Growth conditions of F9 embryonal carcinoma cells affect the degree of DNA methylation

R. P. Erickson[1], S. Ferrucci[1], B. Rahe[1], M. P. Rosenberg[1] & D. Morello[2]
[1] *Department of Human Genetics, Box 015, University of Michigan School of Medicine, Ann Arbor, Mich., USA*
Present address: Imperial Cancer Research Fund Laboratories, P.O. Box no. 123, Lincoln's Inn Fields, London WC2A 3PX, Great Britain
[2] *Département de Biologie Moleculaire, Institut Pasteur, Paris 75024, France*

Abstract

We have investigated differences in C*pG methylation between F9 embryonal carcinoma cells *in vitro* and as tumor cells grown *in vivo* using *Msp* I and *Hpa* II restriction isoschizomers. Southerns were hybridized with two low copy number probes, mouse major β-globin (f7) and a class I, histocompatibility-2 cDNA clone (pH-2^d-4). In each case, the tumor-DNA was hypomethylated while the DNA from F9 cells grown *in vitro* was moderately methylated. We conclude that growth conditions or cell-cell interactions can greatly affect methylation of C*pG sites.

Introduction

Many studies of site-specific methylation of structural genes have found hypomethylation of variably methylated sites in tissues or cells where the gene is expressed (16, 19, 21, 26, 27, 32) and hypermethylation in tissues where they are not expressed. Whether DNA methylation plays a role in the control of gene expression or if methylation occurs after regulation cannot be presently ascertained. In point of fact, many exceptions to this correlation have been found (9, 10, 18, 24, 35).

One of the exceptions to the degree of methylation of certain genes is that which occurs in transformed cells and tissues. Analysis of DNA from lung and colon tumors, with three specific probes to genes not expressed in these tumors, showed them to be hypomethylated when compared to their normal tissue counterparts (6). Because these tumors had not been adapted to tissue culture and were from untreated patients, hypomethylation of normally hypermethylated genes cannot be due to an experimental manipulation. In one case, several metastases were found to have increasingly hypomethylated patterns compared to the primary tumor. In contrast, a comparison of methylated C*pG sites in adult rat liver, a rat hepatoma line, and fetal rat liver using probes to albumin and alpha fetal protein genes showed no correlation in the changes in the methylation pattern with gene activation or repression (24). These data suggest that (1) the rapid growth of tissues affects the methylation pattern of various genes; (2) the relationship of cell-cell interactions in culture versus tumors of various tissues affect the degree of methylation; or (3) the morphology and state of differentiation of tumors change in comparison to their normal counterpart during tumorgenesis and metastasis.

We have investigated the first and second possibility, i.e., that growth and/or cell-cell interactions may influence methylation patterns in tissues and cells from tumors using embryonal carcinoma cells. In order to control for possible differences in differentiated and undifferentiated tissues, we used the F9 mouse embryonal carcinoma (EC) line. F9 is a 'nullipotent' EC cell line originally derived from a transplantable testicular tumor that arose in strain 129/J (1). Although considered 'nullipotent', treatment with *trans*-retinoic acid will stimulate almost all of the cells to differentiate to primitive

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yolk sac cells (33). F9 EC cells grown subcutaneously in syngeneic hosts grow rapidly, forming well-defined tumor masses with only embryonal-like cells apparent.

We recently used F9 *tumor* DNA as a control during studies of DNA methylation during spermatogenesis (25) and found it to be hypomethylated when Southerns were probed with several cDNA clones, including to *H-2*. Unlike many other tumors, EC cells or tumors do not normally express class I histocompatibility antigens (2), *H-2*, unless differentiated (31) or transplanted to non-syngeneic hosts (23). In contrast, Morello *et al.* (22) had found a high degree of methylation of C*pG site at the *H-2* locus in cultured F9 *cells*. In this study we compared the degree of methylation of F9 cells when grown *in vitro* and *in vivo*, using restriction isoschizomers. We present data herein that F9 nullipotent embryonal carcinoma cells are hypermethylated when grown in tissue culture, but hypomethylated when grown *in vivo*. Thus, growth conditions are likely to be an explanation for the variable methylation.

