Variability of DNA methylation pattern in somatic and germ cells in male newt (Amphibia, Urodela) *Triturus cristatus carnifex*

Giovanni Pontecorvo*, Bruna De Felice, Mario Carfagna

Faculty of Biological Science, Department of Life Sciences, II University of Naples, Via Arena, S. Benedetto, 81100 Caserta, Italy

Received 12 May 1998; revised version received 18 June 1998

Abstract In a survey of several mammalian genomes, namely humans, rodents and bovines, the differences in the 5-methylcytosine (m^5C) content show that repeated DNA sequences from sperm were undermethylated and from various somatic tissues were heavily methylated. This report shows a pattern of methylation in male newt (Amphibia, Urodela) *Triturus cristatus carnifex* (*T. c. c.*) unlike that so far described by other authors in mammals. Using methylation sensitive and insensitive enzymes (*Hpa*II and *Msp*I) and successive 3′ terminal labelling (fill-in), we found a greater degree of DNA methylation in premeiotic germ and sperm cells compared to somatic tissue such as hepatocytes. Furthermore the degree of total DNA methylation in spermatozoa appears somewhere between premeiotic germ cells and somatic tissue. Blot hybridization shows that two highly conserved repetitive sequences in amphibian *T. c. c.*, pTvm1 and pTvm8, contribute significantly to the degree of DNA methylation, suggesting a function for these sequences, such as a role in transcriptional regulation.

Key words: DNA methylation; Satellite sequence; *Triturus cristatus carnifex*

1. Introduction

A large number of studies have documented the patterns of methylation in individual genes and repeated sequences from different tissues of various higher eukaryotes, particularly mammals.

Tissue-specific gene sequences in sperm are known to be more methylated, while CpG island sequences are unmodified [1,2]. Reported experiments have demonstrated that a number of repeated sequences in the mouse genome are undermethylated in premeiotic germ cells [3,4]. Overall, sperm shows a lower proportion of 5-methylcytosine (m^5C) than any adult somatic tissue [5] and this is reflected in the hypomethylated state of non-island CpG sites of major and minor satellite sequences [6,7]. In the mouse genome, other repetitive sequence families, such as MUP, IAP [8] and L1 [3] are undermethylated in male foetal germ cells while in mature spermatozoa, spermatids and pachytene spermatocytes these sequences are highly methylated. However, many unique gene sequences are highly methylated in mouse sperm independently of their expression [3,9].

Likewise, in bovines various reports have shown that sperm DNA contains much less m^5C than DNA from a variety of somatic tissues [10,11].

The undermethylation of highly repeated sequences in sperm DNA and the difference in the methylation level between sperm and somatic cell total DNA are borne out by studies of human satellite sequence *Alu*I, particularly rich (9%) in CpG region [12]. Hypermethylation of human Alu elements has been reported for spleen and Kochanek et al. [24] show that Alu elements in the DNA of spermatozoa are much less methylated than in somatic cells. In particular, Alu subset was found to be enriched in young repeats and was almost entirely unmethylated in sperm DNA [13,14]. The unmethylated state of Alu sequences in the male germ line cells may be caused by sperm Alu binding proteins [15].

Most DNA methylation analyses from premeiotic germ cells have been so far limited to mammals. The lack of data regarding amphibians, the ease in obtaining premeiotic tissue, the large number of highly repetitive DNA (up to 60%) within constitutive heterochromatic regions [16] have suggested studies on the methylation degree in total and in repeated highly representative sequences of germ and somatic tissue DNA in *T. c. c.*

In the genomes of different newt species, highly repetitive DNA is organized into families of several sequences that show a large degree of conservation across species. These families are made either of small clusters of many repeats spread over the whole genome or of many tandem repeats clustered in regions of constitutive heterochromatin [16]. Within some newt species, the satellite SatG comprises a highly repetitive sequence *Hin*dIII family, whose repeat units are about 330 bp long (pTvm1) [17]. The most conserved *Bam*HI family, pTvm8, consists of tandemly arranged arrays with basic repeats around 398 bp long [18]. In this study we investigated the methylation level in total DNA, as well as highly conserved pTvm1 and pTvm8 repetitive sequences in premeiotic germ cells, mature sperm and in the hepatocytes from newt, *T. c. c.*

The patterns of methylation in total DNA revealed unequivocally that germ line DNA is substantially fully methylated compared to liver DNA. They also showed that mature sperm DNA is slightly undermethylated compared to premeiotic germ cell DNA. These findings are unlike those so far described in higher organisms.

