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## Genome-Wide Demethylation Promotes Triplet Repeat Instability Independently of Homologous Recombination

Vincent Dion<sup>1</sup>, Yunfu Lin<sup>1</sup>, Brandee A. Price<sup>1,2</sup>, Sharyl L. Fyffe<sup>2</sup>, Andrei Seluanov<sup>3</sup>, Vera Gorbunova<sup>3</sup>, and John H. Wilson<sup>1,2</sup>

<sup>1</sup>Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030, USA

<sup>2</sup>Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030, USA

<sup>3</sup>Department of Biology, University of Rochester, Rochester, New York 14627, USA

### Abstract

Trinucleotide repeat instability is intrinsic to a family of human neurodegenerative diseases. The mechanism leading to repeat length variation is unclear. We previously showed that treatment with the demethylating agent 5-aza-2'-deoxycytidine (5-aza-CdR) dramatically increases triplet repeat instability in mammalian cells. Based on previous reports that demethylation increases homologous recombination (HR), and our own observations that HR destabilizes triplet repeats, we hypothesized that demethylation alters repeat stability by stimulating HR. Here, we test that hypothesis at the *Aprt* (adenosine phosphoribosyl transferase) locus in CHO cells, where CpG demethylation and HR have both been shown to increase CAG repeat instability. We find that the rate of HR at the *Aprt* locus is not altered by demethylation. The spectrum of recombinants, however, was shifted from the usual 6:1 ratio of conversions to crossovers to more equal proportions in 5-aza-CdR-treated cells. The subtle influences of demethylation on HR at the *Aprt* locus are not sufficient to account for its dramatic effects on repeat instability. We conclude that 5-aza-CdR promotes triplet repeat instability independently of HR.

### Keywords

Recombination; APRT; DNA methylation; genome stability; trinucleotide repeats

### INTRODUCTION

Expanded tracts of trinucleotide repeats cause at least 18 human diseases [1,2]. These repeat tracts are unstable and can further expand both in the germline, which causes more severe forms of disease in subsequent generations, and in somatic tissues, which probably contributes to disease progression and pathogenesis in the affected patient[1]. Defining the molecular mechanisms responsible for triplet repeat instability will be critical for development of therapies designed to restrict expansion or promote contraction. Studies in a variety of model organisms have shown that virtually any process that exposes single-stranded DNA—including DNA repair, replication, recombination, and transcription—allows triplet repeats to adopt

Address correspondence to: John H. Wilson, Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-5760. Fax: (713) 796-9438. Email: jwilson@bcm.tmc.edu.

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aberrant secondary structures, which are often misprocessed, changing the length of the repeat [3].

We recently showed that genome-wide demethylation, induced by treatment with 5-aza-2'-deoxycytidine (5-aza-CdR), destabilized CAG triplet repeats in patient-derived cells, causing expansions and contractions [4]. Using a selectable assay for CAG repeat contractions at the *Aprt* (adenosine phosphoribosyl transferase) locus in CHO cells, we found that treatment with 5-aza-CdR, which leads to passive depletion of DNA methylation [5] and destruction of DNMT1 [6], or with hydralazine, which induces demethylation by a different pathway [7], increased repeat contractions by about 1000 and 50 fold, respectively [4]. Stimulation of repeat contraction by two mechanistically distinct agents, in rough proportion to their ability to decrease genome-wide methylation, argues for a causal link between CpG demethylation and CAG repeat instability [4]. In a separate investigation into the interactions between CAG repeats and homologous recombination (HR), we showed that HR significantly altered repeat stability at the *Aprt* locus in CHO cells [8]. Among homologous recombinants, 5% had lost large numbers of repeats, whereas no such changes were observed in more than 250 colonies that had not undergone HR [8]. These repeat contractions were evenly distributed between the two types of HR event—crossover and conversion—detected in our assay [8]. These two studies, coupled with the observations summarized below, led us to propose that 5-aza-CdR treatment destabilizes CAG repeats by stimulating HR [8]. Here, we test that hypothesis.

It has been suggested that a major role of DNA methylation—the addition of a methyl group to cytosine within CpG dinucleotides—is to inhibit HR between repeated sequences [9-11]. Support for this idea is strongest in fungi where crossovers between direct repeats during meiosis are inhibited more than 100-fold by CpG methylation [12]. In mammalian cells, the links between DNA methylation and HR are less direct, and in some cases, contradictory. Treatment with 5-aza-CdR, which induces demethylation, has been shown to stimulate sister-chromatid exchange [5], increase HR within a transgene array [13], and promotes double-minute chromosome dimerization [14]. In mouse ES cells, knockout of DNA methyltransferase 1 (DNMT1), the maintenance DNA methyltransferase, reduces CpG methylation and increases loss of heterozygosity (LOH) at the *Dnmt1* locus, which was attributed to elevated levels of mitotic HR [15]. In the same study, knockout of *Dnmt1* was also shown to increase genomic rearrangements at the *Hprt* (hypoxanthine phosphoribosyl transferase) locus, suggesting that CpG methylation may inhibit nonhomologous recombination, as well [15]. The view that CpG methylation normally inhibits both kinds of recombination can account for several additional observations: patients with mutations in *Dnmt3a* display increased rearrangements of centromeric repeats [16]; ES cells that lack DNMT1 show an increased efficiency of gene targeting [17]; and DNMT1-deficient human cancer cells have higher frequencies of chromosomal rearrangements [18].

