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Aberrantly Silenced Promoters Retain a Persistent Memory of the Silenced State After Long-Term Reactivation

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Abstract

A hallmark of aberrant DNA methylation-associated silencing is reversibility. However, long-term stability of reactivated promoters has not been explored. To examine this issue, spontaneous reactivant clones were isolated from mouse embryonal carcinoma cells bearing aberrantly silenced *Aprt* alleles and re-silencing frequencies were determined as long as three months after reactivation occurred. Despite continuous selection for expression of the reactivated *Aprt* alleles, exceptionally high spontaneous re-silencing frequencies were observed. A DNA methylation analysis demonstrated retention of sporadic methylation of CpG sites in a protected region of the *Aprt* promoter in many reactivant alleles suggesting a role for these methylated sites in the re-silencing process. In contrast, a chromatin immunoprecipitation (ChIP) analysis for methyl-H3K4, acetyl-H3K9, and dimethyl-H3K9 levels failed to reveal a specific histone modification that could explain high frequency re-silencing. These results demonstrate that aberrantly silenced and reactivated promoters retain a persistent memory of having undergone the silencing process and suggest the failure to eliminate all CpG methylation as a potential contributing mechanism.

Keywords

gene silencing; DNA methylation; chromatin; gene reactivation

1. Introduction

The epigenetic modification most commonly associated with transcriptional silencing is DNA methylation. This modification is present at most non-transcribed regions of the eukaryotic genome including the inactive X chromosome, repetitive elements, imprinted regions, and heterochromatin at centromeres and telomeres [1]. In addition to these examples of normal transcriptional silencing, DNA methylation is consistently observed at aberrantly silenced tumor suppressor gene promoters in a diverse array of human tumors and cancer-derived cell lines [2–4]. The cause(s) of aberrant DNA methylation at silenced

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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promoters is not known. One hypothesis is that promoter regions are normally in equilibrium between the spreading of methylation into the promoter and protection of the promoter from this spreading. A boundary would separate these regions and perturbation of the equilibrium could lead to methylation-associated silencing [5]. According to this hypothesis, stochastic spreading of methylation to CpG sites past the boundary creates growing foci of DNA methylation that ultimately result in silencing. A similar hypothesis predicts that random accumulation of methylated CpGs in a promoter region can act as a seed for further accumulation leading to silencing [6]. Alternatively, though not exclusively, promoter silencing associated with histone modifications could provide the conditions that lead to the seeding and spreading of methylation in the promoter region [7,8].

Reversal of DNA methylation-associated silencing is a potential therapeutic approach for cancer [4,9,10] because reactivation of silenced tumor suppressors is commonly observed in cancer cell lines after treatment with the DNA methylation inhibitor 5-deoxyazacytidine (5-aza-dC) [11]. However, induced reactivation is unstable, at least in the short term, because reactivated promoters re-silence rapidly without continued DNA methyltransferase inhibition [12,13]. Repressive histone modifications that remain after inhibition of DNA methylation and/or lack of restoration of active histone marks may also contribute to high frequency re-silencing of activated alleles [14–16], again as seen in short-term experiments.

An open question is whether long-term expression of a reactivated promoter can lead to stable reactivation. To address this question, reactivant subclones were isolated from embryonal carcinoma cells with silenced endogenous mouse *Aprt* alleles and maintained for up to three months under conditions that required *Aprt* expression for survival. The results demonstrated that reactivated alleles retained a susceptibility to undergo re-silencing at very high frequencies despite long-term growth under conditions that required maintenance of promoter expression. Additional work suggested retention of CpG methylation within a normally methylation-free region as a potential mechanism for persistent instability of reactivated *Aprt* alleles.

2. Materials and Methods

2.1 Cell Culture

The mouse embryonal carcinoma cells were cultured in DMEM medium supplemented with 5% fetal bovine serum and 5% Serum Plus (Biosciences, Lexana, KS). The parental P19H22 cell line contains a single expressed *Aprt* allele derived from the C3H mouse strain [17]. The D3 and D3S1 clones were maintained in the presence of 80 μg/ml 2,6-diaminopurine (DAP) (Sigma, St. Louis, MO). The D3 cells were isolated as a spontaneous DAP resistant clone from P19H22 [18]. The D3S1 cells were isolated as a subclone of the D3 cells. Reactivant D3 and D3S1 subclones were selected and maintained in medium containing 10 μg/ml azaserine and 10 μg/ml adenine (Sigma) (AzA medium), which requires *Aprt* expression for cell survival.

