Induction of a Step in Carcinogenesis That Is Normally Associated with Mutagenesis by Nonmutagenic Concentrations of 5-Azacytidine

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The permanent cell line BHK-21/cl 13 can be transformed by mutagenic carcinogens as the result of the induction of a recessive somatic mutation. Yet when these cells were treated with 5-azacytidine under conditions in which no mutants resistant to either ouabain or 6-thioguanine could be detected, they were transformed efficiently. These transformants were induced, not selected. 6-Azacytidine was ineffective at transforming BHK cells; 2'-deoxy-5-azacytidine was exceptionally effective. When tested by cell fusion, transformants induced by 5-azacytidine fell into the same complementation group as those induced by highly mutagenic carcinogens, but they were phenotypically distinct in that they were unstable during prolonged passage and rarely displayed the temperature-limited phenotypes so common among BHK transformants induced by strongly mutagenic carcinogens. These results raise the possibility that a cell can be induced by either genetic or epigenetic means to traverse the same single step in carcinogenesis.

It is generally accepted that mammalian cells can progress toward tumorigenicity as the result of mutagenic changes in base sequences in genomic DNA. Diverse observations suggesting that this is the case (5, 30, 39, 49) have been confirmed by recent demonstrations that oncogenes can be activated effectively by a wide range of structural changes in DNA, including amplification and translocation of genes, insertion of promoters and enhancers, and base substitutions (reviewed in references 3, 34, and 36). An alternative theory proposes that the aberrant gene expression essential for carcinogenesis need not be directly specified by genetic changes but can arise by epigenetic alterations in gene regulation analogous to those which mediate normal development, involving no substantive changes in base sequences (40, 44). This view is less widely supported and has received no confirmation from studies of oncogenes. It is not clear, however, whether epigenetic activation of transforming genes is rare or whether the sample of oncogenes so far available for analysis is artificially restricted by assay methods to those oncogenes activated by point mutations and genetic rearrangements.

The effectiveness of epigenetic changes in inducing neoplasia can be assessed, however, by direct transformation of cells in culture. This paper reports such a study on a baby hamster kidney cell line, BHK-21/cl 13 (55). This immortal cell line is one step removed from being tumorigenic. Recently cloned cells are anchorage dependent and not tumorigenic when injected at doses which exclude spontaneously arising transformants (Bouck, unpublished data). They can be transformed to anchorage-independent, tumorigenic cells in a single step as a result of a recessive mutation (7). Data presented below show that it is also possible to transform these cells with 5-azacytidine, a weakly mutagenic or non-mutagenic agent (35, 38, 46; R. S. Kerbel, P. Frost, R. Liteplo, D. Carlow, and B. E. Elliot, *J. Cell. Physiol.*, in press) which causes undermethylation of cellular DNA and concomitant transcriptional activation of individual genes (12, 14, 48) and of entire developmental programs (4, 50, 58). 5-Azacytidine transforms BHK cells efficiently under condi-

tions in which no parallel mutations are occurring. The 5-azacytidine-induced transformants are phenotypically distinct yet fail to complement dimethylnitrosamine (DMN)-induced transformants, indicating that the same functional step in carcinogenesis is induced by both the mutagenic and the nonmutagenic treatments.

MATERIALS AND METHODS

Cells and media. The cells used were all derivatives of the BHK-21/cl 13 cell line (55). SN-10 cl 3, a subclone of SN-10 (6), was used as normal BHK cells. All were grown in Dulbecco modified Eagle medium containing 10% tryptone-phosphate broth (Difco Laboratories, Detroit, Mich.) and 10% donor calf serum, with serum increased to 20% when cells were at cloning density. Cells plated for growth and stability tests (see Table 2) and fusions (see Table 3) were incubated at 38.5°C. The incubation temperature for all other experiments was 36.5°C.

