Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 7677–7681, October 1989
Biochemistry

Genetic effects of thymine glycol: Site-specific mutagenesis and molecular modeling studies

(ionizing radiation/oxidative damage/hydroxyl radicals)

ASHIS K. BASU*, EDWARD L. LOECHLER†, STEVEN A. LEADON‡, AND JOHN M. ESSIGMANN*§

*Department of Chemistry and Whitaker College of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139; †Department of Biology, Boston University, Boston, MA 02215; and ‡Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720

Communicated by Allan H. Conney, June 26, 1989 (received for review September 22, 1988)

ABSTRACT **The mutational specificity and genetic requirements for mutagenesis by 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol), one of the principal DNA lesions induced by oxidation and ionizing radiation, has been investigated in *Escherichia coli*. Thymine glycol was positioned at a unique site in the single-stranded genome of a bacteriophage M13mp19 derivative. Replication of the genome in *E. coli* yielded targeted mutations at a frequency of 0.3%; the mutations were exclusively T → C. Mutagenesis was independent of SOS and *nth* (*nth* encodes endonuclease III, a thymine glycol repair enzyme). The adduct was not detectably mutagenic in duplex DNA. A chemical rationalization for the mutation observed for thymine glycol was developed by applying molecular modeling and molecular mechanical calculations to the same DNA sequence studied *in vivo*. Modeling suggested that the 5*R*,6*S* isomer of *cis*-thymine glycol, when not base paired, was displaced laterally by ≈0.5 Å toward the major groove in comparison to the position that thymine would otherwise occupy. This perturbation of DNA structure should increase the likelihood of a guanine·thymine glycol wobble base pair during replication, which would explain the mutational specificity of the base observed in the genetic experiments.**

The hydroxyl radical is the principal reactive species responsible for DNA damage by ionizing radiation in aerated aqueous solution (1–3). This and other reactive oxygen species are also generated *in vivo* during normal metabolism (3, 4). Despite some differences, the DNA damages produced by ionizing radiation and cellular oxidation are qualitatively similar (5), with thymine bases being most susceptible to modification (6, 7). The major stable product of thymine modification *in vitro* and *in vivo* is 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol or t′; predominantly the *cis* isomers) (7, 8). Several studies have shown that t′ inhibits DNA synthesis *in vitro* in most sequences (9–12), although certain local contexts allow DNA polymerases to bypass the lesion (12).

The mutational specificity of ionizing radiation has been studied in bacteria and in mammalian cells (13–19). Base substitutions are the most frequently detected mutational event and, although there is no specificity for any one substitution, most studies agree that the major mutational change is the G·C → A·T transition. However, it is estimated that ≈40% of all ionizing radiation-induced mutations in *Escherichia coli* occur at A·T sites.¶ Moreover, *Salmonella typhimurium* strains with deletions in *oxyR*, a gene that positively controls a regulon affording protection against oxidative damage, exhibit a marked enhancement of spontaneous mutations such as T·A → A·T transversions (20). Together, these data show that a significant fraction of oxidant- and radiation-induced mutations occurs at A·T base pairs, and it was the goal of our work to determine the contribution of t′ to this component of the mutational spectrum.

MATERIALS AND METHODS

Materials. *E. coli* strain MM294A (lac^+) was obtained from K. Backman (BioTecnica). Strains GW5100 (M13 plating strain; *lacZ* [ΔM15], *supE*) and AB1157 were from G. Walker (MIT). Strains DE667 [*recA730*, *lexA51*, sup^+, Δ(*chlA–uvrB*), *sulA211*, Δ(*lac–gpt*)5] and DE1018 (*recA430*, *lexA51*, sup^+, Δ(*chlA–uvrB*), *srlC300*::Tn*10*, *sulA211*, Δ(*lac–gpt*)5] were from D. Ennis (DuPont); both were derived from DE274 (21), an AB1157 derivative. BW415 [Δ(*manA–nth-1*) kasugamycin resistant] and BW419 [BW9109, Δ(*xth-pncA*)90/pLC9-9] were AB1157-derived strains from B. Demple (Harvard). The construction of M13-*Nhe*I was described previously (22). Endonuclease III was the gift of R. Cunningham (SUNY, Albany).

Construction of an M13 Genome Containing a Single t′. The details of the synthesis and purification of a hexanucleotide containing t′, d(GCt′AGC), are given in Fig. 1. The hexamer was inserted into a six-base gap in the otherwise duplex genome of M13-*Nhe*I (22), a derivative of M13mp19 with a GCTAGC segment inserted into the unique *Sma* I site. The hexamer constitutes a unique recognition site for *Nhe* I and was positioned in-frame in the polylinker that interrupts the *lacZ* α-fragment. A ds M13-*Nhe*I genome containing a sole t′ residue at position 6275 in the − strand [ds t′-M13-*Nhe*I(−); Fig. 2*A*] was produced by minor modification of the method of Basu *et al.* (22). A pair of ss genomes containing t′ in either the + strand [position 6274; t′-M13-*Nhe*I(+)] or the − strand [position 6275; t′-M13-*Nhe*I(−)] was produced by modification of the method of Green *et al.* (23). In the final step of ss t′-genome construction, t′-containing nicked duplexes were denatured by alkali, heat, or formamide to produce the ss site-specifically modified genomes (Fig. 2*B*).

