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Mutagenicity of 5-Formylcytosine, an Oxidation Product of 5-Methylcytosine, in DNA in Mammalian Cells[1]

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To examine the mutagenicity of 5-formylcytosine (5-fC), an oxidation product of 5-methylcytosine (5-mC), 5-fC was incorporated into predetermined sites of double-stranded shuttle vectors. The nucleotide sequences in which the modified base was incorporated were 5′-AFGCGT-3′ and 5′-ACGFGT-3′ (F represents 5-fC), the recognition site for the restriction enzyme *Mlu*I (5′-ACGCGT-3′). 5-fC was incorporated into the template strand of either the leading or lagging strand of DNA replication. The modified DNAs were transfected into simian COS-7 cells, and the DNAs replicated in the cells were recovered and analyzed after a second transfection into *Escherichia coli*. 5-fC weakly blocked DNA replication in mammalian cells. The 5-fC residues were mutagenic, with mutation frequencies in double-stranded vectors of 0.03–0.28%. The mutation spectrum of 5-fC was broad, and included targeted (5-fC→G, 5-fC→A, and 5-fC→T) and untargeted mutations. These results suggest that the oxidation of 5-mC results in mutations at and around the modified sites.

Key words: 5-formylcytosine, 5-methylcytosine, mutation, reactive oxygen species, replication block.

5-Methylcytosine (5-mC) is a minor component found in most eukaryotic DNA. In humans, 5% of all C residues are enzymatically methylated. This methylation of C occurs postreplicationally, primarily in CpG sequences (*1, 2*). It is involved in the regulation of gene expression (*3–6*) and the silencing of invading viral genomes (*7, 8*). Interestingly, CpG sequences are hot spots for mutations: the predominant mutation in the human p53 gene occurs in a CpG sequence (*9, 10*).

Oxidative DNA damage produced by reactive oxygen species (ROS) is an important source of mutations (*11*). Since ROS are generated during normal cellular respiration and oxygen metabolism, and also by some mutagens/carcinogens, oxidative DNA lesions seem to cause spontaneous mutations and to induce mutagenesis/carcinogenesis pathways that occur in organisms. We hypothesized that the oxidation of 5-mC may be important, because CpG sequences are hot spots for mutations and the oxidation of DNA is an important source of mutations.

We previously demonstrated that 5-formylcytosine (5-fC, Fig. 1A) is the major oxidation product of 5-mC when 5-methyl-2′-dC and DNA fragments containing 5-mC are aerobically treated with Fenton-type reagents (*12*). This damaged base is formed by the oxidation of the methyl group of 5-mC, and is an analog of 5-formyluracil (5-fU, Fig. 1B). Recently, we examined the mutational properties of 5-fU in mammalian cells, and found that 5-fU is weakly mutagenic and induces 5-fU→G and 5-fU→A transversions (*13*). This was the first report that clearly showed the induction of transversion mutations by an oxidized pyrimidine base in the DNA of mammalian cells. Thus, the mutagenic potential of an analogous 5-fC base in mammalian cells is of great interest.

To study the frequency and spectrum of the mutations induced by 5-fC in mammalian cells, we incorporated this oxidized base into unique, predetermined sites in double-stranded (ds) vectors. This base was incorporated into two different sequence contexts, and onto either the leading or lagging template strand. The mutational properties of 5-fC were investigated in simian COS-7 cells. We observed that (i) the 5-fC residue weakly blocked replication. We found that (ii) the 5-fC residue was mutagenic {the mutation frequency (MF) was 0.03–0.28%} in COS-7 cells. Moreover, we discovered that (iii) the mutation spectrum of 5-fC was broad and included targeted and untargeted mutations. Our results show that 5-fC has unique features as compared to those of the analog, 5-fU.

MATERIALS AND METHODS

General—COS-7 cells were from the RIKEN Cell Bank (Tsukuba). *Escherichia coli* strain DH5α cells {F^-, *φ80d lacZ*ΔM15 Δ (*lacZYA-argF*) U169, *endA*1, *recA*1, *hsdS*17 ($r_K^- m_K^+$), *deoR*, *thi*-1, *supE*44, λ^-, *gyrA*96, *relA*1} for $CaCl_2$ transformation were prepared according to the method described in the literature (*14*).

