DNA methylation patterns in tumors derived from F9 cells resemble methylation at the blastula stage

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Abstract **We show here that the genome of F9 teratocarcinoma cells growing in culture is heavily methylated but undergoes massive demethylation in tumors derived by subcutaneous injection of the cells. This demethylation occurs primarily in single copy gene sequences. As a result imprinted genes acquire their characteristic monoallelic methylation patterns while non-imprinted genes undergo demethylation. The overall methylation pattern in the tumors resembles the pattern observed in the mouse preimplantation embryo. The F9 cells in the tumors apparently recognize imprinted genes, distinguish them from non-imprinted genes and change the methylation of both gene classes to the pattern which characterizes the embryo. These cells therefore have the potential of providing an abundant source of protein factors involved in establishing the methylation pattern during embryo development.**

Key words: Imprinted gene; Satellite DNA; Demethylation; De novo methylation; CpG island

1. Introduction

The preimplantation mouse embryo genome is characterized by a very low level of CpG methylation [1]. A quantitative analysis of the global methylation status of *Hpa*II (CCGG) and *Taq*I (TCGA) sites in the blastocyst genome revealed that only 8% of the *Taq*I sites and 14% of the *Hpa*II sites are methylated [2]. Most of the residual methylation observed in the blastula can be accounted for by the partial methylation of repetitive sequences which represent a large proportion of the CpG containing sequences in the mouse genome [3]. Yet, methylation of specific sites in single copy genes was also observed, primarily in differentially methylated regions of imprinted genes [4]. The undermethylated status of the preimplantation embryo genome is the result of a demethylation process which takes place post fertilization and erases the methylation pattern inherited from the gametes [5]. It should be emphasized that specific sites in differentially methylated regions of imprinted genes escape this demethylation [6]. Sites which are inherited methylated from one of the gametes maintain allele-specific methylation throughout embryo development. Other sites, in differentially methylated regions of imprinted genes, undergo monoallelic de novo methylation at different stages of development of the preimplantation embryo [7].

The methylation adjustments in the early mouse embryo have recently been shown to be critical for normal embryo development. Mice which are deficient in DNA methyltransferase (Dnmt) die before the 5–25 somite stage depending on the severity of the mutations in the Dnmt gene [8]. Evidence supporting the role of methylation in the maintenance of monoallelic expression of imprinted genes was also provided by analysis of Dnmt mutant embryos [9].

Here we show that F9 cells which are originally derived from OT 6050 embryoid bodies [10] have a highly methylated genome. F9 cells have the capacity to form solid tumors when injected subcutaneously in 129/Sv syngenic mice [11] and change their methylation pattern. Two low copy number genes, β globin and Class I histocompatibility-2, are hypomethylated in F9-derived tumors [12]. We therefore studied in detail the methylation pattern in the F9-derived tumor cells and found that the genome of F9 tumors is heavily undermethylated and the differential methylation of imprinted genes is reestablished. Therefore, our results suggest that the protein factors which are responsible for establishing the methylation pattern during embryogenesis might be present in the F9-derived tumors. The significance of these findings lies in the possibility of using this system to identify, isolate and characterize these factors.

2. Material and methods

2.1. Cell culture and tumor generation

Mouse F9 embryonal carcinoma cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, (100 μg/ml) penicillin-(100 μg/ml) streptomycin sulfate in a 5% CO_2 atmosphere. To generate tumors, 10^6 F9 cells in 0.2 ml PBS were injected subcutaneously in 129/Sv male mice. Tumors were removed 5–20 days after inoculation and kept at −80°C until use. Tumor cell lines were established by plating minced tumors after incubation in 0.25% (w/v) collagenase in DMEM supplemented with 5% calf serum for 2 h at 37°C. The cells were washed with PBS 1 h after plating and fresh medium added. The cultures were split every 2–3 days. The cells had normal growth and morphology for at least 4 weeks.

