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Short Contribution

Tissue-specific characterisation of DNA methylation in the gonad-specific proto-oncogene, c-*mos*, in the male laboratory mouse

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ABSTRACT The proto-oncogene, c-*mos*, which is expressed only in the germ cells of both testis and ovary, plays an important role in meiotic maturation of these cells. In this research, the methylation status of several CpG sites, present both upstream and within the coding region of the c-*mos* gene, has been studied. The *Hpa*II and *Hha*I sites examined in the 5' half of the coding region were unmethylated in both the c-*mos* expressing and non-expressing tissues. A *Hha*I site, h3, present 380bp downstream of the transcription start site, was unmethylated in germ cells, but was partially methylated in the somatic tissues, inversely correlating with the expression status of the gene. In contrast to these tissues, in the mouse fibroblast cell line L929, all the analysed sites were completely methylated.

KEY WORDS: *DNA methylation, c-mos proto-oncogene, spermatogenesis*

DNA methylation is one of the important controlling mechanisms in the multilevel hierarchy of systems that control gene expression in mammals (Bird, 1995). Research on methylation of several tissue-specific genes has revealed that, in most cases, the CpG dinucleotides in promoter regions are completely methylated in sperm and in other non-expressing tissues. In contrast, the promoter regions of these genes are not methylated in tissues which express the genes (Yeivin and Razin, 1993). The most crucial evidence invoking the role of DNA methylation in gene expression came from *in vitro* gene transfer experiments; when *in vitro* methylated gene sequences are introduced into fibroblasts in culture, they remain methylated and transcriptionally suppressed, whereas unmethylated gene sequences are transcribed as usual (Busslinger *et al.*, 1983). Although there is considerable evidence which correlates DNA methylation with gene expression, there are also examples which do not conform to this (Mckeon *et al.,* 1982; Wilks *et al.,* 1982; Vedel *et al.*, 1983; Benvensity *et al.*, 1985). It is also of considerable interest that in some genes, methylation of only a few CpG sites is important for regulation of expression. (Ariel *et al.,* 1994).

Murine c-*mos* is a 1.1 kb gene with a single exon. In testis, its transcription begins in pachytene spermatocytes and reaches a maximum in the early round spermatids, whereupon it becomes inactivated (Propst and Vande Woude, 1985; Goldman et al., 1987; Propst *et al.,* 1987; Mutter *et al.,* 1988). Sequence analysis has revealed its CpG/GpC ratio to be more than 0.5 in the complete coding region, with a G+C content of 56%. The gene has a higher CpG/GpC ratio (0.64) in the 5' half of the coding region. The total gene sequence meets the criteria therefore for being a CpG island, particularly the 5' half of the coding region. The methylation status of five *Hpa*II (m) and four *Hha*I (h) sites (see Fig. 1), which are present upstream of the transcription start site and in the coding region of the gene, were studied by Southern hybridisation using methyl-sensitive restriction enzymes: *Msp*I, *Hpa*II and *Hha*I. Genomic DNA from enriched fractions of spermatogenic cell types (i.e., spermatogonia, pachytene spermatocytes, spermatid, epididymal sperm), postimplantation whole embryo, fetal and adult somatic tissues were digested first either with *Hpa*II or *Hha*I and then with *Eco*RI to limit the region to be analysed. Restricted DNA was then hybridised with 426 (Probe 1) 172 (Probe 2) and 261bp (Probe 3) PstI fragments of v-*mos*, all of which had 100% homology with c-*mos* (Fig. 1). Physical maps in Figures 2,3 and 4 illustrate locations of *Hpa*II(m) and *Hha*I (h) sites and the lines drawn below the physical map correspond to the fragments expected from complete, partial or lack of methylation at the analysed sites, after hybridisation of the *Hpa*II or *Hha*I digested genomic DNA with the 3 probes.

Expression of murine c-*mos* gene has been shown to be restricted to meiotic and post meiotic germ cell types; no expres-

Abbreviations used in this paper: dpc, days post-coitum.