In contrast to these studies, which generally support a role for CpG methylation in genome stability, other reports either failed to detect an effect, or showed that demethylation decreased HR. CpG-methylated and unmethylated DNA constructs, when transfected into COS1 monkey cells, gave identical frequencies of extrachromosomal recombination in an assay designed to detect single-strand annealing, one kind of HR [19]. Similarly, 5-aza-CdR treatment of HeLa human cells that were modified to carry a chromosomal recombination substrate designed to detect conversion events did not increase the frequency of HR [20]. Finally, DNMT1 deficiency in mouse ES cells, was shown to reduce the rate of loss of an array of selectable transgenes, suggesting that mitotic HR was decreased by demethylation [21].

The lack of agreement among these studies could arise for several reasons: differences between the cell lines, or the species from which they were derived; whether the recombination substrate is extrachromosomal, randomly integrated, or targeted to a specific locus; and the particular

kind of event the substrate is designed to detect—crossover, conversion, or rearrangement. Because our original observations—5-aza-CdR treatment and HR destabilize repeats—were made at the *Aprt* locus in CHO cells, we chose to measure the effects of 5-aza-CdR treatment on HR at the same site, using a well-characterized recombination assay that can simultaneously detect crossovers, conversions, rearrangements, and mutations (Figure 1A) [22]. In this way we can measure the effects of 5-aza-CdR treatment on overall rates of recombination, as well as determine the differential effects, if any, on individual types of events.

By determining whether 5-aza-CdR treatment stimulates HR, we can distinguish between two alternatives for how genome-wide demethylation stimulates CAG repeat contraction. Because 5-aza-CdR treatment and HR each destabilize repeats, we proposed the hypothesis that they operate as part of a common pathway—with 5-aza-CdR stimulating HR, which in turn stimulates repeat contractions. A demonstration that 5-aza-CdR stimulates HR would cleanly distinguish this possibility from the alternative: that 5-aza-CdR and HR have their effects via independent pathways. Here we show that 5-aza-CdR treatment does not significantly alter the rate of HR at the *Aprt* locus, although it does shift the spectrum of events. We conclude that 5-aza-CdR treatment destabilizes triplet repeats by a mechanism that does not proceed via stimulation of HR.

## MATERIALS AND METHODS

### Cell lines and tissue culture conditions

Chinese hamster ovary (CHO) cells lines AT3-2, which contains a single copy of the wild type *Aprt* gene, and GSAA5, which was derived from AT3-2 cells and carries a duplication at the *Aprt* locus, have been described previously [23,24]. Both cell lines carry a functional *Aprt* gene and are phenotypically APRT<sup>+</sup>. These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco), nonessential amino acids, sodium pyruvate, penicillin, and streptomycin. For these experiments, we derived cell populations from individual colonies, and verified the genomic structure of the *Aprt* locus by Southern blotting and PCR (data not shown). To minimize spontaneous APRT<sup>-</sup> cells in the starting APRT<sup>+</sup> cell populations, we propagated the parental cells at low density for 7 to 10 days in ALASA medium (30μM alanosine, 10μM azaserine, 1mM adenine), which does not support the proliferation of APRT<sup>-</sup> cells. Pre-selected populations of cells were frozen in aliquots, which were thawed at the start each experiment.

### Recombination assay

To determine the frequencies of APRT<sup>-</sup> cells, we plated the untreated and 5-aza-CdR-treated cell populations at a density of  $5 \times 10^5$  cells/100mm plates in complete medium supplemented with 400 μM 8-azaadenine, which kills APRT<sup>+</sup> cells. After two weeks, we counted the APRT<sup>-</sup> colonies and used their frequencies to estimate rates, as described previously [4]. The number of APRT<sup>-</sup> colonies was adjusted for cell survival [APRT<sup>-</sup> colonies/(surviving colonies/plated cells)] and then divided by the number of population doublings that the cells underwent prior to applying selection for APRT<sup>-</sup> cells.

The spectra of events that led to the APRT<sup>-</sup> cells were analyzed by picking individual colonies from each independent treated (or untreated) cell population. Two colonies were initially isolated per experiment; if the two clones were identical, only one was counted to ensure independence. Southern blotting and PCR analysis were used to distinguish the four types of event that can give rise to APRT<sup>-</sup> cells (Fig. 1). After digestion of genomic DNA with *Bam*H1, crossovers give rise to a single 4.1-kb band, whereas conversions and mutations give a two-band pattern: a 12.5-kb band from the upstream *APRT* copy, and a 4.1-kb band from the downstream copy. Rearrangements produce a pattern that is distinct from the above patterns;

none were detected in the experiments in this paper. Conversions were distinguished from mutations by the presence of the mutant *EcoRV* site in both copies of the *Aprt* gene. Conversions gave a PCR product that was not cleaved by *EcoRV*, whereas the corresponding PCR product from mutations was cleaved [22]. If the PCR product was cleaved, indicating retention of the *EcoRV* site, the event was categorized as a mutation and was not analyzed further.

