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# Azacytidine-induced tumorigenesis of CHEF/18 cells: Correlated DNA methylation and chromosome changes

(Chinese hamster/trisomy/chromosome 3q/pre-adipocytes/differentiation)

JANIE J. HARRISON*, ANTHONY ANISOWICZ*, INDER K. GADI*, MARK RAFFELD†, AND RUTH SAGER*‡

*Division of Cancer Genetics, Dana–Farber Cancer Institute, 44 Binney Street, Boston, MA 02115; and †Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115

*Contributed by Ruth Sager, July 11, 1983*

ABSTRACT 5-Azacytidine (azaC), a drug that induces decreased methylation of DNA in mammalian cells, was shown previously to induce differentiation of mesenchymal cell types in CHEF/18 cells (Chinese hamster embryo fibroblasts). This paper describes the effectiveness of azaC in inducing tumorigenicity in CHEF/18 cells, previously shown to be nontumorigenic stable diploids. A short exposure of growing cells to 3 μM azaC induced tumor-forming ability in CHEF/18 stem cells. Pre-adipocyte clones and subclones derived from CHEF/18 by prior treatment with azaC were also found to be tumorigenic. Pre-adipocytes previously induced by insulin in the absence of azaC were mostly nontumorigenic, but one clone produced tumors and gave rise to both tumorigenic and nontumorigenic subclones. Karyotype analysis of 41 clones and subclones from azaC-induced and insulin-induced pre-adipocytes revealed a complete correlation between tumor-forming ability and the presence of trisomy for chromosome 3q. In addition, the tumorigenic and tumor-derived lines were demethylated at specific C-C-G-G sites in the preproinsulin, Ha-*ras*, and Ki-*ras* genes as revealed by blot hybridization to *Msp* I- and *Hpa* II-digested DNAs, whereas the nontumorigenic lines resembled the CHEF/18 controls. This three-way correlation between tumorigenicity, trisomy for 3q, and specific demethylation suggests that decreased DNA methylation may be involved both in differentiation and in tumorigenicity, and that azaC may induce chromosomal aberrations as well as altering DNA methylation.

The pyrimidine analog 5-azacytidine (azaC) is a potent effector of decreased DNA methylation in mammalian cells. azaC has been shown to substitute for cytosine residues in DNA (1) and to interfere with maintenance methylation (2)—i.e., the post-replicative methylation of hemimethylated sites that maintains preexisting methylation patterns after DNA replication. Recent evidence summarized by Santi *et al.* (3) has led to the proposal that small amounts of azaC in DNA inhibit methylation extensively by binding the methyltransferase enzyme irreversibly at the azaC sites and thus in effect blocking further activity (3). Wide interest in azaC was stimulated by the reports (4–6) that the drug is effective in inducing fibroblastic cells of embryonic origin to undergo terminal differentiation to myoblasts, adipocytes, or chondrocytes. On this basis, it was suggested that azaC might also stimulate bone marrow differentiation. Recently azaC has been used clinically in the treatment of patients with severe β-thalassemia (7). The therapy was successful in repopulating the bone marrow with erythroid precursors that gave rise to circulating erythrocytes containing fetal hemoglobin. azaC is also used clinically in the treatment of leukemia (8), where it may act by inducing differentiation of normal myeloid cells. The clinical results are consistent with the experimental evidence that azaC induces terminal differentiation of stem cells of mesenchymal origin.