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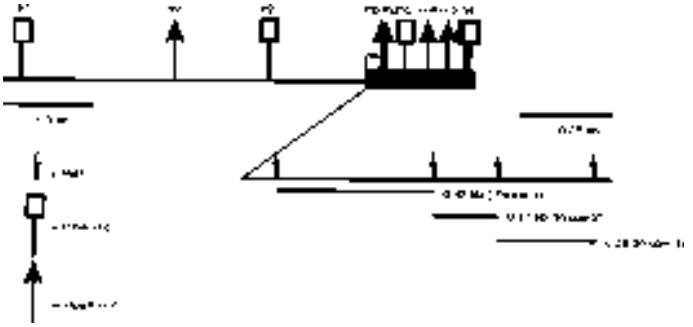

Fig. 1. Physical map of the murine c-*mos* gene (including the promoter region) showing the positions of the *Hpa*II (m) and *Hha*I (h) sites. *The black box represents exon. Nine CpGs (m1, h1, m2, h2, m3, h3, m4, h4, and m5), represented as arrows (HpaII) and rectangles (HhaI), were analysed for their degree of methylation. Below is an extended map of c-mos gene transcription unit showing positions of the PstI sites. The lines drawn below the map are the probes used for evaluation of methylation at the HpaII and HhaI sites.*

sion has been detected in mitotic spermatogonia. In order to understand whether methylation patterns of the c-*mos* gene coincided with its cell-specific transcription, we carried out *Msp*I/*Hpa*II and *Hha*I digestions on fractionated testis germ cell types and epididymal sperm. When *Msp*I/ *Hpa*II digested genomic DNA from testis germ cell types and epididymal sperm was hybridised with Probe 1, fragments of 2.8 kb (m1-m2) and 0.32 kb (m3-m4) were detected both in the *Msp*I and *Hpa*II digested DNA reflecting the lack of methylation of the m1, m2, m3 and m4 sites in all the testis germ cell types and epididymal sperm (Fig. 2A, lanes: 1-5). Since the m2 and m3 sites were separated by just 12 nucleotides, it was not possible to distinguish these two sites for their methylation status. Probe 2 gave the expected 0.2 kb band (m4 and m5) with *Hpa*II/*Msp*I digested DNA from all the testis germ cell types (Fig. 3A, lanes: 1-4) suggesting lack of methylation at the m4 and m5 sites. The *HhaI* digested DNA from testis cell types yielded, in addition to the expected 0.45 kb and 1.5 kb (faint) bands, a prominent 4.0 kb band with Probe 1, which suggested almost complete methylation of the h2 site in all the germ cell types of testis (Fig. 4A, lanes: 1-3). The methylation status at all the analysed *Hpa*II and *Hha*I sites remained the same, therefore, in germ cell types as well as in transcriptionally quiescent epididymal sperm.

The c-*mos* proto-oncogene is expressed at very low levels, if at all, in somatic cells. Therefore, methylation status of c-*mos* was studied in the 9.5 dpc (day postcoitum) whole embryo, fetal and adult somatic tissues. In the postimplantation 9.5 dpc whole embryo, methylation was evaluated at *Hha*I sites. With probe 3, fragments of sizes 4.5 kb (h1-h4), 2.0 kb (h2-h4) and 0.45 kb (h3-h4) were evident (Fig. 4E, lane 2), indicating methylation of h2 and/or h3. This is in contrast to the epididymal sperm where the h3 site was unmethylated (Fig. 4E, lane 1). Since 4.5 kb and 2.0 kb fragments were fainter than the 0.45 kb band, methylation at the h3 site appears to be partial in the somatic tissues. In the 14.5 dpc fetus and adult tissues of liver and brain, hybridisation of *Hpa*II digested DNA with probe 1 gave bands of sizes 2.8 kb and 0.32 kb, as in the *Msp*I digested DNA, exhibiting lack of methylation of m1, m2, m3 and m4 sites (Fig. 2A, lanes: 6-11). The analysis of *Hpa*II digested DNA with probe 2 was performed only in the adult liver and the appearance of the expected 0.2 kb band indicated lack of methylation of m4 and m5 (Fig. 3A, lane 5). Hybridisation of *Hha*I digested adult liver DNA with probe 1 yielded 4 bands of 4.5 kb, 4.0 kb, 1.5 kb and 0.45 kb (Fig. 4A, lane 4) whereas with probes 2 or 3, only 4.5 and 0.45 kb bands were obtained (Fig. 4B, lane 4; Fig. 4E, lane 4). In fetal liver, however, an additional 2.0 kb fragment was noticed when DNA was hybridised with probe 3 (Fig. 4E, lane 3). Appearance of additional 4.5 kb and 2.0 kb bands in the fetal liver and 4.5 kb in the adult liver with probes 2 or 3 suggested a partial methylation at the h3 site. The partial methylation of h3 site was also noticed in the fetal and adult tissues of brain and kidney (Fig. 4D, lanes: 1-4). Taken together, the results indicated that h3 site is unmethylated in germ cell types but partially methylated in the somatic component.

