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# Reverse chemical mutagenesis: Identification of the mutagenic lesions resulting from reactive oxygen species-mediated damage to DNA

(oxygen free radicals/DNA damage/carcinogens/mutagens/human immunodeficiency virus reverse transcriptase)

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ABSTRACT **An understanding of the contribution of reactive oxygen species to mutagenesis has been hampered by the vast number of different chemical modifications they cause in DNA. Even though many of these DNA alterations have been catalogued, the identification of specific lesions that cause mutations has depended on testing one modification at a time. In this study we present another approach to identify key mutagenic lesions from a pool of oxidatively modified nucleotides. dCTP was treated with an oxygen radical-generating system containing $FeSO_4$, $H_2O_2$, and ascorbic acid. The modification products were separated by reverse-phase and anion-exchange HPLC and then incorporated by human immunodeficiency virus reverse transcriptase into a DNA that contains a target gene for scoring for mutations. One of the mutagenic species isolated was identified as 5-hydroxy-2′-deoxycytidine. It is incorporated efficiently into DNA and causes C → T transitions in *Escherichia coli* at a frequency of 2.5%, which is more mutagenic than any previously identified oxidative DNA lesion.**

Many investigations have focused on the potential role of reactive oxygen species-mediated DNA damage in human diseases, in carcinogenesis, and in causing spontaneous mutations (1). Despite intense study, an assessment of the contribution of reactive oxygen species to mutations has been hindered by the multiple sources of damaging species and the vast array of DNA lesions they inflict. The traditional approach to address this complexity has been to catalogue the reaction products formed from oxidation of nucleosides or DNA by using standard methods of chemical identification. Analytical methods, including gas chromatography/MS, HPLC with UV and electrochemical detection, as well as TLC after $^{32}P$-postlabeling are now regularly used to measure formation of specific adducts at biologically relevant levels (2–4). Although each of these techniques detect and identify the types of nucleotide modifications that occur, they do not address which, if any, of these chemical changes are significant to mutagenesis.

The usual scheme to link a chemical alteration with a specific type of mutation has been to introduce a single candidate lesion into one or more sites in a target gene and screen for its mutagenic potential. Site-specific alterations have been introduced into oligonucleotides chemically (5) and enzymatically (6, 7), and genetic assays have been used to characterize the mutations arising secondary to the presence of specific lesions. Data from these experiments have defined the mutagenicity and substitution specificity of 8-oxoguanine, thymine glycol, and a variety of nonoxidative lesions (8–14). Although these studies directly address the mutagenic potential of individual lesions, the vast number of lesions from reactive oxygen species-mediated damage to DNA would make this approach a daunting task.

Previous work in our laboratory has shown that the mutations resulting from transition metal-mediated damage to DNA by reactive oxygen species most frequently occurs opposite cytidine residues and are predominantly C → T transitions (15). When single-stranded M13 DNA is treated with CuCl and $H_2O_2$ followed by transfection and replication in *E. coli*, >60% of all observed mutations were C → T transitions. In similar experiments, using $FeSO_4$ to generate reactive oxygen species, 25% were C → T transitions (16). Damage to DNA by reactive oxygen species also causes large increases in C → T and C → A substitutions by purified mammalian polymerases $\alpha$ and $\beta$ (17, 18). Because modifications of cytosine are mutagenic regardless of the reactive oxygen species-generating system or the DNA polymerase and the lesions that lead to these mutations are not known, we developed a system to identify the cytosine derivatives that are mutagenic. Because the base modifications resulting from the treatment of free nucleotides and DNA with oxidants are qualitatively similar (2, 4, 19, 20), we chose to damage free nucleotides in solution, purify the oxidized products based on their mutagenic potency, and then determine their chemical identities. We have designated this approach reverse chemical mutagenesis.

