mN/meter at concentrations greater than 0.25 μM for wild type; 13 mN/meter at greater than 2 μM for the Pro>Leu revertant; and 4 mN/meter at greater than 5 μM for the deletion mutant signal peptides.
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29. The areas used to calculate insertion energies were 120 to 350 Å^2, which represent the extremes of vertical and horizontal orientations of helical signal peptides in the monolayer. Analysis of surface pressure–area isotherms of peptide monolayers in the absence of lipids from 0 to 10 mN/m gave molecular cross-sectional areas of 350 ± 50 and 275 ± 50 Å^2 for wild-type and pseudorevertant peptides, respectively. Preliminary results suggest that these areas are approximately the same in mixed monolayers of peptide plus phospholipid (J. D. Lear and W. F. DeGrado, unpublished results).
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Reversibility of Progression of the Transformed Phenotype in Ad5-Transformed Rat Embryo Cells

Abstract. *The carcinogenic process is extremely complex and is affected by diverse environmental and host factors. The mechanism for the gradual development of the transformed phenotype (a process termed "progression") was studied in type 5 adenovirus (Ad5)-transformed rat embryo cells. Progression was not correlated with major changes in the pattern of integration of viral DNA sequences. Instead, it was associated with an increased methylation of integrated viral sequences other than those corresponding to the E1 transforming genes of Ad5. A single exposure of progressed cells to the demethylating agent 5-azacytidine (Aza) resulted in a stable reversion to the unprogressed state of the original parental clone. A further selection of cells after growth in agar allowed the isolation of Aza-treated clones that had regained the progressed phenotype. These observations indicate that progression is a reversible process and suggest that progression may be associated with changes in the state of methylation of one or more specific genes.*

On the basis of in vivo and in vitro studies, the neoplastic process has been divided into three (sometimes overlapping) phases termed initiation, promotion, and progression (*1, 2*). The process of progression involves the development of unique phenotypes or the further elaboration of transformation-associated phenotypes during the evolution of a transformed cell (*1*). Specific cellular phenotypes that may be used to monitor progression include stable changes in anchorage independence (the ability of a cell to grow in agar, agarose, or methylcellulose) and stable alterations in a cell's tumorigenic potential (*1*). Progression can occur naturally after treatment with a chemical carcinogen or after viral transformation and repeated subculturing, and can be accelerated by exposing cells to the potent tumor-promoting agent 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (*3, 4*). TPA induces an irreversible acquisition of anchorage independence and increases the ability of carcinogen-treated mouse epidermal cells (*4*) and Ad5-transformed rat embryo cells (*3*) to grow in agar. In addition, TPA can enhance the tumorigenic potential of cells transformed by herpes simplex virus type 2 (*5*). We now present evidence that progression of the transformed phenotype in Ad5-transformed rat embryo cells can be reversed by exposing cells to the demethylating agent 5-azacytidine (Aza) and can be accelerated by a second isolation of clones after growth in agar suspension.

The E11 cell line is a cloned culture isolated from secondary Sprague-Dawley rat embryo cells that had been infected with a temperature-sensitive mutant of Ad5 (H5ts125) (*6*). The biological and biochemical properties of E11 cells are similar to those of rat embryo cells transformed by wild-type Ad5 (*3*). Liquid and filter hybridization analysis has shown that E11 cells contain one complete copy of the Ad5 genome (*3*). The E11-NMT subclones are clones of Ad5-transformed cells reisolated from tumors induced in nude mice by the parental E11 cell line. Passage of E11 cells through agar (in the presence or absence of TPA) or in a nude mouse results in stable subclones of E11 cells displaying further progression of the transformed phenotype, as indicated by their higher cloning efficiency in agar (*7*). Here we have used the stable acquisition of enhanced anchorage independence and enhanced tumorigenic potential in nude mice as markers for progression.

