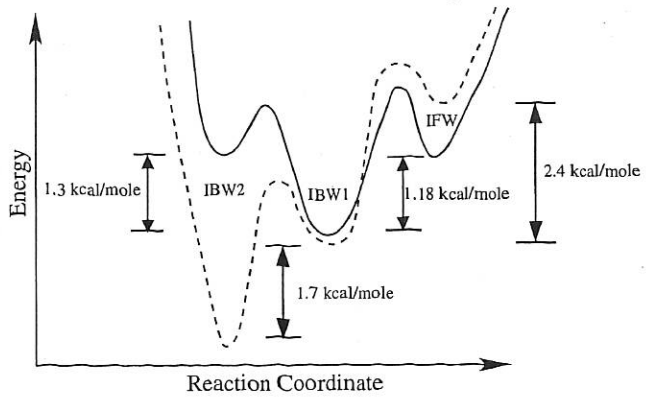

Figure 3. Schematic description of free energy (solid line) and internal energy (dashed line) profiles of interfacial water species. The species are in dynamical equilibrium with themselves and with water present in the bulk region of the micellar solution. The reaction coordinate is arbitrary and does not imply any distance. Barrier heights are also arbitrary.

ween the free and bound species. A large part of this stabilization comes from the stronger hydrogen bonds that bound water molecules form with the surfactant head groups.

Figure 3 provides a schematic of the free energies of the three species, calculated from their average concentrations. Despite the reduced monomer energy arising out of two water–head group hydrogen bonds, the IBW2 state is less stable than the IBW1 state due to entropic considerations (less number of suitable configurations). The reversible reactions between these states of water on such a surface should determine the dynamical response of interfacial water.

Note that Figure 3 describes the free energy and not the binding energy of the three species. The binding energy can be inferred from Figure 2. The total binding energy of the IBW2 species is indeed larger than that of IBW1.

In conclusion, we note that the existence of identifiable bound and free water molecules on the surface can indeed help in developing a phenomenological description of dynamics of water at complex interfaces. The 9 : 1 ratio obtained (for IBW and IFW) is expected to be typical for ionic micelles. However, this ratio is bound to decrease substantially for proteins due to the existence of hydrophobic and less polar amino acid groups on its surface. The water on the protein/membrane surface is expected to play a critical role in the molecular recognition of hydrophobic patches by incoming ligands or drug molecules. Work in this direction is under progress.

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Effect of sodium butyrate on methylation pattern of retinoblastoma (*RB1*) gene in human colon tumour cell line HT29

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Sodium butyrate is known to induce morphological and biochemical changes associated with differentiation in some colon tumour cell lines, including HT29. In this study the HT29 colon carcinoma cells were induced to differentiate with sodium butyrate treatment. High molecular-weight DNA from the untreated and sodium butyrate-treated HT29 cells were isolated at different time periods. The DNA was digested with the restriction enzymes *Msp*I and *Hpa*II to reveal changes in the methylation pattern at the *RB1* gene locus. Sodium butyrate-treated HT29 cells showed an increase in the level of methylation of retinoblastoma (*RB1*) gene compared to the untreated control. The results suggest that the development of human colonic neoplasia may result from hypomethylation of *RB1* gene.

CYTOSINE methylation in CpG dinucleotides is an important control mechanism of the transcriptional regulation in eukaryotes. The transcriptional activity of several

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eukaryotic and viral genes has been found to be inversely correlated to the degree of methylation of cytosine residue of CpG dinucleotides located in the DNA sequences of the gene[1]. DNA methylation is known to alter the expression of specific genes[2–4]. It has been reported that hypomethylation is a consistent biochemical characteristic of human colonic tumours and is an alteration in the DNA that precedes malignancy[4–7].

In all mammalian cells that have been examined so far, the retinoblastoma protein (pRb) is present and is synthesized in actively growing cells and tissues[8–10]. To explain the well-known growth suppressor activity of pRb, it has been suggested that its activity is regulated in a reversible manner to allow passage through the cell cycle. It could be regulated at any level of gene expression, including transcription, mRNA processing, protein synthesis or post-translational modification. It has been convincingly shown that resting, growth-arrested cells have hypophosphorylated pRb, and that growing, proliferating cells of the same type contain phosphorylated pRb[11–13]. pRb becomes transiently hyperphosphorylated in late G1 and early S phases, suggesting an activation of a specific Rb protein kinase or inactivation of a specific Rb phosphatase[14].

