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Gene Silencing by DNA Methylation and Dual Inheritance in Chinese Hamster Ovary Cells

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ABSTRACT

Chinese hamster ovary (CHO) cells strain D422, which has one copy of the adenine phosphoribosyl transferase (APRT) gene, were permeabilized by electroporation and treated with 5-methyl deoxycytidine triphosphate. Cells with a silenced APRT gene were selected on 2, 6-diaminopurine. Colonies were isolated and shown to be reactivated to APRT$^+$ by 5-aza-cytidine and by selection in medium containing adenine, aminopterin and thymidine. Genomic DNA was prepared from eight isolates of independent origin and subjected to bisulphite treatment. This deaminates cytosine to uracil in single-stranded DNA but does not deaminate 5-methyl cytosine. PCR, cloning and sequencing revealed the methylation pattern of CpG doublets in the promoter region of the APRT$^-$ gene, whereas the active APRT gene had nonmethylated DNA. CHO strain K1, which has two copies of the APRT$^+$ gene, could also be silenced by the same procedure but at a lower frequency. The availability of the 5-methyl dCTP-induced silencing, 5-aza-CR and a standard mutagen, ethyl methane sulphonate, makes it possible to follow concomitantly the inheritance of active, mutant or silenced gene copies. This analysis demonstrates "dual inheritance" at the APRT locus in CHO cells.

TWO types of inheritance exist in transformed mammalian cells. The first is due to classic gene mutations that change the DNA sequence or to rearrangements of chromosomal DNA. The second is often referred to as an epigenetic mechanism because heritable changes in gene activity are not due to alteration in DNA sequence (Holliday 1987, 1991; Holliday *et al.* 1996). There is much evidence that such heritable changes can be due to the presence or absence of 5-methyl cytosine in promoter sequences or other nearby sequences (reviewed in Jost and Saluz 1993; Holliday 1996). In almost all cases, DNA methylation is associated with gene inactivity or gene silencing, and it has been shown in many instances that these silent genes can be activated by the DNA demethylating agent 5-aza-cytidine (5-aza-CR) or 5-aza-deoxycytidine. These analogues of cytidine are incorporated into DNA and appear to bring about demethylation by inactivating the DNA methyl transferase, the enzyme that is responsible for the inheritance of any given pattern of DNA methylation (Santi *et al.* 1984). It has been previously shown that 5-methyl deoxycytidine triphosphate (5-methyl dCTP) can be used to silence genes in Chinese hamster ovary (CHO) and V79 cells (Holliday and Ho 1991; Nyce 1991). Electroporation of cells in the presence of 5-methyl dCTP is followed by its uptake into DNA and by the silencing of three genes [*i.e.*, thymidine kinase (TK), hypoxanthine phosphoribosyl transferase (HPRT), and adenine phosphoribosyl transferase (APRT)]. In each case, the enzyme deficient phenotype is stably inherited, and of 51 isolates tested, 49 were reactivated by 5-aza-CR (Holliday and Ho 1991).

Many questions remain about the relationship between DNA methylation of promoter sequences and gene silencing. For example, are there specific sites that when methylated prevent or inhibit transcription? Some studies indicate that such critical sites exist (Tasseron-de Jong *et al.* 1989; Graessman *et al.* 1994), and also it is known that there can be specific interactions between transcription factors and the presence or absence of methylation in the DNA binding sequences (Molloy and Watt 1990; Ehrlich and Ehrlich 1993). In other contexts, it appears that the density of methylation in a given region determines whether or not transcription will be prevented (Boyes and Bird 1991, 1992; Hsieh 1994). It has also become evident that DNA methylation can be very important in silencing tumor suppressor genes during tumor progression (Sakai *et al.* 1991; Ohtani-Fujita *et al.* 1993; Merlo *et al.* 1995; Stirzaker *et al.* 1987). It is essential to have a much better understanding of the mechanism of *de novo* methylation in partially or fully transformed cells and of the relationship between the silencing of tumor suppressor genes and oncogenesis.