Oligodeoxyribonucleotide Synthesis—Oligodeoxyribonu-

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Abbreviations: 5-mC, 5-methylcytosine; 5-fC, 5-formylcytosine; 5-fU, 5-formyluracil; ds, double-stranded; MF, mutation frequency.

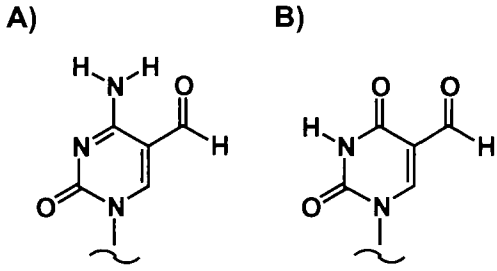

Fig. 1. **Structures of (A) 5-fC and (B) 5-fU.**

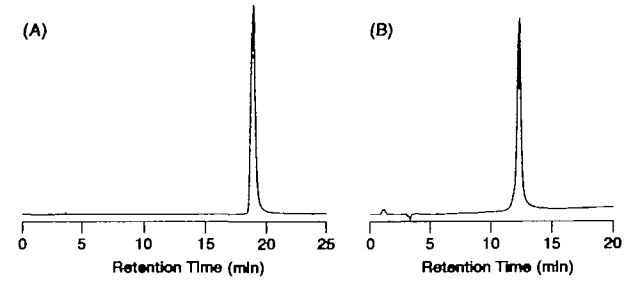

Fig. 2. **Elution profiles of an oligodeoxyribonucleotide containing 5-fC.** (A) Analysis by reverse-phase HPLC. (B) Analysis by anion-exchange HPLC. Elution conditions used were the following: (A) 0 to 25 min, linear gradient of acetonitrile (7.6 to 10.1%) in the presence of 50 mM triethylammonium acetate. (B) 0 to 20 min, linear gradient of ammonium formate (500 to 700 mM) in the presence of 20% acetonitrile.

cleotides containing 5-fC were synthesized by the solid phase phosphoramidite method as described previously (*15*), and were chemically phosphorylated on the support using the Chemical Phosphorylation Reagent 2 (Glen Research). The oligodeoxyribonucleotides synthesized were 5′-dTCTAGAFGCGTTAAC-3′ and 5′-dTCTAGACGFGTTAAC-3′, where F represents 5-fC. These oligodeoxyribonucleotides were synthesized as precursors containing 5-(1,2-dihydroxyethyl)cytosine, and were purified by reverse-phase column chromatography, and reverse-phase and anion-exchange HPLCs. After $NaIO_4$ treatment, the oligodeoxyribonucleotides containing 5-fC were further purified by reverse-phase and anion-exchange HPLCs. After purification, these oligodeoxyribonucleotides were eluted as a sharp single peak in both reverse-phase and anion-exchange HPLCs (Fig. 2 and data not shown). The presence of 5-fC was confirmed by the complete digestion of these oligodeoxyribonucleotides with snake venom phosphodiesterase and calf intestinal alkaline phosphatase, as described (*13, 16*). The unmodified oligodeoxyribonucleotides 5′-dTCTAGACGCGTTAAC-3′, 5′-dTCTAGAMGCGTTAAC-3′, and 5′-dTCTAGACGMGTTAAC-3′ for control experiments, where M represents 5-mC, and the splint 23mer (5′-dAATTGTTAACGCGTCTAGAGGCC-3′) were prepared as described (*17*). These unmodified oligodeoxyribonucleotides were also chemically phosphorylated on the support using the Chemical Phosphorylation Reagent 2, and were eluted as sharp single peaks in both reverse-phase and anion-exchange HPLCs (data not shown). Other oligodeoxyribonucleotides were purchased from Hokkaido System Science (Sapporo) in purified forms.

Construction of Vectors Containing 5-fC and DNA Transfection into COS-7 Cells—Ds vectors were constructed as described (*17*). The constructed ds vectors (4 ng) were transfected into the cultured COS-7 cells using Lipofectamine (Invitrogen), essentially as described previously (*18*). After 48 h, the plasmid amplified in COS-7 cells was recovered by the method of Stary and Sarasin (*19*). The recovered DNA was treated with *Dpn*I to digest the unreplicated plasmids. After the removal of the proteins by passage through a Micropure EZ device (Millipore), the DNA was purified by ethanol precipitation.