Blot hybridization experiments revealed that the repeated sequences pTvm1 and pTvm8 contribute to the tissue-specific differences in estimated end-label DNA analysis. Our study could contribute to the understanding of eukaryotic DNA methylation.

2. Materials and methods

2.1. Animals

The male gonad of *Triturus* has such an anatomical shape that during meiotic standstill (November–March) premeiotic germ cells and mature sperm are both present in different compartments.

In all experiments DNA was extracted from premeiotic germ cells,

*Corresponding author. Fax: +39 (823) 275219.

PII: S0014-5793(98)00798-4

mature sperm and somatic tissue (liver) in *T. c. c.* male. Animals were collected during meiotic standstill from Southern Italy.

2.2. DNA preparation

The fraction of premeiotic germ cells was purified according to Erickson et al. (1993). In all experiments DNA was standardly extracted from premeiotic germ cells and somatic tissue (liver) in *T. c. c.* male. These tissues were digested by proteinase K in the presence of SDS and the DNA was purified by the phenol/chloroform method according to Sambrook et al. [19].

Mature sperm DNA was prepared according to the method of Shiurba and Nandi [20].

2.3. Analysis of DNA methylation by end-labelling

DNA preparations were digested overnight with *Msp*I and *Hpa*II isoschizomers. Completeness of digestion was assured by excess enzyme (about 100-fold) on the minute quantities of DNA involved. Completeness of digestion and lack of degradation were supported by the reproducibility of the results from replicate experiments with independent DNA preparation. The digests were end-labelled with [α-^{32}P]-dCTP [19] using the Klenow fragment of DNA polymerase I eso-free.

This procedure is used to fill and label recessed 3′ ends of double-stranded DNA following cleavage with *Hpa*II and *Msp*I enzymes.

As control, uncut DNA was end-labelled in each experiment to ensure the high molecular weight of the starting DNA preparation and verify the absence of degradation. The end-labelled DNA was subjected to electrophoresis (2 h, 80 V) on a 1.7% agarose gel. The fragment size distribution could be observed by direct exposure of the previously dried gel to X-ray film.

2.4. Hybridization

*Msp*I and *Hpa*II digestions of 6 μg of DNA were subjected to electrophoresis (1.5 h, 80 V) on 1% agarose gels. Afterwards gels were blotted on nylon filters. Oligonucleotide I, which spans positions 120–200 of the pTVm1 satellite (Fig. 1) and oligonucleotide II (Fig. 2), which spans positions 66–145 of the pTVm8 satellite, were terminal transferase labelled with digoxigenin-11-dUTP (Boehringer-Mannheim) and used as specific hybridization probes [19].

2.5. Stringency of hybridization conditions

High stringency hybridization: 2× SSC, 0.02% SDS, 1% blocking reagent, 0.1% *N*-laurylsarcosine, $T = 65$°C. High stringency washing: 2× SSC, 0.1% SDS, 2 washes at room temperature (RT) for 5 min each; 0.5× SSC, 0.1% SDS, 2 washes at 65°C for 15 min each. Immunological detection after hybridization was performed using an antibody conjugate (anti-digoxigenin alkaline phosphatase conjugate, anti-Dig-AP).

A subsequent enzyme-catalysed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitro blue tetrazolium salt (NBT) produces an insoluble blue precipitate, which visualizes the hybrid molecules (Boehringer-Mannheim) [21].