DNA isolation and digestion. High-molecular-weight DNA was extracted in parallel from sister cultures of cells which were either untreated or treated with 10 μ M 5-azacytidine for 24 h and grown for 48 h in drug-free medium. Cells were harvested with trypsin, lysed in 0.5% sodium dodecyl sulfate in the presence of 200 μ g of proteinase K per ml at room temperature for 1 h, extracted twice with phenol, dialyzed at 4°C for 5 h against 10 mM Tris (pH 8.4) with 1 M NaCl, and dialyzed overnight against 10 mM Tris (pH 7.9). The resulting extract was treated for 2 h at 37°C with 50 μ g of RNase per ml and for 3 h at 37°C with proteinase K in sodium dodecyl sulfate, extracted twice with phenol and twice with chloroform-isoamyl alcohol, and dialyzed overnight against Tris containing 1 M NaCl and then against 10 mM Tris (pH 8.0) containing 100 mM NaCl and 1 mM EDTA. DNA was digested sequentially with DNase (Worthington Diagnostics, Freehold, N.J.), nuclease P1 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.; enzyme was preincubated at 95°C to remove adenine deaminase activity) as described by Kuo et al. (33). Samples were frozen until analysis by high-pressure liquid chromatography (HPLC).

HPLC analysis. The HPLC system consisted of a Supelco-

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sil LC-18-DB column (4.6 by 25 cm) (Supelco, Inc., Bellefonte, Pa.), a Perkin-Elmer LC-85 variable-wavelength UV and visible detector set at 267 nm, a Rheodyne 7125 loop injector with a 25- μ l sample loop, a Perkin-Elmer series 2/2 liquid chromatograph, and a Sigma 15 chromatography data station which was programmed by the use of standards to directly analyze peaks corresponding to the four deoxyribonucleosides and 5-methyldeoxycytidine. Samples of 25 μ l were injected and eluted at room temperature with 0.025 M KH_2PO_4 (pH 4.5) containing various concentrations of methanol, as indicated in Fig. 1. The moles percent 5-methyldeoxycytidine was calculated as moles of 5-methyldeoxycytidine divided by moles of 5-methyldeoxycytidine plus moles of deoxycytidine.

5-Azacytidine treatment. Cells were treated in log phase by refeeding with fresh medium containing the indicated concentrations of 5-azacytidine. A 5 mM stock dissolved in 10 mM Tris (pH 6.3) at 4°C was freshly prepared for each experiment. The half-life of the 5-azacytidine under incubation conditions was 6.4 h. Cells were exposed for 24 h and then refed with fresh medium. Survival was measured by cloning cells immediately after the 24-h exposure. Cells were then allowed to grow for 48 to 72 h, harvested, and assayed for relative agar plating efficiency, calculated as the percentage of cells able to clone on plastic which are also able to clone in 0.34% soft agar. Significant increases in transformation frequency were not achieved either by two or three sequential 24-h treatments with the drug or by shortening or

lengthening the growth time after treatment by 1 day. Agar platings consisted of four to eight plates of cells at densities of 1×10^5 to 2×10^5 cells per dish as described previously (5). Samples of cells tested in agar were also tested (i) for cloning efficiency in nonselective media, (ii) for the presence of 6-thioguanine-resistant (6Tg^r) mutants by plating six or more dishes at 1.5×10^5 cells per 100-mm dish in medium containing 40 μ M 6-thioguanine, and (iii) for ouabain-resistant (Oua^r) mutants by plating five or more dishes of cells at 2×10^5 cells per 60-mm dish in medium containing 3 mM ouabain. Transformation and mutation frequencies were calculated by dividing the number of colonies arising in selective medium per cell plated by the cloning efficiency of the cells at the time they were put into the selective medium.

