known. Recent studies suggest that other early adenovirus genes in addition to the E1 transforming region can also affect the ability to grow in agar and oncogenicity (*13*). Our results indicate that although Ad5 can initiate the transformation process, progression of Ad5-transformed cells is not regulated by alterations in the location of the Ad5 genome in cellular DNA. Progression appears to be affected by the methylation of one or more presumably cellular genes which may operate as negative regulators. Direct alterations in expression of Ad5 transforming genes or cellular oncogenes may also influence progression. The ability of Aza to induce a reduction in anchorage independence and to decrease the tumorigenic potential of Ad5-transformed rat embryo cells has been observed in other systems. Aza can reduce the tumorigenic potential of myogenically defective highly tumorigenic T984-15 murine cells (*14*) and Aza treatment results in a loss of the metastatic phenotype in murine Lewis lung carcinoma cells (*15*). Our demonstration that progression can be reversed or accelerated by the appropriate in vitro manipulation will aid in the establishment of a cell culture model system for the molecular analysis of tumor progression.

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Adaptation of the Membrane Lipids of a Deep-Sea Bacterium to Changes in Hydrostatic Pressure

Abstract. *The fatty acid composition of the cell membrane of the barophilic marine bacterium CNPT3 was found to vary as a function of pressure. Greater amounts of unsaturated fatty acids were present in bacteria growing at higher pressures. The results suggest adaptations in the membrane lipids to environmentally relevant pressures. This response to pressure appears to be analogous to temperature-induced membrane adaptations observed in other organisms.*

Phospholipids containing unsaturated fatty acids have lower melting points and greater molecular flexibility than their saturated counterparts (*1*). Consequently, many organisms, including bacteria, plants, and poikilothermic animals maintain optimal membrane fluidity and function as the environmental temperature varies by altering the proportion of unsaturated fatty acids in their phospholipids, a response termed "homeoviscous" adaptation (*2, 3*).

In the ocean, large variations in pressure as well as in temperature are encountered. Some organisms participating in diel or ontogenetic migrations may be subjected to changes in pressure as great as 200 bars (*4, 5*). Marine bacteria may experience similar changes in pressure as a result of attachment to particles or migrating organisms or of vertical transport processes.

High hydrostatic pressures exert effects on membrane systems that are comparable to the effects of low temperature. The more-ordered, less bulky crystalline (gel) state of membrane lipids is stabilized by high hydrostatic pressure, as indicated by the increasing melting points of lipids with increasing pressure (*6*). At constant temperature, high hydrostatic pressure will cause a transition in the lipid bilayer to the gel state (*7*). An organism adapted to high hydrostatic pressure might be expected to adjust its membrane phospholipid composition to offset these lipid-solidifying effects, perhaps by increasing the proportion of unsaturated fatty acids. This would be analogous to homeoviscous adaptation to changes in temperature.

With the recent isolation of barophilic bacteria and the development of methods for their cultivation (*8*), laboratory studies of the biochemistry and physiology of these deep-sea organisms have become possible. We report here changes in the fatty acid composition of the barophilic bacterium CNPT3 as a function of pressure.

This gram-negative, polarly flagellated, facultatively anaerobic marine eubacterium has a guanine-cytosine content of 47.6 mole percent in its DNA (*9*), placing it most likely in the genus *Vibrio* (*10*). In complex medium at 2°C, CNPT3 grows optimally at pressures of 300 to 500 bars (Table 1). Although no definitive statements can be made about the natural distribution of CNPT3, it grows well at the temperatures and pressures found in the cold deep sea and does not survive in warm surface waters (*11*).