3. Results

Results were confirmed by repeated experiments and by independent DNA preparations.

DNA methylation status of the 5′-CCGG-3′ restriction site of *T. c. c*. DNA from various sources (premeiotic germ cells, mature sperm and somatic tissue, liver) was investigated whether in total DNA or in the region of unit repeat pTvm1 (satellite SatG component, comprising a highly repetitive sequence *Hin*dIII family) recognized by oligonucleotide I and in the region of unit repeat pTvm8 (interspersed repetitive sequence *Bam*HI family) recognized by oligonucleotide II, both washed under stringent conditions.

3.1. Analysis of DNA methylation by end-labelling

T. c. c. male DNA extracted from premeiotic germ cells, mature sperm and hepatic tissue was digested with isoschizomeric enzymes *Msp*I and *Hpa*II, end-labelled and subsequently size analyzed by gel electrophoresis (Fig. 3).

An indication of the extent of methylation of the three DNAs may be observed by comparing *Hpa*II digests. Premeiotic germ cells are over-methylated compared to liver DNA, and sperm methylation is somewhere between somatic tissue and premeiotic germ cells.

Since the relevant proportion of repetitive DNA in the *T. c. c*. genome derives from the highly representative satellites pTvm1 and pTvm8, we investigated the contribution of these sequences to the total DNA methylation by Southern blot and hybridization.

3.2. Hybridization to pTvm1

Digestion of DNAs from all sources with a methylation insensitive enzyme (*Msp*I) revealed a multimeric hybridization pattern with a repeat unit of 330 bp (Fig. 4). This reflects the tandem repetition of satellite pTvm1 in the genome when probed with correspondent oligonucleotide I.

The absence of the multimeric pattern in *Hpa*II digests of DNA from premeiotic germ cells and sperm reflects the inability of the enzyme to cut when the internal cytosine in the recognition sequence is methylated. It indicates the extensive methylation of the recognition sequence 5′-CCGG-3′ within satellite DNA. In contrast, *Hpa*II digest of DNA from liver produces multimeric bands comparable to the *Msp*I ones, in-

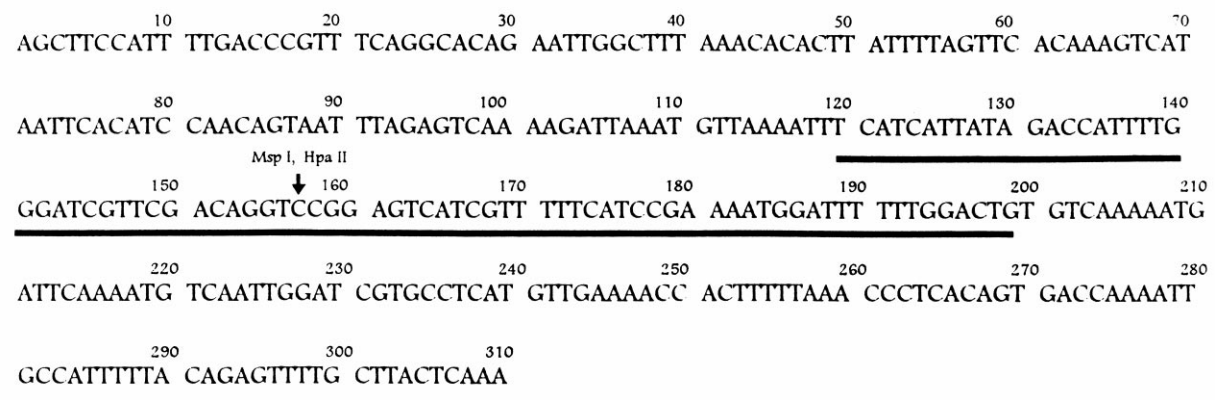

Fig. 1. Sequence of the pTmv1 unit repeat. The position of oligonucleotide probe I is indicated by underlining of the corresponding pTmv1 sequence. Restriction sites for *Msp*I, *Hpa*II are marked by arrows.