2.2. Restriction enzyme analysis by Southern blotting

High molecular weight DNA from F9 cells and their derived tumors was prepared as described [17]. Genomic DNA was digested with the appropriate restriction enzymes and the restriction fragments were separated on agarose gels and transferred to nylon filters. Hybridization was performed at 65°C for 15 h in a solution containing 4% polyethylene glycol, 7% SDS, 0.2 M NaCl, 50 mM sodium phosphate, 1.5 mM EDTA, 75 μg/ml denatured herring sperm DNA and 20 ng heat denatured probes labeled by random priming with [α-^{32}P]dCTP. The filters were washed with buffer and exposed to X-ray film. The intensity of the bands was quantitated by densitometry using a BioImaging analyser (FUJIX) and BAS-1000 system software.

2.3. Isopycnic centrifugation

Main band DNA was isolated by density gradient centrifugation. F9 cells and tumor DNA were supplemented with 30 μg of Hoechst 33258 dye, incubated for 10 min at room temperature and CsCl added to a density of 1.68 g/cm^3. The mixture was centrifuged in a Beckman

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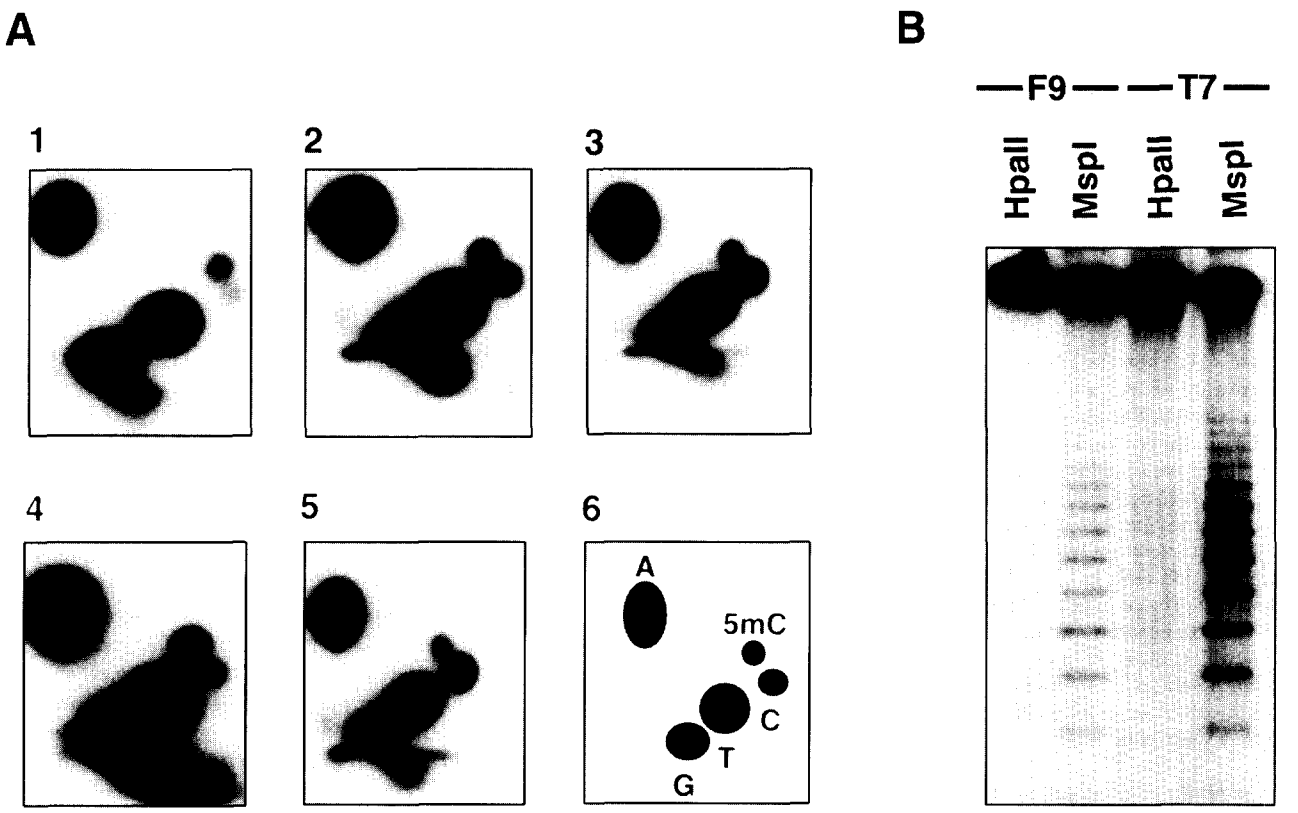