## METHODS

**Purification of Oxidized Products of dCTP.** Oxidation of dCTP was carried out in a 200-$\mu$l reaction containing 5.4 mM [$\alpha$-$^{32}P$]dCTP (692 cpm/pmol of dCTP), 10 mM Tris·HCl (pH, 7.5), 750 $\mu$M $FeSO_4$, 15 mM $H_2O_2$, and 1.5 mM ascorbic acid. Samples were incubated in 1.7-ml tubes at 37°C for 40 min. Reactions were stopped by the addition of 5 $\mu$l of 100 mM deferoxamine (chelating agent), and the mixture was immediately loaded onto a Beckman ODS, reverse-phase HPLC column (4.6 × 250 mm), equilibrated with 50 mM triethylammonium acetate, pH 7.0. Nucleotides were eluted with 15 ml of triethylammonium acetate followed by a linear 45-ml gradient from 0–25% acetonitrile at a constant flow rate of 1 ml/min. Sixty 1-ml fractions were collected and screened for UV absorbance at 254 nm (measured by HPLC UV absorbance detector), for radioactivity and for incorporation into gapped DNA by human immunodeficiency virus type 1 reverse transcriptase (HIV-RT). Fractions that were most efficiently incorporated into DNA and were well separated from undamaged dCTP were pooled and lyophilized. The lyophilized fractions from the $C_{18}$ column were resuspended

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Abbreviations: HIV-RT, human immunodeficiency virus type 1 reverse transcriptase; TMS, trimethylsilyl.

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in 125 mM $KH_2PO_4$/125 mM KCl, pH 4.4, and loaded onto a Whatman Partisil 10 SAX HPLC column (4.6 × 250 mm), which was equilibrated in 125 mM $KH_2PO_4$/125 mM KCl, pH 4.4. An isocratic gradient with a mobile phase of 125 mM $KH_2PO_4$/125 mM KCl, pH 4.4 was run at a flow rate of 2 ml/min. One-hundred and fifty 1-ml fractions were collected and screened for radioactivity and incorporation into gapped DNA.

**Incorporation of Modified Nucleotides.** The purification of oxidized derivatives of dCTP was monitored by measuring incorporation into gapped DNA. The gapped DNA consists of a single-stranded circular M13mp2 DNA containing uracil residues annealed to a linear incomplete complementary strand lacking uracil so as to create a 361-nt gap spanning part of the *lacZ* gene (prepared as described in ref. 17). The reaction mixture (0.02 ml) contains 200 ng of gapped DNA, 60 mM Tris·HCl (pH 8.0), 10 mM KCl, 7 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, 200 μM (each) dATP, dGTP, and TTP, 1 μM [$\alpha$-$^{32}$P]dCTP or modified dCTP (692 cpm/pmol), and 1.25 units of recombinant HIV-RT (21) (1 unit is the amount of enzyme required to catalyze the incorporation of 1 nmol of dNTP into DNA in 1 hr at 37°C). The nucleotide bias in this reaction drives the incorporation of modified cytosine only at sites opposite guanosines in the *lacZα* gene. Samples were extracted once each with phenol and chloroform/isoamyl alcohol, 24:1, followed by ethanol precipitation in the presence of 400 mM NaCl and 10 μg of glycogen (as a coprecipitant). Samples were resuspended in $H_2O$, and gap-filling synthesis was completed with *E. coli* DNA polymerase I, large fragment (pol-1lf) in 20-μl reactions containing the DNA from a reaction with HIV-RT, 50 mM Tris (pH 7.8), 10 mM $MgCl_2$, 200 μM (each) dATP, dCTP, dGTP, and TTP, and 5 units of pol-1lf (provided by Albert Mildvan, Johns Hopkins University, Baltimore). Reactions were incubated at 37°C for 45 min and stopped by the addition of 80 μl of 25 mM EDTA, and incorporation into an acid-insoluble precipitate was determined (17).