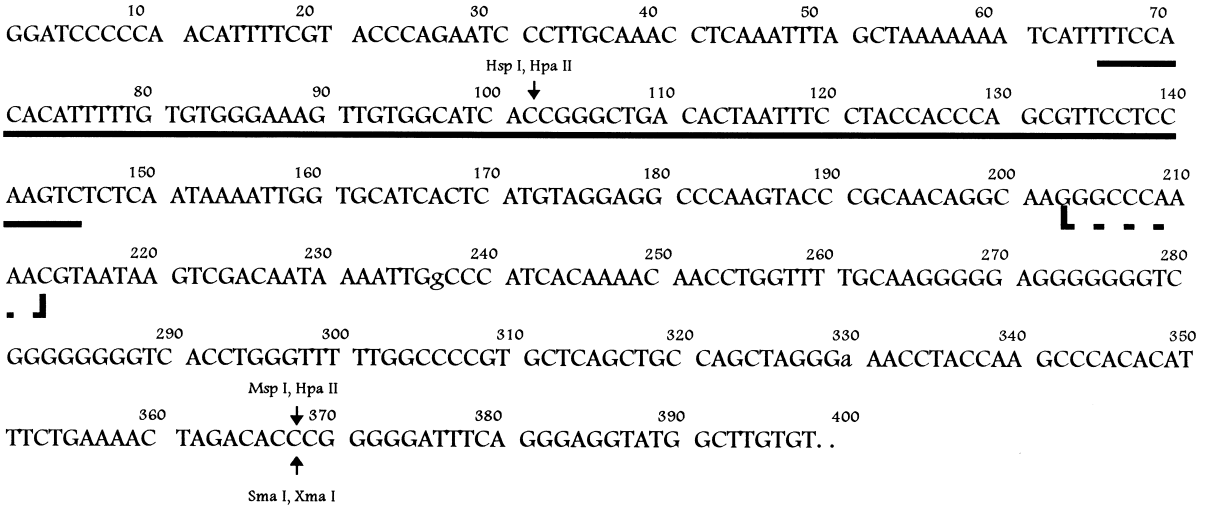

Fig. 2. Nucleotide sequence of pTvm8. The position of oligonucleotide probe II is indicated by underlining of the corresponding pTmv8 sequence. Restriction sites for *Msp*I, *Hpa*II, *Sma*I, *Xma*I are marked by arrows. The sequence between positions 203 and 213, underlined with parentheses, is frequently inverted; in this event the sequence shows the site for *Hpa*II and *Sma*I.

dicating an undermethylation, vs. the other tissues, of the repeat sequence pTvm1 in the hepatic tissue (Fig. 4).

3.3. Hybridization to pTvm8

Fig. 5 shows hybridization of oligonucleotide II which matches site 5′-CCGG-3′ at nucleotide 103 of interspersed repetitive unit pTvm8 in DNA preparation of the three different tissues. DNA was digested with *Bam*HI enzyme, which defines repeat unit pTvm8, with *Hpa*II and *Msp*I and *Hpa*II/*Bam*HI.

Digestions of DNAs from all sources with the enzyme *Bam*HI yielded a series of multimers of 400 bp in unit length (Fig. 5). The *Msp*I lanes show, as expected, 130 and 270 hybridization bands and multimers (Fig. 5). *Hpa*II digests of DNAs from somatic tissue show high and medium molecular weight bands with no less than 400 bp. This indicates a partial undermethylation of the two CpG sites present (Fig. 5).

On the other hand, *Hpa*II digests from germinal tissue show high molecular weight bands, 400-bp band absent, and a smear of hybridization extending down to the bottom of the lanes. Therefore, liver DNA is undermethylated compared to DNA from germinal tissue. In turn DNA from premeiotic germ cells is lightly undermethylated compared to mature sperm.