### 5-aza-CdR treatment and methylation analysis

Pre-selected cells were thawed and allowed to recover for 24 hours before being propagated at low density for 4 days. The exponentially growing cells were subsequently plated at densities of  $2 \times 10^5$ ,  $6 \times 10^5$ , and  $8 \times 10^5$  per 100mm plate for treatment with 0, 0.5, or  $1 \mu\text{M}$  5-aza-CdR (Sigma), respectively. Higher numbers of cells were used in the treated plates to compensate for 5-aza-CdR toxicity [4]. 5-aza-CdR was added to the growth media 24 hr after plating and incubated for three days, with fresh media containing the drug substituted after 24 hrs. For the recombination assay, treated cells were allowed to recover for three days and then they were plated as described above. For global methylation analysis, the cells were harvested, genomic DNA was isolated using GenomicPrep (Amersham), digested with either *MspI* (NEB) or *HpaII* (NEB), and then subjected to electrophoresis on a 1% agarose gel. For analysis of methylation at the *Aprt* locus,  $5 \mu\text{g}$  of *BamHI*-cleaved DNA was digested with either *MspI* or *HpaII*, and the products were separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (Amersham). The membrane was hybridized to the 2.1-kb *XhoI-BamHI* fragment from the 3' end of *Aprt*, which was radiolabelled by random priming with alpha  $^{32}\text{P}$ -dCTP. The positions of the bands were visualized by autoradiography.

### Real-Time RT-PCR

*Aprt* mRNA levels were measured by real-time RT-PCR using the Quantitech Qiagen SYBR green kit as described elsewhere [25]. Briefly, RNA was isolated using RNAeasy (Qiagen) and *Aprt* mRNA was amplified with two different pairs of primers: oVIN-219F (5'-ttg cag gag aga gaa gaa tgg) and oVIN-219R (5'-ggc cag aaa gtg gtt gtt gt), and oVIN-221F (5'-cga gag cat agg agg ctg ac) and oVIN-221R (5'-tgg cca gtc acc tta agt cc). For each sample, the *Aprt* mRNA levels were normalized to vimentin mRNA levels which were obtained using three different pairs of primers: oVIN-222F (5'- ggg cga cct cta tga gga g) and oVIN-222R (5'-gct ctc cgc ttc ctc tct ct), oVIN-223F (5'-aga gag agg aag cgg aga gc) and oVIN- 223R (5'-ctc ctg aat ctg ggc ctg ta), and oVIN-224F (5'-ttc taa gcc cga cct cac tg) and oVIN-224R (5'-ctc att tga ctc ctg ctt tgc).

### Statistics

To compare APRT<sup>-</sup> rates, we used Student's T-test. We used a Chi-square test with Yate's correction to probe the significance of changes in recombination types between crossovers and conversions in treated versus untreated cells. Fisher's exact test was used to obtain the probability values for the distribution of DNA methylation in recombinants.

## RESULTS

### Experimental design

To assay for the effects of demethylation on HR, we used a well-characterized recombination substrate that contains two direct repeats of the selectable *Aprt* gene at its endogenous locus in GSAA5 CHO cells (Fig.1). The upstream copy of the gene is truncated at the 3' end and has a mutated *EcoRV* site in exon 2 [23]. The downstream copy is functional and contains the wild type *EcoRV* site. Selection for APRT<sup>-</sup> cells allows us to detect four types of event—conversion, crossover, rearrangement, and mutation—that inactivate the downstream copy (Fig. 1). As in

our previous studies, these names refer to the products shown in Figure 1; they are not meant to imply (or exclude) any specific mechanism. We can distinguish these events by Southern blotting and PCR (see Materials and Methods). Spontaneous APRT<sup>-</sup> cells arise predominantly by HR, with conversions and crossovers accounting for 95% of all APRT<sup>-</sup> colonies [22,26,27]. To bring about CpG demethylation, we used the cytidine analog, 5-aza-CdR, which is incorporated into DNA and traps DNA methyltransferases. This leads to a passive demethylation of the genome during subsequent rounds of replication [5]. By comparing APRT<sup>-</sup> colony formation in treated and untreated cells, and by analyzing the spectrum of events, we can determine whether 5-aza-CdR treatment significantly affects HR, nonhomologous recombination, or mutation.

### 5-aza-CdR treatment reduces CpG methylation

To confirm our previous findings that 5-aza-CdR treatment of CHO cells reduces DNA methylation genome-wide [4], we grew GSAA5 cells for 3 days in the presence of 0  $\mu$ M, 0.5  $\mu$ M, or 1  $\mu$ M of the drug. We tested 0.5  $\mu$ M and 1  $\mu$ M 5-aza-CdR because they had previously been shown to stimulate triplet repeat contraction 100- to 1000-fold [4]. After drug treatment, genomic DNA was isolated and digested with *MspI*, which cleaves unmethylated and methylated CCGG sites, or with *HpaII*, which only cleaves unmethylated CCGG sites. The digested DNA was displayed by electrophoresis on an agarose gel (Figure 2A). Consistent with our previous findings, 5-aza-CdR treatment makes the genomic DNA much more sensitive to *HpaII* digestion, indicating a substantial loss of CpG methylation. Similarly, we confirmed our previous results [4] that a defined CCGG site at the 3' end of the *Aprt* gene (Figure 2B), which is 100% methylated in untreated cells, is only about 50% methylated in 5-aza-CdR-treated cells (data not shown).