The differentiating effects induced by sodium butyrate are known to change the expression of a wide variety of genes in certain colon cancer cell lines through a general but unknown mechanism of action. Control of cell differentiation can be studied using colon carcinoma cell HT29, which is known to exhibit butyrate-induced changes consistent with cell differentiation[15]. In our previous studies we observed (i) no gross structural change in *RB1* gene locus, (ii) *RB1* gene expression was increased in human colon carcinomas compared to the normal colonic mucosa, and (iii) *RB1* gene expression was increased in established colon tumour cell lines[16–18]. We had also reported that compared to the control, the sodium butyrate-induced and differentiated HT29 cells have decreased levels of *RB1* and *p53* gene expressions[19], and increased levels of underphosphorylated pRb[19], which is known to exhibit the tumour-suppressor function[11–14]. In the present study the HT29 colon carcinoma cell lines were induced to differentiate *in vitro* by sodium butyrate treatment and the methylation pattern of *RB1* gene was analysed. The data presented here show that the sodium butyrate treatment leads to an increase in the methylation of the *RB1* gene. It appears that the observed increase in the methylation of *RB1* gene could be associated with decrease in the *RB1* gene expression and increase in underphosphorylated pRb that we reported earlier[19]. The results indicate that the alteration in the methylation of *RB1* gene locus could be involved in human colonic neoplasia.

The human colon tumour cell line HT29 was obtained from the American Type Culture Collection. The cells were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) from GIBCO BRL (Gaithersburg, MD) supplemented with 10% foetal bovine serum (FBS), penicillin (100 μ/ml) and streptomycin (100 μg/ml) at 37°C in humidified atmosphere of 5% CO_2 and 95% air. Sodium butyrate treatment was carried out according to the procedures described previously[19,20]. Monolayers of 90–95% confluence in 100 mM plastic dishes were harvested after incubation in the above medium containing 2 mM sodium butyrate for up to 7 days. The media, with or without sodium butyrate were changed every other day. Viability of the untreated and sodium-butyrate-treated cells in culture was assessed by trypan blue exclusion[19,20] (data not shown). The monolayers were washed extensively with cold phosphate-buffered saline (PBS, 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per litre, pH 7.4) and lysed in 5 M guanidine thiocyanate for DNA isolation on day 0, 1, 3, 5 and 7.

The total cell lysate was centrifuged through cesium-chloride gradient according to standard procedure[21]. High molecular-weight DNA was isolated from the guanidine isothiocyanate layer of this gradient and was purified further by proteinase K treatment followed by phenol: chloroform extractions and ethanol precipitation[21].

The plasmid pG4-Rb3.8 M containing the 3.8 kb 3′RB1 cDNA was obtained from Dr Y. K. Fung, Children's Hospital of Los Angeles, Los Angeles, California. The probe was excised from the plasmid, purified, radioactively labelled and used for hybridization in Southern blots.

Ten microgram of DNA from control and sodium butyrate-treated HT29 cells was digested with the restriction enzyme *Msp*I or *Hpa*II and separated on a 0.8% agarose gel. After electrophoresis, the DNA was transferred to Nytran (Schleicher and Schuell) membranes by capillary blotting followed by baking at 85°C for 2 h (ref. 21). The membranes were prehybridized in blotto[22] and hybridized in the same solution containing 5×10 cpm/ml of ^{32}P-oligolabelled 3.8 kb 3′RB1 cDNA probe. After hybridization, the filters were washed in 2X standard saline citrate (SSC, 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0) plus 0.1% SDS for 30 min followed by 0.2X SSC plus 0.1% SDS for 1 h at 65°C. Autoradiography of the filters was performed at – 70°C using Kodak XAR-2 film.

The restriction enzymes *Msp*I and *Hpa*II recognize the sequence CCGG. *Hpa*II is sensitive to methylation at both the internal and the 5′ cytosine, while *Msp*I is sensitive only if the 5′C is methylated. Details of the sequences recognized by these enzymes are given in Table 1.

