg) from liver (lanes 1–5) or sperm (lanes 6–10) was digested with *Bam*HI/*Stu* I (lanes 1 and 6), *Bam*HI/*Stu* I/*Xho* I (lanes 2 and 7), *Bam*HI/*Stu* I/*Nci* I (lanes 3 and 8), *Bam*HI/*Stu* I/*Hpa* II (lanes 4 and 9), or *Bam*HI/*Stu* I/*Hha* I (lanes 5 and 10). Samples were electrophoresed in 1% agarose gels, blotted, and hybridized to a 1.4-kb *Eco*RI–*Bam*HI fragment covering the 5′ region of the gene. The map shows the organization of exons (dark boxes) and introns (light box) in the gene. The cDNA has been sequenced (14). The intron region has not been characterized, but is known to be 0.9 kb long. The sites for *Bam*HI (B), *Stu* I (St), *Xho* I (X), and *Nci* (N) are indicated; *Hpa* II and *Hha* I sites are numerous, especially in the island region. The exon part of the island has a CpG content of 6%.

DISCUSSION

Tissue-specific genes that are expressed in somatic cell types have a distinctive pattern of DNA methylation which is correlated with their expression profile. In this scheme, genes that are heavily methylated in all nonexpressing tissues and sperm become stably demethylated in their tissue of expression (1). The studies described here concentrated on the role of DNA methylation during spermatogenesis in the mouse and include data on a variety of gene sequences. The majority of identified genes expressed in the male germ line appear to contain 5′ CpG islands. Three of these, *Int-1, Hox-2.1,* and *Prm-1*, were analyzed and were shown to be unmethylated within the island regions in several somatic tissues, in sperm, and in various stages of spermatogenesis (data not shown). As is the case for many housekeeping genes, the island DNA appears to represent a general signal which is usually recognized and protected from DNA methylation. It is perhaps for this reason that most such genes are expressed constitutively in all cell types and are probably not subject to regulation by DNA methylation *in vivo*. These 5′ islands, however, are not restricted to housekeeping genes and have been found in several well-known tissue-specific sequences such as the human α-globin (31) and mouse Thy-1 (32) genes. In these cases cell-type specificity is probably determined by a system of trans-acting factors and the repression of these genes in the nonexpressing tissue does not require DNA modification.

In addition to the 3 genes investigated in this study, at least 13 additional testis-specific gene sequences from various organisms also have this classical CpG island structure (Table 1). Based on accumulated data for a large number of genes, it can be assumed that the 5′ CpG regions of these testis-specific sequences are also unmethylated in all cell types. Since most tissue-specific genes do not contain 5′ CpG islands, the occurrence of such a large number of these sequences among the genes expressed in the testis is rather unusual. This form may, in fact, have evolved to fit the special needs of spermatogenesis-specific genes, which undergo transcriptional activation and are then immediately passed on to the next generation.

Table 1. Testis-specific genes that have a CpG island structure

Organism	Gene	Ref.
Rat	Cytochrome *c* (testis-specific)	15
	Histone H1 (testis-specific)	16
	Histone H2B (testis-specific)	17
Mouse	*Int-1*	6
	Mos	18
	Abl	19
	T-complex (Tcp1-b)	20
	T-complex (117C3)	21
	Prm-1	11
	RNA helicase (testis-specific)	22
	Mea	23
	Hox-2.1	14
	MH-3	24
	Tyrosine kinase (testis-specific)	25
Human	*ZFX*	26

An exhaustive search of the literature turned up a number of genes from various organisms that are either exclusively expressed in the testis or expressed in several tissues but preferentially in these germ-line cells. Listed are all of the genes that probably have a 5′ island with a CpG content >6%. In some cases, the presence of a classical island was deduced from genomic sequences that include the full upstream regions. For most of the genes, however, only cDNA sequences are available, and the existence of the island is based on high CpG content (7–10%) in the 100–200 nucleotides from the start of transcription. It is presumed that these represent the tail ends of real island regions present in the regulatory portions of these genes. The protamine 1 gene (*Prm-1*) is unusual in this regard, since its CpG island is located within the coding sequence only. Several other gene sequences that could harbor island domains have not been included in the table. For the N-*ras* gene there is no sequence available for its utmost 5′ end. The mouse *ZFY* gene (*Zfy*) has a small domain in its 5′ untranslated region that is ≈3.5% CpG and does not qualify as an island, but further upstream sequences are not available. In the same vein, the human *ZFY* has a short island region (10% CpG) near the 5′ end of the gene. In this case also, there is insufficient upstream sequence data to determine whether it has a real island domain. The *ZFX* gene, which has a similar structure, shows a strong (10% CpG) island region at the most 5′ end of the cDNA, suggesting that the *ZFY* genes will also prove to be of the same nature. Our search revealed six genes, LDH-X (27), *Pgk-2*, Mα3 (α-tubulin), PY353/B (28), transition protein 1 (29), and protamine 2 (30) that are expressed in the testis but do not have a CpG island structure. The methylation patterns of the last two genes in mouse testis have been studied (12). All of the genes covered in the survey are expressed in the germ line and not in other somatic cells present in the testis tissue. *Mea* is the male enhanced antigen gene, and MH-3 is the equivalent of the mouse homeobox gene *Hox-1.4*.

The *Pgk-2* gene represents another category of germ-line-specific sequence. This gene begins to be expressed in meiotic cells (4*N*) and is probably active until the stage of elongated spermatids, at which time all transcriptional activity is halted (34). Although this gene is fully methylated in somatic cells, at least two CpG sites within the body of the gene undergo demethylation at some stage prior to or concomitant with its activation in the testis. Surprisingly, this pattern is transient, and a new round of *de novo* methylation returns the gene to its fully modified form by the final stage of sperm maturation.