2.2 Reactivation and Re-silencing Cell Cloning Assays

To measure *Aprt* reactivation, D3 or D3S1 cells were plated into 100 mm culture plates at densities ranging from 1×10^3 to 1×10^5 cells per plate. The next day the cells were exposed to AzA medium to select for active *Aprt* reactivants. The same protocol was used to measure *Aprt* re-silencing for reactivated subclones, but the medium contained 80 μg/ml DAP to select for cells that had lost *Aprt* expression. Cells were cultured for 10 days in the appropriate selective media before staining live colonies with crystal violet solution. To estimate cloning efficiencies, additional cells were plated under identical conditions as selective plates but at lower densities, 250 to 1000 cells per plate, without selection.

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Silencing or reactivation frequencies were calculated by dividing the number of clones growing under selection by the effective number of cells plated (as determined with the cloning efficiency plates).

2.3 Drug Treatments

Cells were treated overnight (~16 hours) with media containing 300 nM trichostatin A (TSA) (Wako, Richmond, VA) to inhibit histone deacetylation or 3 μM 5-aza-dC (Sigma) to inhibit DNA methylation. Cells were allowed to recover 24 hours in DMEM after drug treatment before harvesting RNA.

2.4 Bisulfite Sequencing Analysis

Bisulfite sequencing of CpGs between −470 and +17 was performed as follows. Genomic DNA was isolated from cell cultures using DNAzol (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. For each treatment, 2 – 4 μg of genomic DNA was digested by restriction enzyme BsrI. Digested genomic DNA was modified in a solution of 6.24 M urea, 4 M sodium bisulfite, and 10 mM hydroquinone as described previously [19]. PCR amplification of modified DNA, cloning of PCR products, and sequence analysis were also described elsewhere [19], with the following exceptions. The primers used in the initial PCR reaction were the sense primer H2+S 5′-GAG GAG GGT ATA TTT TGT TGT AAT G-3′ and the antisense primer ACA+29 5′-AAA AAC AAA AAA AAA ATA AAT ATC AAC AC-3′. PCR product from this initial reaction was used as input in a second reaction with the nested sense primer H2+NS23 5′-AGT GTT TGT GGT TTT AGA GAA GG-3′ and the antisense primer ACA+29. PCR products were cloned using Strataclone PCR cloning kit (Stratagene). Sequence analysis showed all cytosine bases not present in the CpG dinucleotide context were converted to thymine indicating complete bisulfite modification of the genomic template occurred.

2.5 RNA Preparation and Analysis

Total RNA was isolated from cell cultures with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Total RNA samples were converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen) with removal of genomic DNA contamination. 100 ng cDNA was used as input in subsequent quantitative-PCR analysis for either *Aprt* amplification across the exon 2–3 splice site with the sense primer qAprt-F 5′-CTC TTG GCC AGT CAC CTG AAG-3′, the antisense primer qAprt-R 5′-TCT AGA CCT GCG ATG TAG TCG ATC T-3′ and the TaqMan probe 5′-FAM-CAC GCA CAG CGG C-MGB-3′ or *Gapdh* (Mouse TaqMan Endogenous Control, Applied Biosystems, Foster City, CA) with iQ Supermix (Hercules, CA) and a Bio-Rad iCycler. *Aprt* results were normalized in relation to *Gapdh* mRNA levels and displayed relative to expression levels in P19H22 cells.

2.6 Chromatin Immunoprecipitation

ChIP assays were carried out as described previously [7]. Protein-DNA complexes were immunoprecipitated with antibodies to acetyl-H3K9 (07–352, Millipore, Billerica, MA), mono/di/trimethyl-H3K4 (05–791, Millipore), dimethyl-H3K9 (ab1220, Abcam, Cambridge, MA), and trimethyl-H3K27 (17–622, Millipore). Quantitative PCR using an Icycler and iQ Supermix (Bio-Rad) was used to analyze the immunoprecipitated DNA. The *Aprt* promoter was amplified and detected using the sense primer 5′-AAC GTA TGT CGA GGT AGG CGT GTA- 3′, the antisense primer 5′-ATC TCC TTC ATC ACA TCT CGA G-3′, and the TaqMan probe 5′-FAM-TAC CTC CTC CCT GCC TCC TAC A-3′. The active *Gapdh* promoter was amplified using the sense primer 5′-TTG AGC TAG GAC TGG ATA AGC AGG-3′, the antisense primer 5′-AAG AAG ATG CGG CCG TCT CTG GAA-3′, and the