Fusions. Cells were fused while growing in monolayer at a 1:1 ratio with polyethylene glycol 1000 with one parent which was both Oua^r and 6Tg^r. Hybrids were selected 24 h later by a plating in hypoxanthine-aminopterin-thymidine-ouabain medium as described previously (7). Expression of transformation was assessed (i) by plating of the fused population at 24 h postfusion directly in hypoxanthine-aminopterin-thymidine-ouabain medium containing soft agar and calculating the percentage of hybrids which arose on plastic which were able to clone in the selective agar and (ii) by isolating, with cloning cylinders, individual hybrids which arose on plastic, growing and recloning them on plastic in selective medium, and measuring their relative agar plating efficiency. All clones used in fusions were negative for mycoplasma by the Hoechst dye test (9) and had a diploid modal chromosome number.

RESULTS

5-Azacytidine treatment and undermethylation of BHK cell DNA. High-molecular-weight DNA was extracted either from cells which had been treated for 24 h with 10 μ M 5-azacytidine and grown for 48 h without the drug or from untreated cells grown in parallel. It was digested to nucleosides and run on the HPLC system (Fig. 1). Unusually rapid separation with good resolution was obtained by reverse-phase chromatography at room temperature. Digestions were essentially complete and caused no detectable base modifications. In agreement with other systems (e.g., reference 19), 5-azacytidine treatment resulted in a reduction in the moles percent 5-methyldeoxycytidine in the BHK cell DNA from 2.56 ± 0.20 in the untreated cells to 1.71 ± 0.24 .

Induction of transformation by 5-azacytidine in the absence of mutation. Normal BHK cells were treated with 4-nitroquinoline-*N*-oxide (4NQO) or with various concentrations of 5-azacytidine, grown for 3 days, and assayed for the induction of transformation and of mutants resistant to 6-thioguanine (Fig. 2). None of the drug treatments caused significant cell death. 5-Azacytidine induced transformation (Fig. 2) with a frequency comparable to that resulting from treatment of these same cells with common carcinogens at doses giving 20 to 50% survival (5–7) and greater than that induced by most treatments with carcinogens at sublethal doses (Fig. 2; see also previously published extensive dose-response curves for transformation and mutation in response to several carcinogens [6, 7]).

Although in Fig. 2 and in published studies, transformation of BHK cells by mutagenic carcinogens is invariably accompanied by induction of comparable levels of mutations to ouabain resistance and to 6-thioguanine resistance, equivalent induction of transformation by 5-azacytidine failed to induce mutation at either of these loci (Fig. 2 and Table 1). Killing by 5-azacytidine varied somewhat from experiment

FIG. 1. Rapid separation of deoxyribonucleosides by HPLC. Enzymatically hydrolyzed DNA from untreated BHK cells (A) or a mixture of deoxycytidine (dC), 5-methyldeoxycytidine (m^5 -dC), deoxyguanosine (dG), thymidine (dT), and deoxyadenine (dA) at concentrations of 0.12 mM each (B) were applied to a Supercosil LC-18-DB column and eluted with 0.025 M KH_2PO_4 buffer (pH 4.5) containing methanol at the concentrations indicated at 2.5 ml/min at room temperature.

FIG. 2. Induction of transformation by 5-azacytidine. Normal BHK cells treated with 5-azacytidine for 24 h or with 4NQO in phosphate-buffered saline for 1 h were assayed for survival, grown for 72 h, and assayed for anchorage-independent transformants and for mutants resistant to 6-thioguanine.

to experiment. However, even on one occasion when 10 μ M 5-azacytidine gave survival of only 11%, the frequency of induction of 6Tg^r mutants was only 4.9×10^{-6} , less than two times the mutant frequency in the untreated control.

6-Azacytidine does not induce transformation. To test whether or not 5-azacytidine was acting by nonspecific alterations in nucleotide pools, portions of the same cell suspension were treated in parallel with either 5-azacytidine or 6-azacytidine, an analog which is incorporated into DNA but which has minimal effects on DNA methylation (27). No transformation was observed in response to the 6-azacytidine (Table 1).