The opportunity for genetic and epigenetic analysis now exists in CHO cells. We have chosen the APRT gene for a detailed study of gene silencing in relation

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to methylation of the promoter region. We have used the bisulphite genomic sequencing procedure that determines which cytosines are methylated in the region of interest (Frommer *et al.* 1992; Clark *et al.* 1994; Grigg and Clark 1994). APRT is coded for by a fairly small gene with a CpG island at its 5′ end. The hamster gene has been extensively used in molecular studies of mutation (Meuth 1990; Nalbantoglu *et al.* 1983), and both the hamster and mouse genes can be silenced by DNA methylation (Cooper *et al.* 1992, 1993; Holliday and Ho 1991; Mummaneni *et al.* 1995; Stein *et al.* 1982). Mutant APRT strains are resistant to 2, 6-diaminopurine (DAP), or azaadenine, and cannot grow on adenine, aminopterin, and thymidine (AAT) medium (Chasin 1974; Jones and Sargent 1974), whereas the reverse is true for strains with active APRT. Thus, one can select for and against gene activity. To simplify molecular analysis, we have used strain D422, which has only one intact copy of the gene (Bradley and Letovanec 1982; Nalbantoglu *et al.* 1983). We have also used the CHO K1 wild-type strain, which has two copies of the APRT gene, to demonstrate "dual inheritance" in these cells. As was previously shown for the TK gene, it is possible to study inheritance based on gene mutation and also inheritance based on DNA methylation in the same cell populations (Holliday and Ho 1990). Using the APRT gene, we have obtained further evidence for epigenetic changes in gene activity, concomitant with mutations that inactivate the APRT gene.

MATERIALS AND METHODS

Strains and cell culture: CHO K1 cells were the same as previously used (Holliday and Ho 1990, 1991). D422, hemizygous for the $APRT^+$ gene, was kindly supplied by Mark Meuth (University of Utah, Salt Lake City). Cells were grown in Eagle's minimal essential medium (GIBCO BRL, Gaithersburg, MD) supplemented with nonessential amino acids, 10% fetal calf serum, penicillin and streptomycin (0.06 mg/ml and 0.1 mg/ml, respectively) in 25-cm^2 and 75-cm^2 flasks, and incubated at 37° in 5% CO_2. Cells were detached with trypsin and versene (0.16 w/v trypsin and 0.54 mm versene) after washing with phosphate buffered saline (PBS), and counted with a Coulter counter (Harpenden, UK). Viable counts were made by plating cells in 10-cm plates and staining with Giemsa after 7 days of incubation.

Selection procedures: $APRT^-$ cells were selected in 40 μg/ml 2,6-diaminopurine (DAP^R) from populations of 4×10^5 cells in 10-cm plates. Individual colonies were picked using cloning rings or stained with Giemsa to determine the frequencies of DAP^R cells. DAP^R clones were tested for reactivation to $APRT^+$ using 5-aza-CR. 10^5 cells were incubated 24 hr in a 25-cm^2 flask before the addition of 1 μg/ml 5-aza-CR (Sigma, St. Louis), from a freshly prepared solution of 1 mg/ml. After 24 hr, the medium was replaced with MEM (Eagle's minimum essential medium), and the cells incubated for 4–6 days. The semiquantitative method was used to score reactivation (Holliday and Ho 1990). 10^5 treated cells were plated per 10-cm dish, and 2.5×10^4 cells were plated per 5-cm dish in AAT medium (MEM with 10% dialyzed serum, 75 μmol adenine, 0.8 μmol aminopterin and 16 μmol thymidine, by 1:50 dilution of a stock from Sigma). Reactivable DAP^R isolates produced several hundred or several thousand colonies. In all cases, the reactivation of cadmium sensitivity to cadmium resistance (40 μg/ml $CdCl_2 \cdot H_2O$) was checked as a control (Holliday and Ho 1990). To pick individual reactivated $APRT^+$ DAP^S colonies, cells were plated in AAT at lower densities.

Electroporation: The procedure used was the same as previously described (Holliday and Ho 1991), except that the concentration of 5-methyl dCTP was increased to 0.6 –1.2 mm. In some experiments two electroporation pulses were applied (Table 1). Viability of treated cells was 50–70%.

Mutagenesis with ethyl methane sulphonate (EMS): 10^5 cells were incubated for 24 hr in 25-cm^2 flasks, before the addition of 300 μg/ml EMS. The medium was changed to MEM after 24 hr, and the cells subsequently incubated for 4–6 days, before plating on DAP medium. In strain D422, colonies arose at a frequency of $\sim 10^{-3}$, and individual colonies were picked using cloning rings, as required.