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TaqMan probe 5′-FAM-TAT AAA TAC GGA CTG CAG CCC TCC CT-3′. The silenced *Mage-a* promoter was amplified using the sense primer 5′-GTT CTA GTG TCC ATA TTG GTG-3′ and the antisense 5′-AAC TGG CAC AGC ATG GAG AC-3′, and amplification and quantitation was done using iQ SYBR Green Supermix (Bio-Rad). The specific signal from each immunoprecipitation relative to signal from input was calculated for the three promoters, *Aprt*, *Gapdh*, and *Mage*. For activating modifications, levels at *Aprt* are displayed relative to the *Gapdh* promoter; for the repressive modification, dimethyl-K9 H3, results are displayed relative to the *Mage* promoter.

3. Results

3.1 A boundary to DNA methylation is present at the expressed Aprt promoter in mouse embryonal carcinoma cells

The mouse P19H22 embryonal carcinoma cell line contains a single expressed *Aprt* allele due to spontaneous deletion of the other allele [18]. To obtain a detailed picture of the methylation pattern for this region, bisulfite sequencing was used to measure methylation status at 22 CpG sites across a 487 bp region that extends from −470 to +17, relative to the major transcriptional start site. A high level of DNA methylation (50 – 100%%) was present in the upstream region from −470 to −279 at all but one CpG site (Figure 1). The exception was a CpG site within a HpaII site (H2, Figure 1) that a prior Southern blot analysis showed was methylated at a low level (~ 10%) [17]. The bisulfite sequence analysis revealed that the level of methylation at this site was atypical, being significantly lower than surrounding CpG sites. Only rare CpG methylation (1% of all CpG sites examined) was present in the region from −222 to +17 that includes Sp1 binding sites and the transcriptional start site(s). This observation demonstrates a boundary to DNA methylation between positions −279 and −222 that presumably protects the *Aprt* promoter region [5].

3.2 DNA methylation at silenced Aprt alleles breaches the boundary

Spontaneous reactivation frequencies were examined for two clonal cell lines containing silenced *Aprt* alleles. The D3 clone exhibited an exceptionally high spontaneous reversion frequency of 1.9×10^{-1} and the D3S1 clone exhibited a 40-fold lower spontaneous reversion frequency of 4.7×10^{-3}. Relative to expression in P19H22, *Aprt* mRNA levels were reduced to 5% and 1% in the silenced D3 and D3S1 clones, respectively (Figure 2). A bisulfite sequence analysis for the 22 CpG sites from positions −470 through +17 revealed that the methylation boundary was breached and the promoter region methylated extensively in both the D3 and D3S1 cells (Figure 1, Table 1). Essentially complete CpG methylation was observed in the D3 cells until position −79, with sporadic methylation at and after that site. CpG methylation for the D3S1 cells was essentially complete for all sites examined suggesting that increased DNA methylation was responsible for decreased expression and a lower reactivation frequency. High-level methylation of the H2 CpG site was observed in the D3 and D3S1 clones. Inhibiting DNA methylation by 5-aza-dC treatment led to significant reactivation of *Aprt* expression in D3 and D3S1 (approximately 20 to 30% of P19H22) (Figure 2), consistent with DNA methylation associated silencing. Inhibition of histone deacetylase with TSA treatment had only a limited effect (Figure 2), also consistent with a dominant role for DNA methylation in maintaining the silenced state.

3.3 Histone modification changes associated with silencing

Chromatin immunoprecipitation (ChIP) analysis was performed using antibodies against activating modifications, acetylation at H3K9 and methylation at H3K4, and a repressive modification, methylation at H3K9. Histone modifications measured at the *Aprt* promoter in the P19H22 cells were consistent with active expression, i.e., high levels of acetyl-H3K9 (Figure 3A) and methyl-H3K4 (Figure 3B) and low levels of dimethyl-H3K9 (Figure 3C).

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Consistent with epigenetic silencing, the D3 and D3S1 clones exhibited reduced levels of both the acetyl-H3K9 and methy-H3K4 modifications (Figures 3A and 3B). The more stably silenced D3S1 cells had an approximately two-fold higher level of the repressive histone modification dimethyl-H3K9 at the *Aprt* promoter than the level observed in the P19H22 parental cells (Figure 3C). The level of dimethyl-H3K9 for the *Aprt* promoter in the less stably silenced D3 cells was only marginally greater than that for the P19H22 cells.

