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# Next-nucleotide effects in mutations driven by DNA precursor pool imbalances at the *aprt* locus of Chinese hamster ovary cells

(deoxyribonucleoside triphosphate pools/proofreading/mutator gene)

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ABSTRACT **Imbalances of the intracellular pools of the precursors of DNA synthesis, the deoxyribonucleoside triphosphates, produce marked shifts in the spectrum of mutations at the *aprt* locus of Chinese hamster ovary cells. Mutations induced by excess dTTP or dCTP are dominated by misincorporation of the nucleotide in excess, as determined by sequence analysis of cloned mutant genes. The shift in spectrum is also apparently influenced by the nucleotides surrounding the one altered—those 3′ to the nucleotide misincorporated being present in excess in most of the mutant genes characterized. Since next-nucleotide effects are a property of DNA polymerases with "proofreading" activities, our data suggest that this function is part of the mammalian DNA replication complex.**

Imbalances in the supply of the precursors of DNA synthesis, the deoxyribonucleoside triphosphates, have dramatic genetic consequences on cells (1, 2). Thymidylate starvation induces the recA-dependent error prone repair system in *Escherichia coli* (3) and recombination in yeast (4). In cultured mammalian cells, both thymidylate deprivation and excess produce gross chromosomal abnormalities—breakage, deletions, and sister chromatid exchange (5–7)—as well as alterations at the nucleotide base-pair level in the form of mutation (8–11). Precursor pool imbalances also affect the fidelity of DNA synthesis *in vitro* (12–14). Purified DNA polymerases misincorporate deoxyribonucleoside triphosphate supplied in excess to produce predominantly transition substitutions. For bacterial polymerases, this misincorporation frequency is further enhanced by next-nucleotide effects, which diminish the effectiveness of the 3′→5′ exonuclease "proofreading" functions (13, 15), although evidence for such editing functions by mammalian polymerases is limited (16–19).

To determine whether the effects of pool imbalances on the accuracy of DNA replication *in vitro* are sufficient to explain the effects observed on mammalian cells (mutation and chromosome aberrations), we analyzed the mutations induced by pool imbalances at the adenine phosphoribosyltransferase (*aprt*) locus of Chinese hamster ovary (CHO) cells. Making use of a CHO strain having only one copy of the structural gene (20), we made a collection of aprt$^-$ mutants induced by dCTP or dTTP pool imbalances (21). By Southern blot analysis of restriction endonuclease-digested DNA isolated from these strains, a number of mutations were mapped to restriction endonuclease sites (21) within the *aprt* locus. We have now cloned these mutant genes and sequenced the regions to which the mutations were mapped. The data show that the imbalance in dTTP or dCTP pools leads to a dramatic shift in the mutational spectrum to misincorporation of the nucleotide in excess. Furthermore, this misincorporation appears to be significantly influenced by the surrounding nucleotides, suggesting the existence of a proofreading function as part of the mammalian cell replication complex.

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## MATERIALS AND METHODS

**Cell Culture and Mutant Selection.** Cell culture conditions and the cell strains used have been described in detail (9, 10, 21). Mutant aprt$^-$ strains induced by excess thymidine were isolated from the CHO strain D422, which is hemizygous for the *aprt* locus (20). Independent replica cultures were exposed to 10 mM thymidine for 20 hr, followed by growth in nonselective medium to allow expression of the mutant phenotype. Cell survival after this treatment was ≈30%. Treated cultures were then plated in selective medium containing 8-azaadenine. Only one aprt$^-$ mutant was picked from each independently treated culture.

A second collection of aprt$^-$ mutants was obtained from the CHO strain Thy$^-$-2-1, a single-step derivative of D422 (21). Thy$^-$-2-1 has a mutation of CTP synthetase rendering the enzyme resistant to feedback inhibition by the end-product CTP (22). The consequences of this mutation are an increase in the pools of CTP and dCTP and an increase in spontaneous mutational rate directly related to the level of dCTP (10, 22). aprt$^-$ mutant strains were obtained from replica cultures of Thy$^-$-2-1 growing in 4 μM thymidine by plating up to $2 \times 10^6$ cells in 8-azaadenine-selective medium. To ensure that the mutant strains were independent, the replica cultures were begun from only 1000 cells and only one aprt$^-$ mutant was selected from each independent culture. The rate of mutation at the *aprt* locus in Thy$^-$-2-1 grown in such conditions was 7- to 10-fold higher than the rate in the wild-type strain D422 (21).