We report here that azaC is a potent carcinogen for the Chinese hamster embryo fibroblast cell line CHEF/18, which we have previously shown to be stably diploid (9, 10), nontumorigenic (9), and resistant to one-step induction of tumor-forming ability by chemical mutagens and carcinogens (11). We previously found further that CHEF/18 cells could be induced by a single short treatment with azaC to differentiate into myoblasts, adipocytes, or chondrocytes (6) as in other fibroblastic cell lines of embryonic origin (4, 5). In this paper we demonstrate that the cloned and recloned populations of azaC-induced pre-adipocytes that can be driven to differentiate into mature adipocytes by growth with insulin (12) can also give rise to spindle-cell sarcomas when injected into *nude* mice. Specific changes in DNA methylation patterns as well as a particular chromosomal change have been found in the tumorigenic and tumor-derived cells. The results suggest that decreased DNA methylation is a molecular mechanism involved both in terminal differentiation and in tumorigenesis and that azaC may induce chromosomal changes as well. These complex effects of the drug pose a serious dilemma for the clinician who must balance therapeutic effectiveness against the carcinogenic threat.

## MATERIALS AND METHODS

The origin of CHEF/18 cells and their maintenance in culture have been described, as have the *nude* mouse tumor assay and culture of tumor cells (9). In brief, CHEF/18 cells are grown in α minimal essential medium (KC Biological, Lenexa, KS) with 5 or 10% fetal bovine serum (M. A. Bioproducts, Walkersville, MD) at 37°C in a humidified incubator with 6.5% $CO_2$ in air. To test for tumorigenicity, suspensions of 4–10 × $10^6$ cells are injected subcutaneously into *nude* mice. Animals are observed for at least 4 months; mice with nodules more than 0.5 cm in diameter at the site of injection are scored as positive. The pre-adipocyte cell lines were developed as previously reported (6) by incubation of CHEF/18 cells with 3 μM azaC or with bovine pancreatic insulin (Sigma) at 10 μg/ml. Clones and subclones were selected as colonies containing lipid-synthesizing cells and shown subsequently by several criteria to consist of pre-adipocytes (12). Cell lines designated II/1, II/2, etc., were derived from insulin-treated cultures, and lines designated IV/1, IV/2, etc., were from azaC-treated cultures. Subclones designated II/1-1, IV/1-1, etc., come from single-colony isolates of the parental clones. To identify lipid production, cells attached to plastic dishes were stained with oil red O and coun-

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Abbreviations: azaC, 5-azacytidine; Ha-MSV, Harvey murine sarcoma virus; kb, kilobase(s).

‡To whom reprint requests should be addressed.

terstained with hematoxylin (6). For histological studies, tumor sections were fixed in buffered formalin and stained with hematoxylin and eosin in the routine manner. Chromosomes were analyzed by Giemsa banding as described (10).

DNA methylation was assayed by digestion of 10 $\mu$g of total cell DNA with *Hpa* II (Bethesda Research Laboratories) or *Msp* I (New England BioLabs) at 37°C as recommended by the supplier. DNA digests were electrophoresed on agarose gels containing ethidium bromide at 0.5 $\mu$g/ml, transferred to nitrocellulose, hybridized to $^{32}$P-labeled nick translated probes (*ca.* $10^8$ cpm/$\mu$g), and autoradiographed as described (13). Digestion was monitored by incubating an aliquot of the reaction mixture with an equal amount of replicative form DNA of phage $\phi$X174 at 37°C overnight and then electrophoresing on agarose to check for complete digestion of $\phi$X174 DNA. Restriction fragments of DNA from pBR322 and pBR322-containing plasmids of known fragment lengths were used as size standards.

The assay for sedimentable reverse transcriptase activity was performed as described (14), using NIH/3T3 mouse cells infected with Harvey murine sarcoma virus (Ha-MSV) as a positive control.

## RESULTS

**Origin and Morphology of Pre-Adipocyte Cell Lines.** We reported previously that CHEF/18 cells could be committed to the adipocyte pathway of differentiation not only by treatment with azaC but also by insulin (6, 12). Clones and subclones of pre-adipocytes were established and tested for their ability to differentiate terminally into mature adipocytes. Insulin was shown to influence adipocyte differentiation at two distinct stages: stem cell commitment to the adipocyte pathway, leading to formation of pre-adipocytes (stage I), and terminal differentiation of pre-adipocytes to form mature adipocytes (stage II). azaC induces stage I but not stage II.