Since the majority of the CpGs of c-*mos* were unmethylated in somatic as well as germ cells, it was of interest to see its methylation status in *in vitro* cultured cells, where most of the tissue-specific gene functions are absent. The study was performed in the mouse fibroblast cell line L929. EcoRI/*Hpa*II-digested genomic DNA gave a 13 kb fragment with probe 1 as compared to a 0.32 kb *Msp*I fragment (Fig. 4B, lane 2). Similarly, *Hha*I digested DNA also gave a 13 kb fragment (Fig. 4E, lane 5), confirming that unlike the tissues

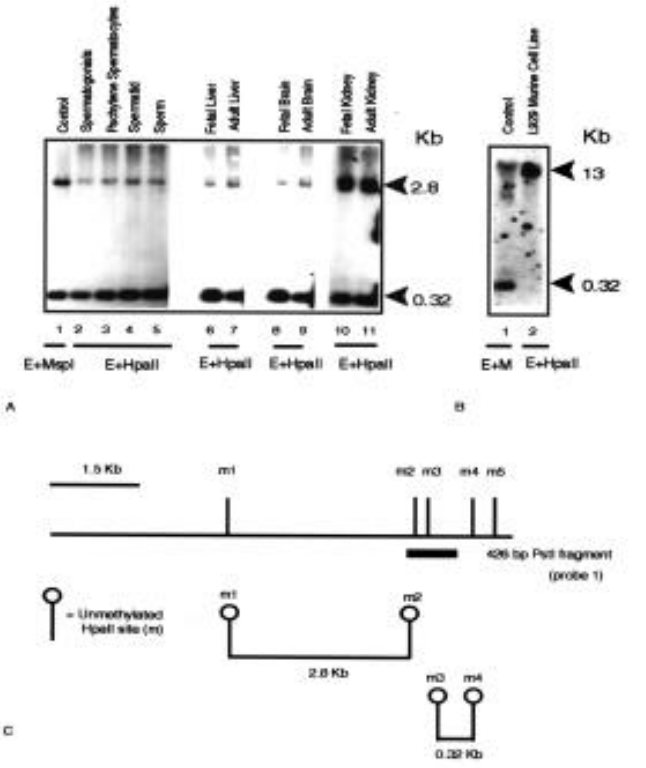

Fig. 2. Methylation status of *Hpa*II sites (m_1-m_2) in testis cell types, fetal and adult somatic tissues and *in vitro* cultured cell line. *Southern blots hybridised with probe 1 showing the methylation status of HpaII sites (m1-m4) in testis cell types and in in vitro cell line, L-929.* **(A)** *Lane 1 contains MspI digested DNA (control), HpaII digested DNA from spermatogonials (lane 2), pachytene spermatocytes (lane 3), spermatids (lane 4), and epididymal sperm (lane 5), fetal liver (lane 6), adult liver (lane 7), fetal brain (lane 8), adult brain (lane 9), fetal kidney (lane 10) and adult kidney (lane 11).* **(B)** *Lane 1 contains MspI digested genomic DNA from L-929 cell line (control), genomic DNA from L929 cell line digested with HpaII (lane 2).* **(C)** *Physical map depicting the positions of HpaII sites. Lines drawn below the map are the fragments resulting from unmethylation of HpaII sites, m1- m4 sites.*