**Southern Blot Analysis of the *aprt* Locus.** Protocols for the extraction of high molecular weight DNA, restriction endonuclease digestions, and blot hybridization were described in detail (20). The probe used for these analyses was the unique 3.8-kilobase (kb) *Bam*HI fragment, which contains all the *aprt* coding sequences as determined by its ability to transform aprt$^-$ cells to aprt$^+$ by transfection (unpublished observations). The probe was labeled to high specific activity ($2 \times 10^8$ dpm/μg) by nick-translation (23).

**Cloning and Sequencing of Mutant *aprt* Genes.** Genomic DNA that had been digested with *Eco*RI and *Hin*dIII was size-fractionated on agarose gels and cloned into *Eco*RI/*Hin*dIII-digested λNM1149 (24). Ligation, *in vitro* packaging, and screening of recombinants were performed according to described protocols (25). Fragments of interest were subcloned directly from λ minipreparations into vectors M13mp18 or M13mp19 (26). Sequencing was carried out by the dideoxy chain-termination method (27) using dATP [α-$^{35}$S] (28). The polymerization reaction was primed by either the 17-mer universal primer (26) or appropriate internal primers (19-mer). Sequencing reaction mixtures were double-loaded onto a 5% polyacrylamide denaturing gel (0.4 mm thick; 65 cm long), allowing the analysis of sequence up to 500 base pairs (bp) from the priming site.

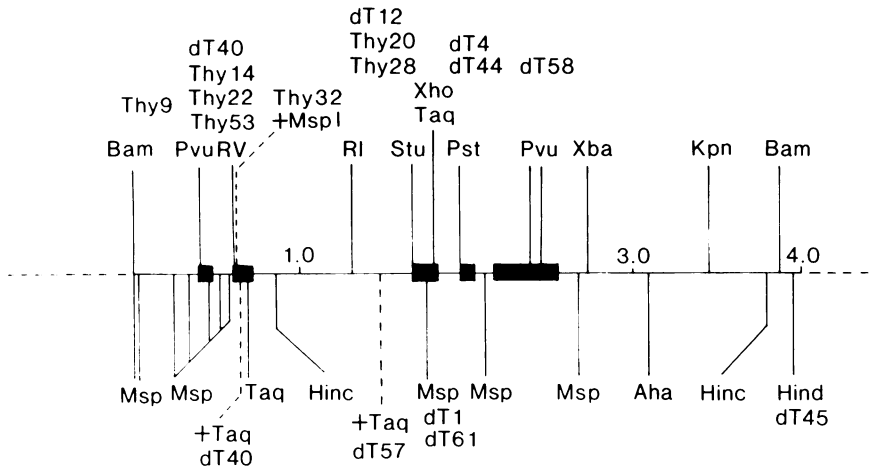


FIG. 1. Restriction map of the *aprt* locus of CHO cells. Map positions of mutations localized by lost or gained sites are indicated by the number of the mutant strain over the affected site. Boxed regions represent exon sequences.

| strain | map site | wild type sequence | mutant sequence | alteration |
|---|---|---|---|---|
| transitions: | | | | |
| dT 4 | lost Pst I | Pst I<br>GTCTGCAGGCTG<br>CAGACGTCCGAC<br>1970 | GTCTACAAGCTG<br>CAGATGTTCGAC | G:C→A:T |
| dT 12 | lost Stu I | Stu I<br>TAACAGGCCT<br>ATTGTCCGGA<br>1680 | TAACAAGCCT<br>ATTGTTCGGA | G:C→A:T |
| dT 44 | lost Pst I | Pst I<br>CTGCAGGCTG<br>GACGTCCGAC<br>1970 | CTGCAAGCTG<br>GACGTTCGAC | G:C→A:T |
| dT45 | lost Hind III | Hind III<br>CCAAGCTTGG<br>GGTTCGAACC<br>3960 | CCAAGTTTGG<br>GGTTCAAACC | C:G→T:A |
| dT57 | gained Taq I | Taq I<br>GTAACCGAT<br>CATTGGCTA<br>1460 | GTAATCGAT<br>CATTAGCTA | C:G→T:A |
| | | CAGGCTGAAC<br>GTCCGACTTG<br>1980 | CAGGTTGAA<br>GTCCAACTT | C:G→T:A |
| dT58 | lost Pvu II | Pvu II<br>TCAGCTGAT<br>AGTCGACTA<br>2430 | TCAGTTGAT<br>AGTCAACTA | C:G→T:A |
| | | TCACCTTAA<br>AGTGGAATT<br>670 | TCACTTTAA<br>AGTGAAATT | C:G→T:A |
| dT61 | lost Msp I | Msp I<br>CATCCGGAA<br>GTAGGCCTT<br>1760 | CATCTGGAA<br>GTAGACCTT | C:G→T:A |
| transversions: | | | | |
| dT 1 | lost Msp I | Msp I<br>ATCCGGAAG<br>TAGGCCTTC<br>1760 | ATCCTGAAG<br>TAGGACTTC | G:C→T:A |
| dT45 | | ATACGCCAA<br>TATGCGGTT<br>3950 | ATACTCCAA<br>TATGAGGTT | G:C → T:A |