3.4 Re-silencing frequencies in reactivant subclones after long-term maintenance of Aprt expression

The above results demonstrated epigenetic silencing of *Aprt* in the D3 and D3S1 clones. Moreover, the levels of DNA methylation and extent of silencing appeared linked because the lower level of promoter region DNA methylation in the D3 clone was associated with a higher spontaneous reversion frequency and vice versa for the D3S1 clone. The ease of isolating spontaneous reactivants from both clones and to maintain *Aprt* expression with continued AzA selection allowed us to determine if reactivated alleles would stabilize, as defined by restoration of silencing frequencies comparable to that for silencing of the naïve *Aprt* allele. (“Naive” allele is defined as an allele is that has never been silenced.) The silencing frequency for the naïve *Aprt* allele in the parental P19H22 cells has been calculated between 10^{-5} to 10^{-6} [17,18].

Aprt re-silencing frequencies were determined for D3 and D3S1 reactivant subclones at one and three months after initiation of selection in AzA medium. At the one-month time point the re-silencing frequencies were remarkably high, on average 18% (18×10^{-2}) for D3 reactivants and 2% (2×10^{-2}) for D3S1 reactivants (Table 1). The difference between the D3 and D3S1 reactivants was statistically significant ($p < 0.001$). The re-silencing frequencies remained high at the three-month time point, decreasing by only 50% from one month to three months. These decreases were not statistically significant ($p = 0.188$ and 0.125 for D3 and D3S1 reactivants, respectively). Thus, the reactivated *Aprt* alleles retained a long-term memory of their previously silenced state, as demonstrated by the exceptionally high re-silencing frequencies despite continuous selection to maintain expression.

3.5 DNA methylation in reactivant subclones

Consistent with *Aprt* reactivation, substantial restoration of *Aprt* mRNA levels was observed in reactivant clones, though in most cases examined the mRNA levels in the D3 reactivants did not reach the levels observed in the P19H22 cells. The *Aprt* levels in the D3S1 reactivants were as high or higher than the levels observed in P19H22 (Figure 4).

Promoter region methylation was examined in detail for one reactivant subclone (D3A5) at the one-month time point by determining the methylation profile for 20 individual alleles (Figure 5). This analysis revealed that restoration of *Aprt* expression was in general associated with loss of promoter region methylation, as would be expected. However, a significant fraction of expressed alleles retained DNA methylation past the boundary. This observation demonstrated that *Aprt* reactivation in D3 cells did not require complete loss of promoter region DNA methylation and that the retained CpG methylation in this region could persist over multiple cell generations (~ 30) despite continuous selective pressure for expression.

A determination of consensus profiles of promoter region DNA methylation for D3 and D3S1 reactivants at different time points extended the observation for the D3A5 reactivants at one month. Promoter region DNA methylation was examined for a number of reactivant subclones growing in AzA medium at two weeks, one month, and/or three months after initiation of selection. With the exception of the D3A5 subclone (see above), 3 to 7 alleles

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were sampled for each reactivant subclone. The data from all reactivant subclones for a given time point were pooled and are presented in Table 2. Over 66% of the alleles from D3 reactivant alleles isolated at the two-week time point exhibited at least one methylated CpG site past the boundary and 37% of all CpG sites in this protected region were methylated. This latter percentage was close to the level of 51% observed for the silenced *Aprt* allele in the D3 clone, which further demonstrates that initial *Aprt* reactivation did not require substantial loss of DNA methylation. The average level of CpG methylation past the boundary dropped by the one month time point in the D3 reactivants to 24% methylation and further still to a level of 9% at the three month time point (Table 2), illustrating that loss of CpG methylation in the promoter region was a gradual process. Nonetheless, even at the three-month time point a majority of reactivant alleles (60%) exhibited methylation of at least one CpG site past the protected boundary. Thus, high frequency re-silencing for D3 reactivant clones (Table 1) correlated with significant retention of DNA methylation past the protected boundary (Table 2).

The levels of DNA methylation were significantly lower in the D3S1 reactivants at all time points than in the D3 reactivants, which is consistent with the nine-fold lower re-silencing frequencies. The DNA methylation levels in the D3S1 reactivants, however, were on average higher than the levels for the naïve *Aprt* alleles in the parental P19H22 cells (Table 2), though the results at the two-week time point demonstrated very rapid demethylation, as compared with the D3 reactivants.