To determine which of the two CpG sites occurring in unit repeat pTvm8 are implicated in the methylation we carried out further analysis.

The CpG site at 370 nt of the repeated pTvm8 is also the site of methylation insensitive and sensitive isoschizomers *Xma*I and *Sma*I (Fig. 2). The results from *Sma*I and *Sma*I/*Bam*HI digestion show that all three tissues present an identical pattern of undermethylation at that site, therefore, the difference in methylation obtained with *Hpa*II among the three tissues cannot be linked to that site (Fig. 6).

The undermethylation of the first site in liver DNA was revealed after *Bam*HI/*Hpa*II digestion, by 300 and 100 bands. These are almost completely absent in mature sperm. There is an intermediate situation in premeiotic germ cells. These results account for the difference of methylation in the three tissues revealed by *Hpa*II digestion (Fig. 5).

4. Discussion

A comparison of gene methylation in various tissues revealed that active genes are undermethylated in the tissue of expression [22] but are heavily methylated where they are not expressed.

Based on these findings it would be expected that germinal tissue DNA, with few active genes, is heavily methylated. In

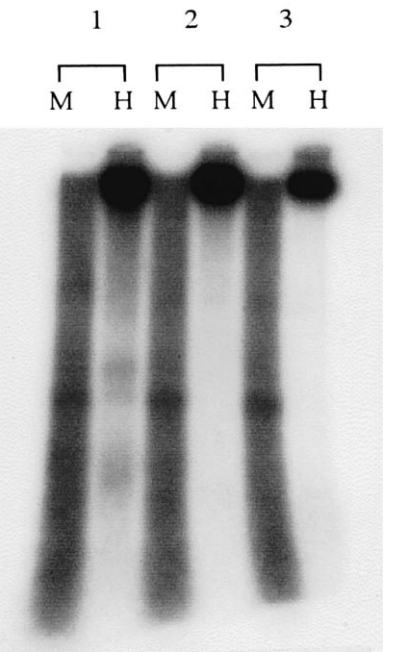

Fig. 3. Autoradiograph of end-labelled *Hpa*II (H) and *Msp*I (M) from liver (lanes 1), mature sperm (lanes 2) and premeiotic germ cell (lanes 3) DNAs.

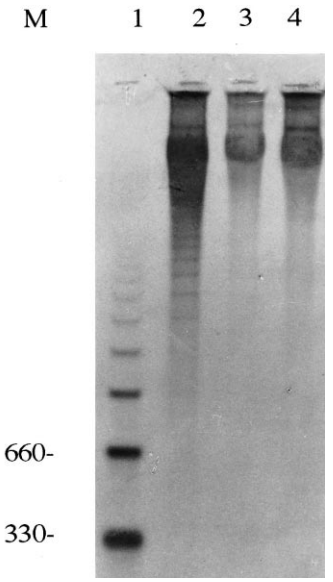

Fig. 4. Southern blot hybridization with oligonucleotide I. Lane 1: *Msp*I digests; lane 2: *Hpa*II digest from liver; lane 3: *Hpa*II digest from mature sperm; lane 4: *Hpa*II digest from premeiotic germ cell DNAs. Marker lengths (M-Dig II) in base pairs are indicated on the left.

almost all cases, satellite sequences were shown to be more methylated in certain somatic tissues than in sperm [14,23,24]; the demethylation of repeated sequences is especially remarkable when contrasted to the increased level of methylation of many individual genes in human and mouse sperm DNA [3,13,14,25,26].

These repeats, which are less methylated in sperm DNA, can be either tandemly organized in satellite sequences [5–7] or interspersed repeats [8]. It was found that the Alu CpGs in sperm total DNA are highly undermethylated [24] and this is sufficient to account for the observed differences in the 5-methylcytosine content of sperm DNA and more highly methylated tissues such as brain [27].

The relevant proportion, up to 60%, of repetitive DNA in the *Triturus* (Amphibia, Urodela) sparks interest in highly repetitive DNA and methylation satellite studies.