Fig. 1. Methylation of CpG residues in the genome of F9 cells growing in culture and F9-derived tumors. (A) Methylation levels of CpG residues in the genome of F9 cells growing in culture and F9-derived tumors was determined by nearest neighbor analysis (Section 2). The analysis was performed on total DNA isolated from F9 cells growing in culture (1), DNA from 7 day old tumors (2), DNA of 13 day old tumors (3), and main band DNA of both F9 (4) and T7 (5) isolated as described in Section 2. The extent of CpG methylation was estimated by densitometry of the 2′-deoxycytosine 3′-monophosphate (C) and 2′-deoxy-5-methylcytosine 3′-monophosphate (mC) spots (6) and calculated from the equation $\%mC=(mC/C+mC)\times 100$. (B) Extent of methylation of major satellite sequences in F9 cells growing in culture and 7 day old tumor DNA was estimated by comparing *Hpa*II and *Msp*I digests of total DNA by Southern blotting. 1 μg DNA was restricted with *Hpa*II or *Msp*I, blotted as described in Section 2 and hybridized with the mouse major satellite specific probe [14].

Ti50 rotor at 32000 rpm for 65 h and at 40000 rpm for 28 h. The bands were illuminated by a UV lamp and extracted by puncturing the tube below the corresponding bands. The dye was removed by seven consecutive extractions with water-saturated isopropanol and salt removed by dialysis against TE buffer. The purified DNAs were subjected to nearest neighbor analysis.

2.4. Nearest neighbor analysis

Determination of the global methylation level at CpG containing sequences was carried out by the nearest neighbor method as previously described [13]. In brief, randomly nicked genomic DNA was end-labeled at the nicks with the Klenow fragment of DNA polymerase I and [α-^{32}P]dGTP as the only nucleotide. The purified labeled DNA was digested with micrococcal nuclease and spleen phosphodiesterase to yield 2′-deoxyribonucleoside 3′-monophosphates. Finally, the nucleotides were separated by two-dimensional chromatography on thin-layer cellulose sheets and autoradiographed. The extent of CpG methylation was calculated from the relative densities of the C and mC radioactive spots using the BioImaging analyser and BAS-1000 system software.

2.5. PCR methylation assay

PCR analysis of individual *Hpa*II or *Cfo*I sites was performed as described [5]. In brief, DNA (20 pg) was cut with either *Eco*RI or *Eco*RI plus a methyl-sensitive enzyme and amplified for 30 cycles with 100 ng of specific flanking primers in a 100 μl PCR reaction mixture supplemented with 2 mM dNTPs and 2 units of Taq polymerase (Promega Co.). The PCR products were electrophoresed and visualized on ethidium bromide stained agarose gels.

3. Results

3.1. Genome-wide demethylation in F9-derived tumor cells

Subcutaneous injection of F9 cells into syngenic 129/Sv mice results in formation of solid tumors within a few days [11]. As a first step towards the elucidation of changes in the methylation pattern of F9 cells in response to the in vivo environment, we have determined the level of methylation of all CpG-containing sequences in the genome of F9 cells and of F9-derived tumors by nearest neighbor analysis [13]. While more than 80% of the CpG-containing sequences in the DNA of F9 cells growing in culture were methylated, the methylation level of DNA extracted from 7 day tumors decreased to a level of around 45%, and in 13 day tumors methylation decreased to as low as 35% (Fig. 1A). To determine whether this demethylation occurs randomly or primarily at single copy sequences, main band DNA was separated from major satellite DNA using isopycnic centrifugation and the methylation level of CpG residues in the main band analyzed by the nearest neighbor method. In the main band DNA fraction isolated from the 7 day tumors, the level of methylation was extremely low (less than 20% of the CpG sequences were methylated) (Fig. 1A). The observed difference between the level of methylation of total DNA as compared to main band DNA suggested that a large proportion of the residual