2'-Deoxy-5-azacytidine is more effective at inducing transformation than the corresponding ribonucleoside. Because 5-azacytidine is incorporated into RNA as well as into DNA and can be a weak mutagen for RNA viruses (22), we looked for enhanced effectiveness of the 2'-deoxy analog as an indication that it was acting on BHK cells via DNA. Portions of the same culture were treated with either 5-azacytidine or 2'-deoxy-5-azacytidine. The deoxyriboside was cytotoxic at doses at which 5-azacytidine was not and transformed cells at 0.1 to 0.2 the concentration of 5-azacytidine required to achieve similar frequencies of transformation (Table 1). Significant induction of 6-thioguanine- or ouabain-resistant mutants was not detected with either compound.

5-Azacytidine induces rather than selects BHK transformants. If 5-azacytidine were not inducing transformation in the treated population of cells but merely acting to select preexisting transformants, then one might expect that transformants, especially those induced by 5-azacytidine, would be more resistant to azacytidine cytotoxicity. However, when cells which had been transformed by the chemical carcinogen DMN (cell line DMN 4A) or by 5-azacytidine at 5 μ M (cell line AzT30 aR) or at 10 μ M (cell line AzT40 aR) were treated for 24 h with 5-azacytidine at 5 to 50 μ M under conditions which induce transformation and then assayed for survival, all transformed cells showed identical cytotoxicity (e.g., survivals of 12, 11, and 12%, respectively, after 20 μ M 5-azacytidine) which did not differ widely from that of the normal parental line.

If selection were the mechanism by which 5-azacytidine transformed, it would also be predicted that if the proportion of transformants existing in the population before treatment is increased, the apparent frequency of induced transformants should rise proportionately. However, when artificial mixtures of transformed and normal cells at ratios of 1:10, 1:100, or 1:1,000 were treated with 5-azacytidine for 24 h and then grown for 48 h and assayed for transformants, the ratios of transformants in the treated population to transformants in the drug-free control were 1.3, 0.6, and 0.4, respectively. No enhancement of the frequency of transformants due to drug treatment could be detected.

5-Azacytidine-induced transformants differ from those induced by mutagenic carcinogens. Long-term stability is a definitive characteristic of somatic cell mutants (53). In contrast, 5-azacytidine-induced phenotypes and methylation patterns are often unstable, particularly in culture (18, 19, 32, 47, 60, 62; Kerbel et al., in press). The same seems to be true of some azacytidine-induced BHK transformants. Two spontaneous, seven 5-azacytidine-induced, and four 4NQO-induced transformants, all isolated from the same starting population of cells and all cloned from agar, were passaged twice a week at 38.5°C and assayed periodically for continued ability to clone in soft agar (Table 2). During growth, care was taken to keep the cells subconfluent to minimize the selective advantage of transformed cells over normal cells. Under these conditions, BHK cells go through about 11 generations per week. During the 77 to 110 generations

TABLE 1. Transformation and mutation in response to azacytidine analogs^a

Drug (μ M)	Transformation frequency	Frequency of mutant:	
		6Tg ^r	Oua ^r
None	7.1×10^{-5}	$<1.8 \times 10^{-6}$	$<2.2 \times 10^{-6}$
5-Azacytidine			
2	6.0×10^{-5}	$<1.8 \times 10^{-6}$	$<1.6 \times 10^{-6}$
5	6.9×10^{-4}	$<2.2 \times 10^{-6}$	$<2.2 \times 10^{-6}$
10	1.0×10^{-3}	$<3.8 \times 10^{-6}$	$<2.8 \times 10^{-6}$
6-Azacytidine			
10	$<4.0 \times 10^{-5}$	5.1×10^{-6}	$<1.3 \times 10^{-6}$
2'-Deoxy-5-azacytidine			
0.2	8.1×10^{-4}	$<2.7 \times 10^{-6}$	$<2.6 \times 10^{-6}$
0.5	1.5×10^{-3}	$<7.9 \times 10^{-6}$	$<1.1 \times 10^{-5}$
2.0	2.2×10^{-3}	$<4.5 \times 10^{-5}$	$<4.5 \times 10^{-5}$

^a Data were derived from the average of two to five experiments, except that for 2'-deoxy-5-azacytidine at 0.2 and 2.0 μ M.