*Msp*I digestions of the high molecular-weight DNA from HT29 cells identified ten bands of varying sizes, they are 9.2, 4.5, 3.0, 2.3, 1.6, 1.5, 1.2 and 0.8 kb and two faint bands of approximately 3.5 and 1.8 kb, when probed with 3′ RB1 cDNA probe (Figure 1). Using 1.0% agarose gel, bands below 2.0 kb region were further resolved. It clearly revealed the presence of two bands of approxi-

mately 1.6 kb and 1.5 kb in the *Msp*I-digested DNA (Figure 2). However, digestion of the high molecular-weight DNA with restriction enzyme *Hpa*II produced only two bands of approximately 23.0 and 1.5 kb, when probed with the same 3′RB1 cDNA probe (Figures 1 and 2). In sodium butyrate-induced and differentiated HT29 cells, the 1.5 kb band disappeared from both *Msp*I and *Hpa*II-digested samples (Figure 2). No evidence of gene amplification or rearrangement was observed in sodium butyrate-treated HT29 cells compared to the untreated control. The sodium butyrate-induced and differentiated HT29 cells showed an increase in the methylation at the *RB1* gene locus, as there was a decrease in the number of bands obtained compared to the untreated control (Figure 2). This could be due to sodium butyrate-dependent methylation of the 5′C residue of the recognition sequence CCGG (Table 1 and Figure 2).

Table 1. Recognition sequences for the restriction enzymes *Hpa*II and *Msp*I

Sequence (5′ to 3′)	Restriction enzyme	Cleavage (yes/no)
CCGG	*Hpa*II	Yes
^{m}CCGG	*Hpa*II	No
C^{m}CGG	*Hpa*II	No
^{m}C^{m}CGG	*Hpa*II	No
CCGG	*Msp*I	Yes
^{m}CCGG	*Msp*I	No
C^{m}CGG	*Msp*I	Yes
^{m}C^{m}CGG	*Msp*I	No

*Hpa*II does not cleave DNA when either 3′C residue is 5-methylcytosine or either C residue is 4-methylcytosine. *Msp*I does not cleave DNA when the 5′C residue is 5-methylcytosine. *Msp*I cleaves when the 3′C residue is 5-methyl-cytosine.

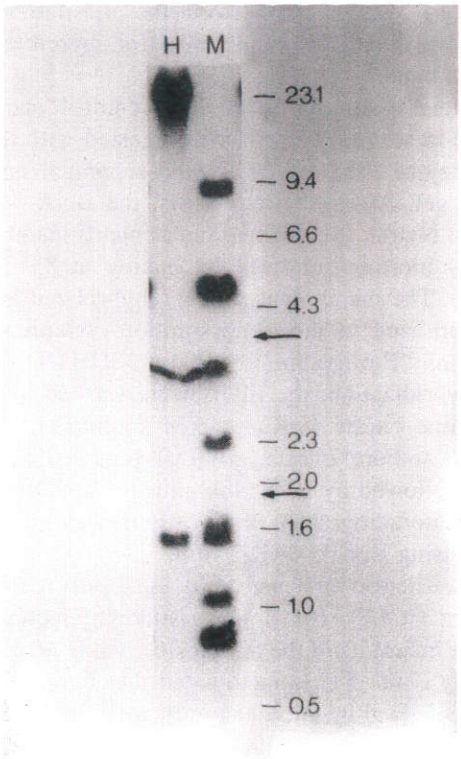

Figure 1. Southern blot analysis of DNA from colon carcinoma cell line HT29. Ten micrograms of the high molecular-weight DNA was digested with *Hpa*II (H) or *Msp*I (M), fractionated on a 0.8% agarose gel and subjected to Southern analysis using ^{32}P-labelled 3.8 kb 3′, RB1 cDNA probe as described in the text. Molecular weight markers in kb are shown on the right hand side. Arrows indicate the position of the two faint bands.

The RB1 promoter has features characteristic of many 'housekeeping' genes, with its ubiquitous expression pattern. The typical TATA and CAAT boxes are absent, although the region is generally GC-rich. The sequences surrounding the transcription-initiation sites have a high GC content[8,9]. Constitutively expressed 'housekeeping' genes generally have demethylated CpG dinucleotides[23–25]. Tissue-specific expression of several genes has been correlated with demethylation of CpG dinucleotides[4,5]. Change in overall methylation is a well-known modulator of gene function[23–25], and is postulated to induce some of the effects of retinoic acid, another differentiating agent, by methylation of promoter sites[26]. The action of sodium butyrate as a 'differentiating' agent in colon carcinoma cell line HT29 has been well-documented[19,27,28], although the mechanism of action remains to be determined. The restriction enzymes *Msp*I and *Hpa*II have varied sensitivity to their recognition sequence, 5′CCGG3′, depending upon the methylation pattern of the C residues (Table 1). Digestion of high molecular-weight DNA using restriction enzymes *Msp*I and *Hpa*II shows ten and two bands respectively, when probed with RB1 3.8 kb cDNA probe under stringent washing condition (Figure 1). This clearly