Materials and methods

F9 EC cellular DNA was obtained from cultures grown in Ann Arbor and Paris. The Ann Arbor cells, originally obtained from Dr. Peter Andrews, were grown in D-MEM (Gibco) containing antibiotics (penicillin and streptomycin) and 5% heat inactivated fetal calf serum. Cells were trypsinized and replated every three days to avoid accumulating spontaneously differentiating cells. The F9 and related PCC4/Aza cellular DNA from Paris was obtained as previously described (22). F9 tumors were induced by injecting approximately 1×10^5 F9 cells (Ann Arbor) subcutaneously into 129/SvJ male mice. Tumors were taken when less than 1 cm in diameter; they were found to be typical of undifferentiated embryonal carcinomas *in vivo*. DNA was purified from both tumors and cells using the proteinase K/phenol method (11).

Restriction digests with *Eco* RI, *Msp* I, and *Hpa* II were performed as recommended by the manufacturer (Bethesda Research Laboratories and PL Biochemicals) using twice the recommended concentration for 24 hrs at 37 °C. The DNA was ethanol precipitated and resuspended in TE buffer and tracking dye. Electrophoresis was carried out in 1% agarose (BRL) at constant voltage (1.6 V/cm) in 'E' buffer as previously described (25). Transfer of DNA to nitrocellulose or Gene Screen® was performed as described by Southern (30). Hybridizations were performed at 42 °C in 5XSSC and 35% formamide.

Low copy number probes

The specific gene probes used were a mouse β-major globin clone, f7, [Dr. Carolyn Jahn, (14)], and a mouse class I, histocompatibility-2 clone, pH-2^d-4, [Dr. Gabriel Gachelin, (15)]. Nick translations of the probes were done with the NEK-004C kit of New England Nuclear using [α^{32}P]-dCTP at 400 mCi/mM. Any one figure represents strips hybridized with the same probe and exposed for the same length of time.

Results and discussion

Hybridization of *Msp* I and *Hpa* II restricted DNA from F9 tumor and cells with the mouse major β-globin probe showed the tumor DNA to be less methylated at C*pG sites (Fig. 1). The *Hpa* II pattern of F9 tumor DNA was nearly identical to that found after restriction with *Msp* I, differing by the presence of a 7.7 kb fragment with *Hpa* II not seen with *Msp* I. A previously prepared batch of F9 tumor DNA studied by these methods and with this probe had shown identical *Hpa* II and *Msp* I patterns (25). Thus, it is probable that there exists variability in the degree of C*pG methylation of DNA extracted from different F9 tumors similar to that existing between different F9 *in vitro* lines. F9 cell line DNA (Ann Arbor) showed *Hpa* II fragments of 13.5 and 7.7 kb and less of the 6.0 and 2.7 kb fragments seen in the *Msp* I digestion. The F9 cell line from Paris seemed even more methylated as only the 6 kb, and fainter higher M.W. bands, were seen with *Hpa* II digestion. All three DNA preparations showed similar *Eco* RI restriction patterns.

A similar result was found when the DNAs were probed with the class I, major histocompatibility complex cDNA clone, pH-2^d-4 (Fig. 2). The F9 tumor showed nearly identical *Msp* I and *Hpa* II digestion patterns while F9, and PCC4 (a multipotent embryonal carcinoma cell line), showed varia-

