DNA base sequence changes and sequence specificity of bromodeoxyuridine-induced mutations in mammalian cells

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ABSTRACT By using a shuttle vector system developed in our laboratory, we have carried out studies on the molecular mechanism by which 5-bromodeoxyuridine (BrdUrd) induces mutations in mammalian cells. The target for mutagenesis in these studies was the Escherichia coli gpt gene that was contained within a retroviral shuttle vector and integrated into chromosomal DNA in mouse A9 cells. Shuttle vector-transformed cells expressing the gpt gene were grown with BrdUrd and cells with mutations in the gpt gene were selected. Shuttle vector sequences were recovered from the mutant cells, and the base sequence of the mutant gpt genes was determined. The great majority of the BrdUrd-induced mutations involving single-base changes were found to be G-C -> A-T transitions. We have shown that mutagenesis by BrdUrd depends upon perturbation of deoxycytidine metabolism. Thus, the current results suggest that BrdUrd mutagenesis involves mispairing and misincorporation of BrdUrd opposite guanine in DNA, driven by nucleotide pool perturbation caused by BrdUrd and the resulting imbalanced supply of triphosphates available for DNA synthesis. The results also revealed a high degree of sequence specificity for the BrdUrd mutagenesis. BrdUrd-induced G-C -> A-T transitions occurred almost exclusively in sequences with two adjacent guanine residues. Furthermore, in ~90% of the cases, the guanine residue involved in mutation was the one in the more 3' position.

Our laboratory has shown that mutagenesis by the thymidine (dThd) analog 5-bromodeoxyuridine (BrdUrd) in mammalian cells involves perturbation of deoxyribonucleotide metabolism (1-3). These studies showed that BrdUrd mutagenesis in mammalian cells is (i) determined by the concentration of exogenous BrdUrd to which cells are exposed and not by the amount of BrdUrd incorporated into DNA, (ii) suppressed by exogenous deoxycytidine, and (iii) quantitatively related to the ratio of BrdUrd to deoxycytidine in the intracellular pools. Thus, we have suggested that BrdUrd mutagenesis in mammalian cells is dependent upon the perturbation of endogenous deoxycytidine metabolism, that mutations could arise from misincorporation of BrdUrd into DNA, driven by the unbalanced deoxyribonucleoside triphosphate (dNTP) pools available for DNA synthesis, and that mutagenesis under such conditions would result primarily in G-C -> A-T transitions (4-6).

We have studied the molecular mechanisms of BrdUrd mutagenesis in mammalian cells, by using a retroviral shuttle vector system developed in our laboratory (7). As the target for mutations, the shuttle vector contains the Escherichia coli gpt gene, coding for the enzyme xanthine guanine phosphoribosyltransferase (5-phospho-alpha-D-ribose-1-diphosphate: xanthine phosphoribosyltransferase; EC 2.4.2.22). The shuttle vector was introduced into mouse A9 cells and a transformed line (A9I-2) was isolated that contains a single copy of the vector integrated into chromosomal DNA. Since A9 cells are deficient in the enzyme hypoxanthine guanine phosphoribosyltransferase (IMP-pyrophosphate phosphoribosyltransferase; EC 2.4.2.8) (8), transformants with mutations in the gpt gene can be selected with 6-thioguanine (sGua). For sequencing, the gpt genes from mutant A9I-2 cells can be recovered by fusion with monkey COS cells (9).

In the present study, the gpt genes were recovered and sequenced from a large number of BrdUrd-induced mutants of A9I-2 cells. We have used this shuttle vector system to analyze mutations that occurred spontaneously or were induced by ethyl methanesulfonate (EtMes) (10, 11). Mutational studies with various shuttle vector systems also have been carried out by other laboratories (12-17). Our system differs from those used in the other studies in that the vector in our system is integrated into chromosomal DNA and clonal selection for cells with mutant genes takes place while the vector is still present in chromosomal DNA.

MATERIALS AND METHODS Shuttle Vector. The construction of the shuttle vector pZipGptNeo has been described (7). The vector was constructed by the insertion of the E. coli gpt gene into the retroviral shuttle vector pZipNeoSV(X), which contains the viral long terminal repeats, the simian virus 40 origin of replication, the pBR322 origin of replication, and the neomycin-resistance gene from Tn5 (18).