Transformation of *E. coli* with ss and ds t′-Containing Phage Genomes. Transformation of *E. coli* with t′-modified ss genomes was performed as described (24), except that 3–6 μg of duplex form II DNA (including the unligated heteroduplex DNA) was used to provide the t′-containing ss genome after denaturation. One to 2 μg of duplex DNA was used for transformation with the ds genome. In some experiments, t′ was excised from the ds M13 genome (1–2 μg) by two 30-min

Abbreviations: t′, thymine glycol; ss, single-stranded; ds, double-stranded; MF, mutation frequency; MF_{SS}, MF in ssDNA; t′-M13-*Nhe*I, M13-*Nhe*I genome containing t′ at position 6274 (minus strand) or 6275 (plus strand); T-M13-*Nhe*I, same as t′-M13-*Nhe*I but containing thymine at these sites.

§To whom reprint requests should be addressed.

¶Calculated from data in refs. 13 and 14 by correcting for spontaneous mutagenesis and target size and making the assumption that the fraction of A·T → G·C mutations is the same in single-stranded (ss) and double-stranded (ds) DNA.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

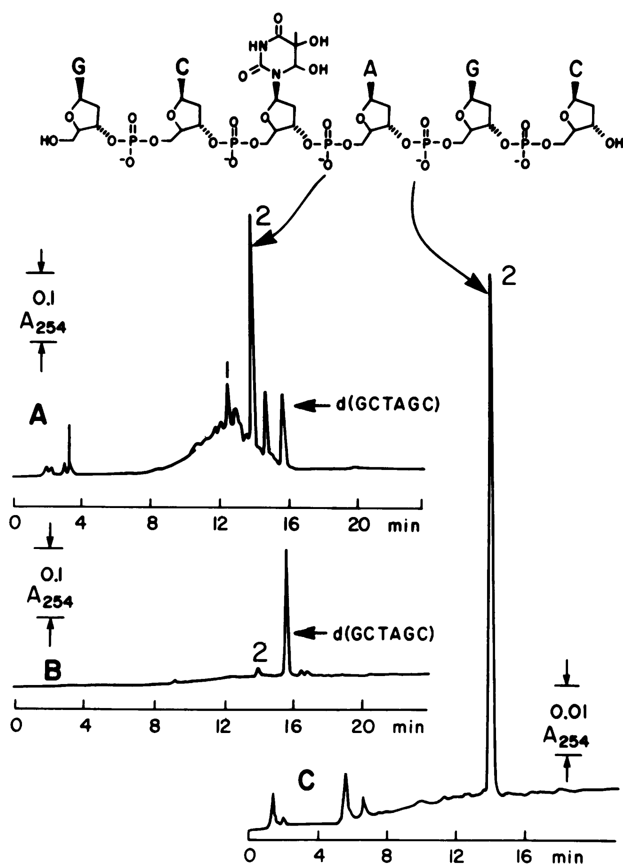


FIG. 1. Oxidation of the deoxyhexanucleotide d(GCTAGC) to d(GCt'AGC) (structure at top). Trace A, reversed-phase HPLC profile of the oxidation of d(GCTAGC) by $KMnO_4$ [5-min incubation of DNA (28 μM) with 1.4 mM $KMnO_4$ at pH 8.6, 37°C; excess permanganate was neutralized with allyl alcohol and the reaction products were held at 0°C for 30 min prior to injection]. Trace B, oxidation of d(GCTAGC) (33 μM) by OsO_4 (2% aqueous solution, 37°C, pH 8.6, 10 min). Excess OsO_4 was extracted with ether and the hexanucleotide was centrifuged through a Sephadex G-25 column before HPLC analysis. Trace C, reinjection of peak 2 collected after $KMnO_4$ oxidation (trace A) subsequent to desalting through a Sep-Pak (Waters) cartridge. Chromatographic conditions for traces A–C: Macherey–Nagel Nucleosil C_{18} (10 μm) column eluted with 0–50% (vol/vol) CH_3CN in 0.1 M ammonium acetate (pH 5.8) over 30 min at 1 ml/min.

treatments with endonuclease III (10 ng) in 50 mM Tris·HCl buffer, pH 7.6/100 mM KCl/1 mM Na_2EDTA/0.1 mM dithiothreitol. Protein was removed by extraction with phenol, and the DNA was precipitated with ethanol. Subsequent denaturation and cell transformation steps were as above.