TABLE 2. Stability of the anchorage-independent phenotype in transformants induced by 5-azacytidine

Cell line	Relative agar plating efficiency (%) in week:						
	1	2	3	4	7	8	10
Spontaneously transformed							
10a	13	9.9	3.3	1.8	0.09	5.6	2.1
10b	23	36	22	28	33		
5-Azacytidine transformed							
30a	11	21	12	4.9	2.0		
30b	13	22	7.4	2.4	3.9	8.7	2.7
30c	18	58	19	19	21		
40a	38	43	24	11	20	21	9.6
40b	5.6	9.5	9.2	0.93	1.2		
40c	13	47	27	9.2	33		
40d	27		27	24	43		
4NQO transformed							
90a	17	45	25	32	44		
90b	28	61	33	31	48		
90c	36	61	37	28	18		
90d	15		25	29	12	22	18

they were carried, one spontaneous line and three 5-azacytidine-transformed lines showed significant drops in relative agar plating efficiency, whereas the relative plating efficiency of all four 4NQO-transformed lines was stable.

To determine whether these drops in plating efficiencies reflected loss of the transformed phenotype, two of these clones (Azc 30b and NQO 90d) were tested further by isolating 10 random subclones from each at passage 15. After expansion for 9 days, the subclones were assayed for relative agar plating efficiency. All 10 subclones derived from the 4NQO-induced transformed line plated with reasonable efficiency in agar. Clones derived from the 5-azacytidine-induced transformant indicated that this line consisted of a mixture of cells with different phenotypes. One subclone was fully transformed (relative agar plating

efficiency of 22%), five clones were weakly transformed (relative agar plating efficiencies between 0.13 and 1.7%), and four clones were apparently of normal phenotype (relative agar plating efficiencies of less than 0.1%; no agar clone in 4,000 cells tested per subclone).

BHK cells are somewhat unusual in that about half of the transformants which arise spontaneously or are induced by a variety of carcinogens which cause mutations in bacteria by inducing base changes are either temperature sensitive or cold sensitive for the ability to clone in soft agar (5). Evidence previously published indicates that these are temperature- and cold-sensitive mutants (5, 7). 5-Azacytidine transformants appear to be different in that they rarely, if ever, show temperature- or cold-sensitive phenotypes. Nine clones of 5-azacytidine-induced transformants were grown for 48 h at either 38.5 or 31.5°C and tested at the temperature grown for the ability to clone in agar. Eight clones showed no temperature restriction; ratios of the relative agar plating efficiency at high temperature to that at low temperature were between 0.6 and 2.9 for all clones except one. Since the frequency of spontaneous transformants in the untreated population carried in parallel to that from which the azacytidine-induced transformants were isolated was 1/50 that in the treated population, this exceptional transformant could be explained by the 1-in-50 chance of unwittingly picking a spontaneous transformant from the treated population of cells. This single cold-sensitive clone was not used in experiments reported in Tables 2 and 3.