To ascertain whether the fatty acid composition of the CNPT3 membrane changes as a function of pressure, we inoculated 1 liter of type 2216 marine medium buffered with 20 m*M* 4-morpholine propanesulfonic acid (*p*H 7) with CNPT3 (5×10^5 cells per milliliter) in the stationary phase. The inoculated medium was distributed into polyethylene bags (170 ml each) that contained mixing marbles and were fitted with polyethylene tubing at one end to allow for sterile subsampling. The bags were placed in pressure vessels, brought to the appropriate pressure, and incubated in a rocker bath at 2°C. Periodically the vessels were decompressed for a maximum of 2

Table 1. Proportion of fatty acids in CNPT3 membrane phospholipids as a function of pressure at 2°C. Values are percentages by weight (means ± standard errors for five separate experiments).

Fatty acid type*	Pressure (bars)				
	1	172	345	517	690
	Generation time (hours)				
	17.2	10.1	7.9	8	17.2
14:1	20.0 ± 0.8	19.6 ± 0.8	17.4 ± 1.0	13.7 ± 0.7	11.5 ± 0.1
14:0	7.5 ± 0.2	5.7 ± 0.3	5.2 ± 0.2	4.8 ± 0.5	3.5 ± 0.4
16:1	40.2 ± 0.7	44.0 ± 1.2	47.9 ± 1.1	46.8 ± 1.6	56.3 ± 0.9
16:0	25.2 ± 0.6	22.2 ± 0.6	20.7 ± 0.3	18.7 ± 1.0	18.5 ± 0.8
18:1	5.5 ± 0.4	5.9 ± 1.8	6.6 ± 1.8	11.0 ± 1.6	7.3 ± 0.5
18:0	1.5 ± 0.2	2.5 ± 0.5	2.1 ± 0.4	4.9 ± 1.1	2.8 ± 0.4

*The number before each colon represents the number of carbons in the fatty acid and the number after the colon denotes the number of double bonds.

minutes and samples were removed to determine cell density. The cells were counted with a Coulter counter and harvested at a density of 2×10^7 to 5×10^7 cells per milliliter, well before entry into the stationary phase. They were then centrifuged and frozen until extraction of the lipids and analysis of the fatty acid content by standard methods (*12, 13*).

The most common fatty acids found in marine vibrios are $C_{16:1}$, $C_{16:0}$, and $C_{18:1}$, with average proportions by weight of 41, 20, and 25.4 percent, respectively (*14*). CNPT3 is different in that its lipids contained greater amounts of $C_{14:1}$ (Table 1). This, however, is not surprising, as increased amounts of shorter chain fatty acids in psychrophilic vibrios have been observed previously (*15*).

CNPT3 grows at a very wide range of pressures, and its fatty acid content did change over this range (Table 1). At higher pressures there were higher proportions of $C_{16:1}$ and $C_{18:1}$, while the relative amounts of $C_{14:1}$, $C_{16:0}$, and $C_{14:0}$ decreased. The ratio of total unsaturated fatty acid to total saturated fatty acid increased from 1.9 at 1 bar (1 atm) to 3.0 at 690 bars (680 atm). This increase in total unsaturated fatty acids is strikingly similar to temperature-induced changes in lipid composition observed in other bacteria (*1, 14*). For example, in *Vibrio marinus*, a marine bacterium, the ratio $(C_{16:1} + C_{18:1})/(C_{16:0} + C_{18:0})$, representing 86 percent of the total fatty acids, changes from 2.6 at 25°C to 3.9 at 15°C (*14*). Since an increase in pressure of 1000 atm has solidifying effects on membranes equivalent to a change in temperature of 15° to 25°C (*16*), the temperature-induced changes in lipid composition in *V. marinus* appear to be comparable to the pressure-induced changes in CNPT3. Furthermore, the major compositional change is in the relative amount of $C_{16:1}$ in both of these marine vibrios.

We cannot conclusively state that these observations represent a homeoviscous adaptation until physical measurements of membrane viscosity are made. However, studies of other organisms have shown strong correlations between temperature-induced changes in lipid composition and maintenance of membrane fluidity and function (*2, 3, 17*). Further studies with techniques such as electron spin resonance spectroscopy or fluorescence polarization should allow us to determine whether the fluidity of the membrane of this barophile is dependent on hydrostatic pressure.