### Effects of 5-aza-CdR treatment on HR

To test whether CpG demethylation stimulates HR, we treated GSAA5 cells with 0  $\mu$ M, 0.5  $\mu$ M, or 1  $\mu$ M 5-aza-CdR, and measured the rates at which APRT<sup>-</sup> colonies arose. As shown in Table 1, treated and untreated GSAA5 cells formed APRT<sup>-</sup> colonies at rates that were not significantly different, suggesting that HR was not altered by CpG demethylation. To verify this conclusion, we isolated several independent APRT<sup>-</sup> colonies to determine the spectrum of events that produced them. In our previous studies of spontaneous events [22,26,27], which are summarized in Table 2, we found that 95% of APRT<sup>-</sup> colonies arose by HR, with a 6:1 ratio of conversions to crossovers, 5% arose by mutation, and less than 0.5% arose by rearrangement. The eight independent APRT<sup>-</sup> colonies isolated from untreated GSAA5 cells, which were all conversions (Table 2), are consistent with the expected distribution of spontaneous events ( $P=0.53$ ). The 15 independent APRT<sup>-</sup> colonies isolated from 5-aza-CdR-treated GSAA5 cells were also generated predominantly by HR (Table 2), but the spectrum of events is significantly different from that of spontaneous events ( $P=0.0055$ ) with only 47% of the events being gene conversions and 40% crossovers. Thus, although 5-aza-CdR does not seem to stimulate HR overall, it apparently promotes crossovers at the expense of conversions.

### Methylation status of recombinants at the *Aprt* locus

Another way to assess the effects of 5-aza-CdR on HR is to determine whether the recombinants arose preferentially from the unmethylated or the methylated subpopulation. We showed previously that about 50% of 5-aza-CdR treated cells lose the methylation mark on the CCGG site at the 3' end of the *Aprt* gene (Figure 2B) [4]. Because DNA methylation patterns are maintained during mitotic division, we can analyze the APRT<sup>-</sup> colonies to track the methylation status of their genomes near the time they underwent recombination. Therefore, we performed Southern blot analysis after digestion with *BamHI*, alone, and in combination with either *MspI* or *HpaII* to determine whether the clones were methylated at the *Aprt* locus. Analyses



of one unmethylated crossover and two methylated conversion events are shown in Figure 2C. As expected, all eight recombinants from untreated cells were methylated. In contrast, nine of twelve recombinants from 5-aza-CdR-treated cells were not methylated (Table 3). These results confirm that the population of 5-aza-CdR-treated cells had in fact undergone demethylation. More importantly, the 1:3 ratio of methylated to unmethylated genomes is not significantly different ( $P=0.4$ ) from the 6:6 ratio expected from the 50% 5-aza-CdR-induced demethylation observed previously at this site [4]. These results support our conclusion that 5-aza-CdR treatment does not stimulate HR.

### Mutagenesis of the APRT locus

In the spectrum of APRT<sup>-</sup> colonies that arose after 5-aza-CdR treatment, we observed more mutations than we had expected (Table 2). Although the difference is small (and not statistically significant), 5-aza-CdR is reported to be a weak mutagen [5]. Therefore, we asked whether 5-aza-CdR treatment increases the rate of mutation at the *Aprt* locus in CHO cells. AT3-2 cells, which carry a single wild type copy of *Aprt* [24], were treated with 0  $\mu$ M, 0.5  $\mu$ M, or 1  $\mu$ M 5-aza-CdR and APRT<sup>-</sup> colonies were selected. As shown in Table 1, 5-aza-CdR treatment had no significant effect on mutation rates ( $P>0.05$ ), consistent with our previous studies [4,28].

### Transcription at the Aprt locus

Loss of DNA methylation can activate gene transcription [29], which has been shown to destabilize triplet repeats [25,30]. To determine whether 5-aza-CdR treatment might stimulate repeat contraction via effects on transcription, we examined *Aprt* mRNA levels using quantitative RT-PCR. We found that *Aprt* mRNA levels were unchanged by 5-aza-CdR treatment (Figure 2), suggesting that the drug does not induce repeat instability by increasing transcription through the repeat tract.

## DISCUSSION

In previous studies, we showed that both 5-aza-CdR treatment and HR stimulate contraction of CAG repeat tract at the *Aprt* locus [4,8]. Because several observations in the literature suggest that demethylation increases HR, we proposed that 5-aza-CdR treatment promotes repeat instability via stimulation of HR [4]. As a critical test of this hypothesis, we measured the effects of 5-aza-CdR treatment on HR, using a well-characterized recombination substrate at the *Aprt* locus. We show here that 5-aza-CdR treatment does not alter the overall rate of HR. These results disprove the hypothesis. We conclude that treatment of cells with 5-aza-CdR increases contraction of CAG repeat tracts via a mechanism that does not involve HR. Thus, demethylation and HR destabilize repeat tracts by independent pathways.

Although 5-aza-CdR treatment did not increase the rate of HR, it did alter the proportions of conversions to crossovers, from the normal (spontaneous) 6:1 ratio to about a 1:1 ratio (Table 2). The increase in crossovers, which is likely to occur via the mechanism of single-strand annealing (SSA) (ref Sargent NAR 28:3771, 2000, plus some others), is intriguing because SSA within the CAG tract could readily generate repeat contractions. However, the magnitude of change observed here—a few fold—is not adequate to account for the 1000-fold effect of 5-aza-CdR on CAG repeat instability observed previously [4]. Moreover, a selective effect of 5-aza-CdR on crossovers does not jibe with our previous observation that HR stimulates CAG contractions about equally among crossovers and conversion events [8]. Thus, the subtle effects of 5-aza-CdR on HR observed cannot account for the dramatic effect 5-aza-CdR has on CAG repeat contraction [4].