3.6 Histone modifications in reactivant subclones

The above results demonstrate residual DNA methylation in the promoter region, which could account for high frequency re-silencing in the reactivant subclones. To determine if the failure to restore activating histone modifications could also play a role, the levels of acetyl-H3K9 and methyl-H3K4 were measured at the *Aprt* promoter in reactivants at the 1 and/or 3 month time points and compared to those in parental P19H22 cells (representative examples in Figure 6). Levels of the repressive dimethyl-H3K9 modification were also examined. In general, the results showed restoration of the activating modifications in the reactivants, though the absolute levels were highly variable including within a clone over time. The levels of the repressive dimethyl-H3K9 modification decreased, but were also variable. Overall, these results failed to reveal a specific histone mark that could account for high frequency re-silencing in the D3 and D3S1 reactivant subclones.

4. Discussion

Epigenetic reactivation is a potential therapeutic approach for cancer treatment because it could restore critical functions necessary to suppress tumor cell growth [4,9]. While the issue of stabilization of reactivated promoters has been addressed in a few reports, the amount of time between induction of expression and analysis was relatively brief (a few days) and selection for maintenance of expression was not used [14,15]. Therefore, those studies could not reveal whether expression of reactivated alleles would stabilize if given sufficient time. Here we examined the issue of epigenetic stabilization by using continuous selective pressure to maintain expression for up to three months for spontaneously reactivated mouse *Aprt* alleles. Despite maintenance of long-term expression, a significant fraction of the reactivated alleles did not achieve stable reactivation, as demonstrated by high frequency re-silencing at one and three months time points. Thus, long-term maintenance of expression was insufficient to stabilize expression of reactivated *Aprt* alleles.

The extent of silencing was different between the D3 and D3S1 clones because the promoter region of the D3S1 clone was essentially fully methylated and its spontaneous reactivation

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frequencies were 40-fold lower. Methylation of the *Aprt* promoter was only partial in the D3 clone. Partial and complete methylation of the human *MLH1* promoter has been reported including normal colinic mucosa in colon cancer patients [20], which demonstrates that partial or complete promoter methylation can be alternative states, as we observed for silenced mouse *Aprt*. A reasonable prediction would be that reactivant subclones from both the D3 and D3S1 clones would behave similarly once reactivation occurred, but this outcome was not observed. Instead, the D3 and D3S1 reactivants differed in two significant ways. The first was that the D3 reactivants exhibited nearly ten-fold higher re-silencing frequencies and the second was that the D3 reactivants retained higher numbers of *Aprt* alleles exhibiting promoter region DNA methylation. Considered together, these results suggest strongly that retention of promoter region DNA methylation is a significant determinant for re-silencing, which is consistent with a model we previously offered to explain the initiation and progression of aberrant DNA methylation-associated silencing [5]. That model envisioned silencing as a process by which the presence of DNA methylation past a protective boundary would lead to decreased transcription, which in turn would allow the spread of more methylated CpG sites in the promoter and eventually extinguishment of expression (i.e., silencing).

The observation that the D3S1 reactivant subclones exhibited lower promoter region DNA methylation than the D3 reactivant subclones suggests that reactivation of the fully methylated promoter may require complete (or nearly complete) loss of DNA methylation, whereas the less heavily methylated promoter in the D3 cells appears capable of reactivation without a significant loss of DNA methylation. In other words, the more heavily silenced a promoter, the greater the apparent requirement for significant demethylation to restore expression. The presence of *Aprt* alleles in D3 reactivant subclones with promoter region DNA methylation at levels similar to those for silenced alleles is consistent with initial reactivation of D3 alleles occurring without significant loss of DNA methylation. This outcome would have been missed if 5-aza-dC has been used to induce reactivation. Constant selective pressure for expression led to the gradual loss of promoter region methylation suggesting a cause and effect relationship that played out over time in the D3 reactivants.

The silenced *Aprt* alleles in the D3 and D3S1 cells had the expected changes in histone modifications (i.e., reductions in methyl-H3K4 and acetyl-H3K9 and increased levels of dimethyl-H3K9), and the expected reversal of these modifications (e.g., reacquisition of acetyl-H3K9) was observed in the reactivants. This observation sets our results apart from reactivation studies in which repressive modifications were maintained in reactivants [14–16], though as noted above in those studies chromatin was examined only a few days after reactivation had been induced by treatment with 5-aza-dC. In contrast, histone modifications were examined in our study at one and three months after continuous selection for promoter expression. These results coupled with the re-silencing frequencies suggest that the reacquired histone modifications in the reactivants were insufficient to stabilize some reactivant alleles. A caveat for interpreting the histone modification results, however, is that the ChIP analysis could only reveal the average modification level for each histone mark examined. In contrast the DNA methylation analysis allowed us to observe individual alleles and individual CpG sites within these alleles.