5-Azacytidine-induced transformation results in a recessive lesion unable to complement that in DMN-induced transformants. To test whether 5-azacytidine-induced transformants and cells transformed by chemical carcinogens have the same functional lesion, cell fusions were performed (Table 3). When either of two independently derived 5-azacytidine-induced transformants (AzT30 aR or AzT40 aR) was fused to normal BHK cells, transformation was suppressed, whether measured at 24 h postfusion or in isolated and recloned hybrids. When these cells were fused to a DMN-transformed clone, transformation was not suppressed and no complementation was seen, although on fusion to normal cells, the DMN-induced transformant can be suppressed (7; Table 3). The 5-azacytidine-induced transformants behave in fusions

TABLE 3. Complementation test between 5-azacytidine-induced and carcinogen-induced transformants

Fusion	Parental line relative agar plating efficiency (%)	% Hybrids expressing transformation at 24 h	Isolated hybrid clones, relative agar plating efficiency (%)
5-Azacytidine transformed clones			
AzT30 aR	33		
× Normal cell ^a	0.0005	0.14	<0.16 (4) ^c
× Carcinogen-transformed cell ^b	44	5.1	23 (4)
AzT40 aR	5.0		
× Normal cell		0.044	0.11 (2)
× Carcinogen-transformed cell		10	15 (4)
Carcinogen-transformed clone NQO90 bR			
× Normal cell	40	0.35	<0.1 (3)
× Carcinogen-transformed cell		10	46 (3)
Normal cell clone SN-10			
× Normal cell	0.0016	0.066	
× Carcinogen-transformed cell		0.14	

^a SN-10 Ouaf 6Tg^r R3.

^b DMN 4A Ouaf 6Tg^r IbR.

^c Number of hybrid clones tested.

just as 4NQO-induced transformants isolated in parallel from the same pool of normal cells.

DISCUSSION

5-Azacytidine effectively induces the transformation of BHK cells. C3H10T1/2 mouse cells can also be transformed by this drug (2, 46), and hamster CHEF/18 cells, when stimulated by 5-azacytidine to begin adipocyte differentiation, may become tumorigenic (24). In both 10T1/2 and CHEF cells, transformation is a multistep process (16, 54), possibly including nonmutational events (28, 52), and it is not possible to tell which step is being facilitated by 5-azacytidine. In contrast, transformation of BHK cells in response to mutagenic carcinogens is clearly due to a single-step mutation (4, 6). Since 5-azacytidine-induced transformants fail to complement those induced by mutagenic carcinogens, it appears that the same functional step in carcinogenesis can be induced by either classical mutagenic carcinogens or by 5-azacytidine.

The transformants induced by 5-azacytidine in BHK cells differ from those induced by strongly mutagenic carcinogens in two ways. They are rarely, if ever, temperature restricted in the expression of the transformed phenotype, whereas half of the transformants induced by a variety of mutagenic carcinogens (with the exception of pure frameshift carcinogens) are temperature restricted (5, 7). In addition, 5-azacytidine-induced transformants are unstable over extended passage in culture, giving rise to clones of normal, anchorage-dependent cells, which are not found among transformants induced by mutagenic carcinogens. These differences argue against the possibility that all BHK transformants have a similar origin and arise in response to hypomethylation induced either by 5-azacytidine or by mutagenic carcinogens acting *in vivo* as they act *in vitro* (61) to inhibit DNA methyltransferase.

Many new phenotypes induced by exposure to 5-azacytidine arise at startlingly high frequencies, often occurring in 10 to 50% of the treated population. These include phenotypes resulting from the induction of differentiation (4, 11, 50), the induction of immunogenic tumor cell variants (Kerbel et al., *in press*), and the activation of endogenous genes (21, 57) or transfected genes (23). Other phenotypes, such as those due to the activation of the transfected thymidine kinase gene in some transformants (10, 45) and of the silent X chromosome in interspecies hybrids (26, 37, 41), occur at lower frequencies (between 10^{-3} and 10^{-6}). Transformation of BHK cells falls into the latter category.

The recessiveness of 5-azacytidine-induced transformation, especially at early times postfusion, suggests that 5-azacytidine-induced transformants have lost some function required to maintain the normal phenotype. In this respect the action of 5-azacytidine on BHK cells is different from that described in most published reports, where it either turns on some function, in one case viral oncogene expression (20), or has no effect (48). This apparent discrepancy may be due either to the fact that azacytidine-induced gene inactivation has seldom been looked for or to as yet unappreciated complexities in BHK cell transformation.