Calculation of the Cytotoxicity in COS-7 Cells—E. coli DH5α cells were transfected with the recovered plasmid by the calcium chloride method (*14*). To measure the cytotoxicity, a small portion of the recovered DNA was used. The numbers of bacterial colonies obtained were used to calculate the cytotoxicities in COS-7 cells, as described (*18*).

Mutant Screening and Sequencing—The plasmid recovered from the COS-7 cells was incubated with *Mlu*I under the conditions recommended by the supplier. The treated DNA was transfected into *E. coli* strain DH5α to obtain a "mutant" pool. Untreated DNA in the same buffer solution was also transfected, and the ratio of (colonies obtained with treated DNA) to (colonies obtained with untreated DNA) was calculated. This value (defined as A) was used to calculate the MF, as described below.

The plasmid DNA was isolated by the alkaline lysis method (*14*) from each colony in the mutant pool. Each plasmid DNA was screened by dot blot hybridization (*17*) using the splint 23mer as a probe. Plasmids that were judged to be effective mutants in this hybridization experiment were treated with the targeted restriction enzyme, *Mlu*I, and the absence of the *Mlu*I site was checked. Plasmids cleaved by this enzyme were not mutants and were excluded from the mutant pool.

The nucleotide sequences of the mutants were analyzed by plasmid sequencing with a primer (5′-dAAAAAAGGGAATAAGGGCGA-3′) and the Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) with an Applied Biosystems model 373A DNA sequencer (Applera).

Calculation of Mutation Frequency—Through screening by hybridization, *Mlu*I treatment, and subsequent sequencing, the plasmids in the mutant pool obtained from the *Mlu*I-treated plasmid fraction were divided into three categories: (i) plasmids with a mutation(s) in the *Mlu*I site, (ii) uncleaved, "normal" plasmids, and (iii) plasmids containing a large (>4 bp) deletion. The plasmids in the first two groups and the cleaved fraction were judged as effective because the third type of event has often been observed in mammalian cells, irrespective of base modification (*18, 20–22*). The colonies containing a normal plasmid (the second group) appeared to be generated by incomplete digestion by *Mlu*I and/or re-ligation of the linearized plasmid DNA in bacterial cells. Typically, the ratios of the plasmids containing a large deletion and the uncleaved, normal plasmids were 2–5 and 1%, respectively, of total *E. coli* colonies. In this study, we judged mutations at the target position, and at the 5′- and 3′-neighboring bases, as actual mutations. The *MF*, (mutant colonies)/(effective colonies), was calculated as follows:

$$MF = (A \times B)/(1-A \times C)$$

where A = (colonies obtained with *Mlu*I-digested DNA)/

(colonies obtained with untreated DNA); B = (mutant colonies)/(colonies screened from the mutant pool); C = (colonies with a large deletion)/(colonies screened from the mutant pool); $1-A \times C$ = the ratio of effective colonies.

RESULTS

Vectors—5-mC is present primarily in CpG sequences (*1, 2*). 5-fC was then substituted into either of the cytosine positions of the CpG sequences in the *Mlu*I recognition site (5′-ACGCGT-3′). Mutants could be selected as plasmids resistant to treatment with this enzyme. Similar methods have successfully been used to screen for mutants induced by 5-fU, 2-hydroxyadenine, the *cis-syn* cyclobutane thymine dimer, and the 6-4 photoproduct of thymine-thymine (*13, 18, 23*). In addition, 5-fC was introduced in either strand of the ds vectors to observe its effects on the leading and lagging strand syntheses of DNA replication (Fig. 3).

We used the following nomenclature: (+)-fC-1 and (+)-fC-2 as the vectors containing 5-fC in the first (5′-side) and second (3′-side) cytosine positions, respectively, of the *Mlu*I sites in the (+)-strand. The (+)-C vector has the same sequence with an unmodified cytosine instead of 5-fC. The (+)-mC-1 and (+)-mC-2 vectors have the same sequence with 5-mC instead of 5-fC. The (–)-fC-1, (–)-fC-2, (–)-C, (+)-mC-1, and (+)-mC-2 vectors are named similarly, although the strand of interest is the (–)-strand (Fig. 3). The (+)- and (–)-strands were assumed to be the template strands for the lagging and leading strand syntheses, respectively (*23*).