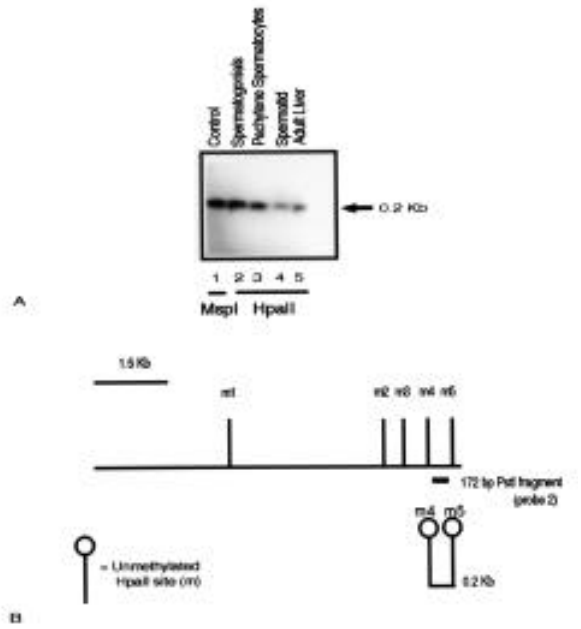

Fig. 3. Methylation status of *HpaII* sites (m4 and m5) in testis cell types and adult liver. (A) *Southern blot probed with probe 2 showing the methylation status of m4 and m5 sites in the testis cell types and liver. Lane 1 contains MspI digested DNA (control), HpaII digested DNA from spermatogonials (lane 2), pachytene spermatocytes (lane 3), spermatids (lane 4) and liver (lane 5).* **(B)** *Physical map showing the locations of HpaII sites. The line drawn below the map is the fragment resulting from lack of methylation of m4 and m5 sites.*

in vivo, all the CpGs of c-*mos* were completely methylated in this cell line.

The analysed *Hpa*II (m2 to m5) and *Hha*I (h3 and h4) sites, therefore, all occurring in the 5' half of the gene, are unmethylated in the male germ line, sperm and in embryonic as well as adult somatic tissues. In contrast, h2, which is present 1.5 kb upstream of the transcription start site, is almost completely methylated and is uniform in both testis cell types and somatic tissues. The only difference between the c-*mos* expressing testis and non-expressing somatic tissues lies in one *Hha*I site, h3, which is unmethylated in the former and partially methylated in the latter. Distinct from all of these tissues, in the mouse fibroblast cell line L929, all the analysed sites are completely methylated (Fig. 5). The role of CpG methylation as one of the modulators of gene expression in mammals has been well documented, the consensus being that the tissue-specific-genes are heavily methylated in sperm and non-expressing tissues and unmethylated in the tissue of expression. The CpG rich regions which form CpG islands, however, remain unmethylated in all tissues (with the glaring exception of those present on the inactive mammalian X-chromosome and in parentally imprinted genes) including the germ line cells, regardless of whether they are house-keeping or tissue-specific (Mckeon *et al.,* 1982; Ariel *et al.,* 1991). The difference in the methylation mostly occurs in the non-island region of tissue-specific genes. Since the abundance of CpGs within the coding region of c-*mos* qualifies the whole gene to act as a CpG island, the lack of methylation of most of the sites (except h2, occurring 1.5 kb upstream of the transcription start site) in testis (the expressing tissue) as well as in the somatic (non-expressing) tissues could be attributed to the CpG island-like structure. In contrast to the tissues *in vivo*, all the examined sites were methylated in the cultured mouse fibroblast cell line L929. The complete methylation of analysed CpGs in the L929 cell line indicates that blocks to methylation *in vivo* have been lost in the *in vitro* cultured cell line. The latter finding attains significance in the light of the observation that transcription factors like SP1, NFkb, GAL4-VP16 induce demethylation in the flanking DNA sequences to their binding sites. It has also been shown that ongoing transcription is dispensable for demethylation (Brandeis *et al.,* 1994; Matsuo, *et al.,* 1998). It is possible, therefore, that the complete methylation of the c-*mos* gene, in the L929 cell line, could be due to absence of specific transcription factors bound to *cis* elements. Moreover, constitutive expression of c-*mos* in somatic cells can lead to cell death or oncogenic transformation (Sagata, 1997).

In the midst of unmethylated sites, h3 (380bp downstream of the transcription initiation site) turns out to be the single site which shows a tissue-specific difference; being unmethylated in all the spermatogenic cells and partially methylated in all the somatic tissues right from the early stages of development. There are ample precedents to prove that methylation (or the lack of it) of a few, or just one site may distinguish between the expressed and unexpressed genes. In the X-linked human *PGK-1* gene, demethylation of just one *Hpa*II site in the promoter region of the gene, shows causal coincidence with its state of expression (Singer-Sam *et al.,* 1992). In c-*mos*, therefore, the methylation of h3 in all the somatic tissues could be a critical epigenetic modification