Our results provide the first example of a bioactive molecule that changes the type of recombination event, without affecting the overall rate. Mammalian somatic cells are thought

to be biased toward conversion events because they permit the beneficial exchange of genetic information during repair processes, without the potential for loss of heterozygosity (LOH) that can accompany crossover events [31]. Little is known, however, about how the cell imposes the preference for conversions over crossovers during HR. Our results, which indicate that small molecules can affect the outcome, suggest that screening chemical libraries could yield significant insights into this process.

This study also raises the possibility that DNA methylation may promote genome stability, not by preventing HR between repetitive sequences as previously suggested [9-12], but rather by helping to ensure that HR proceeds via conversion, which avoids several dangers: LOH by mitotic HR between homologues, deletion of information by HR between repeats on the same chromosome, and rearrangements by HR between repeats on different chromosomes. Previous studies, which all used assays that were specific for a single type of recombination event, would have missed the phenomenon reported here.

Demethylation-induced stimulation of crossovers at the expense of conversions could account for most of the observations in studies that have investigated the role of DNA methylation in chromosomal HR in mammalian cells. The one study that used conversion as an assay for HR detected no increase in DNMT1-deficient ES cells [20]. By contrast, with one exception [21], studies that used crossover as an assay detected a demethylation-induced stimulation of HR [5,13,15,16]. DNA demethylation has also been reported to increase rearrangements [18,21]; however, in neither study were the rearrangements analyzed to determine whether they were generated by HR between similar sequences or by nonhomologous recombination between unrelated sequences. If they arose by HR, then they would represent additional examples of crossover stimulation by demethylation.

Our studies do not define the mechanism of crossover stimulation by 5-aza-CdR treatment. There are several possibilities. First, at the *Aprt* locus, double-strand breaks (DSBs), which greatly increase overall HR frequencies, shift the bias from the 6:1 spontaneous ratio to about 3:1 [23]. Treatment with 5-aza-CdR, however, is unlikely to change the distribution of HR events by inducing DSBs because overall HR frequencies are not affected (Table 1). Second, 5-aza-CdR could act on HR indirectly by altering CpG methylation at distant promoters, thereby changing expression levels of genes such as *Rad51* and *Brca2*, which are known to influence the competition between conversion and crossover [32], or of other DNA repair genes that might alter the ratio. Third, incorporation of 5-aza-CdR into the DNA at the *Aprt* locus, or its covalent attachment to DNMT1, could have direct effects on HR. For example, if 5-aza-CdR-modified DNA interfered with strand invasion or DNA synthesis primed by the invading strand, it would preferentially reduce conversions relative to crossovers, which are thought to occur predominantly by SSA and thus do not require strand invasion. Fourth, by sequestering or destroying DNMT1, 5-aza-CdR could alter the amounts or properties of proteins that are known to bind to DNMT1, including histone deacetylases 1 and 2 [33,34] and histone methyltransferases G9a [35] and SUV39H1 [36], thereby potentially altering the proportion of crossovers via effects on chromatin structure [37,38]. Fifth, local methylation changes at *Aprt* could, in principle, alter recombination via effects on chromatin structure or by influencing the formation or processing of recombination intermediates. It seems unlikely that the single site we have assayed—the only methylated HpaII site in the vicinity of the gene—could have such a profound effect, but there may be additional methylated CpG sites at the *Aprt* locus that we have not detected.

Although our studies rule out an intermediary role of HR in the pathway of 5-aza-CdR-induced stimulation of triplet-repeat instability, they do not further define the mechanism of demethylation-induced repeat instability. Treatment of CHO cells with 5-aza-CdR can increase contraction of a CAG repeat tract by more than 1000-fold, which is the largest effect on triplet-

repeat instability observed to date [4]. Determining its mode of action, although challenging, may be especially relevant for developing therapeutics designed to stimulate contraction of triplet repeats. One well-defined effect of DNA methylation inhibitors, including 5-aza-CdR, is to cause changes in transcription [39]. Since transcription itself destabilizes CAG repeats [25,30], we analyzed transcription at the *Aprrt* locus before and after treatment with 5-aza-CdR, but observed no differences (Figure 2). Thus, it seems unlikely that 5-aza-CdR treatment stimulates repeat instability via changes in transcription through the *Aprrt* locus. It remains possible that 5-aza-CdR alters the expression of other genes—DNA repair genes, in particular—that modulate triplet repeat instability. Further experiments will be needed to define 5-aza-CdR's mode of action leading to triplet repeat instability.

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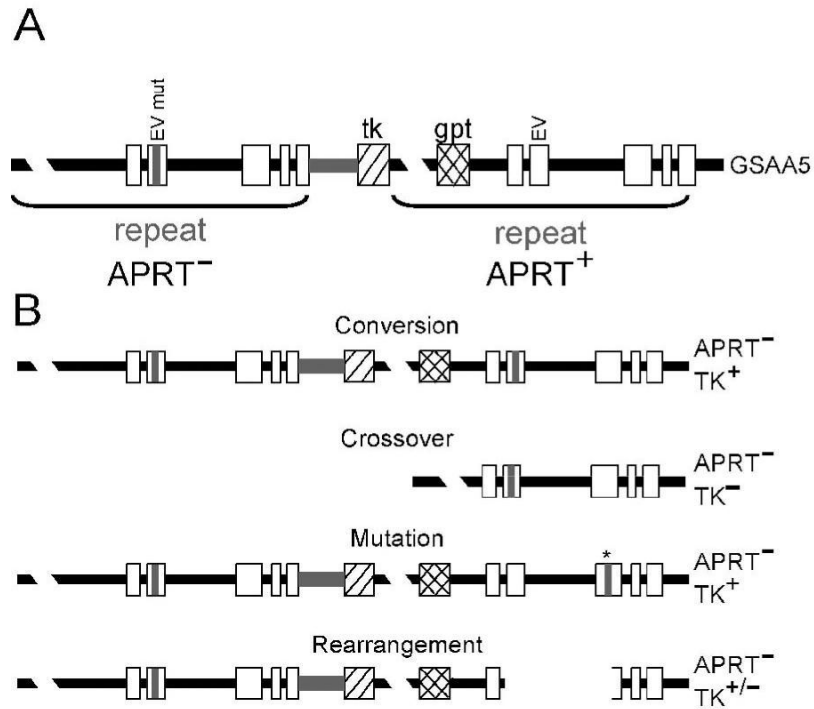
#### REFERENCES