Cytotoxicity of 5-fC in DNA in COS-7 Cells—We first evaluated the cytotoxicities of 5-fC in DNA in COS-7 cells to determine whether this oxidized base blocks DNA replication. We previously observed that the number of *E. coli* colonies and the amount of DNA (the parental vector pSVKAM2) transfected into COS-7 cells shows a linear correlation between zero and 10 ng under our conditions (*23*). This means that the number of bacterial colonies reflects the relative amount of plasmid DNA replicated in COS-7 cells. Thus, the number of *E. coli* colonies is a good indicator for evaluating the cytotoxicity of 5-fC residues in COS cells.

Four nanograms of the modified and unmodified vectors were transfected into COS-7 cells and allowed to replicate in the cells. The plasmid DNAs recovered from the cells were transfected into *E. coli* DH5α cells, and the numbers of colonies formed were counted. In these experiments, 5-mC vectors were used as controls. The numbers of *E. coli* colonies derived from vectors containing 5-fC in the leading and lagging template strands were less than those derived from control vectors containing 5-mC (Table I). The relative numbers of colonies derived from the 5-fC-vectors were 39–90% of those from the control 5-mC vectors. Thus, 5-fC blocked replication weakly. The blockage was more evident during leading strand synthesis than during lagging synthesis. The 5-fC residue in the second (3′-side) cytosine position in the CGCG site on the leading template strand blocked replication most strongly (Table I).

5-fC Is Mutagenic in Mammalian Cells—We introduced a 5-fC residue into a unique restriction enzyme site, the *Mlu*I site, and thus could select mutants grown in bacterial

TABLE I. **The relative transforming efficiencies of vectors containing 5-fC[a].**

Vector	(+), *lagging*	(–), *leading*
AMGCGT (mC-1)[b]	100[d]	100[d]
AFGCGT (fC-1)[c]	66	59
ACGMGT (mC-2)[b]	100[d]	100[d]
ACGFGT (fC-2)[c]	90	39

[a]Percentage of colonies resulting from transformation of *E. coli* DH5α cells with the plasmid DNA recovered from COS-7 cells. [b]M represents 5-mC. [c]F represents 5-fC. [d]This value is defined as 100 for each experiment. The actual number of colonies ranged between 7,000 and 13,500.

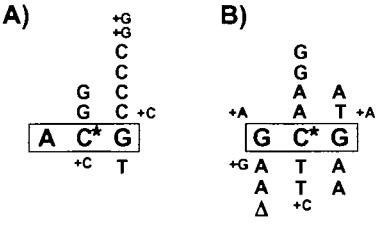

Fig. 4. **Mutation spectra of 5-fC in COS-7 cells.** (A) Mutations observed with (+)- and (–)-fC-1. (B) Mutations observed with (+)- and (–)-fC-2. 5-fC (C*) and the neighboring bases are boxed. The mutations induced by the (+)- and (–)-vectors are shown above and below the sequence, respectively. Δ represents a deletion mutation.

Fig. 3. **Structure of the double-stranded shuttle vectors containing 5-fC (left) and schematic representation of the replication of template strands with 5-fC (right).** The oligodeoxyribonucleotides contain ends that are compatible with the restriction enzyme–cleaved ends within the cloning site. Closed circles represent 5′-phosphate groups. C* indicates the position where the 5-fC, 5-mC, or unmodified cytosine was incorporated. The SV40 origin and the ColE1 origin for replication in COS-7 cells and *E. coli*, respectively, and the ampicillin resistance gene are also shown.