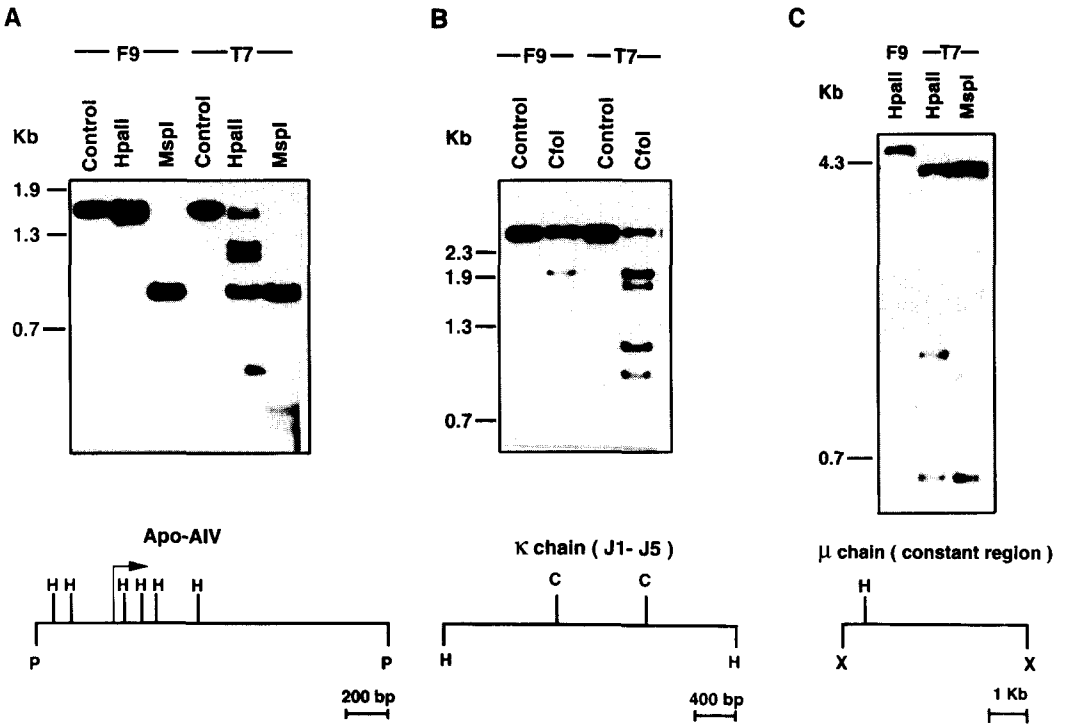

Fig. 2. Demethylation of tissue-specific genes in F9-derived 7 day old tumors. Genomic DNA was extracted from F9 cells growing in culture (F9) and 7 day old F9-derived tumors (T7). (A) The DNAs were digested with *Pvu*II (Control); *Pvu*II+*Hpa*II (HpaII) or with *Pvu*II+*Msp*I (MspI) and subjected to Southern blotting (Section 2). The blot was probed with a radioactive 1.6 kb *Pvu*II fragment of the ApoAIV gene (P-P on the restriction map below the blot). H designates *Hpa*II sites. (B) DNAs were digested with *Hin*dIII (control) or with *Hin*dIII+*Cfo*I (CfoI) and subjected to Southern blotting. The blot was probed with the κ chain *Hin*dIII fragment (H-H on the map under the blot). C designates *Cfo*I sites. (C) DNAs were digested with *Xba*I+*Hpa*II (HpaII) or with *Xba*I+*Msp*I (MspI) blotted and probed with a 4.9 kb *Xba*I fragment of the μ chains (X-X on the map below the blot). H designates the *Hpa*II site.

methylation observed in the tumors is attributable to a high level of methylation of satellite DNA. To verify this conclusion, the level of methylation in the major satellite DNA was determined by restriction enzyme analysis comparing *Hpa*II and *Msp*I digests of the DNA (Fig. 1B). Digestion with *Msp*I revealed a banding pattern that reflects the tandem repetition of the CCGG sites in the satellite DNA whereas *Hpa*II digestion reveals the methylation status of these sites. Although not all repeats harbor CCGG sites as reflected by the upper band in the *Msp*I lane and evident from the sequence [14] the complete resistance of this band to digestion by *Hpa*II with both F9 and tumor DNA clearly indicates that, in contrast to main band DNA which underwent massive demethylation in the tumors (Fig. 1A), the satellite DNA was resistant to this demethylation process (Fig. 1B). These results suggest that the genome-wide demethylation in F9-derived tumors occurs primarily at single copy sequences. This phenomenon is similar to the genome-wide demethylation observed in the very early mouse embryo [5]. Also, in the embryo single copy genes