Several insulin-induced and azaC-induced pre-adipocyte cell lines were compared with respect to morphology, and rate and extent of differentiation. Most insulin-induced pre-adipocytes were similar to CHEF/18 stem cells in their fibroblast-like spindle-shaped morphology in monolayer cultures. In contrast, the azaC-induced pre-adipocytes were elongated, had a rougher appearance (jagged edges), and tended to cross over each other in irregular arrays, which often became distinct foci. Adipocytes could develop in confluent cultures of either type without further insulin treatment, and they were arranged in sectors within the monolayer as shown (6).

When insulin-induced and azaC-induced pre-adipocytes were treated with insulin to accelerate differentiation, subclones within each group were found to vary quantitatively in rate of adipocyte development, number of adipocytes per colony, and amount of oil red O-staining lipid per adipocyte. The only apparent correlation with the mode of induction was that adipocytes appeared more quickly in azaC-induced than in insulin-induced pre-adipocyte cultures. Furthermore, foci in these faster-developing cultures were often composed largely of adipocytes piled upon one another. Thus, the transformed-appearing cells present in foci were capable of differentiating into adipocytes.

**Tumor Formation by Pre-Adipocytes.** Cell populations of 4–$10 \times 10^6$ cells from a series of pre-adipocyte lines of independent origin and subclones derived from them were injected into *nude* mice to test for tumor-forming ability. The results, summarized in Table 1, show that the azaC-induced lines were tumorigenic, whilst most of the insulin-induced lines were not. The exceptions are of interest: insulin-induced pre-adipocyte line II/2 gave tumors at half of the injected sites, and one of three subclones from line II/2 was clearly tumorigenic; a second subclone gave 4 tumors in 16 sites tested, and the third gave rise to no tumors in 6 sites tested. Thus, the subclones were segregating for tumor-forming ability. Similarly, one of the azaC-induced cell lines, IV/2, segregated for tumor-forming ability as noted in Table 1. In general, azaC-induced pre-adipocytes formed tumors faster in *nude* mice than did the insulin-induced cells.

Table 1. Tumor formation by azaC-treated CHEF/18 cells and by pre-adipocyte clones

| Cells | Tumors* | Chromosomes† |
|---|---|---|
| CHEF/18 untreated | 0/36 | D |
| CHEF/18 azaC-treated | 12/26 | — |
| azaC-induced (IV) | | |
| IV/1, IV/1-1, IV/1-2 | 13/18‡ | +3q |
| IV/2, IV/2-2 | 7/8‡ | +3q |
| IV/2-1 | 0/4 | +5 |
| Insulin-induced (II) | | |
| II/2, II/2-1, II/2-2 | 15/28‡ | D/+3q |
| II/2-3 | 0/6 | D |
| II/3, II/3-2, II/3-3 | 0/14 | D |
| II/1, II/5 | 0/8 | D |
| Serum-free growth | | |
| Uncloned: 18ABII, 18BI | 0/10 | D |
| Cloned: 18-57-2, 18-57-4, 18-57-5, 18-57-6 | 1/18‡ | D |

*Tumors/sites; 4–10 × $10^6$ cells injected per site.
†D, normal diploid; +3q, extra copy of chromosome 3q.
‡Tumor-derived cells contained an extra copy of 3q.

Regardless of origin, all pre-adipocyte tumors were composed of pleomorphic spindle-shaped cells, with high mitotic rates and various amounts of necrosis, but no apparent adipocytes as judged by morphology. In general, the azaC-induced tumors appeared more anaplastic than the insulin-induced tumors. In addition, the azaC tumors tended to be more densely cellular in contrast to the insulin-induced tumors, which had a greater amount of intercellular stroma. An example of an azaC-induced tumor is shown in section in Fig. 1.