## RESULTS

**Mutant *aprt* Genes with Altered Restriction Endonuclease Sites.** The isolation of aprt$^-$ mutants of D422 induced by excess dTTP has been reported (21). In these experiments, cell cultures were exposed to high concentrations of exogenous thymidine, resulting in an intracellular accumulation of dTTP. As a result of the complex allosteric regulation of ribonucleotide reductase (29), the increase in dTTP leads to a corresponding increase of dGTP, a depletion of dCTP, and a decrease in the rate of DNA replication (30). As an apparent consequence of these perturbations, the frequency of mutations is increased 10- to 50-fold at several genetic loci, including *aprt* (8, 21). Increased levels of dCTP were obtained in a mutant strain of D422, Thy$^-$-2-1, having altered regulation of CTP synthetase (21). As a consequence of this alteration, the dCTP pool in Thy$^-$-2-1 is increased 2- to 10-fold over that in the wild type. This imbalance of the dCTP pool has no observable deleterious effect other than a 5- to 300-fold increase in the rate of mutation at independent genetic loci (and a 7- to 10-fold increase in the rate of mutation at the *aprt* locus; ref. 21).

DNAs purified from 58 aprt$^-$ strains induced by dTTP and 45 from the Thy$^-$ background were digested with restriction endonucleases, fractionated on agarose gels, and transferred to nitrocellulose before being probed with the unique genomic *aprt* fragment. Most of the mutant strains analyzed showed no alteration of the pattern of *aprt* gene fragments in various digests. A few showed alterations in one digest consistent with a mutation causing loss of a restriction endonuclease site. In this manner, nine mutations induced by excess dTTP and seven induced by excess dCTP in the Thy$^-$ mutator strain were mapped (Fig. 1).

To analyze these events at the nucleotide level, mutant genes in which alterations had been mapped were cloned from genomic libraries produced in the λ phage vector NM1149. The regions bearing the mutations were subcloned into M13 and sequenced. The results of these analyses are presented below.

**Simple Base-Pair Substitutions Induced by Excess dTTP.** The most frequent event detected among the mutations induced by excess dTTP was simple base-pair substitution (Fig. 2). As a result of multiple mutations occurring in some

FIG. 2. Base-pair substitutions at the *aprt* locus induced by excess thymidine. The nucleotide substitutions identified at each strain are boxed. Those that alter restriction endonuclease sites are indicated. Numbering of nucleotides represents the positions of those nucleotides within the 4.0-kb *Bam*HI *aprt* fragment.

```
          580        590        600        610
TTTCCCCGGCCACCAGCCTCTCCTTGTTCCCAGGGATATCTCGCCCCTCC    w.t.
:::::::::::::::::::::  :::::  ::::::::: ::: :  ::
TTTCCCCGGCCACCAGCCTCTTTTTGTTTTCAGGGATATTTCGTCTTTCT    dT40

620        630        640        650        660
TGAAGGACCCCGCCTCCTTCCGAGCTTCCATCCGCCTCCTGGCCAGTCAC    w.t.
:::::::  : : :: ::: ::::::::::  :: :  :::  ::: :
TGAAGGATTCTGTCTTCTTTCGAGCTTCCATTTGCTTTTTGGTTAGTTAT    dT40

670        680        690        700        710
CTTAAGTCCACGCATGGCGGCAAGATCGACTACATCGCAGGCGAGTGGCC    w.t.
 ::::::::::::::::::::::::::::::::::::::::::::::::::
TTTAAGTCCACGCATGGCGGCAAGATCGACTACATCGCAGGCGAGTGGCC    dT40
```