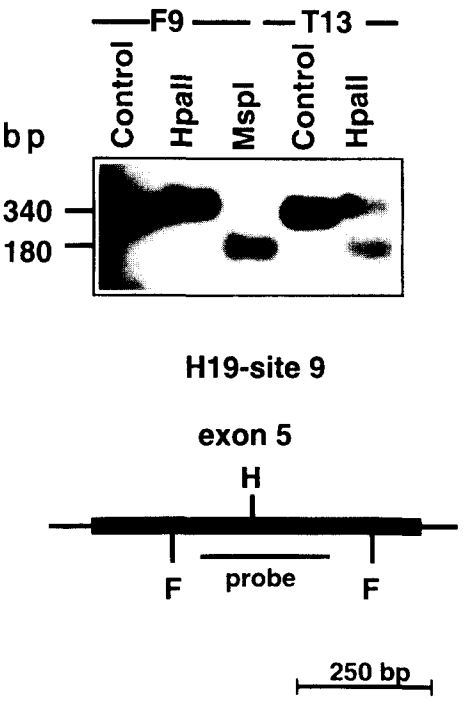

Fig. 3. Methylation status of a differentially methylated site in exon 5 of H19 gene in 13 day old tumors. Genomic DNA was extracted from F9 cells growing in culture and 13 day old F9-derived tumors (T13), digested with *Fok*I (Control), *Fok*I+*Hpa*II (HpaII) or *Fok*I + *Msp*I (MspI), blotted and hybridized with a radioactive PCR fragment including the *Hpa*II site 9 of H19 exon 5. ACTTCATCATCTCCCTCCTG(5′primer)GCATGTTGAACACTTTATGA(3′ primer). F designates *Fok*I; H designates *Hpa*II/*Msp*I.