Genomic DNA isolation: A culture of 5×10^7–2×10^8 cells was washed in PBS, trypsinized, washed in PBS again and pelleted by centrifugation at 1000 rpm for 2 min. Cells were resuspended by hand agitation in 2 ml of ice-cold N-lysis solution (10 mm $MgCl_2$, 30 mm HEPES pH 7.0, 10% (w/v) sucrose) to lyse the outer cell membrane. Intact nuclei and cellular organelles were pelleted by centrifugation at 7000 rpm for 5 min. The pellet was resuspended by hand agitation or pipetting in 200 μl of N-lysis solution, then lysed in 1.4 ml of lysis solution [10 mm Tris-HCl, 100 mm EDTA, 50 mm NaCl, 0.5% (w/v) SDS] containing 1 mg/ml proteinase K added from powdered stock. The lysate was transferred to a 15-ml polypropylene tube and incubated at 37° overnight on a roller or with gentle shaking. The overnight lysate was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) w/v, and once with chloroform-isoamyl alcohol (24:1 w/v). Powdered cesium chloride (Chemetall AG, Frankfurt, Germany) was added to an optical density of 1.391 (achieved by adding exactly 1.65 g of cesium chloride to 1.5 ml of lysate, then adding 100 μl of 10 mg/ml of ethidium bromide). The solution was sealed into a mini ultracentrifuge tube (Beckman Instrs., Inc., Fullerton, CA) and isopycnic cesium gradient ultracentrifugation performed overnight at 100,000 rpm or 100,000 rpm for 6 hr. The DNA band was removed from the gradient by a syringe fitted with a clean 19-gauge needle. The DNA was transferred to an Eppendorf tube and extracted repeatedly with an equal volume of CsCl-saturated isopropanol until the ethidium bromide was removed. The solution was transferred to a 15-ml polypropylene tube. DNA fibers were spooled, washed in 70% (w/v) ethanol, air-dried, and dissolved in a minimal volume of TE buffer (10 mm Tris, 0.1 mm EDTA), usually 200 μl in an Eppendorf tube. DNA concentration was measured by $A_{260/280}$.

Primer synthesis: The primers were synthesized on a 391 synthesizer (Applied Biosystems, Foster City, CA) as per the manufacturer's recommended protocol. The oligonucleotides were deprotected by incubating in concentrated ammonia solution at 55° overnight. A 50-μl aliquot was dried by vacuum centrifugation, redissolved in 80% acetic acid for detritylation, dried, redissolved in distilled water and then used directly for PCR.

Oligonucleotides complementary to bisulphite-converted DNA were synthesized based on the sequence obtained from the cloned APRT gene (30) and tested on bisulphite-treated DNA of the plasmid pD422B, kindly provided by Dr. Mark Meuth (University of Utah, Salt Lake City).

Bisulphite primers: first round primers were A4, GGATTTG GATAATATTTATATTGGTTT, (upper strand) and B7, CAC CAACTACAACTCAAATTCCACCATAAC (lower strand). Second round: A1, AAAATATCAACAAACTAAAATCATACCAA (upper strand) and B2, AGGTTTAGAAAGTTGGTTTTGT GAGAAG (lower strand).

Bisulphite genomic sequencing: This was based on published procedures (Frommer *et al.* 1992; Clark *et al.* 1994; Grigg and Clark 1994). The bisulphite conversion reaction was carried out on 2 μg of cesium-purified genomic CHO DNA digested with the enzyme *Eco*RI. The DNA was denatured by adding freshly prepared NaOH (BDH, Port Fairey, Victoria, Australia) to a final concentration of 0.3 N and incubating for 15 min at 37° in a final volume of 22 ml. A solution of 2 m sodium metabisulphite (BDH), 10 mm hydroquinone (BDH) pH 5.0 was prepared immediately before use and 208 ml added to the denatured DNA. The mixture was overlaid with mineral oil and incubated at 55° for 16 hr. DNA was recovered from under the oil, and free bisulphite removed from the modified DNA using a desalting column (Wizard DNA Clean Up System, Promega, Madison, WI) according to the manufacturer's instructions. TE buffer was added to bring the volume of the sample up to 200 ml.

Freshly prepared NaOH (BDH) was added to a final concentration of 0.3 N and incubated at 37° for 15 min. The solution was neutralized by addition of NH_4OAc to 3 m. The DNA was ethanol precipitated, dried and redissolved in 100 μl of DNA buffer and stored at −20°.

PCR Amplification was performed in two rounds with nested primers, in 25-μl reaction mixtures containing 200 ng of treated genomic DNA, 200 μmol dNTPs (Boehringer Mannheim, Indianapolis), 1 μmol primers, commercially supplied buffer (Advanced Biotechnologies, Surrey, UK) with 2.5 units thermostable DNA polymerase (Advanced Biotechnologies) stabilized beforehand by the addition of Taqstart antibody (Clontech, Palo Alto, CA) as per the manufacturer's instructions. A FTS 320 thermocycler (Corbett Research) was used for the PCR under the following conditions: 94°/3 min × 1 cycle; 94°/1 min, 55°/2 min, 72°/3 min × 5 cycles; 94°/0.5 min, 50°/2 min, 72°/1.5 min × 25 cycles; 72°/5 min × 1 cycle.