Medium and Cells. The basic cell culture medium was Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum. HAT medium is DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM dThd (19). G418 medium is supplemented with G418 at 1 mg/ml (20). sGua was used at a concentration of 18 μM to select for sGua-resistant (sGua') cells.

The derivation and properties of the A9I-2 cell line have been described (7). The expression of the gpt gene enables the cells to grow in HAT medium and renders them sensitive to sGua. The expression of the neomycin-resistance genes enables the cells to grow in G418 medium and also provides a kanamycin resistance marker for selection of bacterial transformants.

Induction and Selection of Mutants. A9I-2 stock cultures were maintained continuously in HAT medium. Prior to BrdUrd treatment, the cells were cultured in DMEM containing 100 μM hypoxanthine and 16 μM dThd (HT medium) for 3 days to ensure the removal of residual aminopterin.

Abbreviations: BrdUrd, 5-bromodeoxyuridine; dThd, thymidine; FdUrd, 5-fluorodeoxyuridine; sGua, 6-thioguanine; EtMes, ethyl methanesulfonate; sGua', sGua resistance(t).

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cells then were trypsinized and 150-mm dishes were inoculated with $3 \times 10^4$ cells in DMEM plus the appropriate drugs for mutagenesis. The cells were exposed to BrdUrd in the presence of dThd and 5-fluorodeoxyuridine (FdUrd), an inhibitor of de novo dThd biosynthesis. Typically the cells were exposed to 10 $\mu$M FdUrd plus BrdUrd and dThd at a molar ratio of 3:2, so that $\approx 60\%$ of the dThd residues in newly replicated DNA were replaced by BrdUrd (21). BrdUrd was used at concentrations from 30 to 300 $\mu$M, and with each experiment involving several series of dishes at various BrdUrd concentrations. Control cultures contained FdUrd and dThd alone, with the dThd concentration ranging from the lowest to the highest total pyrimidine concentration in the dishes containing BrdUrd.

After a 4-day exposure to BrdUrd, cells were cultured in HT medium (to ensure removal of residual FdUrd) supplemented with G418 at 1 mg/ml (to prevent growth of cells that had lost expression of the neomycin-resistance gene in the vector). As necessary, cells in each series were harvested and subcultured in medium containing G418, to allow a total of 10 days for expression of mutations in the gpt gene prior to selection of mutants.

To select for Gpt$^+$ mutants, cells in each series were harvested and three 100-mm dishes were inoculated with $10^5$ cells in DMEM plus sGua. To determine the plating efficiency in the absence of selection, three 100-mm dishes were inoculated in parallel with 100 cells in DMEM. After 10–14 days, colonies of sGua$^+$ cells were isolated and maintained in the presence of sGua. The dishes then were stained and colonies were counted. In general, only one sGua$^+$ colony was isolated from a given series. When two colonies were isolated from the same series, if the sequencing data were the same for the two mutants, the isolates were considered to be siblings and one of the mutants was excluded from the analysis.

The cells were shielded at all times from exposure to wavelengths of light below 550 nm, as described (22).

**Recovery and Analysis of Vector DNA.** To recover the vector sequences from mutant cells, sGua$^-$ cells were fused with COS cells as described (7). Low molecular weight DNA was extracted and used to transform E. coli strains DH-1 or DH-5, and kanamycin-resistant transformants were selected (7). The structure of the recovered plasmid molecules was determined with small-scale plasmid preparations from 2-ml overnight cultures of the kanamycin-resistant transformants, as described (7).

**DNA Sequencing.** Plasmid DNA for sequencing was extracted from 15-ml overnight cultures by the alkaline lysis method (23), treated with RNase A, extracted with phenol chloroform, and concentrated by ethanol precipitation. Di-deoxy sequencing reactions were carried out at 45°C with $\approx 1 \mu$g of plasmid DNA and avian myeloblastosis virus reverse transcriptase. A series of four oligonucleotide primers located on the 5' side of and within the gpt gene was used for sequencing. Wedge shaped 8% polyacrylamide sequencing gels containing 8 M urea were used to reliably resolve 150–200 bases.