The tumors were also examined by electron microscopy, and they were found to be clearly mesenchymal rather than epithelial in origin. Cells were closely spaced, with little extracellular material. No desmosomal attachments, typical of epithelial cells, were seen. Individual tumor cells contained abundant rough endoplasmic reticulum, mitochondria, occasional bundles of fine filaments, and occasional lipid droplets. In particular, evidence was sought for the presence of lipoblasts, but only rare lipoblast-like cells were seen. Thus the tu-

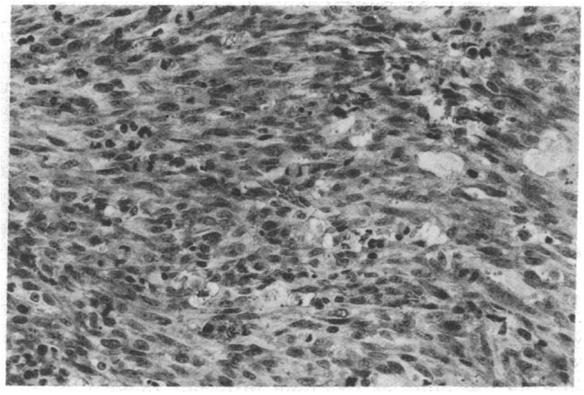

FIG. 1. Histological section of an azaC-induced sarcoma. The tumor is highly cellular and is composed of spindle cells. Numerous mitotic cells are present. (Hematoxylin and eosin; ×160.)

mors were relatively undifferentiated sarcomas in their *in vivo* morphology.

Cultured cells from excised tumors displayed bizarre shapes from round blast-like cells to fibroblast-like cells and grew in an unorganized and loosely attached manner. All tumor-derived populations examined have been capable of lipid production (detected by oil red O staining) in the presence or absence of insulin. However, most of the cells remained spindle-shaped with tiny droplets of fat even in the presence of insulin. A few percent of cells became large and round with huge globules of fat obscuring the nucleus. Occasionally cells with normal adipocyte morphology arose in the cultures, but their arrangement bore little resemblance to the sectored pattern in pre-adipocyte cultures; lipid-containing cells appeared randomly throughout the cultures. Often the most lipid synthesis occurred in foci as opposed to the monolayer cells.

The possibility that azaC might induce tumorigenesis by activating an endogenous retrovirus was tested by assaying for the appearance of sedimentable reverse transcriptase activity (14). The following lines were tested: CHEF/18 with and without azaC treatment, II/2, IV/1-2, IV/1-2 T1, IV/2-2, and IV/2-2 T1, as well as Ha-MSV-transformed NIH/3T3 cells as a positive control. Over 50,000 cpm was obtained in 30 min with the virus-transformed 3T3 cells, whereas none of the other lines gave values significantly higher than the reaction blank. Thus no retrovirus was detected in any CHEF cells by this method.

**Chromosome Changes in Tumor Cells.** Banded karyotypes of 41 cell lines were examined, including azaC-induced and insulin-induced pre-adipocytes with tumors derived from them. The results are summarized in Table 1. A detailed analysis will be published elsewhere. The outstanding regularity is the presence of an extra copy of the long arm of chromosome 3 (trisomy for 3q) in all tumor-derived lines and in tumorigenic parental lines as well. Clone IV/2 is of particular interest because it produced tumors with trisomy for the intact chromosome 3 and segregated one subclone (IV/2-1) trisomic for chromosome 5 but diploid for chromosome 3 that was nontumorigenic, as well as a subclone (IV/2-2) and its tumor-derived progeny that were trisomic for 3q. Most tumor-derived lines from azaC-treated cells had few additional rearrangements.

Among the insulin-induced pre-adipocytes, several clones of independent origin were examined, and only one gave rise to any tumors. Clones II/1, II/3, and II/5 were diploid and nontumorigenic, as were several cell lines derived from pre-adipocytes induced in serum-free medium (12). In contrast, clone II/2 was a mixture of diploid cells and cells containing an extra 3q. All tumors derived from II/2 and from its tumorigenic subclones II/2-1 and II/2-2 contained an extra 3q. Further chromosome changes were present, including a t(6q;7q) reciprocal translocation in some tumors and a t(3q;6q), t(3q;5q), or t(4q;7q) in others.