FIG. 3. Complex nucleotide substitution at the *aprt* locus occurring in mutant dT40. The sequence in dT40 is aligned with the corresponding wild-type (w.t.) sequence. Numbering of nucleotides represents the positions of these nucleotides within the 4.0-kb *Bam*HI fragment.

mutant genes, 12 of these events were characterized in the aprt$^-$ strains having altered restriction endonuclease sites. Ten of these were C·G→T·A transitions. G·C→T·A transversions were responsible for the remaining two. dT4 had two C·G→T·A transition mutations separated by 2 bp. dT45 also had two mutations separated by 5 bp, a C·G→T·A transition, eliminating the *Hin*dIII site, and a G·C→T·A transversion.

Three of the mutations produce missense mutations while four others result in modification of consensus splice junctions. The remaining four occur in either intron (the mutation in dT57 at nucleotide 1461) or downstream noncoding sequence (dT58, a nucleotide 2434, and the two alterations in dT45). In two of these strains, an additional base-pair substitution was detected in coding (dT58 at position 670) or consensus splicing (dT57 at nucleotide 1975) sequences some distance from the mutation localized by the altered restriction endonuclease site. No further alterations were detected in ≈1000 bp sequenced from the mutant gene of dT45, although we have not pursued this rigorously by sequencing the entire 4.0-kb *aprt* locus. In view of the multiple alterations occurring in the thymidine-induced mutants, ≈500 additional bp were sequenced in each of the previously described mutants to determine whether other cryptic substitutions were present. However, no further changes were detected.

The nucleotides surrounding those altered in the mutant genes showed some bias. In 7 of the 10 transitions, thymine was 3′ to the misincorporated TMP on the newly synthesized strand. The remaining three had guanine, adenine, or cytosine. Of nucleotides 5′ to the one misincorporated, five were cytosine, four were guanine (which is also present in excess), and one was adenine.

**A Complex Mutation Induced by Excess dTTP.** Fine structure restriction endonuclease mapping of the mutant *aprt* gene in dT40 suggested that multiple mutations had occurred in the G+C-rich 5′ portion of the gene leading to two alterations of restriction endonuclease sites (21). Sequence analysis of the cloned mutant gene clearly confirmed this mapping (Fig. 3). TMP is substituted for cytosine at a number of sites in register with the template to produce runs of thymine where previously mixed stretches of thymine and cytosine were present. This is particularly evident in sites where the cytosine residues are surrounded by thymine residues. This high frequency of substitution (25 C·G→T·A substitutions) covered an 80-bp region of *aprt*, although other G+C-rich regions were not affected.

**Mutations Induced by Excess dCTP in a Thy$^-$ Mutator**

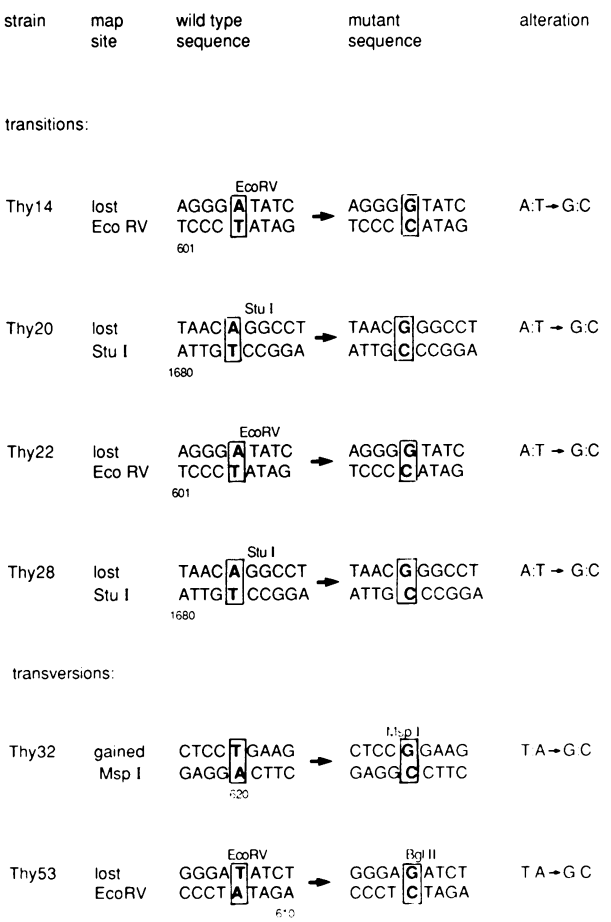


FIG. 4. Base-pair substitutions at the *aprt* locus occurring in the Thy$^-$-2-1 mutator strain of CHO cells. The alterations detected are boxed and the altered restriction endonuclease sites are indicated, as is the nucleotide position of the mutational event.