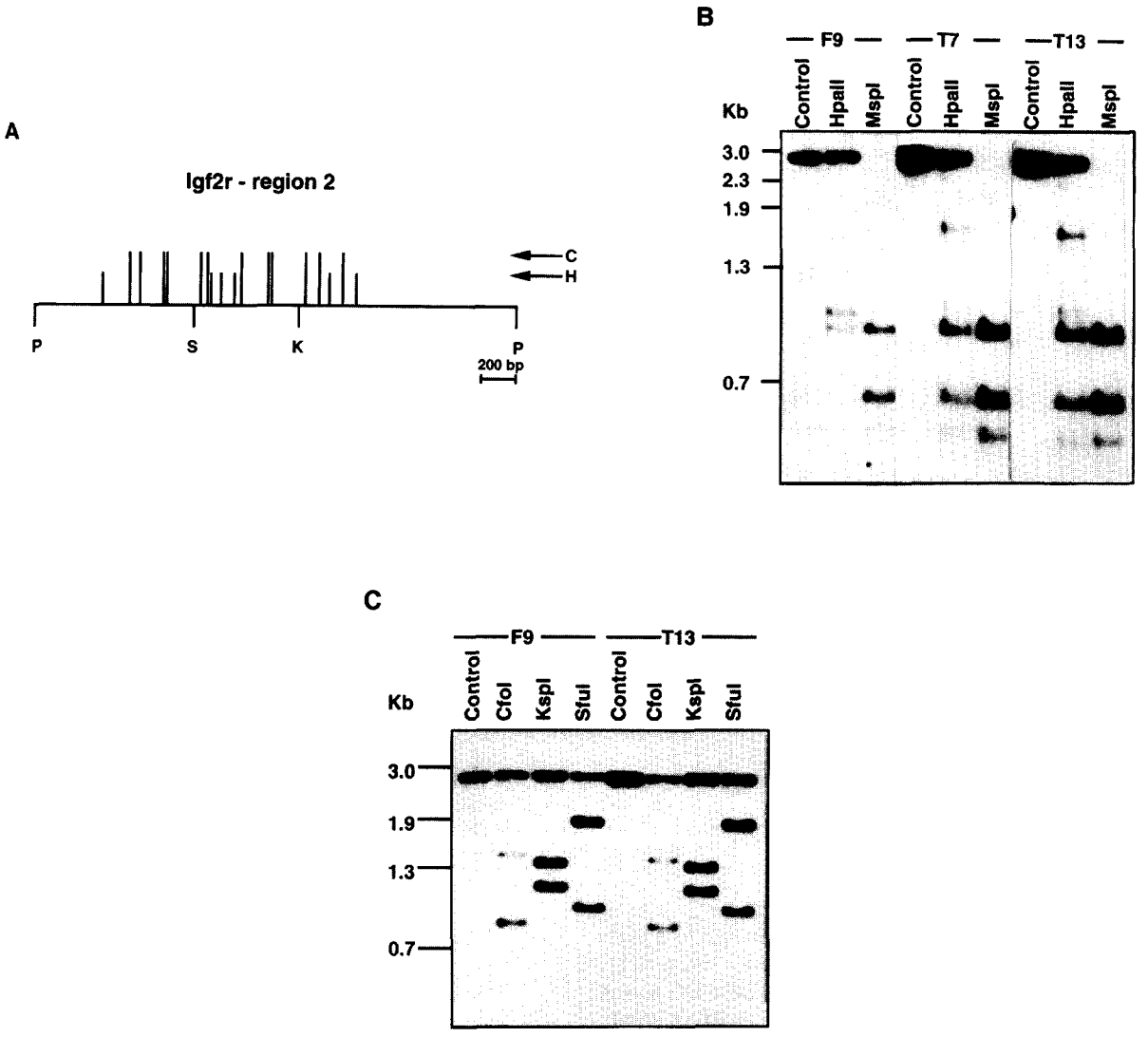

Fig. 4. Methylation pattern of the differentially methylated region 2 of the Igf2r gene in F9 cells growing in culture and in T7 and T13 tumor DNA. (A) Restriction map of Igf2r region 2. A 3 kb region positioned 27 kb downstream of the transcription start. Restriction sites are marked by vertical lines. The shorter lines designate H, *Hpa*II/*Msp*I sites; the longer lines designate C, *Cfo*I sites; S designates *Sfu*I; K, *Ksp*I and P, *Pvu*II. (B) Genomic DNA was extracted from F9 cells growing in cuture (F9) and from 7 and 13 day old tumors (T7 and T13, respectively). The DNAs were subjected to restriction enzyme digestion with *Pvu*II (Control), *Pvu*II+*Hpa*II (HpaII) or *Pvu*II+*Msp*I (MspI). The restriction digests were Southern blotted using the 3 kb *Pvu*II fragment (P-P as described in A) as probe. (C) Genomic DNA was extracted from F9 cells growing in culture (F9) and 13 day old tumors (T13), digested with *Pvu*II (Control); *Pvu*II+*Cfo*I (CfoI); *Pvu*II+*Ksp*I (KspI); *Pvu*II+*Sfu*I (SfuI), Southern blotted and probed with the *Pvu*II (P-P) fragment.

undergo demethylation while repetitive sequences maintain their methylation status [3].

3.2. *Methylation patterns of tissue-specific genes in F9 cells and F9-derived tumor cells*

Fig. 2 shows a comparison of methylation patterns in F9 and 7 day tumor DNAs of 3 different murine tissue-specific genes; apolipoprotein AIV (ApoAIV), immunoglobulin κ light chain and the constant region of the immunoglobulin μ chain. The patterns of methylation were analyzed by restriction enzyme digestion with *Hpa*II (CCGG) or *Cfo*I (GCGC), followed by Southern blotting. The blots clearly show that the sites in ApoAIV and κ chain are heavily methylated in F9 cells growing in culture and substantially hypomethylated in the tumors. On average 70% demethylation of individual sites in ApoAIV and κ chain was observed as judged by densitometry of the blots (Fig. 2A,B). A single *Hpa*II site in the constant region of the immunoglobulin μ chain was methylated in F9 cells and unmethylated in the tumors (Fig. 2C). Similar results were obtained with other single copy genes such as PGK2, α spectrin and c-abl (data not shown). The demethylation observed with the specific gene sequences is in accord with the substantial demethylation of the main band DNA in the tumors.