The highly repeated DNA of *Triturus* is organized in two ways [16]. In the first case a satellite is made of clustered sequences associated with heterochromatin and found on specific sites along the chromosome [28]. In the second, a satellite is made of sequences scattered throughout the entire set of chromosomes [29], as the Alu family in humans [30] and the two correlated MIF-1 families in the mouse and L1 in humans [31]. The pTVM1 repeat [16] is a clustered sequence in the pericentric zone of the chromosome (the only clustered family conserved in the *Triturus* genus and abundant in the *T. c. c.* genome) presenting a 5′-CCGG-3′ site. On the other hand, the pTvm8 satellite [18] is a scattered sequence largely conserved with two 5′-CCGG-3′ sites and representing 1% of the *T. c. c.* genome.

We studied methylation levels in premeiotic germ cells, mature sperm and hepatocytes of *T. c. c.* from total DNA and repeat DNA sequences (pTvm1 and pTvm8), where we found a situation unlike that described in mammals. Total DNA analysis showed that somatic tissue DNA was undermethylated vs. germinal tissue DNA. The results obtained by the analysis of repeated sequences of pTvm1 and pTvm8 accounted for the prior analysis.

In other words, the only cut site 5′-CCGG-3′ occurring in the pTvm1 sequence was undermethylated in somatic tissue

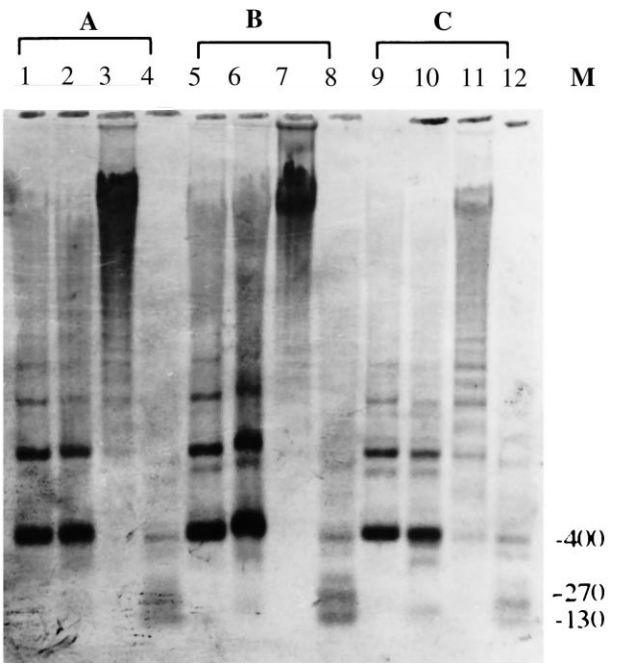

Fig. 5. Southern blot hybridization with oligonucleotide II. Lanes A–C represent, respectively, digestion from premeiotic germ cell, mature sperm and liver DNAs. Genomic DNA digests are: lanes 1, 5, 9: *Bam*HI; lanes 2, 6, 10: *Bam*HI/*Hpa*II; lanes 3, 7, 11: *Hpa*II; lanes 4, 8, 12: *Msp*I. Marker lengths (M) in base pairs are indicated on the right.