Taken together, these germ-line-specific genes reveal an interesting pattern of methylation usage. In every case, the methylation profile of these genes in somatic tissues is

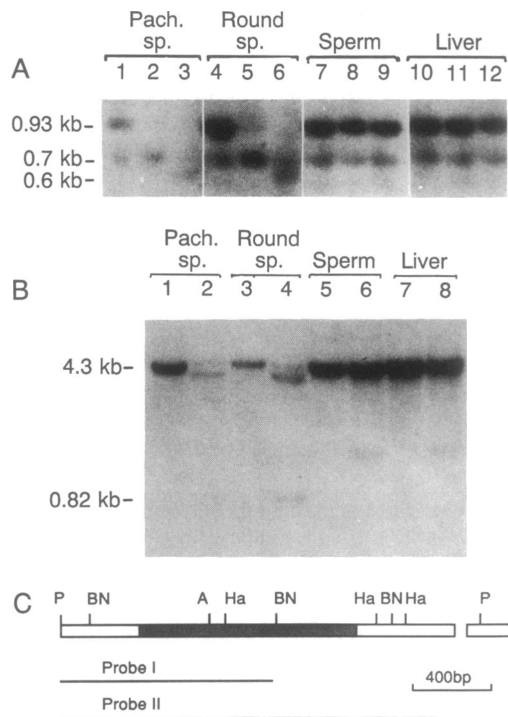

FIG. 4. Methylation pattern of the testis-specific *Pgk-2* gene. (*A*) Genomic DNA (20 μg) from pachytene spermatocytes (lanes 1–3), round spermatids (lanes 4–6), sperm (lanes 7–9), and liver (lanes 10–12) was digested with *Bst*NI (lanes 1, 4, 7, and 10), *Bst*NI/*Hha* I (lanes 2, 5, 8, and 11), or *Bst*NI/*Aha* II (lanes 3, 6, 9, and 12). Samples were electrophoresed in 1% agarose gels, blotted, and hybridized to a 1.1-kb *Pst* I/*Bgl* I fragment (probe 1). In this system, the product of *Aha* II digestion is a 0.6-kb fragment, which can be clearly seen in lanes 3 and 6. The product of *Hha* I digestion is a 0.7-kb band, which cannot be visualized due to the presence of an artifactual band that appears in all of the lanes. (*B*) DNA samples as in *A* were digested with *Pst* I (lanes 1, 3, 5, and 7) or *Pst* I/*Hha* I (lanes 2, 4, 6, and 8) and hybridized with a 1.9-kb *Pst* I/*Bgl* II fragment (probe II). This system detects the expected bands resulting from demethylation of the 5′ *Hha* I site and also shows that all sites, including the 3′ *Hha* I loci are fully methylated in sperm and liver. (*C*) Map shows the location of *Pst* I (P), *Bst*NI (BN), *Hha* I (Ha), and *Aha* II (A) sites. The *Bgl* I site is very close to the *Bst*NI locus. Dark box represents the coding sequence, which is not interrupted by any introns (33). The existence of the 3′ *Hha* I sites is inferred from cloned DNA and not from a genomic sequence.

identical to that found in sperm. Thus, unlike other cell types, the DNA that emerges following germ-line development does not seem to carry tissue-specific alterations in its methylation state. This is consistent with the idea that DNA from somatic cells has reached a dead end, while that from sperm must be used to supply genetic and, perhaps, epigenetic information to the next generation. For most of these genes this is accomplished because of the presence of CpG islands that are constitutively unmethylated. *Pgk-2* is a most illuminating example of this hypothesis, since it undergoes demethylation over a small window of development but is restored to its fully methylated state before the appearance of mature sperm cells. In a similar manner, the testis-specific mouse protamine 2 gene (*Prm-2*) appears to be undermethylated in spermatogonia but eventually becomes further modified during progressive stages of germ-line development (12). Unlike these examples, the gene encoding testis-specific α-tubulin has a fully methylated pattern at many sites within the gene and this is preserved throughout postmeiotic germ line development and in somatic tissues (unpublished results).

The same process of methylation restoration as seen for *Pgk-2* may be responsible for correcting modification patterns in other, non-testis-specific genes. Groudine and Conklin (35) reported that both *ev-3* (avian endogenous virus) and *Tk-1* (thymidine kinase) sequences underwent generalized partial demethylation in their 3′ regions during embryonic development and that these methyl moieties were then restored in later stages of germ-line development, so that the sperm DNA once again contained the fully methylated pattern. This *de novo* methylation apparently occurs at an early stage of postnatal spermatogenesis prior to the modification of *Pgk-2*, since these sequences were already fully methylated in pachytene spermatocytes.

In addition to these examples of sequence-specific *de novo* methylation, there are indications that the germ-line DNA undergoes dramatic and massive alterations in its methylation pattern during prenatal stages of spermatogenesis, since total DNA from primordial germ cells is extremely undermethylated as compared with DNA from the mature sperm (36). The mouse repeated sequences L1, IAP, and MUP, which are partially unmethylated in the early embryo, may represent one example of DNA that becomes *de novo* methylated during early stages of spermatogenesis (37), and more recent studies suggest that this process may also occur on several tissue-specific genes (38). Thus, *de novo* methylation is not only prevalent in male germ-line cells but takes place in a developmentally programmed manner that is probably both temporal and sequence-specific. The pattern that emerges from these studies suggests that biochemical processes during spermatogenesis are directed toward remethylating non-island CpG residues and thus regenerating the somatic cell-type modification profile. Since little is known about the state of DNA methylation of genomic DNA in the oocyte or in cells during early embryogenesis, it is difficult to evaluate the reasons for a methylated paternal genome, but this may be necessary to ensure proper development.

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