**Frequency and Spectrum of Mutations.** Mutations produced by the incorporation of oxidized derivatives of dCTP into gapped DNA were identified by reduced α-complementation of β-galactosidase activity. Logarithmic-phase MC1061 [hsdR, mcrR, araD, (139Δ$^-$araABC$^-$leu) 7679ΔlacX74, galU, galK, rspL, thi$^-$] was prepared for electroporation by washing the cells four times with sterile $H_2O$. Cells were then pelleted and resuspended in a volume of $H_2O$ equal to that of the packed cells. Fifty nanograms of the filled-gap DNA product was mixed with 300 μl of cells and subjected to electroporation using a Bio-Rad Gene Pulser (25 mF, 400 W, 2.0 kV, and time constants between 6.0 and 7.5). Samples were placed on ice and mixed with 1 ml of LB medium. Aliquots (10–40 μl) of the transfection mixtures were added to 3 ml of molten top agar (42°C) consisting of 0.9% NaCl, 0.8% Bacto Agar, 0.08% 5-bromo-4-chloro-3-indolyl β-D-galactoside dissolved in dimethylformamide, and 0.2 ml of CSH50 [Δ(proBlac)/F'+raD36, thi$^-$, ara$^-$, proAB, lacI$^q$Z$^-$-Δm15] cells in logarithmic phase, and layered onto plates containing 30 ml of solidified M9 medium supplemented with 1.5% Bacto Agar and 15 mM isopropyl β-D-thiogalactoside. Phenotypes were confirmed by mixing mutant phage with an equal quantity of wild-type M13mp2 phage and replating. Phage DNA was isolated (22) and sequenced by the method of Sanger (23) using a 15-mer oligodeoxyribonucleotide primer complementary to the +179 to +194 segment of the *lacZα* coding sequence.

## RESULTS

**Identification of dCTP Alterations by Reactive Oxygen Species Using Reverse Chemical Mutagenesis.** In these initial studies we examined the oxidation products of cytidine. We aerobically incubated [$\alpha$-$^{32}$P]dCTP in a Fenton reaction consisting of $FeSO_4$, $H_2O_2$, and ascorbic acid and separated the oxidation products on a reverse-phase ($C_{18}$) HPLC column. The column fractions were used in place of dCTP for DNA synthesis reactions. The DNA template was a double-stranded M13mp2 molecule with a 361-nt single-stranded gap spanning part of the *lacZ* gene for bacterial β-galactosidase (Fig. 1). The uracil-containing strand is rapidly hydrolyzed upon transfection in *E. coli*, resulting in a $10^4$-fold preferential expression of the strand lacking uracil and preventing the repair of altered cytidines incorporated into the strand lacking uracil. DNA synthesis was catalyzed by HIV-RT in the presence of 200 μM dATP, dGTP, and TTP and 1 μM of each fraction from the reverse-phase column. HIV-RT was used for the initial synthesis step because it incorporated modified nucleotides more efficiently than other polymerases (6, 11). Partially double-stranded DNA products were purified, and gap-filling synthesis was completed using the Klenow fragment of *E. coli* DNA polymerase I. Incorporation of oxidized cytosine derivatives was quantified by scintillation counting, and the toxicity of the fractions was assessed by comparing the transfection efficiency of DNA synthesized by using fractions containing the altered nucleotides to that of DNA synthesized with dCTP passed through the same column. The mutagenicity of each fraction was measured by scoring forward mutations in the *lacZ* gene for β-galactosidase (22).

The HPLC elution profiles and relative incorporation into DNA of the oxidized nucleotides is shown in Fig. 2. With the reverse-phase column, not all of the $^{32}$P-containing species are incorporated into DNA, presumably because they are no longer a substrate for HIV-RT. dCTP and dUTP eluted from the reverse-phase column in fractions 29–31 and 34–35, respectively. The presence of mutagenic compounds in these fractions cannot be ruled out; however, in these initial studies, we purified fractions 12–14, which were highly mutagenic and lacked undamaged nucleotides. Fractions