The absence of an extra 3q in the single azaC-induced subclone that was non-tumorigenic, IV/2-1, is strong evidence in support of the hypothesis that trisomy for 3q is required for tumor formation by these cells. Further support comes from the insulin-induced pre-adipocytes, in which only those clones with 3q trisomic cells were tumorigenic and in which all tumor-derived populations consisted of cells trisomic for 3q or, rarely, for an intact extra chromosome 3.

**Methylation Changes.** To look for methylation changes in the pre-adipocyte clones and subclones, we used several different gene probes (cDNA) chosen on the basis of their availability, in the hope of scanning different regions of the genome. Six genes were chosen: β-actin (15), β-globin (16), dihydrofolate reductase (17), preproinsulin (18), Ki-*ras* (19), and Ha-*ras* (19). The methylation changes were monitored by comparing restriction fragment patterns after digestion of aliquots of genomic DNA with *Msp* I and *Hpa* II. The original CHEF/18 cells were taken as controls, on the assumption that decreased methylation of *Msp* I/*Hpa* II sites (C-C-G-G) could be detected by comparing the restriction patterns of CHEF/18 DNA with those of DNAs from the various pre-adipocyte and tumor-derived cell lines. The patterns generated by digestion with *Msp* I were the same with all genes and with all cell lines, showing that no detectable changes had occurred in methylation of the 5′ C in the C-C-G-G sequence, a change that confers resistance to cleavage by *Msp* I (data not shown).

The results summarized in Table 2 are therefore limited to changes seen in *Hpa* II digestion patterns resulting from methylation changes at the inner C of C-C-G-G, a site at which methylation confers resistance to *Hpa* II digestion. Decreased methylation was seen in all six genes, but a detailed analysis of the results with β-actin, β-globin, and dihydrofolate reductase was complicated by the use of heterologous probes, and by the presence of many fragments. Further analysis awaits the selection of Chinese hamster cDNA probes for these and other genes. On the other hand, the results with preproinsulin, Ki-*ras*, and Ha-*ras* were more readily interpretable. With the preproinsulin probe, decreased methylation at a single *Hpa* II site was seen in all azaC-treated cells and in the tumor-derived cells from insulin induced pre-adipocytes, but not in the untreated controls or in the nontumorigenic insulin-induced pre-adipocytes. A similar pattern was seen with the Ha-*ras* probe for most cell lines, whereas with the Ki-*ras* probe the decreased methylation was more variable than with Ha-*ras*.

An example of the results obtained with the preproinsulin probe is shown in Fig. 2. After *Hpa* II digestion, insulin-induced clones (II) showed the same single fragment of 4.9 kb as in the CHEF/18 control, whereas the four different azaC-induced pre-adipocytes (IV) had either the two smaller bands of 1.7 and 3.2 kb seen in the *Msp* I digests or all three bands. The outer *Hpa* II sites, called $H_1$ and $H_3$, were unmethylated in all lines examined, thus producing the 4.9-kb fragment or the two fragments of 1.7 and 3.2 kb. The inner *Hpa* II site, $H_2$, was methylated to different extents in various cell lines, accounting for the presence of three bands. For further clarification, the relative intensities of the bands were compared by densitometry; they were found to fall into distinct classes. In cell lines showing three *Hpa* II fragments, the density of the uncut 4.9-kb band was either about $^1/_3$ or about $^2/_3$ of the total density of