Amplified DNA was cleaned using a Wizard PCR preps purification kit (Promega) and treated with 5 units of P*fu* polymerase/1 mm dNTPs at 72° for 30 min to produce blunt ends. The blunt-ended product was kinased with 20 units of polynucleotide kinase (Boehringer Mannheim), buffer and 1 mm ATP. The mixture was incubated at 37° for 1 hr, heated to 75° for 15 min, phenol chloroform extracted, ethanol precipitated, and ligated into commercial, SmaI cut dephosphorylated pUC18 (Pharmacia, Uppsala, Sweden). The ligation mix was used to transform commercial competent *E.coli* XL-1 Blue (Stratagene, La Jolla, CA). Clones were sequenced using the fmol cycle sequencing kit (Promega).

Cell extracts and APRT assay: A total of 1–2 × 10^7 cells were harvested, pelleted and washed twice with isotonic saline. The pellet was suspended in 3 ml 0.03 m Tris-HCl buffer (pH 7.4) and the cells disrupted by sonication. A supernatant extract was prepared by 20-min centrifugation at 12,000 *g* at 4°. Protein was determined by the A260/280 nM absorption procedure.

The enzyme assay is based on the procedure of Chasin (1974). The reaction mixture contained 0.25 m Tris-HCl (pH 7) containing 1.2% bovine serum albumin; 0.1 m $MgCl_2$; 0.1 m NaN_3; 0.01 m 5-phosphoryl-ribose-1-pyrophosphate; 0.01 m cold adenine with ^{14}C adenine (Amersham International, Buckinghamshire, UK; 0.175 μCi per assay), in a total volume of 0.05 ml. The same volume of cell-free extract was added and the assay mix was incubated for 1 hr at 37°. The reaction was terminated by the addition of 1 ml of 0.05 m cold Na acetate (pH 5.0) containing 2.0 mm K_2PO_4, and 0.2 ml 0.5 m $LaCl_3$, with mixing on ice. After 20 min, the precipitated radioactive adenosine monophosphate was collected on a glass fiber filter, washed with water, dried and counted in Aquasol scintillation fluid. All biochemicals were obtained from Sigma (St. Louis).

RESULTS

Inactivation and reactivation of the APRT gene in strain D422: We have used electroporation in the presence of 5-methyl dCTP to silence the APRT gene. These isolates are selected in medium containing DAP. Table 1 illustrates the frequencies of DAP^R colonies under different inducing conditions. A number of clones were isolated, subjected to 5-aza-CR treatment, and plated on AAT medium. We used the semiquantitative assay for detecting reactivation, and in all cases reactivation of the silent metallothionein gene was also checked by plating treated cells in medium containing cadmium (Holliday and Ho 1990). The reactivation test gives, in effect, an all or none result. Thus, 5-aza-CR treatment can reactivate a methylated gene at a frequency of 1–10%, whereas a nonmethylated mutant gene produces 10^{-5} or less colonies on AAT medium. Of 17 $APRT^-$ clones picked, 16 were reactivated by 5-aza-CR. Eight of these isolates were used for subsequent molecular analysis. Clones 1C, 3C and 8C were from Experiment 2 (Table 1), 1A from Experiment 3 and 2B, 4B, 5B and 6B were from Experiment 4.

Genomic sequencing of the promoter of the APRT gene: The bisulphite genomic sequencing procedure is able to distinguish cytosine from 5-methyl cytosine

TABLE 1

Induction of DAP^R colonies in strain D422 by electroporation and treatment with 5-methyl dCTP

Experiment No.	5-Methyl dCTP (mm)	Viable cells plated	DAP^R colonies	Frequency
1	0.6	7.00×10^5	126	1.80×10^{-4}
2	0.8[a]	2.84×10^6	384	1.35×10^{-4}
3	0.8[a]	9.36×10^5	77	8.23×10^{-5}
4	0.8	1.22×10^6	82	6.72×10^{-5}
5[b]	1.2	1.06×10^6	137	1.29×10^{-4}
6[b]	1.0	3.26×10^6	214	6.56×10^{-5}
Controls (5 experiments)		1.12×10^7	28	2.50×10^{-6}

[a] Cells electroporated twice in the presence of 5-methyl dCTP.
[b] Populations derived from a single clone.