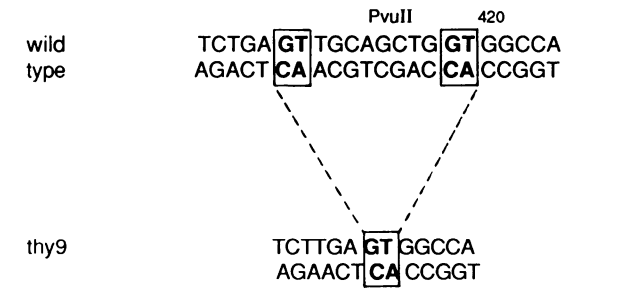


FIG. 5. Small deletion mutation occurring in aprt$^-$ mutant Thy-9. Wild-type and mutant sequences are indicated, as is the site of the mutation. The very short direct repeats bounding the deleted nucleotides are boxed.

**Strain.** Of seven mutant *aprt* genes analyzed from Thy$^-$ strains, four were T·A→C·G transitions (Fig. 4). Two of these, Thy-14 and -22, occurred at the *Eco*RV site at nucleotide position 605, while the other two, Thy-20 and -28, eliminated the *Stu* I site through a mutation at nucleotide 1684. Both occurred in runs of cytosines, but in the case of Thy-14 and -22 the cytosines were 3′ to the one altered, while in Thy-20 and -28 the cytosines were 5′ to the mutation. Two transversions were characterized, both A·T→C·G (Fig. 4). One of these, Thy-53, occurred in the same *Eco*RV site as Thy-14 and -22. The transversion in Thy-53 also produced a novel *Bgl* II site, which was confirmed by Southern blotting (data not shown). Thy-9 was different from the other *aprt*$^-$ mutations produced in the Thy$^-$ background in that it was a small 10-bp deletion eliminating the *Pvu* II site (Fig. 5). The deleted nucleotides are flanked on both sides by 2-bp repeats, only one copy of which is retained in the mutant allele. No further alterations were found in ≈500 bp sequenced in each of the mutant genes. Four of the mutations result in missense mutations, and consensus splice sequences were altered in the remaining two.

## DISCUSSION

Our data show that the predominant type of mutation induced by dCTP or dTTP pool excess is simple base-pair substitution, apparently "driven" by the nucleotide in excess. The substitutions occurring in the presence of excess dCTP in the Thy$^-$ mutator background are the reverse of those induced by excess dTTP for both transitions and transversions. In a collection of spontaneous mutations (isolated by growth conditions producing intermediate pool levels) a mix of base-pair substitutions was observed (Table 1). Neither type of pool imbalance produced the large deletions or insertions observed in the spontaneous collection. Multiple mutations, on the other hand, were frequent among the thymidine-induced mutants, probably as a consequence of both the large pool imbalance of dTTP over dCTP and the retardation of the replication fork caused by the depletion of dCTP (30). These multiple mutations are particularly evident in dT40 where 25 C·G→T·A substitutions were found in an 80-bp stretch in the G+C-rich 5′ coding region of *aprt*.

Our results further indicate that local sequence context influences the positions at which the base-pair substitutions caused by DNA precursor pool imbalances occur. Even in our analyses limited to mutations at restriction endonuclease sites, the nucleotide in excess is present 3′ to the one misincorporated in well over half the transition mutations characterized. In the case of mutations induced by excess dTTP, 7 of the 10 transitions had a thymine 3′ to the misincorporated thymine. If the events were simply random, one would expect a much lower figure as only about one-third of potential cytosine residue targets are flanked by a 3′ thymine. Next-nucleotide effects are a predicted outcome of a competition between the polymerization and proofreading activities of replication complexes (32). *In vitro* analyses support this prediction since, in the presence of an excess of a nucleotide 3′ to the one misincorporated, polymerization of the next nucleotide is favored rather than excision of the incorrect one, thereby sealing in the error (13). As next-nucleotide effects are characteristic of polymerases with 3′→5′ exonuclease proofreading functions, our observations suggest that a proofreading activity is part of the mammalian replication complex.