**RESULTS**

**Induction of Mutants.** BrdUrd-induced sGua$^-$ mutants of A9I-2 cells were isolated from 11 experiments, comprising a total of 61 independent series of mutagenized cultures. Mutants were isolated from cultures treated with BrdUrd at concentrations from 30 to 300 $\mu$M. For these experiments, the average frequency of mutants induced by BrdUrd was $1.6 \times 10^4$, and the average increase above the spontaneous background was 6.5-fold.

Table 1 shows the results of a typical mutagenesis experiment. After treatment with 60 $\mu$M BrdUrd, there was an increase in mutant frequency of $\approx 5$-fold above background. In this experiment there appeared to be a tendency for a further increase in mutant frequency as the BrdUrd concentration increased. However, this increase did not occur in all experiments.

A total of 32 independent sGua$^-$ A9I-2 mutants were isolated from the mutagenesis experiments. Plasmid was recovered from all of the 32 mutants, and the plasmids (six from each mutant line) were analyzed by Kpn I restriction digestion. As observed (7), $\approx 50\%$ of the recovered plasmids exhibited a three-band Kpn I restriction pattern (3.5-, 0.9-, 0.7-kilobase bands), which is indicative of the presence of an intact gpt gene. Plasmids with this pattern were isolated from all 32 mutants and used for DNA sequencing. (The other 50% of the recovered plasmids exhibited a variety of restriction patterns.)

**DNA Sequence Analysis.** The gpt genes recovered from all 32 BrdUrd-induced mutants were sequenced in their entirety and compared with the wild-type gpt sequence (24). Among the genes recovered from the 32 mutants, there were single-base changes and deletions. No mutant gene showed more than one change in the DNA sequence.

The great majority of the mutant genes sequenced (27 of 32 genes) exhibited single-base changes. However, five mutants contained deletions of 2, 3, 3, 12, and 269 base pairs (bp). These deletion mutations may primarily represent spontaneous background mutation. Since BrdUrd in these experiments caused an average increase in mutant frequency of $\approx 6.5$-fold above background, $\approx 15\%$ of the mutants isolated should represent the spontaneous background. Thus, among the 32 sGua$^-$ mutants isolated in this study, the number of spontaneous mutations should be $\approx$five. In the previous study on spontaneous mutations in the gpt gene in A9I-2 cells (10), it was observed that $\approx 70\%$ of the spontaneous mutations (29 of 43 mutations) involved deletions. Thus, among the five spontaneous mutations expected in the current study, there should be 3 or 4 deletion mutations. This is close to the number of deletion mutations actually observed, i.e., five. Furthermore, three of the five deletion mutations observed in this study had deletions of <10 bp, similar to the majority of deletion mutations observed in the study on spontaneous mutations (10). In addition, many of the deletion mutations in these studies were at a 3-bp deletion 'hotspot'. Among the deletion mutations observed in the current study, two of the five mutations involved that same 3-bp deletion and the 12-bp deletion spanned that deletion. All these considerations strongly suggest that most if not all of the five deletion mutations obtained in the present study arose spontaneously rather than being induced by BrdUrd.

<table>
<thead>
<tr>
<th>BrdUrd, $\mu$M</th>
<th>Mutant frequency</th>
<th>Plating efficiency, %</th>
<th>Corrected mutant frequency</th>
<th>Increase above background</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.65</td>
<td>35</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>5.3</td>
<td>53</td>
<td>10</td>
<td>5.3</td>
</tr>
<tr>
<td>120</td>
<td>4.7</td>
<td>36</td>
<td>13</td>
<td>6.8</td>
</tr>
<tr>
<td>180</td>
<td>2.0</td>
<td>28</td>
<td>7.1</td>
<td>3.7</td>
</tr>
<tr>
<td>300</td>
<td>8.7</td>
<td>44</td>
<td>20</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Table 1. Induction of mutants by BrdUrd
The base changes, numbers of independent isolates, positions, and codon changes are indicated for all of the BrdUrd-induced mutants with single-base changes. The first base of the gpt coding sequence is considered as position number 1.