Table 2. Decreased methylation in azaC- and insulin-treated cells and derived tumor populations

| Cells | Methylation changes* | | |
|---|---|---|---|
| | INS† | KI† | HA† |
| CHEF/18 | | | |
| Untreated | − | − | − |
| azaC-treated | + | − | + |
| Tumor-derived (from azaC-treated) | + | + | ++ |
| azaC-induced pre-adipocytes | | | |
| Pre-adipocyte clones (3 lines) | + | + | + |
| Tumor-derived (7 lines) | + | + | ++ |
| Insulin-induced pre-adipocytes | | | |
| Pre-adipocyte clones (6 lines) | − | − | −‡ |
| Tumor-derived (9 lines) | + | − | +/− |

* *Hpa* II restriction fragment patterns in untreated controls compared with experiment-derived cells. −, Same pattern as control; +, decreased methylation at one (+) or several (++) sites; +/−, heterogeneity among lines.

† INS, preproinsulin cDNA probe; KI, v-Ki-*ras* probe (HiHi3); HA, v-Ha-*ras* probe (BS-9).

‡ Clone II-2 was demethylated in the HA site.

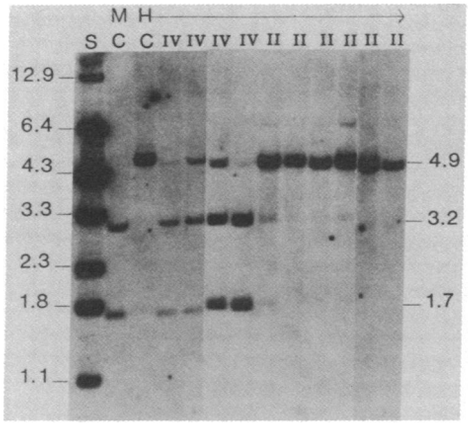


FIG. 2. Hybridization of *Msp* I and *Hpa* II digested pre-adipocyte DNAs with preproinsulin cDNA. DNAs from CHEF/18 cells (C), azaC-induced pre-adipocytes (IV), and insulin-induced pre-adipocytes (II) were digested with *Msp* I (M) or *Hpa* II (H), electrophoresed on 1.0% agarose, transferred to nitrocellulose, and hybridized with preproinsulin cDNA (pRI2). Fragment lengths [kilobases (kb)] were standardized (lane S) with restriction fragments of pBR322 and pBR322-containing plasmids of known size.

all three bands. Assuming that the relative densities reflect the frequency of cleavage at $H_2$, the results are most simply interpreted as evidence for the presence of three copies of the gene.

CHEF/18 stem cells and insulin-induced pre-adipocytes gave only the 4.9-kb *Hpa* II fragment. Tumor-derived lines from induced pre-adipocytes had one unmethylated copy (65% of total fragments were 4.9 kb), whereas tumor-derived lines from azaC-induced pre-adipocytes had either two or three unmethylated copies. A few exceptions were seen, indicating heterogeneity of methylation of one copy (15–24% of fragments were 4.9 kb).

In Fig. 3, a comparison of *Hpa* II-digested DNAs hybridized with the v-Ha-*ras* probe shows that the insulin-induced lines (II) resemble the CHEF/18 control, whereas the azaC-induced (IV) and tumor-derived (T) lines have lost the principal band at 1.03 kb partially or completely and show a new band at 0.89 kb. There is also a new band at 0.62 kb as well as a pair of small