Biochemical evidence for proofreading functions in mammalian cells is limited. No 3′→5′ exonuclease was found to be associated with the replicative polymerase α complexes recovered by immunoaffinity chromatography (33, 34). However, an exonuclease cosedimenting with polymerase α was recently reported (19), reminiscent of the associated proofreading subunit of the *E. coli* pol III (35). Another form of DNA polymerase appears to have a 3′→5′ exonuclease residing on the same protein (18).

Next-nucleotide effects could also provide an explanation for the locus specificity of the Thy$^-$ mutator gene. Some target loci coding for essential gene products, such as the emetine drug-resistance locus, which encodes the 40S ribosomal subunit protein S20 (36), or the ouabain-resistance locus, which encodes the $Na^+,K^+$-dependent ATPase (37), may have limited numbers of target sites since total inactivation of the protein leads to cell death. Consequently, mutational rates may be either greatly enhanced or even decreased, depending on the nucleotides surrounding the targets.

Transversions accounted for an unexpectedly high proportion of the mutations. These events are also detected by *in vitro* systems, although at a much lower frequency relative to transitions (38). Since the transversion pathway stimulated by dTTP is the opposite of that stimulated by high dCTP, it appears that the transversions are also promoted by misincorporation of the nucleotide in excess, even though such misincorporation would result in energetically unfavorable pyrimidine–pyrimidine interactions. One explanation is that these events are the result of misincorporation of the nucleotide in excess at apurinic sites. However, it is also possible that any mismatch repair in mammalian cells simply functions less efficiently on the pyrimidine–pyrimidine mispairs and, as a result, the proportion of transversions is increased (39).

Two of the mutations were notably unusual: the small deletion in Thy-9 and the multiple base-pair substitutions in dT40. The deletion in Thy-9 strongly resembles those occurring spontaneously—i.e., there are two short direct repeats, one of which is retained in the mutant allele (25). This deletion may well have been of spontaneous origin since mutational rates are increased only 7- to 10-fold at the *aprt* locus by the thy background.

The mutation in dT40, on the other hand, is quite revealing. In a G+C-rich region of *aprt*, dTMP is frequently misincorporated in place of dCMP in register with the template, particularly in stretches of thymine interspersed with cytosine. It is remarkable that the large dTTP/dCTP pool imbalance produces such a dramatic breakdown of the ability of the polymerization complex to discriminate between the correct and mispaired nucleotides. An intriguing possibility is that the large pool imbalance causes transient dissociation of a proofreading subunit from the replication complex, resulting in long runs of misincorporated dTMP. A precedent for this suggestion is the observation that the altered proofread-

Table 1. Comparison of mutations occurring spontaneously or in the presence of deoxyribonucleotide triphosphate pool imbalance

| | Mutants in collection | Base-pair alterations determined | Transitions | | Transversions | | | Rearrangements | |
|---|---|---|---|---|---|---|---|---|---|
| | | | A·T→G·C | G·C→A·T | G·C→T·A | A·T→C·G | Other | <25 bp | >25 bp |
| Spontaneous* | 120 | 18 | 3 | 3 | 1 | 0 | 1 | 4 | 8 |
| Thymidine-induced | 58 | 12 | 0 | 10 | 2 | 0 | 0 | 0 | 0 |
| Thy$^-$ mutator | 45 | 7 | 4 | 0 | 0 | 2 | 0 | 1 | 0 |

*The properties of these mutants have been published (25, 31).

ing subunit of *MutD* strains of *E. coli* is acutely sensitive to fluctuations of thymine (and most likely, therefore, dTTP; ref. 40). Whether this is due to promoting dissociation at the altered subunit or some other modification has not been explored.

Clearly these long stretches of mismatches in mutant genes, if attacked by single-stranded endonucleases, could lead to double-strand breaks and the chromosome abnormalities that have been reported for cells treated with high levels of thymidine. The accumulation of these gross chromosomal aberrations combined with the multiple mutations at essential loci might also explain the extensive lethality of this agent.


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