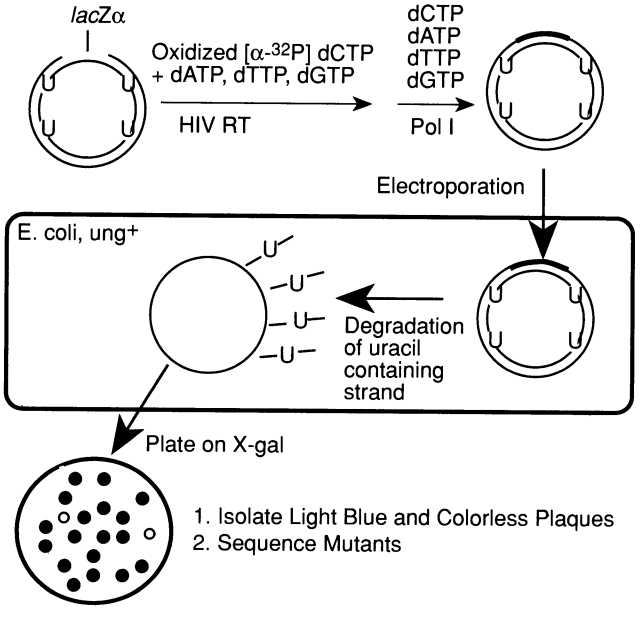


FIG. 1. Protocol for reverse chemical mutagenesis. The template is a circular M13mp2 DNA containing uracil residues annealed to a linear incomplete complementary strand lacking uracil, so as to create a 361-nt gap spanning part of the *lacZ* gene. Incorporation of oxidized dCTP derivatives was done with HIV-RT. The partially double-stranded DNA products were purified, synthesis was completed with the large fragment of *E. coli* DNA polymerase (Pol I), and the extended product was transfected into *E. coli* (CSH50). Mutations in *lacZ* were identified and sequenced. X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

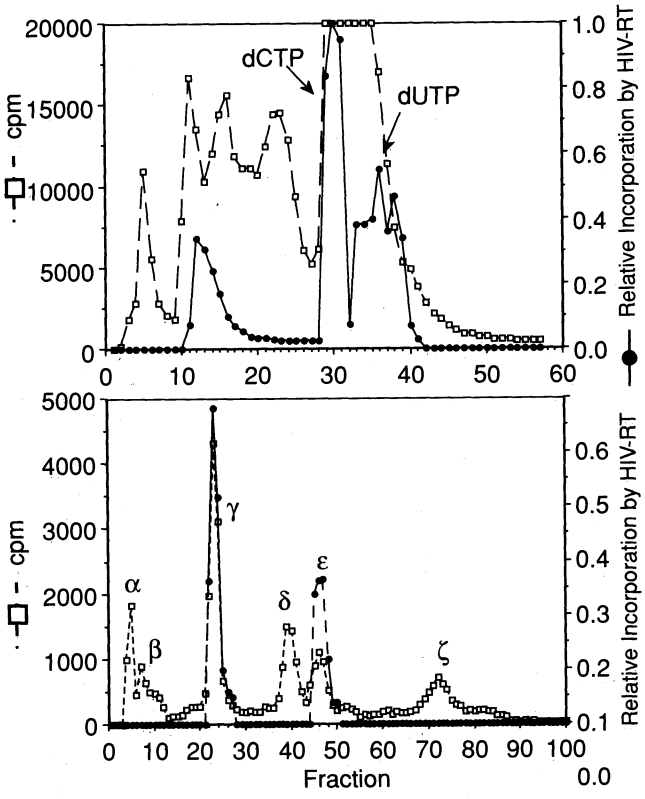


FIG. 2. Purification of dCTP oxidation products. □, radioactivity ($^{32}P$) of each fraction; ●, efficiency with which HIV-RT uses the fraction as a nucleotide substrate relative to undamaged dCTP. (*A*) Separation by $C_{18}$ HPLC. Nucleotide-damage reactions and reverse-phase chromatography were done as described. Radioactivity in 10 μl of each fraction was measured, and each fraction was tested as a substrate for HIV-RT. Under identical conditions undamaged dCTP and dUTP elute in fractions 29–31 and 34–37, respectively. (*B*) Separation by strong anion-exchange chromatography. Fractions 12–14 from the $C_{18}$ column were combined, chromatographed on a Partisil 10-SAX column using an isocratic gradient of 0.125 M $KH_2PO_4$/0.125 M KCl, pH 4.4, and analyzed for radioactivity and incorporation. Based on radioactivity, six peaks were separated and are designated as α, β, γ, δ, ε, and ζ, two of which, γ and ε, are incorporated into DNA by HIV-RT.