(5-mC) in genomic DNA (Frommer *et al.* 1992; Clark *et al.* 1994; Grigg and Clark 1994). In brief, the bisulphite treatment deaminates cytosine to uracil, while 5-mC is unaffected. When the region is amplified by PCR, using appropriate primers the uracil residues are replaced by thymidine and the 5-mC residues are replaced by cytosine. The PCR product is cloned and sequenced. In our experiments, only one strand of the PCR product is sequenced, and in most experiments this comprised 323 bases.

The promoter region of the APRT gene is within a CpG island at the 5′ end of the gene. Genomic DNA from the D422 DAP^S strain consistently showed an absence of cytosine methylation in the promoter region. Genomic sequencing of CHO K1 DNA was also carried out, and the promoter region was consistently shown to be nonmethylated.

D422 DAP^R isolates that are reactivable by 5-aza-CR, were grown to a population size of approximately 2×10^7 cells in selective medium, and the DNA was isolated. A single DNA preparation was subjected to bisulphite treatment and several clones from the PCR product were sequenced (PCR clones). We detected DNA methylation in the promoter region of all these isolates, and in almost all cases the 5-mC residues were in CpG doublets. These results are presented in Table 2. The sequence analyzed is 323 base pairs in length and this contains 16 CpG doublets. In some clones (1A, 1C, 3C and 5B), the pattern of methylation was the same in all PCR clones, in others (2B and 6B) a majority of PCR clones had the same pattern, and in two (4B and 8C) there was considerable heterogeneity among the PCR clones. This variability presumably arose by the loss or gain of methylation during the time the original isolate was growing prior to the extraction of DNA. The lowest number of methylated CpG doublets was 5 or 6 in clone 2B, and the highest was 16 in clone 4B(iv) and 4B(ix). Two CpG sites (180 and 292) were methylated in all cases.

Non-CpG methylation was occasionally seen, at random sites, which was probably the result of the failure of deamination of cytosine by bisulphite. We also examined DNA from 5-aza-CR reactivable DAP^R isolates from CHO K1. In this case, one would expect two different methylated genes to be present, each with a somewhat different pattern. This demands more exhaustive analysis, and although we confirmed that these isolates contained methylated promoter DNA (results not shown), we decided to concentrate on the more straightforward investigation of the D422 hemizygous strain.

Dual inheritance at the APRT gene: The D422 strain provides a simple system in which the APRT can be inactivated by DNA methylation and reactivated by 5-aza-CR. The CHO K1 strain with two APRT genes makes it possible to examine both methylated epimutants and mutations induced by EMS. Previously we reported that 5-methyl dCTP produced DAP^R colonies with a frequency of about 7×10^{-4}, which was comparable to the frequencies of BrdU^{R}TK$^-$ and TG^R HPRT$^-$ colonies (Holliday and Ho 1991). There is only one copy of the HPRT gene as it is *X*-linked, and the TK gene is hemizygous (Holliday and Ho 1990). In subsequent studies with APRT, we obtained a much lower frequency of DAP^R APRT$^-$ isolates from CHO K1 ($\sim 3.7 \times 10^{-5}$, see Table 3). We assume, but cannot prove, that in the earlier populations the APRT gene had become spontaneously hemizygous by *de novo* methylation, in at least a substantial proportion of the population, whereas in the more recent populations this had not happened.

The DAP^R colonies obtained by 5-methyl dCTP-induced silencing in strain K1 presumably have two methylated APRT genes (Figure 1C). Of 22 isolates tested, all were reactivated by 5-aza-CR (Table 3; Figure 1D), but one would expect that in most cases only one of the genes would have become active, since this is sufficient for growth on AAT medium. Hemizygosity was confirmed by treatment with 5-methyl dCTP. DAP resistant strains arose with a frequency of 7×10^{-4} (Table 3; Figure 1E), which is about 20-fold higher than step C in Figure 1 and Table 3.

One of the hemizygous APRT isolates was treated with EMS, under conditions that induce TG^R HPRT$^-$ mutants at a frequency of about 10^{-3}. DAP^R colonies were obtained at a comparable frequency (Table 3; Figure 1F) and these should now contain one silent and one mutant gene. If so, they should be reactivable by 5-aza-CR. Sixteen out of twenty such isolates were shown to be reactivated by this procedure (Table 3; Figure 1G). At this point in the pathway, the cells should contain one inactive mutant gene, and one active gene. One of these isolates was treated with EMS, and again DAP^R colonies were obtained (Table 3; Figure 1H). The prediction is that these isolates have 2 mutant genes, which either do not complement each other, or complement weakly. Twenty colonies were picked and twelve were found to be leaky, that is, they grew on AAT medium. It is possible that this growth is due to interallelic complementation. The remaining eight colonies were found to be nonreactivable by 5-aza-CR (Table 3).