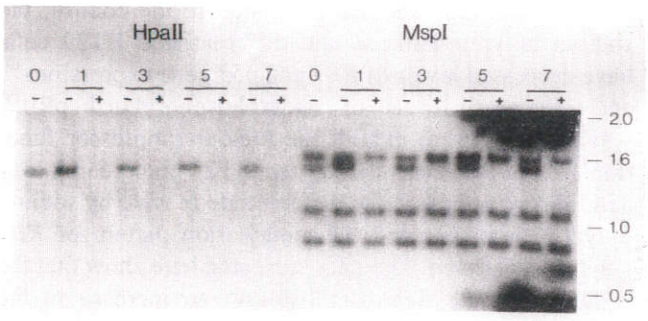

Figure 2. Southern analysis of DNA from sodium butyrate-treated and untreated HT29 cells. Sodium butyrate treatment and DNA isolation were done as described in the text. Ten micrograms of the DNA was digested with the enzymes *Hpa*II or *Msp*I (as indicated on the top of the lanes) fractionated on 1% agarose gel and subjected to Southern analysis as described in Figure 1. Molecular-weight markers in kb are shown on the right hand side. Numbers above each lane indicate the days of treatment with sodium butyrate. –,Control without sodium butyrate treatment; +,Cells treated with 2 mM sodium butyrate.

indicates that some of the 3′C residues in the recognition sequence (CCGG) of these two enzymes are already present in methylated (C^mCGG) form in the *RB1* gene of the HT29 cells. Absence of the 1.5 kb band from both *Msp*I and *Hpa*II-digested DNA from sodium butyrate-treated cells (Figure 2) indicates that specific methylation at the 5′C residue in the CCGG sequence takes place as a result of sodium butyrate treatment. This could result in either ^mCCGG or $^mC^mCGG$, that cannot be cleaved by either enzymes used in our study (Table 1). The fact that only a minority of CpG residues within a sequence can be recognized by methylation-sensitive restriction endonucleases, and we used only a 3.8 kb 3′RB1 cDNA probe to observe this change, shows that the *RB1* gene is relatively hypomethylated in control HT29 colon carcinoma cells and sodium butyrate treatment leads to its hypermethylation. Hypomethylation of DNA in benign and malignant human colon tissues has been well documented[2]. Studies on the quantitation of genomic 5′-methylcytosine content in human colonic adenomas and adenocarcinomas have shown an apparent reduction in methylation[3].

Colon tumours have increased *RB1* and *p53* gene expressions compared to the normal colonic mucosa[16,17]. In addition, the cultured human colon cells have increased expression of *RB1* and *p53* genes compared to the normal cell lines WI38 and WS1 (refs 17, 18). In our previous study we have reported a decrease in the expression of *RB1*, *p53* and histone *H3* genes after sodium butyrate treatment[19]. Decrease in the expression of the *RB1* gene could be due to change in the methylation pattern reported here[19]. The expression of histone *H3* gene is finely regulated and is limited to the S phase of the cell cycle[29–31]. The apparent reduction in the level of histone *H3* reported in our previous study indicates that the number of cells entering the cell cycle has decreased considerably due to sodium butyrate treatment-associated differentiation[19]. We also showed that the sodium butyrate treatment resulted in an increase in the level of underphosphorylated form of pRb compared to the untreated control[19]. Underphosphorylated form of pRb is known to have the tumour-suppressor function[11–14]. Thus it appears that the sodium butyrate treatment could modulate the phosphorylation of pRb and this could lead to differentiation of the treated HT29 colon carcinoma cells. The exact mechanism by which sodium butyrate induces differentiation in colon tumour cell lines remains unclear. This study indicates that sodium butyrate could modulate the methylation of *RB1* gene in colonic epithelial cells and thus could induce differentiation.