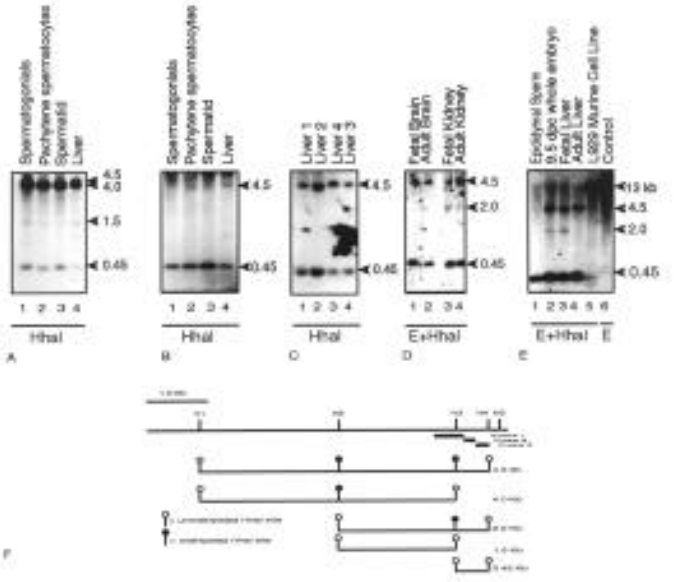

Fig. 4. Methylation status of *HhaI* sites (h1-h4) in testis cell types, fetal and adult somatic tissues and *in vitro* cell line. (A) *Southern blot showing the methylation analysis of HhaI sites (h1, h2, h3 and h4) in testis cell types (lanes 1-3) and liver (lane 4).* **(B)** *Blot in Figure 4A reprobed with probe 2.* **(C)** *Lanes 1-4 show the hybridisation pattern of HhaI digested genomic DNA from four different liver samples with probe 2.* **(D)** *Southern hybridisation of genomic DNA digested with HhaI from fetal and adult tissues of brain (lanes 1,2) and kidney (lanes 3,4) with probe 3.* **(E)** *Southern blot analysed with probe 3 shows unmethylation at the h3 site in the EcoRI+HhaI digested epididymal sperm DNA (lane 1) and partial methylation in the 9.5 dpc whole embryo (lane 2), fetal liver (lane 3) and adult liver (lane 4) DNAs. Lane 5 contains HhaI DNA from the mouse fibroblast cell line, L929, which shows complete methylation of HhaI sites (h1, h2, h3 and h4). Lane 6 (control) has EcoRI digested DNA.* **(F)** *Physical map showing the locations of HhaI sites. Lines drawn below the map are the fragments expected to result from methylation, partial methylation or unmethylation of HhaI sites, h1-h4.*

Fig. 5. Schematic drawing showing the relative extents of methylation at the individual CpGs in testis cell types, postimplantation whole embryo (e9.5 dpc), fetal (14.5 dpc) and adult somatic tissues. *The boxes, lined against the respective CpG sites, are filled according to the extent of methylation at individual sites.*

for the inactivation of the gene. The curious feature, however, is the partial methylation of h3 in all the somatic tissues. That this partial is not technical (e.g., due to partial digestion by the restriction enzymes) has been shown by the reproducibility of results in more than four samples of liver. One possible reason for the partial methylation could be that methylation of h3 is effected in only a limited number cells in each tissue. Since this investigation is restricted to the analysis of a few specific CpG sites, the possibility of certain other CpGs being tissue-specifically methylated cannot be ruled out. In brief, the present research provides clear insight into epigenetic modifications in the gonad-specific proto-oncogene, c-*mos* in expressing and non-expressing tissues.

Experimental Procedures

Random bred mice of Parkes strain reared under constant dark and light cycles were used throughout this study. Postimplantation whole embryos [9.5 days post coitum (dpc)] and various somatic tissues viz., liver, brain and kidney from fetus (14.5 dpc) and adult (above 70 days old) were collected, taking the date of vaginal plug formation as day 0.5.

Separation of enriched fractions of spermatogenic cells

Testes from 5, 18 and 70 days old mice were used for obtaining populations of, spermatogonials, primary spermatocytes and round spermatids, respectively and were separated using as protocol previously the one described (Das and Raman, 1994).

Genomic DNA extraction, restriction digestion and Southern hybridisation

Genomic DNA extraction, restriction digestion and Southern hybridisation were carried out according to Sambrook *et al.* (1989).

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