In the previous study on spontaneous mutations (10), ~25% (11 of 43 mutations) of the mutations exhibited single-base changes. Given the five deletion mutations obtained in the present study and the ratio of deletion mutations to single-base change mutations observed among the spontaneous mutations in the previous study, the spontaneous background in the present study should not contribute more than 1 or 2 mutants with single-base changes. Thus, essentially all the mutants with single-base changes isolated after BrdUrd treatment can be considered as resulting from BrdUrd mutagenesis.

The DNA sequence changes for the BrdUrd-induced mutations with single-base changes are presented in Table 2. Of the 27 mutations with single-base changes, 22 were G→A transitions, 4 were A→T→G→C transitions, and 1 was an A→T C→T A transversion. Of the 27 single-base changes, 12 of them (45%) involved a G→A transition at position 92. There were three other positions at which two mutations were observed at each position: two involved G→A/T transitions (positions 91 and 402) and one involved an A→T→G→C transition (position 419). All other mutations were represented only once.

The distribution of the single-base change mutations induced at the various BrdUrd concentrations is presented in Table 3. The results indicate that the various types of base changes were induced at all the various BrdUrd concentrations. There is no apparent specificity for a given base change being induced at a particular concentration of BrdUrd.

The DNA base sequences surrounding the various G→A/T transition mutations were examined and these sequences are presented in Table 4. Based on our working hypothesis that these mutations occurred as the result of BrdUrd mispairing with a guanine residue in DNA, the table presents the DNA strand containing the guanine residue presumably involved in mispairing with BrdUrd. This analysis reveals a high degree of sequence specificity for BrdUrd-induced G→A/T transitions. When all the G→A/T transition mutations are considered, ~95% occurred in a sequence with two adjacent guanine residues. Only 1 out of 22 mutants had a mutation at the site of a single guanine residue. Even when considering only different sites of mutation (the main effect of which is to reduce the impact of the hotspot mutants), ~90% of the mutation sites involved the sequence 5' GNN 3', where N is any nucleotide. Thus, almost all guanine residues presumably involved in mispairing with BrdUrd appear to be adjacent to another guanine residue. The results also indicate a high degree of specificity for which guanine residue in a GG doublet is involved in the mispairing with BrdUrd. Of the 21 total mutations in which BrdUrd mispairing occurred within a GG doublet, the 3' guanine residue was the one involved in mispairing ~90% of the time. Considering only the different sites of mutation, the 3' guanine residue was the one involved in mispairing >85% of the time.

### Table 2. BrdUrd-induced base substitution mutations

<table>
<thead>
<tr>
<th>Base change</th>
<th>Number of isolates</th>
<th>Position</th>
<th>Codon change</th>
</tr>
</thead>
<tbody>
<tr>
<td>G→C → A→T</td>
<td>2</td>
<td>91</td>
<td>GGC → AGC</td>
</tr>
<tr>
<td>G→C → A→T</td>
<td>12</td>
<td>92</td>
<td>GGC → GAC</td>
</tr>
<tr>
<td>G→C → A→T</td>
<td>1</td>
<td>116</td>
<td>GGU → GAU</td>
</tr>
<tr>
<td>G→C → A→T</td>
<td>1</td>
<td>128</td>
<td>GGU → GAU</td>
</tr>
<tr>
<td>G→C → A→T</td>
<td>1</td>
<td>170</td>
<td>ACC → AUC</td>
</tr>
<tr>
<td>G→C → A→T</td>
<td>1</td>
<td>262</td>
<td>GAU → AAU</td>
</tr>
<tr>
<td>G→C → A→T</td>
<td>1</td>
<td>278</td>
<td>ACC → AUC</td>
</tr>
<tr>
<td>G→C → A→T</td>
<td>2</td>
<td>402</td>
<td>UGG → UGA</td>
</tr>
<tr>
<td>G→C → A→T</td>
<td>1</td>
<td>412</td>
<td>CCG → UCG</td>
</tr>
<tr>
<td>A→T → G→C</td>
<td>1</td>
<td>56</td>
<td>CUC → CCC</td>
</tr>
<tr>
<td>A→T → G→C</td>
<td>1</td>
<td>269</td>
<td>CUG → CCG</td>
</tr>
<tr>
<td>A→T → G→C</td>
<td>1</td>
<td>419</td>
<td>GAU → GGU</td>
</tr>
<tr>
<td>A→T → T→A</td>
<td>1</td>
<td>119</td>
<td>CUG → CAG</td>
</tr>
</tbody>
</table>

The base changes, numbers of independent isolates, and codon changes are indicated for all of the BrdUrd-induced mutants with single-base changes. The first base of the gpt coding sequence is considered as position number 1.