Molecular Modeling of t' in DNA. Molecular modeling was done on an Evans and Sutherland PS300 picture system using the program PSFRODO (25), version 6.4. Molecular mechanical calculations were performed on a VAX 11/750 using the program AMBER (26, 27). Geometric and energetic parameters for t' (26, 27) are available upon request. Assessment of t'-induced base wobble was as described (28). Both the crystal coordinates (29) and a planar version of the t' monomer provided the initial coordinates for energy minimization.

RESULTS

Deoxyhexanucleotide Containing a Single t'. Oxidation of d(GCTAGC) with either $KMnO_4$ or OsO_4 yielded more polar products as detected by HPLC (Fig. 1). Permanganate oxidation at pH ≤ 7 yielded peak 1 as the major product, suggesting by analogy to the observations of others (30) that its sole thymine was converted to 5-hydroxy-5-methylbarbituric acid. At pH 8.6, a second product (peak 2) predominated; this peak contained t'. By a slight modification of a published procedure (30), $KMnO_4$ converted >80% of the starting material to products (Fig. 1A). Osmium tetroxide gave peak 2 almost exclusively, although the extent of oxidation was less than 10% (Fig. 1B).

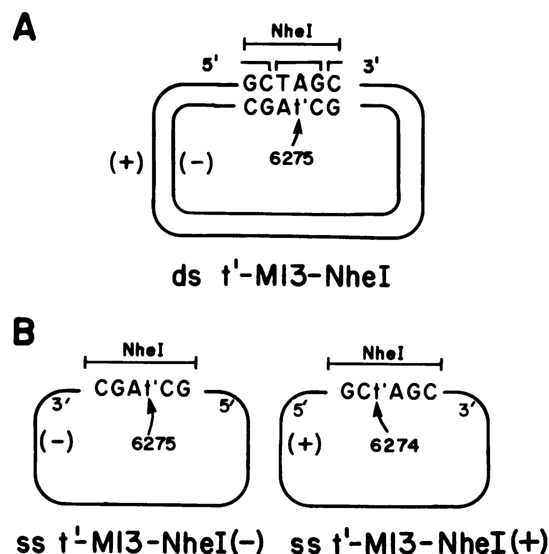


FIG. 2. ds (A) and ss (B) derivatives of M13mp19 containing a solitary t' at an amber codon of the unique *Nhe* I site. The precursors to the genomes in B were a pair of duplexes in which the strand opposite t' was nicked at the *Bgl* II site. Denaturation of the nicked duplex gave rise to the structures shown. In one experiment, the nicked duplex was treated with endonuclease III prior to denaturation. If endonuclease III nicked the t'-containing strand, the product of denaturation would be linear ssDNA, which should be nonviable.

The following data constituted the evidence that peak 2 was the oligonucleotide containing t'. The efficient formation of peak 2 by $KMnO_4$ at pH 8.6 (but not at lower pH values) and its selective production by OsO_4 suggest a t'-containing oligonucleotide (6, 30). Enzymatic digestion (22) of peak 2 to deoxynucleosides followed by HPLC revealed the anticipated amounts of dA, dG, and dC, but no detectable dT. As expected, thymidine glycol was undetectable because of its weak UV absorbance (30). However, HI treatment of the lyophilized fractions from the chromatographic region where thymidine glycol was known to elute regenerated thymine in a manner consistent with the characteristic reduction of thymidine glycol (or t') (8). By contrast, no thymine was detected by identical treatment of the same chromatographic fraction from digests of either peak 1 or d(GCTAGC). Significantly, 5-hydroxy-5-methylbarbituric acid and ring-opened derivatives of pyrimidines, such as methyltartronylurea, decomposed under HI reduction conditions. Finally, a monoclonal antibody highly selective for t' (31) reacted with peak 2 better than peak 1 and d(GCTAGC) by at least 1500-fold (data to be published elsewhere).

The hexanucleotide for the control experiments was obtained either by mock treatment (no $KMnO_4$) followed by HPLC purification of d(GCTAGC) or by HPLC recovery of the unmodified hexamer from the oxidation reactions.

Mutation Frequency and Lethality of t'. ss or ds genomes containing t' (Fig. 2) were replicated in *E. coli* strains MM294A [nth^+; *nth* is the gene encoding endonuclease III, which has t'-*N*-glycosylase and AP endonuclease activities (32–34)], AB1157 (nth^+), BW415 (Δ*nth*), BW419 (an overproducer of endonuclease III), DE1018 (a *recA430* strain with impaired SOS inducibility owing to defective cleavage of LexA and UmuD), and DE667 (a *recA730* strain that consti-

tutively expresses SOS functions). The control for these experiments, as well as those on t′ mutagenesis, was T-M13-*Nhe*I, which was produced by insertion of 5′-phosphorylated d(GCTAGC) into the M13-*Nhe*I phage genome. The presence of t′ failed to reduce the numbers of either progeny phage or infective centers in any of these strains. Typically, $>10^4$ infective centers were obtained per transformation. That there was no decrease in viability implied efficient bypass of t′ by the polymerase, repair, or conversion of t′ to a secondary lesion that did not lead to genome inactivation.