Thus, the results obtained are consistent with the pathways indicated in Figure 1. It should be noted that the mutation pathway (Figure 1, A and B) was previously documented (Chasin 1974; Jones and Sargent 1974). In those studies, the hemizygous strains were shown to have almost 50% of the initial APRT enzyme activity (100%) and the full mutant had no activity. We have examined the enzyme in the initial K1 strain (100% activity) and have shown that the doubly methylated strain (Figure 3C) has no activity. Hemizygous strains have up to 50% activity. (Note that reactivation to give growth on AAT does not require full gene expression.)

DISCUSSION

Many studies of DNA methylation state that there is a correlation between the methylation of cytosines in

TABLE 2

The distribution of nonmethylated and methylated CpG doublets in the promoter region of the APRT gene

Strain	No. of clones	59	74	80	113	180	210	233	238	243	253	264	275	287	292	320	336	No. sites methylated
D422	6	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	0
1A	6	○	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	14
1C	7	○	●	○	●	●	●	○	●	●	●	●	○	●	●	●	●	13
2B(i)	12	○	●	○	○	●	○	○	○	○	○	○	●	●	●	●	○	6
2B(ii)	1	○	○	○	○	●	○	○	○	○	○	○	●	●	●	○	●	5
3C	6	●	●	●	●	●	●	○	○	●	●	●	●	●	●	●	●	14
4B(i)	5	●	○	○	●	●	○	○	○	○	○	●	●	○	●	●	●	8
4B(ii)	3	●	●	●	●	●	●	○	●	●	●	●	●	●	●	●	●	15
4B(iii)	4	○	●	●	●	●	●	○	●	●	●	●	●	●	●	●	●	14
4B(iv)	2	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	16
4B(v)	1	○	●	●	●	●	○	○	●	●	●	●	●	●	●	●	●	14
4B(vi)	1	●	●	●	●	●	●	○	●	●	●	●	●	●	●	○	●	14
4B(vii)	2	○	●	●	●	●	●	○	●	●	●	●	●	●	●	●	●	14
4B(viii)	4	●	●	●	●	●	●	○	●	●	●	●	●	●	●	●	●	15
4B(ix)	1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	16
4B(x)	2	○	●	●	●	●	○	○	●	●	●	●	●	●	●	●	●	13
4B(xi)	2	○	●	●	●	●	●	○	●	●	●	●	●	●	●	●	●	14
4B(xii)	1	○	●	●	●	●	●	○	●	○	●	●	●	●	●	●	●	13
5B	3				●	●	●	●	○	●	○	●	●	●	●			9/11
6B(i)	12	○	○	○	○	●	●	○	○	●	●	●	●	●	●	●	●	10
6B(ii)	3	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	15
8C(i)	1					●	○	○	○	●	●	●	●	●	●	●	●	9/12
8C(ii)	9	●	●	●	●	●	○	●	●	○	●	○	●	●	●	●	●	13
8C(iii)	2	●	○	●	○	●	●	●	●	○	●	○	●	●	●	●	●	12
8C(iv)	1	○	○	●	○	●	●	●	●	○	●	○	●	●	●	●	●	11
8C(v)	1	●	●	●	○	●	●	●	●	○	●	○	●	●	●	●	●	13
8C(vi)	1	●	●	●	○	●	○	●	●	○	●	●	●	●	●	●	●	13

Open circles are nonmethylated sites, closed circles are methylated sites. The 16 CpG sites are a 323-base pair region of part of the CpG island of the gene. The numbers refer to the cloned fragment from strain D422 (Nalbantoglu et al. 1986). The transcription start sites are in the region 320–330 (Park and Taylor 1988). The DAP^R clones are 1A, 1C, 2B, 3C, 4B, 5B, 6B and 8C. The roman numerals refer to the different sequences identified among the cloned PCR products. (The sequencing of 5B and 8C(i) is incomplete.)