The mechanism by which temperature regulates membrane phospholipid composition in *Escherichia coli* is well understood (*18*). The major site of control resides in 3-oxoacyl-[acyl-carrier-protein]synthase II (E.C.2.3.1.41). This enzyme shows increased activity in catalyzing the elongation of palmitoleoyl acyl-carrier-protein to *cis*-vaccenoyl acyl-carrier-protein at lower temperatures, resulting in increased incorporation of *cis*-vaccenic acid (the major unsaturated fatty acid in *E. coli*) into phospholipids. If fatty acid synthesis in CNPT3 is analogous to that in *E. coli*, a similar, pressure-sensitive fatty acid synthase may exist. At high pressures an increase in the activity of this enzyme might result in elevated incorporation of palmitoleic acid into phospholipids. The site of control CNPT3 would be one step earlier in the elongation process, however. The decrease in $C_{14:1}$ and $C_{16:0}$ observed with the increase in $C_{16:1}$ at higher pressures suggests that such a process might indeed be occurring.

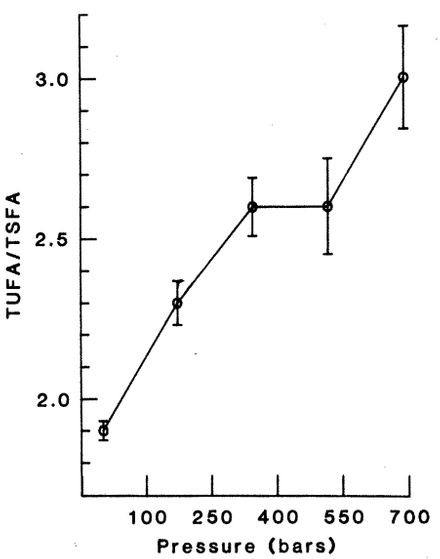

Fig. 1. Ratio of total unsaturated fatty acids (TUFA) to total saturated fatty acids (TSFA) as a function of pressure. Data points are means ± standard errors for five separate experiments.

High hydrostatic pressure has several effects on biological processes (*19*). For example, there is evidence that the brain myelin membranes of deep-sea fish undergo adaptive changes (*16*). Our study indicates that changes in hydrostatic pressure alter membrane lipid composition in a marine bacterium. Hence it appears that homeoviscous adaptation of membrane lipids occurs not only in response to temperature fluctuations but to variations in pressure as well.

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identified initially by comparing the retention times with those of known standards. Positive identification of FAME's was made by gas chromatography–mass spectroscopy with a 3 percent Dexsil column on a Finigan automated system.
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Induction of Hepatitis B Virus Core Gene in Human Cells by Cytosine Demethylation in the Promoter

Abstract. *A recombinant human cell line constructed by transfection of epithelial cells with a plasmid containing the hepatitis B virus core gene* (HBc) *was used to study the regulation of* HBc *gene expression. Methylation of a single Hpa II site 280 base pairs upstream from the structural gene was found to regulate the expression of the core gene. Expression increased in cells treated with 5′-azacytidine as a result of cytosine demethylation at this site, and there was a fivefold increase in the number of* HBc *gene transcripts in total cellular messenger RNA. The varied life cycle of hepatitis B virus in diseases such as viral hepatitis and liver cancer may therefore be attributable to the site-specific regulation of the gene involved in replication of the viral DNA and to the cytopathic effects elicited by this gene in human cells.*

Hepatitis B virus (HBV) infection is a serious worldwide health problem that is pathologically linked to viral hepatitis and liver cancer (*1*, *2*). There are an estimated 200 million individuals whose serum is positive for the surface antigen, HBsAg (*3*). The surface antigen and the core antigen (HBcAg) have different roles during HBV infection. Serologic data suggest that the constitutively regulated (*4*) gene for HBsAg (*HBs*) is needed for expansion of the focus of viral infection because antibodies to HBsAg are required for protection against the virus by immunization and are present during recovery from infection (*5*, *6*). In contrast, antibodies to the HBV core gene (*HBc*) product do not appear consistently during recovery and are frequently associated with virus replication and consequent infectivity of patients' sera (*7*).