The mutation frequency (MF) of t′ was determined by taking advantage of the placement of the adduct in a *Nhe* I site. This site was positioned in the phage *lacZ′* region such that the unique thymidine was part of an amber codon (GCTAGC). In the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, M13-*Nhe*I produced light-blue plaques in *supE* strains. A point mutation at the unique thymidine of the *Nhe* I site generated dark-blue plaques, denoted B_1, which were easily distinguishable from the M13-*Nhe*I parental phenotype. In addition to B_1, a background of dark-blue plaques, denoted B_2, was generated by the genetic engineering techniques used to construct t′-M13-*Nhe*I; these were unrelated to t′ mutagenesis and were easily eliminated.[||] The MF of t′ was $[B_1/(B_1 + \text{light-blue plaques})] \times 10^2$ after the complete elimination of B_2 phage DNA.

The MF of t′ was 0.2–0.4% in ssDNA (Table 1*A*). The modest yield of mutants from t′ in ssDNA made it essential that we rigorously establish that the mutations were not derived from trace synthetic impurities or t′ breakdown products. While none of the experiments described below is conclusive alone, the collective weight of the evidence argues that the mutations observed *in vivo* were indeed due to t′. First, a center cut on the d(GCt′AGC) peak was collected after one, two, and three serial HPLC purifications; the material in each peak was subsequently ligated into M13, and MF was determined in MM294A. The MFs were 0.20%, 0.42%, and 0.35%, respectively; these numbers are virtually identical in our system. The failure to observe a decrease in MF with purification demonstrates that if an impurity were responsible for the mutations, it had chromatographic properties identical to the t′-containing hexamer. Second, the MF of t′ and the type of mutation were the same when d(GCt′AGC) was prepared by $KMnO_4$ or OsO_4 oxidation. This comparison was made because the different oxidation routes should generate different levels and possibly different types of impurities in the preparations of d(GCt′AGC) used. Third, d(GCt′AGC) and thymidine glycol were treated under conditions identical to those described for 5′-phosphorylation and ligation as well as to the conditions used to generate ssDNA. HPLC analysis revealed no evidence of degradation by these treatments. Fourth, treatment of the t′-containing genome with exaggerated conditions of duplex DNA denaturation (100°C for 2–5 min; 0.1 M NaOH for 1–5 min; or formamide) yielded no detectable change in MF, suggesting that whatever breakdown products might have been generated were not significantly mutagenic as compared to t′.

Biochemical evidence suggesting that the mutations were derived from t′ was their elimination by treatment of the ds precursor to ss t′-M13-*Nhe*I (Fig. 2*B*) with purified endonuclease III prior to transformation into *E. coli* [ds- rather than ssDNA was treated because the enzyme is more active on a ds substrate (35)]. Without endonuclease III treatment, t′-M13-*Nhe*I produced a phage population, ≈3% of which produced dark-blue plaques. Most of these were B_2,[||] while the remainder (≈0.3%) were the putative t′-induced mutants (B_1). After endonuclease III treatment none of the phage in the dark-blue plaques had the B_1 genotype, implying that the lesion giving rise to the B_1 mutants (the T → C transitions described below) was endonuclease III sensitive. This result would be expected if the premutagenic lesion were t′. Furthermore, the ratio of light-blue to dark-blue plaques from t′-M13-*Nhe*I was reduced ≈80-fold upon endonuclease III treatment (ratio reduced from 32:1 to 0.4:1) Because $B_2 >> B_1$, the light-blue plaques (i.e., wild-type progeny) must also have been derived from the endonuclease III-sensitive, t′-containing vector.

t′ was not detectably mutagenic in dsDNA (Table 1*A*). Two possibilities are that t′ blocked replication of the strand in which it resided, so that few if any progeny were issued from the adducted strand (see ref. 36) or that t′ was rapidly repaired in duplex DNA. The former possibility seems the less likely, since t′ did not reduce the survival of ss t′-M13-*Nhe*I genomes.