TABLE 3

Genetic and epigenetic changes in the APRT gene

Step	Treatment and result	No. of experiments	Frequency range (average)	5-aza-CR induced reactivation	
				No. tested	No. reactivated
A	Mutation to 50% enzyme activity }		—	—	—
	}	Chasin (1974) and Jones and Sargent (1974)			
B	Mutation: }				
	$APRT^+ \rightarrow APRT^-$ }		—	—	—
C	5-methyl dCTP (methylation) $APRT^+ \rightarrow APRT^-$	4	$1.63 - 5.66 \times 10^{-5}$ (3.71×10^{-5})	—	—
D	5-aza-CR (reactivation) $APRT^- \rightarrow APRT^+$	1	ND[a]	22	22
E	5-methyl dCTP (methylation) $APRT^+ \rightarrow APRT^-$	5	$2.92 \times 10^{-4} - 2.17 \times 10^{-3}$ (7.00×10^{-4})	—	—
F	EMS (mutation) $APRT^+ \rightarrow APRT^-$	3	$2.23 - 3.08 \times 10^{-3}$ (2.69×10^{-3})	—	—
G	5-aza-CR (reactivation) $APRT^- \rightarrow APRT^+$	1	ND[a]	20	16
H	EMS (mutation) $APRT^+ \rightarrow APRT^-$	3	$1.31 - 4.88 \times 10^{-3}$ (2.77×10^{-3})	8	0

For steps A–H see Figure 1.
[a] Not determined; reactivation is in the range 1–10%.

or near a gene and its lack of activity, and between the absence of methylation and gene activity. Where appropriate genetic analysis can be done, it is now reasonable to draw much stronger conclusions than this. The existence of epimutagens such as 5-methyl dCTP and 5-aza-CR makes it possible to silence and reactivate a given gene. There are similarities and differences between these procedures and the use of mutagens. The latter can inactivate a gene by forward mutation at reasonably high frequency and reactivate by back mutation, usually at very much lower frequency. Very successful genetic analysis was done over many decades using such mutations, without any molecular documentation. It was sufficient to have different heritable phenotypes, which could be characterized unambiguously. The same is now true of DNA methylation. Genes can be shut off or silenced by DNA methylation and activated by removing methylation. It is now reasonable to say that DNA methylation causes inactivation of genes, which results in a heritable phenotype (Holliday 1996). One does not say that a mutation is correlated with a phenotype, because in innumerable instances, it causes the phenotype, which was demonstrated by genetic analysis during the period when the molecular structure of the gene and its product were unknown.

Turning genes on and off by changing DNA methylation does not mean that the molecular basis for the heritable epigenetic effects is fully understood. In an earlier molecular study, using a different genomic sequencing technique, the CpG island and promoter region of the phosphoglucokinase gene in the inactive X chromosome and active *X* chromosome were examined (Pfeiffer *et al.* 1990). In the region studied, 35 CpG sites were methylated in the inactive *X* and all were unmethylated in the active *X*. In cells treated with 5-aza-CR, about half the CpG sites in the inactive *X* were unmethylated, but the gene remained inactive. In our experiments, we commonly found that a high proportion of CpG sites were methylated. Two of the 16 sites were in the 323-bp region of the CpG island of the silenced APRT gene were always methylated, but it is not known whether they are critical for turning off gene activity. A detailed follow-up study of 5-aza-CR treated cells, with or without APRT activity, should determine whether or not critical sites exist. The data which we have obtained so far favor the possibility that DNA methylation is a nonspecific effect, namely, that it is only necessary to have a given number of methylated cytosines in a given region to shut off transcription. This is in agreement with other evidence that density of methylation and proteins that bind to these sites prevent transcription (Boyes and Bird 1991, 1992; Hsieh 1994).

It is surprising that 5-methyl dCTP treatment produces such a high density of DNA methylation in the promoter region of APRT (Table 2). According to the results of Nyce (1991) with V79 cells, about 20% of cytosines are methylated after electroporation in the presence of 5-methyl dCTP, and most of these would be lost as they are not in CpG sites. However, he examined total DNA from a random population of cells. 5-methyl dCTP can silence APRT in a hemizygous strain at frequencies of about 10^{-4}, and obviously the most highly methylated strains will be selected in DAP medium. It seems likely that the small minority of cells, which are actually replicating the APRT gene, are the ones that will incorporate a high level of 5-methyl dCTP.