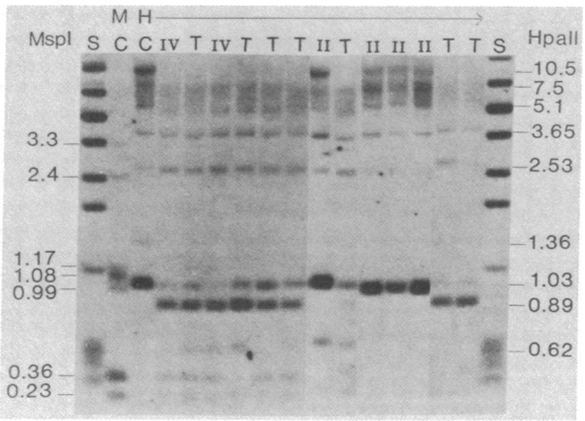


FIG. 3. Hybridization of *Msp* I- and *Hpa* II-digested pre-adipocyte DNA with Ha-MSV DNA. DNAs from CHEF/18 cells (C), azaC-induced preadipocytes (IV), insulin-induced pre-adipocytes (II), and tumors (T) derived from IVs and IIs were digested with *Msp* I (M) or *Hpa* II (H), electrophoresed in 1.4% agarose, transferred to nitrocellulose, and hybridized with nick-translated Ha-MSV DNA. Fragment lengths (kb) were standardized (S) with restriction fragments of pBR322 and pBR322-containing plasmids of known size.

fragments at 0.23 and 0.36 kb present in the *Msp* I digest of CHEF/18 DNA as well as in all the IVs and Ts but not in the IIs. Thus, the results with the HA probe are qualitatively in line with those obtained with the preproinsulin probe, as summarized in Table 2, although quantitation of the v-Ha-*ras* bands was not practicable. (The faint bands at high molecular weights in Fig. 2 probably correspond to crossreacting sequences of other *ras* family genes.)

## DISCUSSION

This paper reports the tumorigenic effectiveness of azaC on the stably diploid, nontumorigenic CHEF/18 cell line. Unselected populations of azaC-treated cells, grown up in the absence of the drug, as well as clones of azaC-induced pre-adipocytes, were found to be tumorigenic in the *nude* mouse (Table 1). We have previously shown that either azaC or insulin in the absence of azaC can induce pre-adipocyte formation—i.e., commitment to the adipocyte pathway of differentiation. Comparisons of azaC-induced and insulin-induced pre-adipocytes have revealed only minor differences between them, for example, in the rate of terminal differentiation to adipocytes (12).

We show here that azaC-induced and insulin-induced pre-adipocytes differ significantly in tumor-forming ability. Most of the insulin-induced pre-adipocytes were nontumorigenic, whereas all but one subclone of azaC-treated cells produced tumors rapidly after subcutaneous injection into *nude* mice (Table 1). These results demonstrate that commitment of CHEF/18 stem cells to the adipocyte pathway of differentiation is separable from tumorigenicity but that the two potentialities may co-exist in the same cloned cell populations. Tumor-derived cells of pre-adipocyte origin retained some adipocyte-like properties, indicating that tumor formation did not eliminate their ability to differentiate.

CHEF/18 cells are very resistant to becoming tumorigenic (11). No spontaneous occurrence of tumorigenicity has been detected in over 36 tests ($10^7$ cells per site) in *nude* mice, representing a frequency of less than $3 \times 10^{-9}$, and after mutagenesis it was still less than $10^{-8}$ (11). In contrast, the frequency of spontaneous single gene mutations (e.g., $hprt^+ \rightarrow hprt^-$) in CHEF/18 is about $5 \times 10^{-7}$ and in ethyl methanesulfonate-treated cells it is 100-fold higher. Thus, CHEF/18 cells do not become tumorigenic as the result of a single gene mutation. The effectiveness of azaC in inducing tumor-forming ability needs to be evaluated in light of this resistance to classical mutagens.

**Trisomy for Chromosome 3q.** When the karyotypes of the 41 cell lines involved in this study were examined, as summarized in Table 1, a complete correlation was observed between tumorigenicity and the presence of an extra copy of chromosome 3q. This correlation includes the segregating subclones (IV/2-1 and II/2-1) in which the loss of the extra copy of 3q paralleled the loss of tumorigenicity.

Trisomy for chromosome 15 has been closely correlated with tumor forming ability in mouse T-cell leukemia (20). Recent identification of c-*myc* sequences on chromosome 15 raises the possibility that trisomy for this oncogene contributes to tumorigenicity. The c-*myc* region is also involved in translocations characteristic of mouse plasmacytomas (21), in parallel with analogous translocations present in Burkitt lymphoma (reviewed in ref. 22). Translocations involving 3q have also been seen in some of the tumors described in this paper (unpublished results), and it will be of interest to identify genes at the break points in the other Chinese hamster chromosomes involved in these translocations.