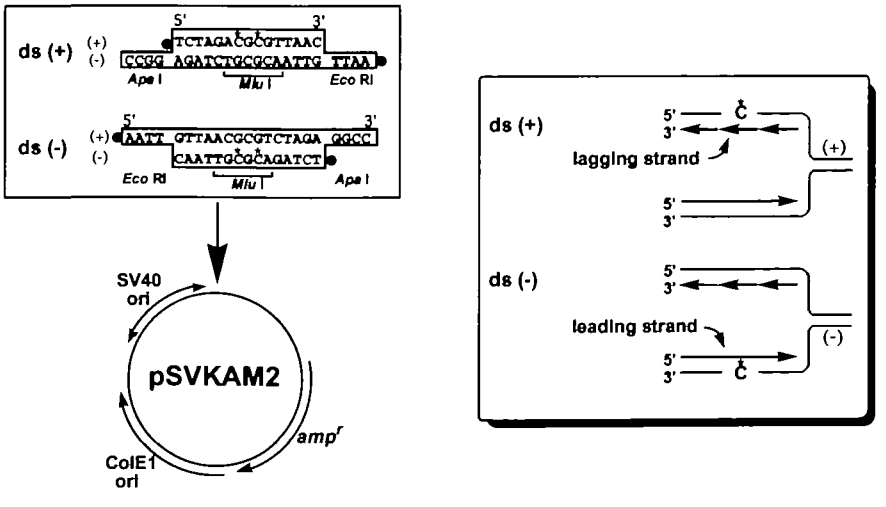

TABLE II. **Mutation frequencies of 5-fC in COS-7 cells.**[a]

Vector	(+), *lagging*		(−), *leading*	
	exp. 1	exp. 2	exp. 1	exp. 2
ACGCGT (C)	ND[d]	<0.03	ND[d]	<0.02
AMGCGT (mC-1)[b]	<0.04	ND[d]	<0.03	ND[d]
ACGMGT (mC-2)[b]	<0.04	ND[d]	<0.05	ND[d]
AFGCGT (fC-1)[c]	0.20	0.28	0.03	0.04
ACGFGT (fC-2)[c]	0.12	0.10	0.09	0.07

[a]Percentage of colonies containing a plasmid mutated at the target position, and at the 5′- and 3′-neighboring bases. Mutants containing large deletions were excluded from the calculation, as described in the "MATERIALS AND METHODS." [b]M represents 5-mC. [c]F represents 5-fC. [d]ND, not determined.

cells as plasmids resistant to this restriction enzyme. However, almost all of the *Mlu*I-resistant plasmids in the *E. coli* colonies contained random deletions that occurred in the mammalian cells (*18, 20–22*). We used dot blot hybridization to select against this type of event, as described previously (*18*). By this technique, we obtained plasmids mutated within the inserted linker region. The existence of the mutation was confirmed by digestion of the plasmid with the targeted restriction enzyme.

Table II shows the MFs of the (+)- and (−)-vectors with 5-fC. The 5-fC residues induced mutations with an efficiency of 0.03–0.28%. The maximum MF was expected to be 50%, because the complementary strand is also replicated. Thus, we estimate that at least 0.06–0.5% of the 5-fC residues induced the misincorporation of nucleotides during DNA replication. These values are higher than the MFs of 5-fU in ds vectors in COS-7 cells (*13*).

Mutation Spectra of 5-fC in COS-7 Cells—The mutations elicited by 5-fC are shown in Fig. 4. Interestingly, untargeted mutations at 5′- and 3′-neighboring bases occurred more frequently than targeted mutations. Of 28 colonies sequenced, 8 colonies contained targeted base substitutions. All possible substitutions were observed at the modified site [5-fC→G (four clones), 5-fC→A (two clones), and 5-fC→T (two clones)]. Base substitutions were found at 3′-neighboring sites more frequently than at 5′-flanking sites (Fig. 4). In addition, insertions and a deletion were observed. Since no mutations were found in the cases of the control vectors, these targeted and untargeted mutations appeared to be elicited by 5-fC. Most of the same mutations were observed with different transfection experiments in COS-7 cells, indicating the reproducibility of the mutation spectra.

DISCUSSION

5-fC is the major oxidation product when 5-mC is treated with ROS-generating reagents (*12*). In addition, the formation of 5-fC from 5-mC by UV irradiation in the absence and presence of a photosensitizer has been reported (*24, 25*). We recently reported that an analog, 5-fU, induces 5-fU→G and 5-fU→A transversions in COS-7 cells, and this is the only oxidized pyrimidine base in DNA that induces transversion mutations (*13*). Thus, it is interesting to examine the mutational properties of 5-fC to test whether it also elicits transversions.

To investigate the mutational properties of 5-fC in mammalian cells, we used a site-specific mutagenesis approach that has been successfully employed for several DNA lesions (*13, 18, 23*). The vector used in this study contains the SV40 origin (Fig. 3), and the (+)- and (−)-strands near the region where the 5-fC residues were inserted appeared to be replicated during lagging and leading strand syntheses, respectively.