Genetic Requirements for t′ Mutagenesis. The influences on t′ mutagenesis of host SOS functions and of defects in t′ repair were investigated (Table 1*B*). The MF of t′ in ss M13

Table 1. Thymine glycol mutagenesis

Genome	DNA strandedness*	*E. coli* host (relevant genotype)	MF,† %
A. Mutagenesis of t′ in ss and ds DNA‡			
T-M13-*Nhe*I	ss	MM294A	≤0.02
T-M13-*Nhe*I	ds	MM294A	≤0.01
t′-M13-*Nhe*I ($KMnO_4$)	ss	MM294A	0.33
t′-M13-*Nhe*I ($KMnO_4$)	ds	MM294A	≤0.01
t′-M13-*Nhe*I (OsO_4)	ss	MM294A	0.42
t′-M13-*Nhe*I (OsO_4)	ds	MM294A	≤0.01
B. Genetic requirements for t′ mutagenesis§			
t′-M13-*Nhe*I	ss	AB1157	0.24
t′-M13-*Nhe*I	ss	DE1018 (*recA430*)	0.21
t′-M13-*Nhe*I	ss	MM294A + UV	0.23¶
t′-M13-*Nhe*I	ss	DE667 (*recA730*)	0.21¶
t′-M13-*Nhe*I	ss	BW415 (Δ*nth*)	0.19
t′-M13-*Nhe*I	ss	BW419 (*nth*⁺)	0.28

*Thymine- or t′-M13 DNA, in either ss or ds form, was transfected into *E. coli* cells.

†MF of transfected DNA determined as described in text. Values in section *A* are the averages of three to five independent determinations, except the value for OsO_4-generated t′, which was derived from a single determination. Values in section *B* are based on a single MF determination, except for those for AB1157, BW415, and BW419 cells, which are the average of two determinations. The standard deviation for ss t′-M13-*Nhe*I ($KMnO_4$) ($n = 4$) was ±0.19.

‡$KMnO_4$ and OsO_4 denote the oxidant used to prepare the t′-containing oligonucleotide.

§t′-containing genome (t′ produced by $KMnO_4$) was transfected into the hosts indicated. MM294A + UV indicates cells treated with UV light at 50 J/m² to induce SOS functions.

¶The absolute MF_{ss} of t′ was 0.6–0.8% in the SOS-constitutive strain, DE667. This enhancement was not attributed to t′, however, since the spontaneous MF_{ss} of T-M13-*Nhe*I in DE667 cells was also high (≈0.5%; data not shown). DNA sequencing revealed that only one-third of the mutations from t′-containing vectors were T → C, whereas the remaining mutations from t′-containing vectors and nearly all mutations from T-containing genomes were large deletions. The value reported here is the frequency of T → C transitions only; the spontaneous SOS-induced deletions were not included. The same correction was made for MM294A + UV cells.

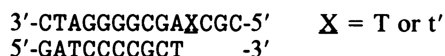
||The dark-blue plaque (B_2) background had the *Nhe* I sequence of M13-*Nhe*I deleted, and the product was M13mp19 with its intact *Sma* I site. The B_2 mutants arose either by religation of residual *Sma* I-linearized M13mp19 replicative form (RF) or by joining of the nucleotides abutting the six-nucleotide gap of the gapped heteroduplex genome (e.g., ref. 24). The RF DNA of the B_2 phage was eliminated by treatment with *Sma* I; the RF DNAs from progeny of M13-*Nhe*I (and its t′-containing analog) were refractory to this enzyme.

genomes (MF_{SS}) in the *recA430* strain, which is unable to undergo SOS mutagenesis, was the same as in the control cells. The MF_{SS} also was unchanged in the SOS-constitutive strain, DE667, and in MM294A cells that had their SOS functions induced by UV light. Finally, the MF_{SS} was also ≈0.2–03.% in *E. coli* strains that are defective in t′ glycosylase activity (BW415) or that overproduce the same enzyme (BW419); in neither strain was the adduct detectably mutagenic in dsDNA, even upon induction of SOS functions (data not shown). Collectively, these experiments failed to identify repair states in which t′ mutagenesis was increased or decreased relative to the level in physiologically normal cells.

Mutational Specificity of t′. Mutants from the progeny of ss t′-M13-*Nhe*I were selected by the insensitivity of their dsDNA to *Nhe* I. The selective procedures generated a population of t′-induced mutant phage (>98%) producing dark-blue plaques.** No dark-blue mutants were found in similar experiments utilizing DNA in which unmodified d(GCTAGC) was incorporated into M13-*Nhe*I. Eighty-seven mutants from 13 independent experiments were sequenced, and only t′·A → C·G mutations were observed.†† Mutants derived from t′ in the (+) strand (position 6274) were ≈3-fold more abundant than those from the (−) strand (position 6275).