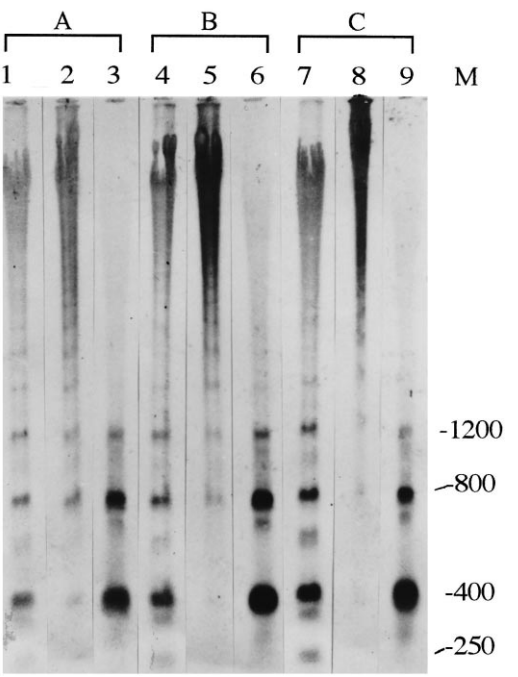

Fig. 6. Southern blot hybridization with oligonucleotide II. Lanes A–C represent, respectively, digestion from premeiotic germ cell, mature sperm and liver DNAs. Genomic digests are: lanes 1, 4, 7: *Xma*I; lanes 2, 5, 8: *Sma*I; lanes 3, 6, 9: *Sma*I/*Bam*HI. Marker lengths (M) in base pairs are indicated on the right.

compared to germ tissues. There were no significant differences between DNAs from two germinal tissues. On the other hand, the 5′-CCGG-3′ sites in pTvm8 sequence were more undermethylated in somatic tissues than in germinal tissue. Moreover, the DNA from premeiotic germ cells was more demethylated than DNA from mature sperm. Our findings, supported by other authors [16], showing the pTvm1 sequence transcription on lampbrush chromosome from oocytes, suggest that the CpG sites from these satellites can play a role in transcriptional regulation. This is likely because these sequences can interact with non-histone protein, as previously described for other satellite sequences [32,33].

Hence, we are studying the role of repeated pTvm1 and pTvm8 sequences in transcriptional regulation.

The transcription patterns in the highly repeated DNA families vary and some have peculiar transcriptional patterns [34,35]. Others report that these transcripts come from promoters that control the expression of structural gene sites upstream to the satellite sequences [36,37]. In contrast, Hellmann-Blumberg et al. [14] reported that there is no evident relationship between Alu repeat demethylation and transcriptional activation of neighboring genes, because of their ubiquity and abundancy. The sequence-specific differences between the methylation pattern of Alu repeat in sperm DNA and those in DNAs from other tissues make them candidates for genomic imprinting [14].

Studies on both endogenous and exogenous imprinted genes in the mouse have shown that these loci are characterized by regions of allele-specific methylation, which suggests that DNA modification may play a role in the regulation of genomic imprinting [38]. It is worth noting that our findings may be correlated with imprinting in that the pTvm1 sequences are transcribed on oocytes lampbrush [16]. In fact, methylation of these sequences in male germ tissue from *T. c. c.* may indicate a turn-off of transcription.

Our findings could make a small contribution to the occurrence of methylation and demethylation events in amphibian newts. The functional significance of our results, however, remains largely elusive.