Analysis of the pattern of viral DNA integration by DNA filter hybridization (*8*) in parental E11 cells and E11 subclones isolated from agar and nude mouse tumors indicated that progression was not associated with detectable alterations in the state of integration of the Ad5 genome in cellular DNA (Fig. 1). In addition, hybridization of Xba I–cleaved cellular DNA with the Xho I-C fragment of Ad5 (0 to 15.5 map units), which contains the primary region of Ad5 DNA involved in transformation (*9*), indicated that this region of viral DNA was conserved during progression (*10*). Chromosomal DNA from agar- and tumor-derived E11 subclones was then cleaved with the restriction endonuclease Hpa II, which recognizes the tetranucleotide sequence C-C-G-G (C, cytosine; G, guanine) but does not cleave if the internal C is methylated (*11*). The migra-

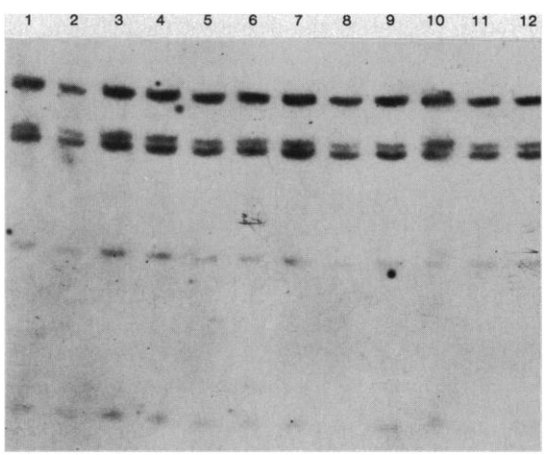

Fig. 1. Analysis of Ad5 DNA sequences persisting in clone E11-L and its agar-, tumor-, and Aza-derived subclones. E11-L (lane 1); E11-L nude mouse tumor (lane 2); E11-AT-1a (lane 3); E11-AT-1a nude mouse tumor (lane 4); E11-NMT subclones Cl 1, 16, 18, and 12 isolated from monolayer culture (lanes 5 to 8, respectively); E11-NMT-Aza subclones 10-1, -6, -9, and -11, isolated from E11-NMT cells treated for 24 hours with 10 μM Aza (lanes 9 to 12, respectively). DNA (10 μg) was cleaved with the restriction endonuclease Xba I, separated by electrophoresis in 0.7 percent agarose, transferred to nitrocellulose sheets by blotting, and hybridized to Ad5 DNA that had been labeled with ^{32}P by nick-translation.

Table 1. Agar cloning efficiency of subclones of E11-NMT cells treated with 5-azacytidine (Aza).

Cell type	Agar cloning efficiency (%)*
E11	2.5
E11-NMT	29.7
Control subclones	
E11-NMT cl 1	36.2
E11-NMT cl 3	27.4
E11-NMT cl 6	24.3
E11-NMT cl 8	31.2
E11-NMT cl 12	29.1
E11-NMT cl 15	35.1
E11-NMT cl 19	30.7
Aza treated subclones	
E11-NMT Aza 10-1	3.5
E11-NMT Aza 10-5	1.2
E11-NMT Aza 10-6	2.8
E11-NMT Aza 10-9	4.6
E11-NMT Aza 10-11	2.4
E11-NMT Aza 10-13	5.2
E11-NMT Aza 10-17	1.4

*Agar cloning assays were performed as described (*3*, *7*). The number of colonies in triplicate plates varied by ≤15 percent.

tion of Ad5-specific fragments on agarose gels indicated that integrated Ad5 sequences were methylated (Fig. 2). Analysis of 11 additional E11 subclones (four isolated from agar lacking TPA, three isolated from agar containing TPA at 100 ng/ml, and four separate nude mouse tumor isolates) also indicated that Ad5 sequences were hypermethylated (*10*). Each of the E11 subclones had a different pattern of methylation of Ad5 sequences, suggesting that progression may not be associated with methylation of specific sequences. In contrast, hypermethylation of Ad5 DNA sequences was not observed when DNA from the parental E11 clone was cut with Hpa II (Fig. 2). When E11 and its subclones were cleaved with the isoschizomer enzyme Msp I, which recognizes C-C-G-G sequences and cleaves both methylated and unmethylated nucleotides, a similar series of DNA fragments was generated (Fig. 2). To determine if the transforming genes of Ad5 were methylated in the progressed clones, we hybridized Hpa II–cleaved DNA with a nick-translated ^{32}P-labeled Hpa I fragment of Ad5 DNA (0 to 4.5 map units). This region of the integrated viral DNA remained unmethylated (*10*) as would be predicted if it is involved in maintenance of the transformed phenotype.