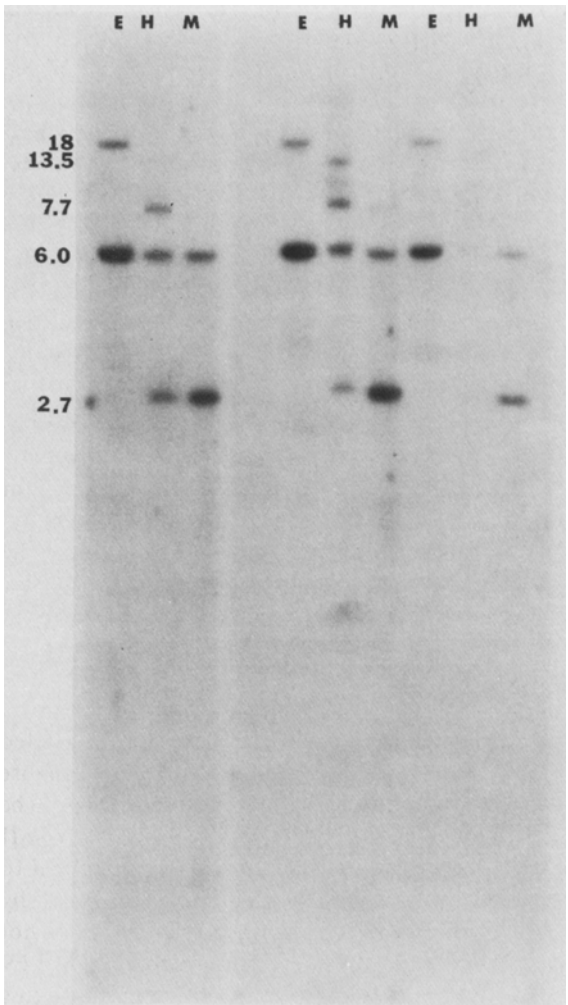

Fig. 1. Hybridization of β-major globin probe (f7) to *Eco* RI (E), *Hpa* II (H) and *Msp* I (M) restricted DNA from F9 tumor DNA (left three lanes), F9-Ann Arbor cellular DNA (middle three lanes) and F9-Paris cellular DNA (right three lanes).

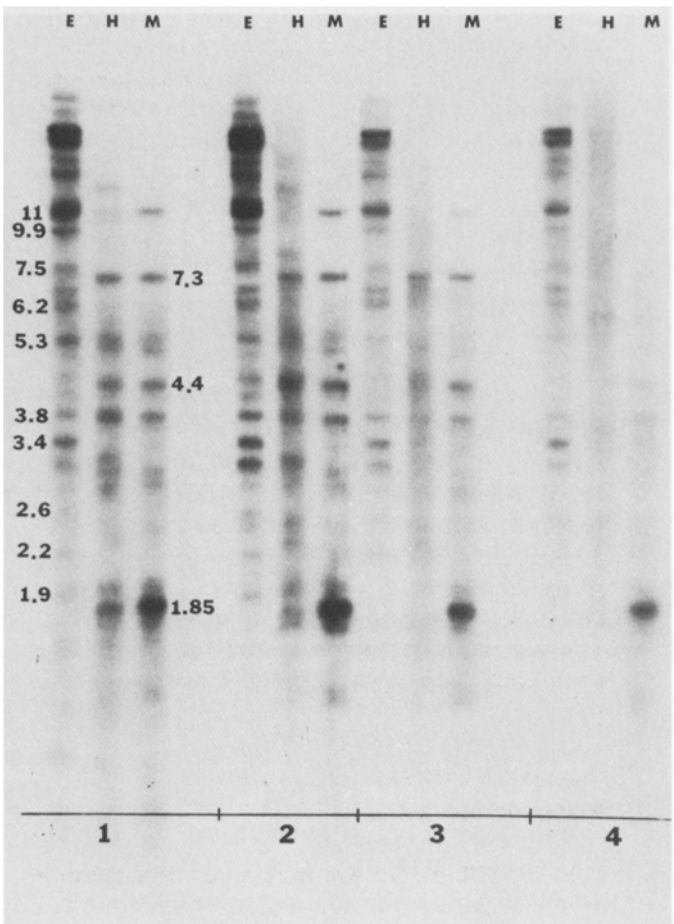

Fig. 2. Hybridization of *H-2* complex probe (pH-2^d-4) to *Eco* RI (E), *Hpa* II (H) and *Msp* I (M) restricted DNA. DNAs were obtained from F9 tumors (set 1), F9-Ann Arbor cellular DNA (set 2), F9-Paris cellular DNA (set 3), and PCC4/Aza cellular DNA (Paris, set 4).