The Structural Basis of Mutation Induced by t′. The most straightforward origin of the t′ → C mutation would be for t′ to become paired with guanine during DNA replication, possibly in a structure resembling a T·G wobble base pair (37). Molecular mechanical calculations were performed to test the feasibility of the model. The starting point for determination of the base wobble induced by t′ was either the crystal coordinates of t′ (29) or a planar version in which the local structure of DNA molded the pucker of the t′ ring. In the latter, initial coordinates were employed such that the six-membered ring of t′ was planar and, during energy minimization, the structure of DNA in the vicinity of t′ established an optimal conformation for the six-membered ring. It is noteworthy that the resultant structure (shown in Fig. 3*a*) had a pucker opposite to that derived from the crystal coordinates of the t′ monomer. Nevertheless this conformation seemed reasonable because the C5 methyl of t′ assumed an equatorial position in which it was accommodated best (i.e., with the least local distortion) by the 5′-adjacent base (cytosine in Fig. 3*a*).

In the first step of the molecular modeling, idealized coordinates for 14 base pairs of ds B-DNA were generated, and then four bases were removed from the 3′ end of one strand to form a ss/ds DNA junction. Thymine (or t′) was placed in the template such that it was the next base to be copied by DNA polymerase.



This structure was then refined by molecular mechanical calculation. The structure refined by this method has a minimized energy, and in this case it might be a reasonable approximation of the structure that DNA polymerase encounters when it is about to copy a thymine (or t′) residue at the ss/ds junction. Unfortunately, the approximation procedure ignores any effect DNA polymerase may have on local DNA architecture.

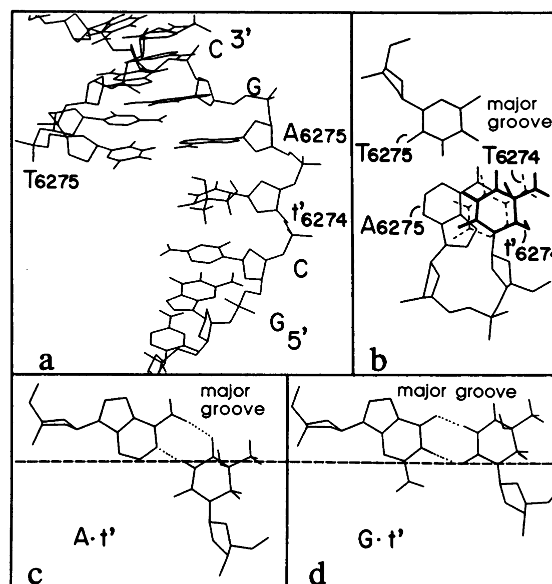


FIG. 3. Structure of t′ in DNA deduced from molecular modeling and molecular mechanical calculations. (*a*) t′ (5*R*,6*S* isomer) located at position 6274 in the genome of M13-*Nhe*I. The structure has four base pairs that are ds at the top of the panel, and four bases that are ss at the bottom. t′ is located in the template strand adjacent to the ds domain; it is the next base to be copied by DNA polymerase. The view is from the major groove. The structure has been refined by potential energy minimization. (*b*) Portion of the structure from *a* viewed along the helix axis from below. *b* includes t′ (bold lines), the base pair on the immediate 3′ side of t′ (i.e., A_{6275}·T_{6275}), and thymine (broken lines; from a structure computed separately). t′ is displaced toward the major groove as compared to thymine. (*c*) Structure of the A·t′ base pair, when a dAMP residue is added opposite t′ in the structure in *a* (following minimization). (*d*) Structure of the analogous G·t′ base pair when a dGMP residue is added opposite t′. The broken horizontal line through *c* and *d* is for reference and lies just below C2 of both adenine (*c*) and guanine (*d*). t′ is primarily below and above the broken line in *c* and *d*, respectively, which indicates that t′ must be displaced upward (i.e., toward the major groove) in a G·t′ vs. an A·t′ base pair.

As seen in Fig. 3, after minimization t′ has been displaced by ≈0.5 Å toward the major groove compared to the position otherwise occupied by thymine. This orientation would be expected to facilitate the mispairing of t′ with guanine during replication. Interestingly, while the base wobble observed for t′ was similar in magnitude when either the crystal structure or the planar t′ structure provided the initial spatial coordinates, the propeller twists of the two possible starting structures differed (data not shown). The preference of t′ for mispairing with guanine, compared to the analogous T·G mispair, was also observed in a second way. By calculation, t′ favored pairing with adenine by 4.3 kcal/mol (1 kcal = 4.18 kJ) compared with guanine, while thymine favored adenine over guanine by 5.5 kcal/mol; i.e., both t′ and thymine interacted best with adenine, but the t′·G interaction was calculated to be ≈1.2 kcal/mol more favorable than the T·G mispair.

Modeling also enabled the estimation of how bases flanking t′ affected the calculated extent of base wobble. While the 5′-flanking base had little influence, the nature of the 3′ base did markedly affect the displacement of t′ toward the major groove. The displacement increased as the 3′ base was changed in the order G < A < T ≈ C. This order is rationalized by the observation that pyrimidines have atoms that project further into the major groove than purines. Our results are in accord with those of Clark *et al.* (38), who found

**The remaining mutants, which produced colorless plaques, were found to be missing one guanine of the otherwise intact *Sma* I site of M13mp19. The same mutation was detected in both t′-containing genomes and in the control DNA. The frameshift could have originated from a contaminating exonuclease activity of *Sma* I during the digestion step prior to gapped-duplex formation and should be unrelated to t′ mutagenesis.