Finally*, Aprt* is not a tumor suppressor gene, but its promoter region includes elements commonly found in and near tumor suppressor promoters [21–26]. These elements include heavily methylated repetitive elements within 1 kb upstream of the promoter [19,27] and Sp1 binding sites that apparently create a boundary to protect the promoter from DNA methylation [28,29]. Thus, the results reported here should be applicable to a wide range of tumor suppressor promoters.

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5. Conclusion

The results demonstrate that epigenetically silenced and then reactivated promoters are not equivalent to a naïve version that has never undergone silencing. The salient difference is that the reactivated promoters exhibit exceptionally high frequencies of re-silencing despite being retained under conditions that require promoter expression for cell survival. Retention of residual promoter region DNA methylation may account for high frequency silencing, though instability of histone modifications as a contributing factor cannot be ruled out at this time. Methods to stabilize reactivated alleles are needed because stable expression of reactivated promoters represents an important requirement for epigenetic cancer therapies. Our system may be useful for identifying these methods.

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Figure 1. DNA methylation profiles for *Aprt* promoter region in P19H22, D3, and D3S1 cells
Average DNA methylation levels at 22 CpG sites extending from position −470 to position +17, relative to the major transcriptional start site. The locations of CpG sites within a HpaII site (H2) and three Sp1 bindings are shown. An apparent, though imperfect, boundary to the spread of methylation is shown as a red box.

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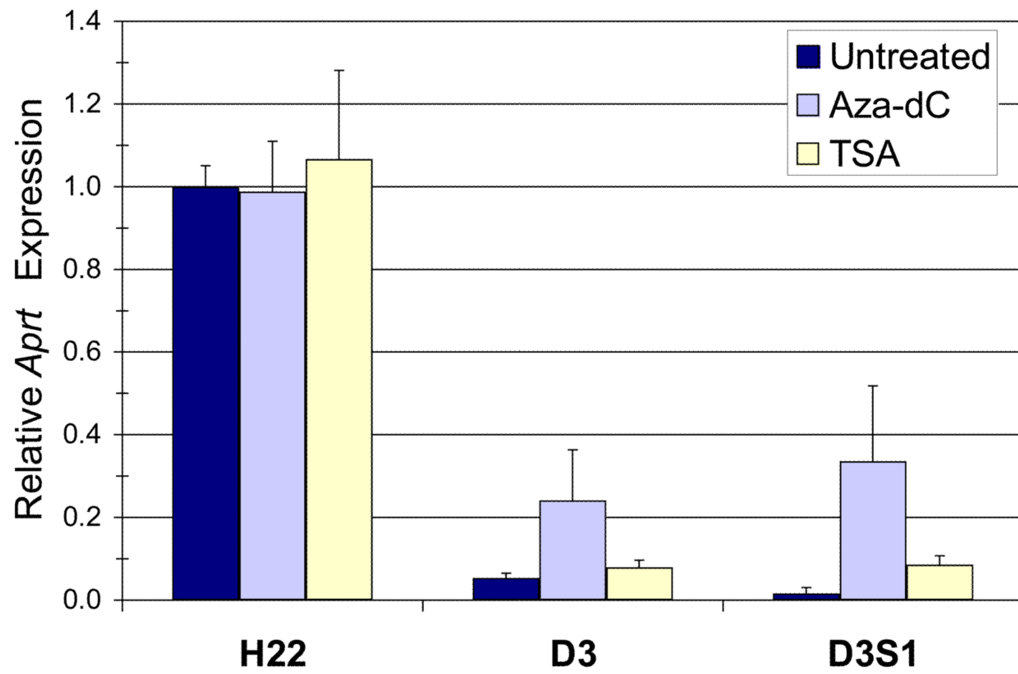

Figure 2. *Aprt* mRNA levels and effects of drug treatments

The relative levels of *Aprt* mRNA were determined with qRT-PCR for untreated P19H22 (H22), D3, and D3S1 cells and after overnight treatment with 3 μM 5-aza-dC (Aza-dC) or 300 nM trichostatin A (TSA) and a 24 hour recovery period.