Vectors with 5-fC replicated less efficiently than the control (unmodified) vectors (Table I). Thus, the presence of 5-fC weakly inhibits leading and lagging strand synthesis during replication in mammalian cells. The 5-fC residues in mammalian cells were mutagenic, and the MF of 5-fC was 0.03–0.28% (Table II). It was previously shown that the MFs of 8-OH-Gua and 2-OH-Ade in ds DNA are 0.1–1% in mammalian cells (*18, 26, 27*). In contrast, the MFs of 5-fU in ds DNA are 0.01–0.04% (*13*). Thus, the mutagenic potential of 5-fC is slightly lower that those of 8-OH-Gua and 2-OH-Ade and higher than that of 5-fU in mammalian cells.

The 5-fC residues induced various mutations (Fig. 4), including targeted mutations and untargeted mutations at 5′- and 3′-neighboring bases. Of the eight colonies containing targeted base substitutions, six clones had transversion mutations as 5-fU (5-fC→G, four clones; and 5-fC→A, two clones). This result suggests the participation of the 5-formyl group in hydrogen bonding between C and T, as we proposed for 5-fU (*13*). The presence of an intramolecular hydrogen bond has been shown between the carbonyl of the 5-formyl group and the 4-amino function (*28, 29*). This interaction may affect the equilibrium between the amino- and imino-tautomers. The induction of 5-fC→T (two clones) transitions may result from this putative equilibrium shift to the imino-tautomer. The "targeted" MF, calculated by multiplying the averaged MF (0.12%) and the ratio of the targeted mutations (8 of 28), is 0.03%. This value is comparable to the MF of 5-fU (*13*). Thus, 5-fC is as mutagenic as 5-fU for mutations at the modified positions.

The induction of untargeted mutations has been found for several DNA lesions (*26, 27, 30–32*). Since the oligodeoxyribonucleotides used in this study were purified extensively (Fig. 2), the mutations at the 5′- and 3′-neighboring bases appeared to be induced by 5-fC itself, not by impurities.

In vitro DNA synthesis experiments with a 5-fC-containing template and the exonuclease-deficient Klenow fragment have indicated that this polymerase inserts nucleotides opposite 5-fC with similar fidelity to insertions opposite C or 5-mC (*15*). The order of incorporation of nucleotides opposite 5-fC is G ≫ A > T > C (*15*). These findings are in contrast to the present results that 5-fC is mutagenic and that it induces 5-fC→G, 5-fC→A, and 5-fC→T mutations, and untargeted mutations (Table II and Fig. 4). These discrepancies may be due to the involvement of low-fidelity, specialized DNA polymerases (*33*) in translesion synthesis across 5-fC. The bypass efficiency and the MF were higher during lagging strand synthesis than during leading strand synthesis (Tables I and II). These results may be due, in part, to differences in the specialized DNA polymerases and/or in the frequency of their loading to the blocked site.

It is possible to speculate that 5-fC is recognized and released by a DNA glycosylase(s) and that the resulting abasic sites induce the mutations described in this study. To date, the involvement of a DNA glycosylase(s) in repair of 5-fC in mammalian cells is unknown, and thus, it is unclear whether an abasic site and other repair intermedi-

ates are generated from 5-fC. In mammalian cells, abasic sites in ds DNA elicit mutations to A at modified and adjacent positions (*32*). The observation that 5-fC induces mutations at the flanking positions is consistent with the putative formation of abasic sites. However, mutations to A were not detected for (+)- and (–)-fC-1 DNAs (Fig. 4A). Because abasic sites induce mutations to A at both modified and adjacent positions, it is unlikely that abasic sites formed from 5-fC in cells caused the mutations in the case of (+)- and (–)-fC-2 DNAs (Fig. 4B). Although we cannot exclude this possibility completely, the mutations observed in this study appear to be due to mispairing properties of 5-fC.

In this study, we found that 5-fC in DNA is mutagenic in COS-7 cells and induces various mutations, including 5-fC→G, 5-fC→A, and 5-fC→T mutations, and untargeted mutations. These features are of great interest, and their mechanisms will be reported elsewhere.

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