An inverse relationship has been found between the transcriptional activity of several genes and their state of methylation (*12*). Our observations suggest that progression in Ad5-transformed rat embryo clones might be associated with an inhibition by methylation in expression of one or more genes which, when unmethylated, code for a repressor protein or proteins that prevent expression of the progressed phenotype. To determine if the degree of methylation controls the progressed phenotype, we seeded E11-NMT cells at low density (200 to 1000 cells per 5-cm plate), exposed them for 24 hours to solvent or Aza (1 to 20 μ*M*), and refed with drug-free medium. Clones were isolated by means of steel cloning cylinders 14 days later (*6*). When retested for growth in agar, Aza-treated E11-NMT clones displayed the same low level of anchorage independence as the original unprogressed E11 parental clone, whereas solvent-treated E11-NMT clones could grow in agar as well as E11-NMT cells (Table 1). Furthermore, Aza treatment did not alter the pattern of integration of viral DNA sequences in the E11-NMT subclones, and no correlation was observed between specific changes in the pattern of methylation of Ad5 sequences and reversal of the progressed phenotype in Aza-treated E11-NMT clones. However, the changes in methylation were different for each subclone and no obvious similarity in the patterns of demethylation were apparent among the subclones. These findings suggest that changes in cellular gene expression governed by the degree of methylation, as opposed to changes in the methylation of specific integrated Ad5 sequences, may be responsible for regulating progression of the transformed phenotype in Ad5-transformed rat embryo cells.

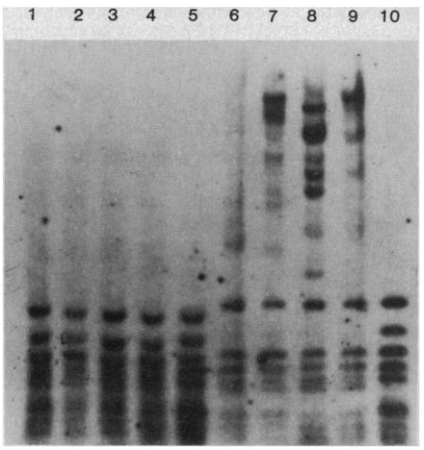

Fig. 2. Methylation pattern of Ad5 DNA integrated in clone E11-L and the agar- and tumor-derived subclones. DNA (10 μg) extracted from E11-L (lanes 1 and 6), E11-L nude mouse tumor (lanes 2 and 7), E11-AT-1a (lanes 3 and 8), and E11-AT-1a nude mouse tumor (lanes 4 and 9) was digested with the isoschizomer restriction endonuclease pair MspI (lanes 1 to 5) or Hpa II (lanes 6 to 10). The fragments were then separated by electrophoresis and hybridized to Ad5 DNA as described in Fig. 1. Lanes 5 and 10 contained 10 μg of DNA from uninfected rat embryo cells and one genome-equivalent of Ad5 DNA per diploid cell genome.

Aza-treated clones were then grown in agar and subclones were reisolated and established in monolayer culture to determine if Aza treatment had resulted in an irreversible suppression of anchorage-independence. The majority (six of ten) of the agar-derived E11-NMT-Aza subclones regained the level of anchorage independence of the progressed E11-NMT clone. When injected subcutaneously into nude mice a direct relationship was observed between agar cloning ability in vitro and the time required for tumor formation in vivo (Table 2).

The mechanisms involved in regulating progression of the transformed phenotype in cells transformed by chemical carcinogens or viruses are not yet

Table 2. Anchorage-independent growth and tumorigenicity of cloned Ad5-transformed rat embryo cells.

Cell type	Derivation	Agar cloning efficiency (%)	Tumor induction in nude mice*	
			Time (days)	Animals, tumors
E11	H5ts125 transformed rat embryo clone	2.7	38	3, 3
E11-NMT	Nude mouse tumor-derived E11 subclone	24.6	18	3, 3
E11-NMT Aza 10-11	Subclone of E11-NMT isolated following a single exposure to 10 μ*M* Aza	2.2	53	3, 3
E11-NMT Aza 10-11 R5	Agar-derived revertant of E11-NMT Aza 10-11	20.5	20	3, 3

*One million cells were injected subcutaneously into 4-week-old nude mice. The average time for the production of a palpable tumor is indicated. Largest tumor size was observed in animals injected with E11-NMT or E11-NMT Aza 10-11 R5 cells.

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