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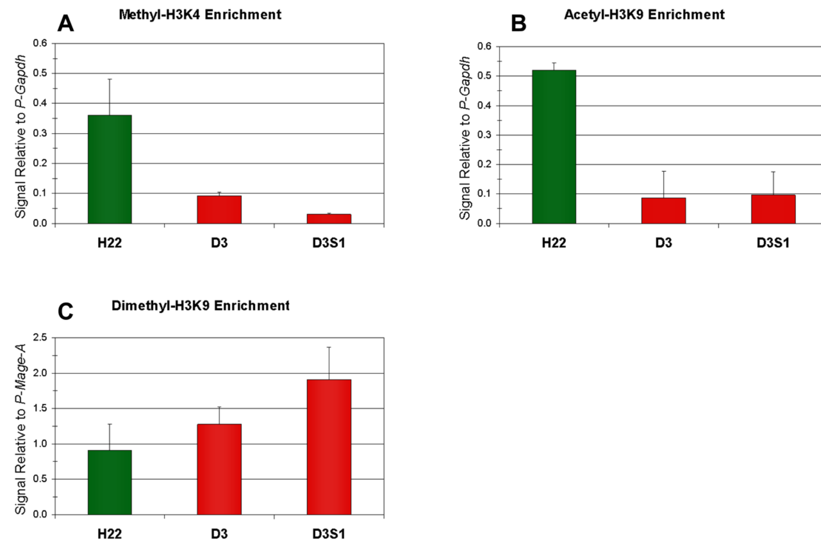

Figure 3. Chromatin immunoprecipitation (ChIP) assay for *Aprt* promoter region in P19H22, D3, and D3S1 cells

Relative levels of the methyl-H3K4 (**A**), acetyl-H3K9 (**B**) and dimethyl-H3K9 (**C**) were determined with the ChIP assay.

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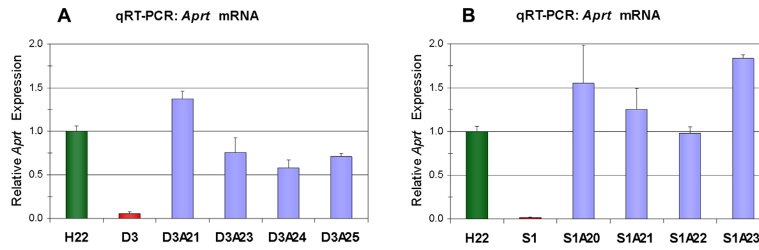

Figure 4. *Aprt* mRNA levels in reactivant clones

Aprt mRNA levels were determined for reactivant clones isolated from the D3 cells **(A)** and D3S1 cells **(B)** and compared with levels in the P19H22 cells.

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D3A5

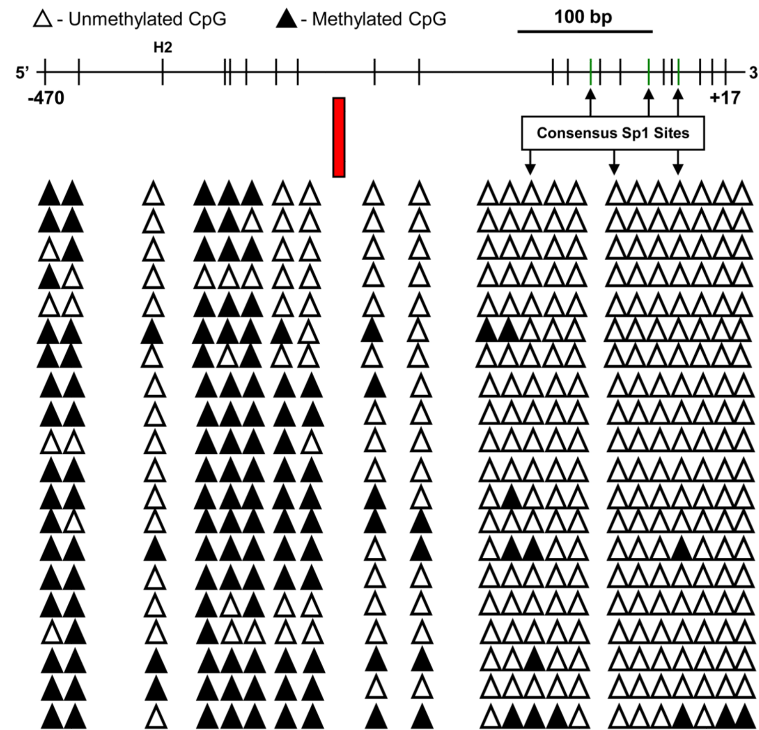

Figure 5. DNA methylation profile in a D3 reactivant clones at one month after selection began
Bisulfite sequence analysis is shown for D3A5 reactivant clone one month after initiation of selection for reactivant cells with AzA medium. Open triangles indicate unmethylated CpG sites and closed triangles indicate methylated CpG sites. The boundary to the spread of DNA methylation for expressed *Aprt* alleles in the P19H22 cells is shown with a red box. Sp1 indicates CpG sites within consensus Sp1 binding sites. H2 is a HpaII site (see text).