### Table 4. Sequence specificity for BrdUrd-induced G→A/T transitions

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA GGC ATT</td>
<td>92</td>
<td>12</td>
</tr>
<tr>
<td>AAA GGC ATT</td>
<td>91</td>
<td>2</td>
</tr>
<tr>
<td>ACC TGG ATT</td>
<td>402</td>
<td>2</td>
</tr>
<tr>
<td>GGC GGT CGT</td>
<td>116</td>
<td>1</td>
</tr>
<tr>
<td>CCG GGT GCG</td>
<td>128</td>
<td>1</td>
</tr>
<tr>
<td>AAC GGT ATC</td>
<td>170</td>
<td>1</td>
</tr>
<tr>
<td>ATT GAT GAC</td>
<td>262</td>
<td>1</td>
</tr>
<tr>
<td>ACC GGT ATC</td>
<td>278</td>
<td>1</td>
</tr>
<tr>
<td>CCA CGT CTG</td>
<td>412</td>
<td>1</td>
</tr>
</tbody>
</table>

The wild-type base sequences surrounding the various BrdUrd-induced G→A/T transitions are presented. The sequence is presented for the guanine containing DNA strand presumed to be the template for BrdUrd mispairing. The boldface guanine residue indicates the position at which the mutation occurred. All sequences are written in the 5' → 3' direction for that particular strand.

### DISCUSSION

The present studies were undertaken to elucidate the molecular mechanisms by which BrdUrd induces mutations in mammalian cells. By using the shuttle vector system developed in our laboratory (7), the gpt genes were recovered and sequenced from 32 independently isolated sGua' A91-2 mutants induced by BrdUrd. Among the 27 mutations with single-base changes, >80% had G→A/T transitions, ~15% had A→T→G→C transitions, and <5% had transversions. Over the range of BrdUrd concentrations tested, G→A/T transitions predominated at all concentrations. These results are in agreement with the prediction based on our studies on BrdUrd mutagenesis and nucleotide pool perturbation (5).

Among the G→A/T transitions, there was a strong hotspot at position 92. However, mutations involving single-base changes were observed at 12 other sites in the gene, and the mutations at ~70% of those sites also involved G→A/T transitions. Thus, even without considering the occurrence of multiple mutations at certain sites, G→A/T transitions appear to be the major alteration induced by BrdUrd.

Our results suggest the following mechanism for BrdUrd mutagenesis in mammalian cells. The first step in mutagenesis would be the induction of a high BrdUTP/dCTP molar ratio (3, 4), due to the inhibition by BrdUTP of the ribonucleotide reductase catalyzed reduction of CDP to dCDP (25).
The next step would be the mispairing and misincorporation of BrdUrd opposite guanine residues in DNA, due to the large excess of BrdUTP and the lack of dCTP, the correct nucleotide. Thus, BrdUrd-induced G-C → A-T transitions would be due to base mispairing driven by nucleotide pool perturbation caused by BrdUrd and the resulting imbalanced supply of triphosphates available for DNA synthesis. This mechanism is consistent with the observation that imbalances in the availability of dNTPs can decrease the fidelity of DNA replication in vitro (26).

The results of the present study cannot be explained solely on the basis of BrdUrd having a tendency to mispair with guanine residues in DNA (cf. ref. 27), since our results clearly demonstrated (1-4) that BrdUrd is a mutagen only when nucleotide pool perturbation occurs. On the other hand, the results cannot be explained solely on the basis of base misincorporation driven by nucleotide pool perturbation, since we have also shown that dThd by itself can produce imbalanced pool levels like BrdUrd but is only weakly mutagenic (3, 21).