12–14 were combined, loaded onto a strong anion-exchange (SAX) column, and six species, α, β, γ, δ, ε, and ζ, were separated (Fig. 2*B*). Compounds γ and ε represent 0.11% and 0.04% of the dCTP starting material, respectively, and are incorporated into DNA by HIV-RT with efficiencies of ≈56% and 27% of that for dCTP. Neither species is incorporated appreciably by mammalian DNA polymerase-α, and only γ is a substrate for mammalian DNA polymerase-β (18% relative to dCTP). As ε is also highly detrimental to phage replication (each molecule incorporated into DNA causes a 44% reduction in transfection efficiency), we concentrated on characterization of γ.

Fractions from successive steps in the purification of γ were analyzed by TLC (Fig. 3). Each lane contains an equal quantity of radioactive material: dCTP (lane 1), dCTP after oxidative damage (lane 2), pooled fractions 12–14 of the reverse-phase column (lane 3), and pooled fractions 22–24 of the strong anion-exchange column (lane 4). Oxidative damage to cytidine can result in >40 modified nucleotide species (3, 24), as manifested by the broadly distributed material in lane 2. The reverse-phase step separates γ species from most of the other products, and the strong anion-exchange column further purifies it.

Mutagenic potential of γ was assessed by using a forward mutation assay. To reduce the frequency of mutations arising

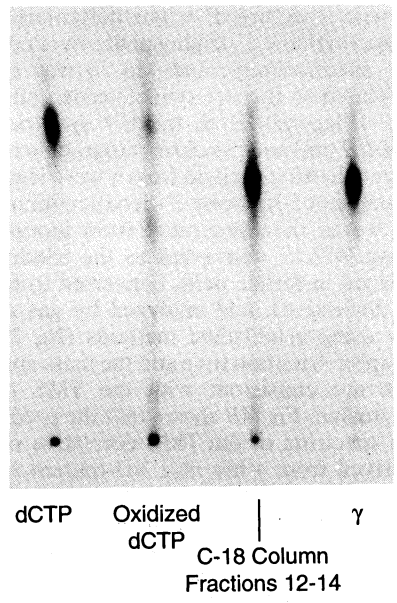


FIG. 3. Polyethylenimine-cellulose TLC of oxidized dCTP. Undamaged dCTP (lane 1), crude oxidized dCTP (lane 2), pooled fractions 12–14 from the $C_{18}$ column (lane 3), and γ (lane 4), were applied to a polyethylenimine-cellulose plate and chromatographed in a 1.3 M LiCl mobile phase. Equal amounts of the nucleotide products were added to each lane based on radioactivity.

from the incorporation of noncomplementary nucleotides by HIV-RT, this incubation was done for only 2 min. Under these conditions, HIV-RT incorporated approximately five molecules of γ or nine molecules of undamaged dCTP per molecule of template. The gapped DNA was then completely filled by pol-Ilf (the large fragment of *E. coli* DNA polymerase I) using the four undamaged nucleotides as substrates. The transfection efficiencies and mutation frequencies are given in Table 1. In reactions without HIV-RT, 1040 transfectants were obtained per ng of DNA, whereas with HIV-RT and undamaged dCTP ("purified" by reverse-phase and strong anion-exchange chromatography) the yield was 165 transfectants per ng of DNA. Substitution of γ for dCTP further reduced transfection efficiency, 73 transfectants per ng of DNA. In the absence of HIV-RT the mutation frequency is $4.6 \times 10^{-4}$ (line 1, Table 1). The reaction with HIV-RT and dCTP increases mutation frequency 2.4-fold, presumably from the high frequency of misincorporation by HIV-RT (21, 25) and the nucleotide pool bias (21) in the reaction mixture. Most importantly, the mutation frequency secondary to the incorporation of an average of five molecules of γ per template DNA is $1200 \times 10^{-4}$, an increase of 109-fold. The relatively low mutation frequency in the control reaction with only 3 nt (line 4, Table 1) indicates that the mutagenesis induced by γ does not result from the lack of dCTP in the mixture.