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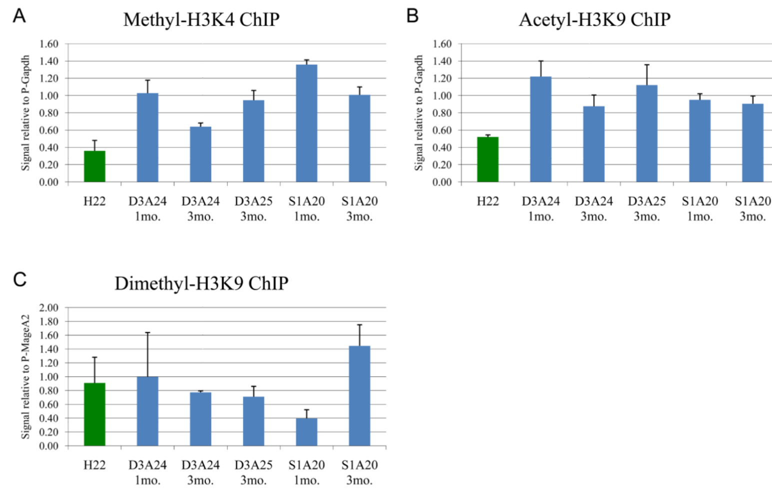

Figure 6. Chromatin immunoprecipitation (ChIP) assay for *Aprt* promoter region in reactivant clones

Results from ChIP assay are shown for representative reactivant subclones isolated from the D3 and D3S1 clones. Results are shown for the aectyl-H3K9 (**A**), methyl-H3K4 **(B)**, and dimethyl-H3K9 histone modifications **(C)**.

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Table 1

Re-silencing frequencies for reactivant clones[a].

D3 reactivant re-silencing frequencies		
Clone	**one month**	**three months**
D3A1	4.7×10^{-2}	
D3A4	5.2×10^{-2}	
D3A5	26.8×10^{-2}	
D3A20	6.7×10^{-2}	6.5×10^{-2}
D3A21	21.7×10^{-2}	3.7×10^{-2}
D3A23	13.2×10^{-2}	14.2×10^{-2}
D3A24	47.6×10^{-2}	16.0×10^{-2}
D3A25	19.8×10^{-2}	6.0×10^{-2}
Average	18.2×10^{-2}	9.3×10^{-2}
D3S1 reactivant re-silencing frequencies		
Clone	one month	three months
D3S1A1	2.0×10^{-2}	
D3S1A3	1.1×10^{-2}	
D3S1A4	1.4×10^{-2}	
D3S1A20	1.9×10^{-2}	0.7×10^{-2}
D3S1A21	1.5×10^{-2}	0.1×10^{-2}
D3S1A22	3.7×10^{-2}	1.5×10^{-2}
D3S1A23	2.2×10^{-2}	1.6×10^{-2}
Average	2.0×10^{-2}	1.0×10^{-2}

[a]Reactivant clones were selected and maintained in medium containing azaserine and adenine for the amount of time shown and then were selected for silencing frequencies in medium containing 2′ 6-diaminopurine.

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Table 2

DNA methylation levels at expressed, silenced, and reactivated *Aprt* alleles[a].

	Methylated alleles[b]	Methylated CpG sites[c]
P19H22	14% (2/14)	1% (2/196)
D3	100% (14/14)	51% (100/196)
D3S1	100% (14/14)	98% (192/196)
D3 Reactivants		
2 weeks (N = 5)	69% (18/26)	37% (133/364)
one month (N = 9)	47% (37/71)	24% (234/994)
three months (N = 3)	53% (9/17)	7% (17/238)
D3S1 Reactivants		
2 weeks (N = 4)	6% (1/16)	1% (2/224)
one month (N = 5)	33% (9/27)	6% (22/378)
three months (N = 3)	21% (4/19)	2% (5/266)

[a]P19H22 contains a single and naïve (with respect to silencing) *Aprt* allele; D3 and D3S1 contained silenced *Aprt* alleles; D3 and D3S1 (two weeks, one month, and three months) represent pooled data from reactivant clones at 2 weeks, one month, and three months after initiation of selection in medium containing azaserine and adenine.

[b]The percentage of alleles with at least one methylated CpG site past the protective boundary.

[c]The percentage of CpG sites past the methylation boundary that were methylated.