††All mutants showing dark-blue plaques, whether selected by *Nhe* I resistance or screened by plaque color (35 independent mutants screened), were T → C mutations.

that t′ bypass by DNA polymerase *in vitro* occurs only in sequences with a 3′ purine. Thus, the molecular modeling predictions are consistent with experimental results in that t′ contexts with the smallest base wobble (purines 3′ to t′) are the ones in which DNA polymerase is likely to bypass the lesion.

DISCUSSION

There were two goals to our studies. The first was to characterize the type, amount, and genetic requirements for mutagenesis by t′. Our second goal was to use computational tools to rationalize the mutation observed in the genetic studies. t′ was chosen for these studies (*i*) because cells are likely to be confronted by a low-level challenge from this modified base owing to its formation under conditions of normal oxidative stress (8), and (*ii*) because it is a major product of ionizing radiation damage (1–3).

There is evidence that oxidation damage in DNA is lethal, possibly because of the presence of t′. Osmium tetroxide treatment of ss and ds viral DNAs to t′ levels of 2.8 or 7–8 residues per genome, respectively, constitutes one lethal hit (39, 40). It is unknown whether the observed lethality results from t′ itself or from other DNA lesions formed by the oxidative treatment. In the present study, no reduction in survival was observed with one t′ per ss genome, implying that t′ bypass was efficient, or that repair (or modification) of t′ left a readily bypassable entity. The t′-bypass model is supported by the sequence context, 5′-Ct′A-3′, which is one of two known to be traversed by DNA polymerase I *in vitro* (12).

It is noteworthy that others recently have concluded that oxidation-derived t′ lesions present in randomly modified ss phage genomes are not detectably mutagenic (41). It is unlikely, however, that the level of mutagenesis we observed (≈0.3%) would have been detectable in their system.

We observed solely T → C transitions from replication of the genome containing t′. Since ionizing radiation (13, 14) induces a multiplicity of mutations at A·T sites, only one of which is T → C, we conclude that t′ could be responsible for some but not all of the changes at A·T base pairs. This result is not surprising because a large number of lesions are induced in DNA by ionizing radiation (1, 7), and it is likely that the broad spectrum of mutations results from the participation of multiple lesions in the mutagenic process. The failure of T → C transitions to dominate the ionizing radiation-induced mutations at A·T base pairs may reflect the fast and accurate repair of t′, a process that presumably involves endonuclease III *in vivo* (32–34). This enzyme, when purified, removes t′ lesions at least 50-fold less efficiently from ss- than from dsDNA (35). If a similar preference for dsDNA occurs *in vivo*, this finding could explain our observation that mutations derived from t′ in dsDNA were less than 1/30th as frequent as those derived from t′ in ssDNA (Table 1). The relative inactivity of endonuclease III toward t′ in ssDNA might also explain the observation that the MF of t′ was unaltered in *E. coli* strains that either over- or underproduced this enzyme *in vivo*. The inability of the *nth* gene product to modulate t′ mutagenesis *in vivo* suggests either that 0.3% is the maximum attainable MF of the lesion or that repair factors in addition to endonuclease III protect *E. coli* from the effects of low levels of t′ *in vivo*. Finally, we observed that the SOS system did not influence the biological effects of t′. It is possible that the SOS system would affect the MF in a sequence context in which t′ blocked DNA polymerase, although we note that radiation-induced mutagenesis has both SOS-dependent and SOS-independent components (14).

One of the key results to come from our studies is the prediction that the MF of t′ may be sequence dependent. Molecular modeling suggested that the extent of base wobble and hence the extent of mutagenesis should increase as the base on the 3′ side of t′ is changed in the order G < A < T ≈ C. This prediction is testable.

We are grateful to L. J. Niedernhofer and F. Traynor for assistance and to R. Cunningham for the gift of endonuclease III. This work was supported by Grants CA33821 and ES03775 from the National Institutes of Health.