It seems likely that the extra copy of chromosome 3 arose by nondisjunction and that centromeric fission occurred subse-

quently, producing a 3p that was lost and a 3q that was retained. One may ask whether loss of chromosome 3p is a prerequisite for tumorigenesis. Out of 26 tumor-derived cell populations examined in this study, only 2 were trisomic for the intact chromosome 3 rather than 3q. A third tumor contained a mixture of cells, some with trisomy for 3 and some for 3q. This result suggests that the loss of 3p is advantageous in the establishment of tumorigenicity.

The high correlation observed between trisomy for 3q and tumor-forming ability has led us to suggest that an oncogenic gene or gene region may be located on chromosome 3q. In previous studies in this and other laboratories, changes in chromosome 3 have been noted in tumorigenic cells (23–26), but nowhere else was the correlation so high and the karyotypes so uncomplicated by other chromosome changes as in the present study.

**Methylation Changes.** Because azaC has been shown in several systems to stimulate differentiation by interfering with DNA methylation (1–3), it seemed reasonable to postulate that azaC might also stimulate tumorigenicity by the same mechanism. We have tested this hypothesis by examining methylation changes at *Hpa* II sites, using a set of cDNA gene probes chosen at random to represent a variety of genomic locations and gene products. Decreased methylation of *Hpa* II sites was observed at six loci in the tumorigenic and tumor-derived cells compared with CHEF/18 controls. In particular, parallel losses of methylation at specific sites were seen at two functionally unrelated loci, the preproinsulin gene and the Ha-*ras* gene; somewhat less complete correlation was seen with the Ki-*ras* gene. DNAs from nontumorigenic preadipocytes resembled those of the CHEF/18 controls. Thus, the methylation patterns followed the same distribution as did tumor-forming ability (Table 2).

In the human genome, the preproinsulin and Ha-*ras-1* genes are closely linked on chromosome 11 (27). If this linkage is conserved in the Chinese hamster, then it may be that the same demethylation event affected both loci, although the intergenic distances are very long on the molecular scale. The Ki-*ras*-2 gene is on human chromosome 12 (28), but in the Chinese hamster genome, which contains only half the number of chromosomes of most mammals, it is possible that Ha-*ras* and Ki-*ras* are linked on the same chromosome. Thus, the similar methylation changes in preproinsulin, Ha-*ras*, and Ki-*ras* could reflect a structural continuity in the three genes.

Several lines of evidence in this paper support the speculation that preproinsulin, Ha-*ras*, and Ki-*ras* are all located on chromosome 3q: (*i*) the concordance of methylation changes in these three genes; (*ii*) the likelihood that an activated oncogene is located on chromosome 3q, based on the correlation of trisomy for 3q with tumorigenicity; and (*iii*) the evidence that three copies of the preproinsulin gene are present in the azaC-induced pre-adipocytes and tumor-derived cells. There is, however, no evidence from DNA·RNA hybridization studies that either preproinsulin or Ha-*ras* is transcribed at an increased rate in these cell lines. Thus changes in expression of these genes may be too low for easy detection, or the decreased methylation may encompass other linked transforming genes as yet unidentified, whose expression is altered.

In summary, the effectiveness of azaC in potentiating tumor-forming ability in CHEF cells has been demonstrated in this study. Specific changes in DNA methylation and in the chromosome complement have been identified, supporting the speculation that both processes are involved in tumorigenesis. How insulin promotes either adipocyte differentiation or tumorigenesis remains unknown. Nonetheless, the similarities in chromosome and methylation changes in the tumorigenic insulin-induced and azaC-induced pre-adipocytes and in their tumor-derived descendants support the speculation that a common mechanism of tumorigenicity involving DNA methylation and chromosome changes is activated with high probability by azaC and rarely by insulin.

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