Table 1. Mutagenesis by γ

| HIV-RT | Cytosine substrate for HIV-RT | Transfection efficiency, plaques per ng | Mutation frequency |
|---|---|---|---|
| − | − | 1040 | $4.6 \times 10^{-4}$ (41) |
| + | − | 183 | $31 \times 10^{-4}$ (91) |
| + | dCTP | 165 | $11 \times 10^{-4}$ (54) |
| + | γ | 73 | $1200 \times 10^{-4}$ (196) |

HIV-RT was used to incorporate dATP, dGTP, and dTTP alone, with dCTP, or with γ, as shown in column 2. Transfection efficiencies and mutation efficiencies are shown in columns 3 and 4. Number of mutants isolated for each class of reactions appear in parentheses. Data is cumulative for several experiments.

The chemical structure of γ was determined to be 5-hydroxy-2′-deoxycytidine 5′-triphosphate by a combination of HPLC, UV spectroscopy, and MS. γ was enzymatically dephosphorylated to the deoxynucleoside and analyzed by HPLC (Fig. 4, legend). Both the UV spectra (absorbance maximum of 292 nm) and the chromatographic retention time of the deoxynucleoside derived from γ were identical with an authentic sample of 5-hydroxy-2′-deoxycytidine (Fig. 4*A*). A fraction of γ was then separated from inorganic salts by reverse-phase HPLC, converted to the triethylammonium salt, hydrolyzed in formic acid, converted to the trimethylsilyl (TMS derivative), and analyzed by gas chromatography/MS by using established methods (26, 27). Both the chromatographic retention time and the mass spectrum of the γ derivative are consistent with the TMS derivative of 5-hydroxycytosine. Fig. 4*B* shows that the predominant ions in the mass spectrum of the TMS derivative of 5-hydroxycytosine derived from γ are *m/z* 343 (parent ion), *m/z* 342 $[M-H]^+$, and *m/z* 328 $[M-CH_3]^+$, consistent with published data. Previously 5-hydroxycytosine has been identified in