The relation between *HBc* gene expression and the pathology of HBV infection is indicated by the cytopathologic effects of induced *HBc* gene expression in cells of the human epithelial cell line GTC2 (*8*). This line was constructed by transfection of carcinoma cells with a plasmid (pKYC200) that contains the *HBc* gene sequence without other HBV genes (*8*). This obviates the problems caused by the inability to infect mammalian cells with HBV in vitro and permits the study of *HBc* gene expression in human cells without interference from other HBV genes.

The importance of 5′-methyl cytosine to *HBc* gene regulation was first indicated by the cytotoxicity that follows induction of HBcAg expression in GTC2 cells or in an HBV-containing hepatocellular carcinoma cell line (*8*) [PLC/PRF/5 (*9*)] after treatment with 5′-azacytidine. The high degree of methylation of *HBc* gene sequences in PLC/PRF/5 (*10*) indicates that methylation of the *HBc* gene is biologically important. The demethylation of 5′-methyl cytosine at Hpa II–Msp I sites [CCGG (C, cytosine; G, guanine)] in total genomic HBV DNA has been studied in connection with generalized increases of gene expression in mammalian cells after they are treated with 5′-azacytidine (*11*). However, the low copy number of *HBc* gene inserts in GTC2 cells, the absence of other HBV sequences, and the ability to release the *HBc*-containing fragment from cellular DNA by digestion with Bam HI or Ava I (*8*) provide a model to determine the biologic importance of specific methylation sites within the 1.8-kilobase (kb) Bam HI fragment containing the *HBc* gene. Two Hpa II sites have been identified by sequence analysis of this Bam HI fragment of plasmid pKYC200 and have been mapped relative to the AUG start site (A, adenine; U, uracil) for *HBc* translation. One of these potential sites for regulation of methylation occurs 280 base pairs (bp) upstream from the *HBc* structural gene sequence ($HpaII^{-280}$), and the other site occurs 479 bp downstream from the sequence ($HpaII^{+479}$).

The methylation state at $HpaII^{-280}$ and $HpaII^{+479}$ was determined by digestion of DNA from nuclei of GTC2 cells with Bam HI or Ava I to release the transfected *HBc* gene. The DNA was subsequently digested with Hpa II before separation of the fragments by gel electrophoresis for Southern hybridization. The HBV-hybridizing fragments predicted at these two sites for each possible methylation state are listed in Table 1. Evaluation of site-specific methylation in the *HBc* gene of GTC2 cells after treatment with 5′-azacytidine is possible because the ratio of the HBV-hybridizing DNA fragments varies directly with the methylation state at the relevant Hpa II sites (Table 1). Internalization of the Hpa II sites being tested for methylation by digestion of cellular DNA with Bam HI or Ava I provides an internal control for measurement of methylation at these sites without interference from fragmented *HBc* gene inserts or from partially digested restriction fragments. This is because the fragments selected for analysis can only be produced by Hpa II digestion of complete *HBc* gene segments that do not contain inserted sequences (Table 1, Fig. 1). However, control digests of plasmid DNA indicated that the reaction conditions for Bam HI and Hpa II yielded

Table 1. Predicted sizes (in base pairs) of HBV-containing DNA fragments generated by enzymatic digestion of GTC2 cellular DNA. The methylation state at the Hpa II site is indicated by the presence (+) or absence (−) of 5′-methyl cytosine.

Restriction enzyme	Methylation state		Predicted size (bp)
	$HpaII^{-280}$	$HpaII^{+479}$	
Bam HI–Hpa II	+	+	1854
	−	−	171, 759, 924
	+	−	924, 930*
	−	+	171, 1683†
Ava I–Hpa II	+	+	975
	−	−	107, 109, 759
	+	−	109, 866
	−	+	107, 868

*Fragments at $HpaII^{+479}$. †Fragment at $HpaII^{-280}$.