- [1]. Pearson CE, Edamura KN, Cleary JD. Repeat instability: mechanisms of dynamic mutations. *Nat. Rev. Genet* 2005;6:729–742. [PubMed: 16205713]
- [2]. Gatchel JR, Zoghbi HY. Diseases of unstable repeat expansion: mechanisms and common principles. *Nat. Rev. Genet* 2005;6:743–755. [PubMed: 16205714]
- [3]. Mirkin SM. Expandable DNA repeats and human disease. *Nature* 2007;447:932–940. [PubMed: 17581576]
- [4]. Gorbunova V, Seluanov A, Mittelman D, Wilson JH. Genome-wide demethylation destabilizes CTG.CAG trinucleotide repeats in mammalian cells. *Hum Mol Genet* 2004;13:2979–2989. [PubMed: 15459182]
- [5]. Haaf T. The effects of 5-azacytidine and 5-azadeoxycytidine on chromosome structure and function: implications for methylation-associated cellular processes. *Pharmacol Ther* 1995;65:19–46. [PubMed: 7536332]
- [6]. Ghoshal K, Datta J, Majumder S, Bai S, Kutay H, Motiwala T, Jacob ST. 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Mol Cell Biol* 2005;25:4727–4741. [PubMed: 15899874]
- [7]. Segura-Pacheco B, Trejo-Becerril C, Perez-Cardenas E, Taja-Chayeb L, Mariscal I, Chavez A, Acuna C, Salazar AM, Lizano M, Duenas-Gonzalez A. Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. *Clin Cancer Res* 2003;9:1596–1603. [PubMed: 12738711]
- [8]. Meservy JL, Sargent RG, Iyer RR, Chan F, McKenzie GJ, Wells RD, Wilson JH. Long CTG tracts from the myotonic dystrophy gene induce deletions and rearrangements during recombination at the APRT locus in CHO cells. *Mol Cell Biol* 2003;23:3152–3162. [PubMed: 12697816]
- [9]. Colot V, Rossignol JL. Eukaryotic DNA methylation as an evolutionary device. *Bioessays* 1999;21:402–411. [PubMed: 10376011]
- [10]. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 2003;33(Suppl):245–254. [PubMed: 12610534]
- [11]. Krickler MC, Drake JW, Radman M. Duplication-targeted DNA methylation and mutagenesis in the evolution of eukaryotic chromosomes. *Proc Natl Acad Sci U S A* 1992;89:1075–1079. [PubMed: 1736289]
- [12]. Maloisel L, Rossignol JL. Suppression of crossing-over by DNA methylation in *Ascomobolus*. *Genes Dev* 1998;12:1381–1389. [PubMed: 9573054]