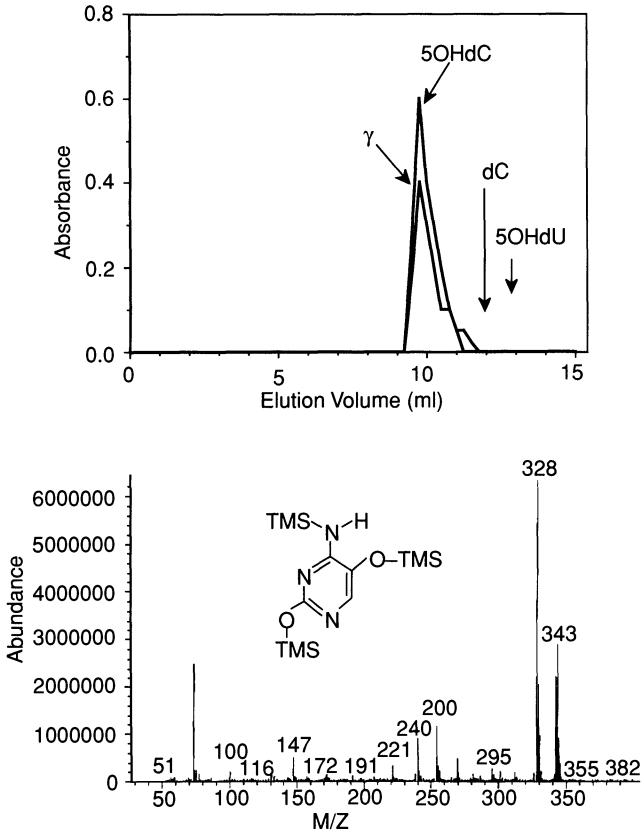


FIG. 4. Identification of γ. (*A*) Identical retention of γ and 5-OH-dC. Authentic 5-OH-dC was obtained from John Essigman (Massachusetts Institute of Technology), and γ was treated with phosphodiesterase and phosphatase to convert each to a nucleoside derivative. One hundred microliters of 12 mM solution of each in 0.125 M $KH_2PO_4$/0.125 M KCl, pH 4.4, was treated with 0.0005 unit of bovine intestinal phosphodiesterase and 0.5 unit of calf intestinal phosphatase for 30 min at 37°C. The reaction was stopped by addition of 25 μl of 100 mM EDTA and transferred to 70°C for 10 min. The sample was loaded onto a Beckman ODS, reverse-phase HPLC column (4.6 × 250 mm), equilibrated with 50 mM triethylammonium acetate, pH 7.0/10% (vol/vol) acetonitrile, and an isocratic gradient was run at a flow rate of 1 ml/min. One hundred microliters of 12 mM 5-OH-dC in 0.125 M $KH_2PO_4$/0.125 M KCl, pH 4.4, was loaded onto the same column and eluted with the same gradient. Each was loaded on a reverse-phase HPLC column and eluted in an isocratic gradient of 50 mM triethylamine acetate, pH 7.0/10% acetonitrile. (*B*) Mass spectrum of γ. Preparation of sample and generation of the mass spectrum were done by standard methods (10).

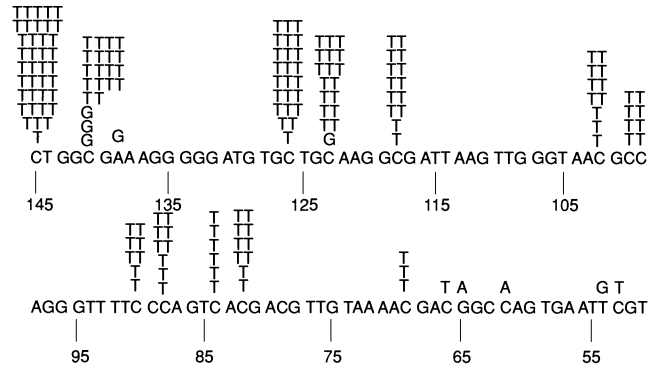


FIG. 5. Mutations induced by γ/5-OH-dCTP. The sequence is the coding strand of wild-type M13mp2; sequence positions are numbered relative to the transcription start site of *lacZ*. The first position of gap-filling nucleotide incorporation is +145, and synthesis is from right to left. Letters above the line each represent separately identified substitution.

acid hydrolysate of oxidized DNA; however, it was presumed to arise from dehydration of cytosine glycol during acid hydrolysis (10). The UV spectrum of γ, however, clearly shows that the base moiety was 5-hydroxycytosine and not cytosine glycol before acid hydrolysis (data not shown).

The mutation spectrum resulting from the incorporation of γ/5-hydroxy-2′-deoxycytidine 5′-triphosphate into the *lacZ* target gene is shown in Fig. 5. We sequenced ≈200 nt within the gap in each of 92 of the 196 mutants isolated: 78 (85%) had detectable sequence changes, and all of the observed changes were base substitutions. Of the mutants, 18 (19.5%) had a single substitution; 28 (30.4%) had two, 24 (26.1%) had three, 6 (6.5%) had four, 1 (1.1%) had five, and 1 (1.1%) had six substitutions. Of the 181 observed substitutions, 178 (98.3%) occurred at cytosines (opposite guanines in the template strand of the gapped DNA). This result indicates that despite the pool bias against dCTP, γ/5-hydroxy-2′-deoxycytidine 5′-triphosphate nearly invariably base-pairs with guanine during DNA synthesis by HIV-RT. Of the mutations, 173 (95.6%) were C → T transitions, showing the strong specificity of mutations caused by γ/5-hydroxy-2′-deoxycytidine 5′-triphosphate. The high frequency of mutations with multiple substitutions is atypical. It probably results from the presence of second mutations that occur at sites that do not yield white or blue colonies. In fact, single C → T substitutions at positions 126, 123, 102, 100, 99, and 66 have not previously been shown to yield mutant plaques (T. Kunkel, personal communication). One possible mechanism for C → T substitutions would be if the 5-OH group can stabilize the imino tautomer of 5-OH-cytosine (28), which base-pairs with adenine. Another possibility is that γ/5-hydroxy-2′-deoxycytidine is similar to an abasic site and causes the incorporation of deoxyadenosine by the *E. coli* replicative apparatus (29). This mutagenic specificity is particularly intriguing, as C → T transitions occur extremely frequently when oxidatively damaged DNA is replicated in *E. coli* (15), and the chemical nature of the lesion responsible for this substitution is unknown.