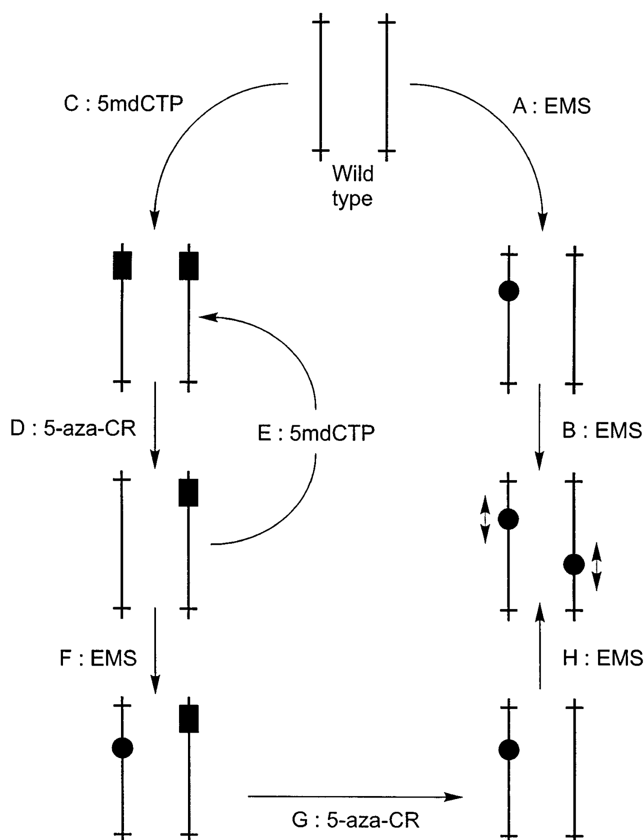

Figure 1.—Dual inheritance at the APRT locus in CHO K1 cells. The starting wild-type cell line has two active copies of the gene. Filled circles indicate mutations induced by standard mutagens, and filled rectangles the silencing of the gene by DNA methylation of CpG doublets in the promoter region (see Table 2). The events A and B are described in Chasin (1974) and Jones and Sargent (1974). The events C–H are documented in Table 3, and see text.

This also implies that the nucleotide has only a transient existence in the cell and could also explain why the treatment kills only about 30% of the cells. If a high level of 5-methyl dCTP was incorporated throughout the genome, presumably lethality would be very much higher than we observe. This interpretation explains why the frequency of APRT gene silencing in cells with one or two copies of the APRT$^+$ are not more different. If silencing was a totally random, cycle independent event, then the frequency of step C in Table 3 and Figure 1 should be the sequence root of the frequency of step E. The fact that this is not the case, supports the interpretation that a subset of the population, at a particular time during S phase, provides sensitive targets for 5-methyl dCTP treatment.

Nevertheless, it is likely that the initial methylation produced by incorporation of 5-methyl dCTP is much less than is seen in the molecular analysis. This methylation may lead to a secondary spreading of methylation throughout the promoter region In particular, it may be that the initial methylation of the binding sites for the ubiquitous transcription factor SP1 (Kadonaga *et al.* 1986) in the region leads to such spreading. It has been shown that mutation, or deletion, of Sp1 sites in the APRT gene of mouse or hamster cells results in *de novo* methylation of the promoter region (Brandeis *et al.* 1994; Macleod *et al.* 1994). It is therefore possible that the initial methylation of such sites also leads to a secondary methylation of other CpG sites. Spreading of initial methylation has been seen in integrated adenovirus DNA, but over a much longer time scale (many passages) than our experiments (Toth *et al.* 1989, 1990; Orend *et al.* 1991).

The dual inheritance we have demonstrated at the TK and APRT loci may be a unique feature of transformed cells. Even though these cells can maintain a given pattern of methylation during normal growth (Holliday 1987, 1993), they can also acquire *de novo* methylation and thereby shut off the activity of dispensable genes. It is probable that they also lose methylation at a given rate (Pfeifer *et al.* 1990); the overall result is to maintain the constant overall level that is seen in established cell lines. Normal diploid cells behave very differently. They do not appear to have *de novo* DNA methylation that can shut off gene activity, but they do lose overall methylation during the course of their *in vitro* lifespan (Wilson and Jones 1983). It has been recently shown that 5-methyl dCTP can be used to inactivate the HPRT gene on the *X* chromosome in normal human fibroblasts, but at a significantly lower frequency than in CHO cells (Holliday and Ho 1995).

The importance of studies of dual inheritance in transformed cell lines is its obvious relevance to tumor progression *in vivo.* Much of the attention in this field has focused on the existence of gene mutations in oncogenes or tumor suppressor genes. Several recent studies have demonstrated the importance of DNA methylation events in the silencing of tumor suppressor genes, such as Rb and p16 (Sakai *et al.* 1991; Ohtani-Fujita *et al.* 1993; Merlo *et al.* 1995; Stirzaker *et al.* 1997). It is not yet clear whether these are primary or secondary events during oncogenesis. Also, the exact mechanism of *de novo* methylation of such genes is far from clear. Much can be learned about changes in methylation in relation to gene activity in well characterized established cell lines such as CHO cells.

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