The complete *RB1* gene is approximately 200 kb long and the 5′ region of this gene is known to be rich in CG bases[8,9]. Thus, it is quite likely that there could be similar methylation of C residues in *RB1* gene, which we obviously could not identify as we used only a 3.8 kb 3′RB1 cDNA probe. It would be interesting to study the butyrate-induced changes in methylation pattern in the 5′ region of the *RB1* gene and its effect on gene expression. Several studies in other systems have shown that methylation of the 5′ flanking regulatory sequences is more critical in gene expression than methylation of coding exons[32–35]. In tumours with wild-type *p53*, apoptosis is controlled by methylation of downstream genes in the *p53*-mediated apoptosis pathway[36]. It has been also reported that epigenetic changes may contribute to the formation and spontaneous regression of certain tumours[37]. Similarly, alteration in the methylation pattern of *RB1* gene could be involved in the initiation and progression of malignant tumours. Further studies on epigenetic modification of the genome may help better understand the real cause of the disease. It may be more difficult to reverse a mutation, but it may be relatively easier to reverse a modification-like methylation by changing the cellular environment with diet, drugs, etc. It is important to understand the mechanism of such modifications and develop tools to screen genome-wide for such alterations.

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Molecular logistics of cytokines in cervical cancer

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Effective immune response against cancer relies on the orchestration of the interrelated immune cells and their products. Several important aspects like the activation and proliferation of T cells, recruitment of phagocytes to the tumour site and influence of cytokines on cancer progression are areas of current investigation. The capacity of T cells to secrete IL-2 for self-propagation, the prognostic significance of the inflammatory cytokines IL-6 and IL-8 along with MIP3α, a chemo attractant, has been analysed elaborately in our study by employing RT–PCR and immunohistochemical methods with thrust on biology of cancer uterine cervix. This indeed opens up a new avenue for further studies.

THE cytokine family of proteins has been shown to have a vital role in the control of normal cellular differentiation, mitosis and motility[1]. They are important immunological mediators of cell-mediated defences against tumours. But, given the multiplicity of cytokine function, it is not surprising that cytokines also play a role in the pathophysiology of cancer. They are capable of promoting tumour aggressiveness and metastasis by promoting angiogenesis, tumour-cell adhesion and inducing proteolytic enzymes[2]. Many cytokines and chemokines are inducible by hypoxia, which is a major physiological difference between a tumour and a normal tissue[3]. IL-6 is one such cytokine, whose role in promoting cancers is being discussed widely, though with conflicting views[4,5]. According to an ICMR report (1997), cervical cancer is the most prevalent form of cancer in Barshi, Bhopal and Chennai, and the second-most prevalent form in Bangalore, Delhi and Mumbai following breast cancer. The incidence of cervical cancer in India is two to three times higher compared to the West.

We have attempted to understand the role of cytokines in cervical carcinogenesis. The activation status of T cells evaluated by their expression of the α-subunit of the receptor for IL-2 (CD25), which is necessary for their clonal expansion, and the expression of inflammatory cytokine IL-6 along with MIP3α, a chemo attractant for dendritic cells needed for T cell stimulation, were analysed by RT–PCR, before and after treatment. Cases that showed high levels of IL-6 and MIP3α were immunostained for $CD3^+$ T cells, IL-6 and the inflammatory cytokine IL-8, to observe the actual localization of these proteins and better define their roles in influencing these cancers.

For RT–PCR analysis, biopsy samples were collected when the patients reported to the Out Patient Department and the samples were stored at – 70°C immediately. This was cleared by the Institute Research Committee. Part of the biopsy samples collected for pathological studies was used for *in vitro* studies with no intervention. Twenty-nine patients who underwent treatment in our institute were selected. All the patients underwent 6 MeV X-ray beam therapy of 50 Gy to the total pelvis followed by an intra-cavitary application of 20 Gy to point A. On completion of treatment, they were reviewed after six weeks and longer periods. Smears with cervical brushes were collected at that time. For RT–PCR analysis, total RNA was extracted by the method of Chomczynski and Sacchi[6]. cDNA was synthesized from 5 μg RNA using the Boehringer Manheim RT–PCR kit with oligo dT primers. Next 2.5 μg cDNA was utilized for amplification. The primers used for IL-6, MIP3α and CD25 were purchased from Microsynth (Switzerland). The sequences are as follows:

IL-6: forward 5′ACG AAT TCA CAA ACA AAT TCG GTA CA, reverse 5′CAT CTA GAT TCT TTG CCT TTT TCT GC

IL-2Rα: forward 5′TT CTG CAG AGA AAG ACC TCC GCT, reverse 5′GGT TGC AGC CAT TTC TGT CTG TAT

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