A small fraction of the BrdUrd-induced mutants in the present study were observed to have A-T → G-C transitions. These mutations also can be explained by a mechanism involving base mispairing driven by pool perturbation. There are two modes for BrdUrd mutagenesis in mammalian cells and these two modes can produce reciprocal genetic events, as judged by reversion analysis (6, 28). The protocol for mutagenesis used in the current study favors the induction of mutations during the incorporation of BrdUrd into DNA, with mutagenesis dependent upon the ratio of BrdUTP to dCTP in the intracellular pools. This is referred to as incorporation mutagenesis. An alternative protocol favors the incorporation of mutations during the replication of BrdUrd previously incorporated into DNA, with mutagenesis in this case apparently dependent upon the establishment of a high ratio of dGTP to dATP. This is referred to as replication mutagenesis. Although the conditions used in the present study should favor incorporation mutagenesis, replication mutagenesis would not be entirely excluded. Previous results from this laboratory showed (4) that the conditions of incorporation mutagenesis result in an increase in the dGTP/dATP molar ratio. However, this ratio apparently increases to a much smaller extent during incorporation mutagenesis than replication mutagenesis (6). Thus, the occurrence of a small number of A-T → G-C transition mutations in the present study could be due to a low level of replication mutagenesis, and these mutations could be explained by the following mechanism. After the establishment of an increased dGTP/dATP ratio (due to allosteric effects of BrdUrd on the enzyme ribonucleotide reductase), mispairing and misincorporation of guanine opposite BrdUrd residues in DNA would result from the unbalanced supply of the triphosphates available for DNA synthesis. It is of interest to note that sequence analysis of mutations induced by bromouracil in λ phage revealed a predominance of A-T → G-C transitions (29). This suggests that bromouracil mutagenesis in λ phage (and E. coli) primarily involves replication of BrdUrd-containing DNA, rather than incorporation of BrdUrd into DNA, in contrast to the results of the present study. This may reflect differences in nucleotide pool regulation between mammalian and prokaryotic systems.

It is informative to compare the single-base change mutations induced by BrdUrd in the present study with those observed in our previous studies on spontaneous and EtMes-induced mutations in the same A91-2 cells (10, 11). In the study on spontaneous mutations, mutations involving single-base changes were divided approximately equally between transitions and transversions (10). In the study on EtMes-induced mutations, all the mutations involving single-base changes were G-C → A-T transitions (11). However, the mutations induced by EtMes did not exhibit the G-C → A-T transition hotspot observed at position 92 in the BrdUrd-induced mutants. The comparison of the results from these studies suggests that the mutations involving single-base changes resulted from three mechanisms for spontaneous, EtMes-induced, and BrdUrd-induced mutagenesis. The spectra of mutations observed in these studies also indicate that the mutations identified through the use of our shuttle vector system reflect the mutagen treatment applied and are not artifacts of the recovery of the gpt gene from the mammalian cells.

The analysis of the DNA sequence in the region of the BrdUrd mutagenesis hotspot (at position 92) has not revealed any features to explain its very frequent occurrence. However, there are two features shared by almost every BrdUrd-induced mutation with a G-C → A-T transition observed in the present study (including the hotspot mutants). There was a high degree of sequence specificity for BrdUrd-induced G-C → A-T transitions, as ≈95% of the mutations occurred in a sequence containing 2 adjacent guanine residues. In addition, there was a high degree of specificity as to which of the two guanine residues in the sequence was involved in mispairing with BrdUrd. In ≈90% of the cases in which BrdUrd mispaired occurring within the sequence NGGN, the 3' guanine residue represented the position at which the mutation occurred. Thus, BrdUrd mispairing in this sequence would appear to be highly nonrandom, as mutations should occur at equal frequencies at both positions if BrdUrd mispaired randomly with guanine residues within the sequence NGGN. These results may be slightly skewed because of the degeneracy of the genetic code. For two mutations (involving positions 170 and 278), if the mutations had involved the more 5' guanine residue and resulted in a change in the more 3' cytosine residue in the codon as read (ACC), there would have been no mutation detected because no amino acid change would have occurred. However, for all other mutations involving alterations in the sequence NGGN, changes involving either guanine residue would have resulted in an amino acid change or a stop codon. Even if these two mutations are discounted because alterations at the adjacent guanine residue would have been nondetectable, the results still indicate a strong preference for mutations involving the more 3' guanine residue.