3.3. *Methylation patterns of parentally imprinted genes in F9 cells and F9-derived tumors*

In addition to heavy methylation of repetitive sequences, specific regions in imprinted genes are monoallelically methylated in the mouse blastocyst genome. It was therefore of interest to analyze the methylation pattern of these differentially methylated sites in the F9-derived tumors. One such differen-

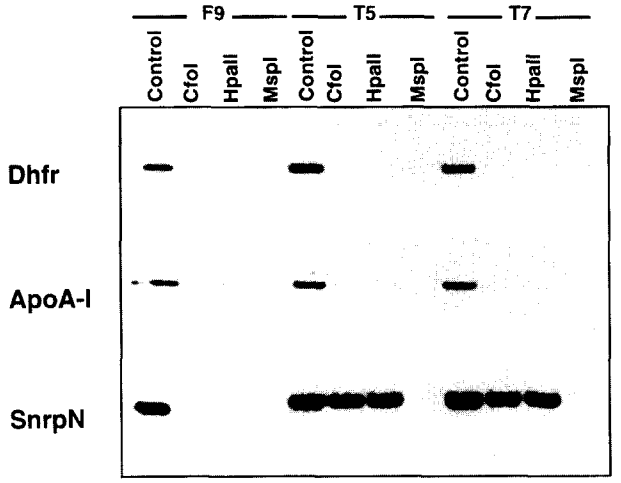

Fig. 5. Methylation analysis of CpG islands in F9 cells growing in culture and 7 day old tumors. DNA extracted from F9 cells growing in culture (F9), 5 day old tumors (T5) and 7 day old tumors (T7) was digested with *Eco*RI (Control), *Eco*RI plus *Cfo*I (CfoI), *Eco*RI+*Hpa*II (HpaII), *Eco*RI+*Msp*I (MspI). 20 pg of digested DNA were amplified with the following primer pairs: Dhfr-ACTGGAAGCACCAAGACCTCA(5′primer)GCAGAGAAGGGT-AAAGTACGGC(3′primer), ApoAI-GATGGTGCAACTGCCTTA-(5′primer)-ATTCTGTTCTCTGTGCCC(3′primer), SnrpN-CCCTC-TCCCACATAGTAAAAATCTGT(5′primer)CGTCCCAGGCAAT-GGCTGC(3′primer). For each pair of primers 'no DNA' controls were added (not shown).

tially methylated site is site 9 in exon 5 of the H19 gene which is methylated in sperm, unmethylated in the oocyte and paternally methylated throughout development [7]. The results presented in Fig. 3 show that this site is fully methylated in culture growing F9 cells and 50% methylated in 13 day old tumors. To verify this observation with differentially methylated sites in another gene, we analyzed the intronic CpG island located 27 kb downstream of the promoter region of the Igf2r gene (Fig. 4A). This region shows maternal methylation in the blastocyst [4]. Seven *Hpa*II sites within this region were found to be almost completely methylated in F9 cells growing in culture. In the tumors, within 7 days this region underwent massive demethylation, leaving 50% of the molecules methylated at all seven sites (upper band). This newly established methylation pattern was also observed in the 13 day old tumors (Fig. 4B). Other sites in this region, 12 *Cfo*I sites, one *Ksp*I and one *Sfu*I site, were 50% methylated in the culture growing F9 cells and remained 50% methylated in the 13 day tumors (Fig. 4C). This observation strongly suggests that demethylation is not random but rather operates to establish a monoallelic methylation pattern. The results presented in Figs. 3 and 4 taken together demonstrate that the tumor cells adjust the methylation to a pattern which is characteristic to the preimplantation embryo. This argument is further supported by the results shown in Fig. 5 (see below).