## DISCUSSION

The traditional approach for understanding the biological consequences of chemical damage to DNA has required elucidation of the reaction chemistry first, followed by biochemical studies. The "predominant" reaction product might be viewed as the most important, as it would be the one most likely to occur *in vivo*. However, the predominant chemical product might be of minor biologic importance, whereas a chemically minor product might prove highly

mutagenic. In this context, it is well established that the predominant products of DNA alkylation are N-alkyl derivatives, whereas the mutagenically important derivatives are O-alkyl products that represent only a minor fraction of the alkylated bases (30).

To circumvent this problem, we have developed an approach called reverse chemical mutagenesis. The principle is that one exposes deoxynucleotide substrates to chemically reactive agents and then selects the most interesting reaction products on the basis of mutagenesis before determining their chemical structures. This assay can be used to search for mutagenic lesions derived from many types of DNA damage, including oxidative and alkylation damage. The advantage is that one can identify highly mutagenic species among a large repertoire of chemically altered species. Although the assay is limited to lesions that can be incorporated into DNA enzymatically, the incorporation and mutation assays are extremely sensitive, and the lack of stringency in selection by HIV-RT allows one to incorporate a wide variety of altered nucleotides. A comparison of incorporation by and mutagenicity of modified nucleotides with HIV-RT and eukaryotic DNA polymerases could serve to identify altered nucleotides preferentially incorporated by HIV-RT. These altered nucleotides could be selected on the basis of either chain termination or enhanced mutagenesis and thus might be of value in the therapy of AIDS (21).

γ/5-OH-dCTP constitutes both a lethal and a mutagenic lesion. The mutational specificity of γ/5-OH-dCTP is C → T transitions. Our system measures the induced mutations at several sites; this specificity is not sequence-context dependent. We cannot, however, address the sequence context effects on the formation or repair of oxidatative lesions that will certainly effect mutagenesis. In the mutation assay, incorporation of γ/5-OH-dCTP in place of dCTP by HIV-RT causes a 56% loss in transfection efficiency (Table 1, row 4). As this results from the incorporation of an average of five molecules of γ (determined by measurement of $\alpha$-$^{32}$P), each lesion confers an average 16% chance of lethality. Knowing the number of lesions, the total number of observed substitutions, and the frequency of lethal events, we can estimate the mutational efficiency of γ/5-OH-dCTP. γ/5-OH-dCTP causes transition mutations in our system ≈2.5–2.9% (Table 1) of the time it is encountered by the DNA replicative apparatus of *E. coli*. This value may be an underestimation of the mutagenicity of γ/5-OH-dC because the host strain of *E. coli* is completely competent for repair. The only other oxidative lesions for which mutational efficiencies have been estimated are thymine glycol and 8-oxoguanine, which cause substitutions in similar systems with efficiencies of 0.3% (10) and 0.6% (31), respectively. 5-Hydroxycytosine has been found as a product of DNA oxidation; however, it was thought to arise from the dehydration of cytosine glycol during acid hydrolysis (32). For this reason, using the traditional chemical approach, 5-hydroxycytosine would likely be one of the last candidates proposed for biochemical studies. However, Wagner *et al.* (33) recently identified 5-hydroxycytidine as a major alteration in DNA after enzymatic hydrolysis and electrochemical detection. Treatment of DNA with systems that generate reactive oxygen species increased the level of 5-hydroxycytidine by as much as 430-fold. We have used reverse chemical mutagenesis to identify 5-hydroxycytosine as a lesion that is considerably more mutagenic than previously studied oxidative DNA damage products. On the basis of its presence in oxidized DNA (31) and on the types and frequency of mutation it produces, 5-hydroxycytidine could be a key lesion in the production of mutations by oxygen free radicals.

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