References

[1] Yisraeli, J. and Szyf, M. (1984) in: DNA Methylation: Biochemistry and Biological Significance (Razin, A., Cedar, H. and Riggs, A.D., Eds.) pp. 352–370, Springer-Verlag, New York, NY.
[2] Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Many, T., Scialky-Gallili, N. and Cedar, H. (1984) Proc. Natl. Acad. Sci. USA 81, 2275–2279.
[3] Monk, M., Boubelik, M. and Lehnert, S. (1987) Development 99, 371–382.
[4] Sanford, J., Forrester, L., Chapman, V.M. and Rossant, J. (1987) Genes Dev. 1, 1039–1046.
[5] Gama-Sosa, M.A., Wang, R.Y.-H., Kuo, K.C., Gehrke, C.W. and Ehrlich, M. (1983) Nucleic Acids Res. 11, 3087–3095.
[6] Sanford, J., Forrester, L., Chapman, V., Chandley, A. and Hastie, N. (1984) Nucleic Acids Res. 12, 2823–2836.
[7] Ponzetto, Zimmermann, C. and Wolgemuth, D.J. (1984) Nucleic Acids Res. 12, 2807–2820.
[8] Howlett, S.K. and Reik, W. (1991) Development 113, 119–127.
[9] Rahe, B., Erickson, R.P. and Quinto, M. (1983) Nucleic Acids Res. 11, 7947–7959.
[10] Kaput, J. and Sneider, T.W. (1979) Nucleic Acids Res. 7, 2303–2322.
[11] Vanyushin, B.F., Tkacheva, S.G. and Belozersky, A.N. (1970) Nature 225, 948–949.
[12] Jurka, J. and Milosavlyevic, A. (1991) J. Mol. Evol. 32, 105–121.
[13] Schmid, C.W. (1991) Nucleic Acids Res. 19, 5613–5617.
[14] Hellman-Blumberg, U., Mc Carthy Hintz, M.F., Gatewood, J.M. and Schmid, C.W. (1993) Mol. Cell. Biol. 13, 4523–4530.
[15] Chesmokov, I. and Schmid, C.W. (1995) J. Biol. Chem. 270, 18539–18542.
[16] Barsacchi, G. (1991) in: Symposium on the Evolution of Terrestrial Vertebrates (Ghiara, G. et al., Eds.) pp. 171–198, Selected Symposia and Monographs, Mucchi, Modena.
[17] Barsacchi-Piloni, G., Batistoni, R., Andronico, F., Vitelli, L. and Nardi, I. (1986) Chromosoma 93, 435–446.
[18] Vignali, R., Rijli, F.M., Batistoni, R., Frattta, D., Cremisi, F. and Barsacchi, C. (1991) Chromosoma 100, 87–96.
[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[20] Shiurba, R. and Nandi, S. (1979) Proc. Natl. Acad. Sci. USA 76, 3947–3951.
[21] Holtke, H.J. (1995) Cell. Mol. Biol. 41, 883–905.
[22] Razin, A. and Riggs, S.D. (1980) Science 210, 604–610.
[23] Sano, H. and Sager, R. (1982) Proc. Natl. Acad. Sci. USA 79, 3584–3588.
[24] Kochanek, S., Renz, D. and Doerfler, W. (1993) EMBO J. 12, 1141–1151.
[25] Driscall, D.J. and Migeon, B.R. (1990) Somatic Cell Mol. Genet. 16, 267–275.
[26] Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., Mc Carrey, J., Cedar, H. and Razin, A. (1992) Genes Dev. 6, 705–714.
[27] Shemer, R., Kafri, T., O'Connel, A., Eisenberg, S., Breslow, J.L. and Razin, A. (1991) Proc. Natl. Acad. Sci. USA 88, 11300–11304.
[28] Baldvwin, L. and Macgregor, H.C. (1985) Chromosoma 92, 100–107.
[29] Batistoni, R., Vignali, R., Negroni, A., Cremisi, F. and Barsacchi-Pilone, G. (1986) Cell Biol. Int. Rep. 10, 486.
[30] Schmid, C.W. and Jelinek, W.R. (1982) Science 216, 1065–1070.
[31] Singer, M.F. and Skowronski, J. (1985) Trends Biochem. Sci. 10, 119–122.
[32] Levinger, L. and Varshavsky, A. (1982) Proc. Natl. Acad. Sci. USA 79, 7152–7156.
[33] James, T.C. and Elgin, S.C.R. (1986) Mol. Cell. Biol. 6, 3862–3872.
[34] Epstein, L.M., Mahon, K.A. and Gall, J.G. (1986) J. Cell. Biol. 103, 1137–1144.
[35] Miklos, G.L.G., Matthaei, K.I. and Reed, K.C. (1989) Chromosoma 98, 194–200.
[36] Diaz, M.O., Barsacchi-Pilone, G., Mahon, K.A. and Gall, J.G. (1981) Cell 24, 649–659.
[37] Bromley, S.E. and Gall, J.G. (1987) Chromosoma 95, 396–402.
[38] Ariel, M., Robinson, E., Mc Carrey, J.R. and Cedar, H. (1995) Nat. Genet. 9, 312–315.