1. Huttermann, J., Kohnlein, W., Teoule, R. & Bertinchamps, A. J., eds. (1978) *Effects of Ionizing Radiation on DNA* (Springer, New York).
2. Hutchinson, F. (1985) *Prog. Nucleic Acid Res. Mol. Biol.* **32**, 115–154.
3. Pryor, W. A., ed. (1976–1982) *Free Radicals in Biology* (Academic, New York), Vols. 1–5.
4. Ames, B. N. (1983) *Science* **221**, 1256–1264.
5. Frenkel, K., Chrzan, K., Troll, W., Teebor, G. W. & Steinberg, J. J. (1986) *Cancer Res.* **46**, 5533–5540.
6. Beer, M., Stern, S., Carmalt, D. & Mohlhenrich, K. H. (1966) *Biochemistry* **5**, 2283–2288.
7. Teoule, R., Bonicel, A., Bert, C., Cadet, J. & Polverelli, M. (1974) *Radiat. Res.* **57**, 46–58.
8. Cathcart, R., Schwiers, E., Saul, R. L. & Ames, B. N. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5633–5637.
9. Rouet, P. & Essigmann, J. M. (1985) *Cancer Res.* **45**, 6113–6118.
10. Ide, H., Kow, Y. W. & Wallace, S. S. (1985) *Nucleic Acids Res.* **13**, 8035–8052.
11. Clark, J. M. & Beardsley, G. P. (1986) *Nucleic Acids Res.* **14**, 737–749.
12. Hayes, R. C. & LeClerc, J. E. (1986) *Nucleic Acids Res.* **14**, 1045–1061.
13. Glickman, B. W., Rietveld, K. & Aaron, C. S. (1980) *Mutat. Res.* **69**, 1–12.
14. Brandenburger, A., Godson, G. N., Radman, M., Glickman, B. W., van Sluis, C. A. & Doubleday, O. P. (1981) *Nature (London)* **294**, 180–182.
15. Shinoura, Y., Kato, T. & Glickman, B. W. (1983) *Mutat. Res.* **111**, 43–49.
16. Kato, T., Oda, Y. & Glickman, B. W. (1985) *Radiat. Res.* **101**, 402–406.
17. Ayaki, H., Higo, K. & Yamamoto, O. (1986) *Nucleic Acids Res.* **14**, 5013–5018.
18. Glickman, B. W. (1982) in *Environmental Mutagenesis and Carcinogenesis*, eds. Sugimura, T., Kondo, S. & Takebe, H. (University of Tokyo Press, Tokyo), pp. 712–728.
19. Grosovsky, A. J., De Boer, J. G., De Jong, P. J., Drobetsky, E. A. & Glickman, B. W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 185–188.
20. Storz, G., Christman, M. F., Sies, H. & Ames, B. N. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8917–8921.
21. Ennis, D. G., Fisher, B., Edmiston, S. & Mount, D. W. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3325–3329.
22. Basu, A. K., Niedernhofer, L. J. & Essigmann, J. M. (1987) *Biochemistry* **26**, 5626–5635.
23. Green, C. L., Loechler, E. L., Fowler, K. W. & Essigmann, J. M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 13–17.
24. Loechler, E. L., Green, C. L. & Essigmann, J. M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6271–6275.
25. Jones, T. A. (1982) *Computational Crystallography* (Clarendon, Oxford), pp. 303–317.
26. Weiner, P. K. & Kollman, P. A. (1981) *J. Comp. Chem.* **2**, 287–303.
27. Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Ghio, C., Alagona, G., Profeta, S., Jr., & Weiner, P. (1984) *J. Am. Chem. Soc.* **106**, 765–784.
28. Loechler, E. L. (1989) *Biopolymers* **28**, 909–927.
29. Flippen, J. L. (1973) *Acta Crystallogr. Sect. B* **29**, 1756–1762.
30. Iida, S. & Hayatsu, H. (1970) *Biochim. Biophys. Acta* **213**, 1–13.
31. Leadon, S. A. & Hanawalt, P. C. (1983) *Mutat. Res.* **112**, 191–200.
32. Demple, B. & Linn, S. (1980) *Nature (London)* **287**, 203–208.
33. Kow, Y. W. & Wallace, S. S. (1987) *Biochemistry* **26**, 8200–8206.
34. Breimer, L. H. & Lindahl, T. (1984) *J. Biol. Chem.* **259**, 5543–5548.
35. Breimer, L. H. & Lindahl, T. (1985) *Biochemistry* **24**, 4018–4022.
36. Koffel-Schwartz, N., Maenhaut-Michel, G. & Fuchs, R. P. P. (1987) *J. Mol. Biol.* **193**, 651–659.
37. Brown, T., Kennard, O., Kneale, G. & Rabinovich, D. (1985) *Nature (London)* **315**, 604–606.
38. Clark, J. M., Pattabiraman, N., Jarvis, W. & Beardsley, G. P. (1987) *Biochemistry* **26**, 5404–5409.
39. Hariharan, P. V., Achey, P. M. & Cerutti, P. A. (1977) *Radiat. Res.* **69**, 375–378.
40. Moran, E. & Wallace, S. S. (1985) *Mutat. Res.* **146**, 229–241.
41. Hayes, R. C., Petrullo, L. A., Huang, H., Wallace, S. S. & LeClerc, J. E. (1988) *J. Mol. Biol.* **201**, 239–246.