3.4. Methylation status of sites located in CpG islands in F9 cells and F9-derived tumor cells

CpG islands in general are unmethylated in all adult tissues, in the gametes and at all stages of embryo development [5]. Exceptions to this rule were found in CpG islands of imprinted genes which are frequently methylated on one of the parental alleles [15]. One such island is present at the 5′ end of the mouse imprinted gene SnrpN. This CpG island is methylated in oocytes, unmethylated in sperm and the differential methylation pattern is preserved throughout embryogenesis [16]. To analyze specific sites within CpG-rich regions we used the PCR method as we previously described [5]. DNA was extracted and digested with *Hpa*II or *Cfo*I and primers flanking the sites were used to generate a PCR product. As shown in Fig. 5 the sites analyzed in CpG islands of non-imprinted genes (ApoAI and Dhfr) were, as expected, unmethylated in F9 cells and stayed unmethylated in the tumors. In contrast, *Hpa*II and *Cfo*I sites in the SnrpN CpG island were unmethylated in F9 cells and methylated in the 7 day tumors. The de novo methylation of the sites in the SnrpN island was observed in the tumors as early as 5 days after injection, testifying to the existence of methylation adjustment mechanisms in the tumors including the one involved in establishing differential methylation in imprinted genes. The de novo methylation observed in the CpG island of SnrpN is striking in light of the global demethylation of genes that is observed in the tumors.

4. Discussion

The methylation pattern of the mammalian genome is created anew in each individual during early embryo development. The first step in this process is genome-wide demethylation to erase the different methylation patterns which are inherited from the gametes. The undermethylated state of the genome at the precavitation stage is maintained through the blastula followed by global de novo methylation of the genome at the pregastrula [1,5]. An exception to this rule are specific sites in differentially methylated regions of imprinted genes. Differential methylation of some of these sites may be inherited from the gametes and maintained as monoallelic methylation throughout development of the preimplantation embryo. Other sites become methylated soon after fertilization and others at later stages of development [7]. These specific and dynamic changes of methylation in the preimplantation embryo suggest that a set of stage-specific protein factors exists in the early embryo to perform the genome-wide demethylation and at the same time protect specific sites from undergoing demethylation. Other proteins must be used to distinguish between the paternal and maternal alleles and specifically methylate one of the alleles. It is clear that in order to advance our understanding of the processes involved in the adjustment of methylation in the early embryo it is necessary to identify, isolate and characterize the proteins involved. It is equally clear that this task cannot be carried out with the small amount of material available in early developmental stages of the mouse embryo. Embryonal cells in culture such as F9 or ES cells are also not the choice to solve this problem since these cells have apparently lost in culture the methylation characteristics of the preimplantation embryo.

We describe in this report the methylation characteristics of DNA extracted from tumors derived by subcutaneous injection of F9 cells. The overall methylation level of the tumor genome is low. The residual methylation is accounted for, in part, by the high methylation level of satellite DNA as is the case in the blastocyst. The methylation analysis of non-imprinted genes revealed a complete unmethylated state at CpG island sequences and substantial demethylation of non-island sequences. As is the case in the blastocyst, specific sites

in imprinted genes are 50% methylated suggesting monoallelic methylation. It is interesting that sites which are differentially methylated in imprinted genes and were completely methylated in F9 cells growing in culture regained the level of 50% methylation in the tumor cells while other differentially methylated sites such as those in the CpG island of the SnrpN gene, which were unmethylated in the F9 cells, underwent de novo methylation in the tumor cells. Sites that are 50% methylated in F9 cells, such as those of Igf2r region 2, retain this partial methylation in the F9 tumors. These observations suggest that the machinery which is responsible for the recognition of differentially methylated regions and adjustment of methylation levels in the preimplantation embryo might be present in the tumor cells. Since the F9 cells lost this capacity while growing in culture our results suggest that it can be regained when the F9 cells are returned to the mouse environment. It should be emphasized that the methylation characteristics of the F9 cells in the tumors are maintained for at least several weeks in cultures prepared from disintegrated tumors as described in Section 2 (data not shown).

These interesting findings imply that the F9 cells in the tumors might be useful in the identification, isolation and characterization of the protein factors involved in the specific methylation and demethylation events that take place in the early embryo.

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