The mechanism by which 5-azacytidine transforms BHK cells is not yet clear. If its action is dependent on incorporation into DNA, as the data suggest, then it could be transforming by causing one of four general types of changes. (i) Induction of a point mutation is perhaps the least likely possibility because 5-azacytidine is only a weak mutagen in bacteria (25, 38), has not produced significant increases in mutant frequencies in a number of mammalian

cells tested (35, 38, 46), and is not able to increase the frequency of either ouabain- or 6-thioguanine-resistant mutants in BHK cells at the concentrations effective at inducing a 100-fold increase in transformation. In addition, the differences in phenotype range and in stability between transformants induced by 5-azacytidine and those induced by alkylating carcinogens suggest different mechanisms of induction. (ii) Chromosomal rearrangements in mouse cells (2) and in two populations of CHEF hamster cells (24) after treatment with 5-azacytidine have been reported and could cause transformation of BHK cells by inactivating a function responsible for maintenance of the normal phenotype. Although causing mutations in the broad sense of the word, this type of damage might not induce ouabain-resistant mutants where only subtle alterations are compatible with mutant viability or 6-thioguanine-resistant mutants where preservation of an intact functional X chromosome may be required for mutant survival. However, neither chromosome rearrangement nor chromosome loss offers an easy explanation for the delayed instability observed in half of the azacytidine-induced transformants. (iii) Enhancement by 5-azacytidine of somatic recombination (8) or gene conversion could generate BHK transformants since these cells behave as if they are functionally hemizygous at the locus involved in the induction of transformation. In procaryotes, decreased methylation can stimulate recombination (31); however, either of these events would be expected to result in perfectly stable transformants. (iv) The instability of 5-azacytidine-induced BHK transformants is easily accounted for if one assumes that the drug transforms by inducing epigenetic changes in gene regulation which are not accompanied by any change in the base sequence or structure of the DNA. It is presumably by such mechanisms that normal mammalian development unfolds and that 5-azacytidine activates genes at high frequency and induces differentiation programs. Although epigenetic changes have not been defined in molecular terms in mammalian cells, the high correlation of hypomethylation with gene activity (12) and the ability to directly inhibit transcription by methylation of specific base positions (59) suggest that 5-azacytidine may cause these changes by its ability to decrease DNA methylation (51). This in turn may influence DNA conformation (43) and the binding of specific regulatory molecules (17) or of chromatin proteins. More complex mechanisms of action mediated, for example, by specific alterations of nucleotide metabolism as demonstrated for bromodeoxyuridine (1, 13) have not yet been excluded.

Although the transformation of BHK cells to anchorage independence does not, of course, recapitulate the multistep process of *in vivo* carcinogenesis, BHK cell transformation does appear to represent one step in this process. Anchorage-independent BHK transformants induced by strongly mutagenic carcinogens are tumorigenic at doses at which the normal parental population is not (Bouck, unpublished data). Tumorigenicity studies now in progress on 5-azacytidine-induced transformants indicate that the two early-passage transformed clones tested are as tumorigenic as DMN-induced BHK transformants. No tumors have yet arisen in animals injected with parental cells or with azacytidine-induced transformants which have, after prolonged passage, lost their ability to clone in agar. In addition, 5-azacytidine itself has been shown in two reports to be carcinogenic *in vivo* (42, 56), and thus it is in a class with such compounds as L-ethionine, which also alters methylation patterns and gene expression (23) and is an effective carcinogen (15) without appearing mutagenic in a battery of current tests (25, 29).

If the unstable BHK transformants induced by 5-azacytidine are interpreted as resulting from changes in gene regulation, then these results raise the possibility that the same single step in transformation can be induced by either genetic mutations or by epigenetic changes and, at least for this one step, render moot the long-standing debate as to whether cancer results from genetic or epigenetic alterations.

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