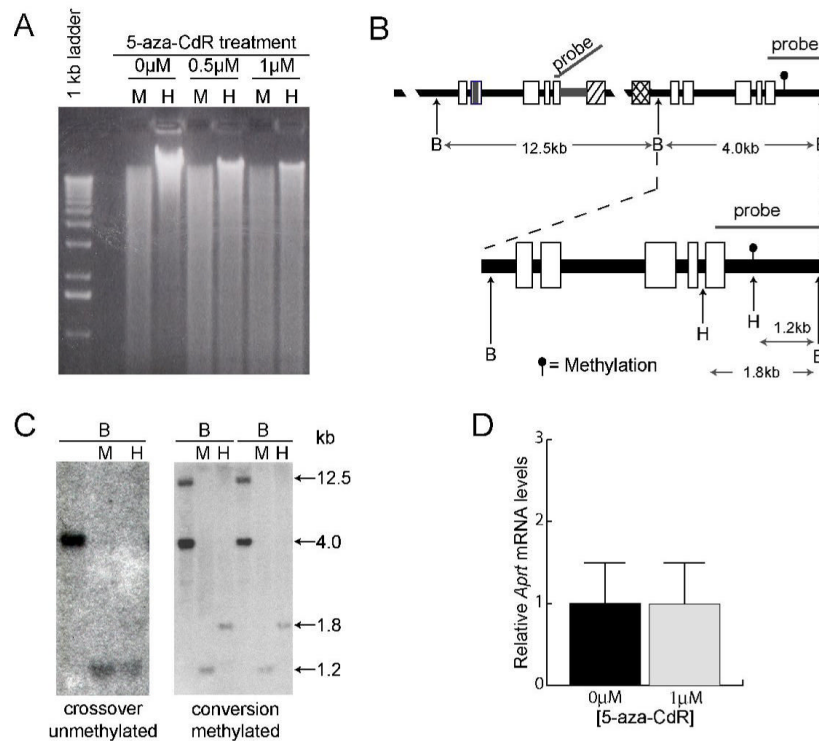


- [13]. McBurney MW, Lau S, Jardine K, Yang X, Davies B. Reexpression of a cluster of silenced transgenes is associated with their rearrangement. *Genes Chromosomes Cancer* 2001;32:311–323. [PubMed: 11746972]
- [14]. Rizwana R, Hahn P. CpG methylation reduces genomic instability. *Journal of Cell Science* 1999;112:4513. [PubMed: 10574701]
- [15]. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. *Nature* 1998;395:89–93. [PubMed: 9738504]
- [16]. Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, Viegas-Pequignot E. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* 1999;402:187–191. [PubMed: 10647011]
- [17]. Dominguez-Bendala J, McWhir J. Enhanced gene targeting frequency in ES cells with low genomic methylation levels. *Transgenic Res* 2004;13:69–74. [PubMed: 15070077]
- [18]. Karpf AR, Matsui S. Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. *Cancer Res* 2005;65:8635–8639. [PubMed: 16204030]
- [19]. Liang F, Jasin M. Studies on the influence of cytosine methylation on DNA recombination and end-joining in mammalian cells. *J Biol Chem* 1995;270:23838–23844. [PubMed: 7559561]
- [20]. Cuzzo C, Porcellini A, Angrisano T, Morano A, Lee B, Pardo AD, Messina S, Iuliano R, Fusco A, Santillo MR, Muller MT, Chiariotti L, Gottesman ME, Avvedimento EV. DNA Damage, Homology-Directed Repair, and DNA Methylation. *PLoS Genet* 2007;3:e110. [PubMed: 17616978]
- [21]. Chan MF, van Amerongen R, Nijjar T, Cuppen E, Jones PA, Laird PW. Reduced rates of gene loss, gene silencing, and gene mutation in Dnmt1-deficient embryonic stem cells. *Mol Cell Biol* 2001;21:7587–7600. [PubMed: 11604495]
- [22]. Sargent RG, Merrihew RV, Nairn R, Adair G, Meuth M, Wilson JH. The influence of a (GT)<sub>29</sub> microsatellite sequence on homologous recombination in the hamster adenine phosphoribosyltransferase gene. *Nucleic Acids Res* 1996;24:746–753. [PubMed: 8604319]
- [23]. Sargent RG, Brennenman MA, Wilson JH. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol Cell Biol* 1997;17:267–277. [PubMed: 8972207]
- [24]. Adair GM, Carver JH, Wandres DL. Mutagenicity testing in mammalian cells. I. Derivation of a Chinese hamster ovary cell line heterozygous for the adenine phosphoribosyltransferase and thymidine kinase loci. *Mutat Res* 1980;72:187–205. [PubMed: 6449663]
- [25]. Lin Y, Dion V, Wilson JH. Transcription promotes contraction of CAG repeat tracts in human cells. *Nat. Struct. Mol. Biol* 2006;13:179–180. [PubMed: 16388310]
- [26]. Kilburn AE, Shea MJ, Sargent RG, Wilson JH. Insertion of a telomere repeat sequence into a mammalian gene causes chromosome instability. *Mol Cell Biol* 2001;21:126–135. [PubMed: 11113187]
- [27]. Sargent RG, Rolig RL, Kilburn AE, Adair GM, Wilson JH, Nairn RS. Recombination-dependent deletion formation in mammalian cells deficient in the nucleotide excision repair gene ERCC1. *Proc Natl Acad Sci U S A* 1997;94:13122–13127. [PubMed: 9371810]
- [28]. Lin Y, Dion V, Wilson JH. A novel selectable system for detecting expansion of CAG.CTG repeats in mammalian cells. *Mutat Res* 2005;572:123–131. [PubMed: 15790495]
- [29]. Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* 2006;31:89–97. [PubMed: 16403636]
- [30]. Lin Y, Wilson JH. Transcription-induced CAG repeat contraction in human cells is mediated in part by transcription-coupled nucleotide excision repair. *Mol Cell Biol* 2007;27:6209–6217. [PubMed: 17591697]
- [31]. Johnson RD, Jasin M. Double-strand-break-induced homologous recombination in mammalian cells. *Biochem Soc Trans* 2001;29:196–201. [PubMed: 11356153]
- [32]. Stark JM, Pierce AJ, Oh J, Pastink A, Jasin M. Genetic steps of mammalian homologous repair with distinct mutagenic consequences. *Mol Cell Biol* 2004;24:9305–9316. [PubMed: 15485900]
- [33]. Rountree MR, Bachman KE, Baylin SB. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat Genet* 2000;25:269–277. [PubMed: 10888872]

- [34]. Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet* 2000;25:338–342. [PubMed: 10888886]
- [35]. Esteve PO, Chin HG, Smallwood A, Feehery GR, Gangisetty O, Karpf AR, Carey MF, Pradhan S. Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. *Genes Dev* 2006;20:3089–3103. [PubMed: 17085482]
- [36]. Fuks F, Hurd PJ, Deplus R, Kouzarides T. The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res* 2003;31:2305–2312. [PubMed: 12711675]
- [37]. Zhang Z, Fan HY, Goldman JA, Kingston RE. Homology-driven chromatin remodeling by human RAD54. *Nat Struct Mol Biol* 2007;14:397–405. [PubMed: 17417655]
- [38]. Cummings WJ, Yabuki M, Ordinario EC, Bednarski DW, Quay S, Maizels N. Chromatin Structure Regulates Gene Conversion. *PLoS Biol* 2007;5:e246. [PubMed: 17880262]
- [39]. Yoo CB, Jones PA. Epigenetic therapy of cancer: past, present and future. *Nat Rev Drug Discov* 2006;5:37–50. [PubMed: 16485345]