This observation of a strong preference for mutations involving the more 3' guanine residue in a G-G doublet was seen to hold true for the sequence containing the BrdUrd mutagenesis hotspot at position 92. In this case, alterations at both the 3' guanine residue (position 92) and the 5' guanine residue (position 91) lead to amino acid changes and the isolation of sGua' mutants. However, there were 6 times more mutations involving the 3' guanine residue than the 5' guanine residue. This is in agreement with the sequence preference observed when considering the BrdUrd-induced mutants altogether. Since mutations at both positions 91 and 92 clearly lead to the generation of sGua' cells, these results also argue strongly that the specificity observed is not an artifact of the degeneracy of the genetic code.

To assess more fully the sequence specificity for BrdUrd mutagenesis, an analysis of the potentially mutable guanine residues in the gpt gene was performed. There are 238 G-C pairs in the coding region of the gene. Of these, 162 sites are mutable by a G-C → A-T transition, such that an altered amino acid or nonsense codon would result. These mutable sites include 88 single guanine residues (out of 139 in the gene), 23 guanine residues at the 5' position and 34 at the 3' position of the sequence NGGN (out of 39 such sequences in the gene), and 5 at the 5' position, 7 at the middle position, and 5 at the 3' position of the sequence NGGGGN (out of seven such sequences). Thus, of the mutable guanine residues in the gpt gene, <20% occurred at the 3' position of the sequence.
NGGN. However, \(\approx 85\%\) of the G-C \(\rightarrow\) A-T transitions were seen at such positions. Clearly, the distribution of mutations, relative to the distribution of mutable sites, is highly nonrandom, and the sequence specificity observed cannot be explained by the lack of other available sites for mutagenesis.

The comparison of the BrdUrd-induced and EtMes-induced G-C \(\rightarrow\) A-T transitions that we have observed in the gpt gene is again informative. As shown in Table 4, 19 of 22 BrdUrd-induced transitions occurred at the 3\' guanine residue of the sequence NGGN. In contrast, only 10 of 25 EtMes-induced transitions occurred at such positions (11). The difference in sequence distribution with the two mutagens suggests that the sequence specificity observed with BrdUrd is not an artifact but instead reflects the mechanism by which BrdUrd induces mutations in mammalian cells. Although we did not observe the same sequence specificity for mutagenesis with EtMes as with BrdUrd, preferential mutagenesis at the 3\' guanine residue in the sequence NGGN has been observed with other alkylating agents in various systems (17, 30, 31).

The results of the present study support a mechanism of BrdUrd mutagenesis in which mutations arise as the result of base mispairing driven by BrdUrd-induced nucleotide pool perturbation. However, the results do not provide evidence to explain the sequence specificity observed for BrdUrd mutagenesis. It should be noted that the DNA templates containing the sequence NGGN are presented in the 5\' \(\rightarrow\) 3\' direction in Table 4. Replication of the complementary strand, in which BrdUrd misincorporation would occur, would proceed in the opposite direction (5\' \(\rightarrow\) 3\' for its own strand). Thus, in terms of the direction of DNA replication on the newly synthesized strand, it is the first of the two guanine residues to be replicated that is preferentially involved in BrdUrd mispairing leading to mutations.

It is possible that the mutations that are observed after BrdUrd mutagenesis in mammalian cells represent the overall result of DNA replication, which produces mutations by base mispairing, and a proofreading mechanism that removes incorrectly incorporated bases and incorporates the correct base. If such a proofreading mechanism were functioning in these cells, then the mutations that are observed are those that have escaped proofreading. It is conceivable that a mechanism of mutagenesis involving base mispairing and subsequent proofreading would contribute to the sequence specificity for BrdUrd mutagenesis observed in the present study. Further analysis could provide insight into not only mechanisms of chemical mutagenesis but also the question of proofreading in mammalian cells.

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