ble degrees of C*pG methylation. The Ann Arbor F9 cell line showed the least difference between *Msp* I and *Hpa* II digestions while the Paris F9 cell line was markedly resistant to *Hpa* II digestion and only showed the 7.3 *Hpa* II band and smears of hybridization from 3–12 kb. The PCC4 line hybridized poorly after *Msp* I and *Hpa* II digestions but the hybridization which occurred was at higher M.W.'s with *Hpa* II. All four DNA preparations gave similar *Eco* RI restriction patterns.

It has been postulated that decreased C*pG methylation is correlated with altered gene expression and lack of normal growth controls in malignant tissue. Measurements of total 5-methylcytosine by high performance liquid chromatography in a large variety of tumors also revealed a correlation of malignancy with decreased 5-methylcytosine content (7) and hybridizations with unique sequence probes to unexpressed genes confirmed this (6). Azacytidine can induce tumorigenesis of cultured cell lines at the same time that it results in DNA hypomethylation and chromosome changes

(13). However, these correlations might be causally unrelated and the hypomethylation might be secondary to some aspect of malignancy, such as high growth rate. Our results show that a particular, homogeneous cell line, F9 embryonal carcinoma, is less methylated at two unexpressed sequences when grown *in vivo* (presumed optimal growth conditions) than *in vitro*. The data are most easily interpreted as showing that altered growth conditions are responsible for the difference.

It has been observed that subclones of one cell line show large differences in methylation, whether studied in terms of C*pG methylation of total DNA (29) (by density scanning electrophoretograms of restricted DNA) or for particular unique sequences (28). Our data suggest that there may be differences in C*pG methylation between F9 cells grown in Paris and those grown in Ann Arbor but genetic drift (the cell lines have been separated for several hundred generations) or slight differences in culture technique may be responsible. While selection for expressed genes may alter DNA methylation of those genes (12), our use of probes for genes which are unexpressed *in vitro* or *in vivo* makes it unlikely that selective changes were invoked in the differing degrees of DNA methylation.

Our results seem to be in conflict with those recently reported by Tanaka *et al.* (34) who found an association of hypermethylation of a class I, histocompatibility gene and its expression in F9 cells. As stated in the Introduction F9 cells do not normally express *H-2* antigens; Tanaka *et al.* (34) studied unusual cell lines. Differentiated clones of F9 were selected after retinoic acid treatment which express *H-2* antigens (6 months treatment was necessary to find a line with amounts of *H-2* typical for splenocytes) and these were used by these authors. Since the conclusions of Tanaka *et al.* (34) are based on the comparison of several derived lines differing in *H-2* expression, the unusual direct correlation of *H-2* expression with several C*pG methylation sites they found may be an artifact of divergence in methylation between long separated cell lines which is unrelated to the gene expression. Alternatively, the sites they have studied may not have been detected in our study.

While differences in C*pG methylation found between malignant and normal cells may be related to variables such as growth rate rather than to genetic control, detailed studies of DNA methylation in the globin system suggest the possibility of a role for C*pG methylation in control of transcription of some genes. Many studies (5, 16, 20, 27, 35) have consistently found decreased methylation of expressed globin genes. It is now becoming apparent that the sites whose methylation is most correlated with transcription are near the 5′ end of the globin genes (4). On the other hand, methylation had no effect on the transcription of *Xenopus* globin genes injected into *Xenopus* oocytes (3), or on the transcription of ribosomal genes (17). Thus, even in the case of globin genes, the correlation may be secondary rather than causal.

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