**Figure 1.** Recombination substrate and products. A. The duplication at the *Aprt* locus in GSA A5 cells. The upstream copy of *Aprt* contains a mutated *EcoRV* site (EV mut) in exon 2, as well as a truncated exon 5, rendering it nonfunctional. The downstream copy contains the normal *EcoRV* site (EV) and is functional, making GSA A5 cells APRT<sup>+</sup>. The herpes virus thymidine kinase (TK) and the guanine phosphoribosyl transferase (*gpt*) genes served as additional selective markers useful in generating the GSA A5 cell line and for characterizing the recombination products. B. Illustrations of the types of APRT<sup>-</sup> colonies that arise in GSA A5 cells. After selection against APRT function, it is possible to recover clones that underwent HR (conversions or crossovers), mutation of the wild-type copy of the *Aprt* gene (indicated by the \* above exon 3), or rearrangement of the *Aprt* gene (shown here as a deletion of a portion of the *Aprt* gene). Each of these events can be distinguished by a combination of Southern blot and PCR analysis (see Materials and Methods).



**Figure 2.** Methylation analysis of the *Aprt* locus. **A.** Analysis of global methylation in GSAA5 cells. Genomic DNA was isolated from untreated cells and from cells treated with 5-aza-CdR, digested with *MspI* (M) or its methyl-sensitive isoschizomer *HpaII* (H), and displayed by electrophoresis on an agarose gel. Decreased methylation in treated cells is shown by the more complete digestion of DNA by *HpaII*. A 1-kb marker ladder is shown on the left. **B.** Restriction maps and probe used for analysis of methylation at the *Aprt* locus. The complete recombination substrate is shown on top, with an expanded view of the 3' copy of *Aprt* shown below. The methylated *HpaII* (H) site, which is indicated by the filled circle in the downstream copy of *Aprt*, is not present in the upstream copy. The probe hybridizes to the 12.5- and 4.0-kb *BamHI* (B) fragments, which contain the upstream and downstream copies of *Aprt*, respectively. **C.** Representative Southern blots showing the structure and methylation status of a crossover and two conversions. All samples were digested with *BamHI* (B) alone or in combination with *MspI* (M) or *HpaII* (H). Sizes of the digestion products (in kb) are shown on the right. **D.** Relative *Aprt* mRNA levels after 0 and 0.5 μM 5-aza-CdR treatment are shown. Error bars represent one standard deviation.

**Table 1**

Rates of APRT colony formation.

Cell lines	5-aza-CdR ( $\mu\text{M}$ )	Rates of APRT <sup>+</sup> colony formation ( $10^{-7}$ ) <sup>a</sup>	P value vs 0 $\mu\text{M}$	P value vs 1 $\mu\text{M}$
GSAA5	0	48 $\pm$ 7		0.7
	0.5	36 $\pm$ 2	0.15	0.4
	1	62 $\pm$ 24	0.7	
AT3-2	0	5.0 $\pm$ 0.5		0.8
	0.5	3.5 $\pm$ 0.2	0.06	0.6
	1	4.6 $\pm$ 2	0.8	

<sup>a</sup>Results represent at least three independent experiments. In each experiment, three to four different plates were treated in parallel and the mean rates for each experiment were averaged. The standard error is shown.



Table 2

Distribution of events that gave rise to APRT<sup>-</sup> colonies.

Cell line	5-aza-CdR (μM)	Conversion <sup>b</sup>	Crossover <sup>b</sup>	Mutation <sup>b</sup>	Rearrangement	Total
G5AA5	0	8 (100)	0 (0)	0 (0)	0	8
G5AA.5	0.5 & 1	7 (47)	6 (40)	2 (13)	0	15
Other <sup>d</sup>	0	172 (82)	28 (28)	10 (5)	0	210

<sup>a</sup>These numbers are the sum of published data for a variety of other CHO cell lines (GSB2, GSB5, GSC4, GSC6, GSE1, GSE3, GS21-15, and AK209) that each carries the duplication substrate at the *Appt* locus [22,26,27]. These numbers define the expected distribution for spontaneous events at the duplicated *Appt* locus.

<sup>b</sup>The number of APRT<sup>-</sup> colonies identified in each category is shown, with the percentage of the total indicated in parentheses.

Table 3

Analysis of methylation in recombinants.

Cell line	5-aza-CdR ( $\mu$ M)	Conversion <sup>a</sup>		Crossover <sup>a</sup>		Total <sup>a</sup>	
		Mel	Unmet	Mel	Unmet	Mel	Unmet
GSAAS	0	8 <sup>b</sup>	0	0	0	8	0
GSAAS	0.5 & 1	2	9	1	2	3	9

<sup>a</sup> APRT<sup>-</sup> colonies that arose by HR were analyzed by Southern blotting (Figure 2C) to determine whether the 3' *HpaII* site (Figure 2B) was methylated (Mel) or unmethylated (Unmet).

<sup>b</sup> One clone was found to be methylated at both *HpaII* sites shown in Figure 2B.