Bisulfite Induces Tandem Double CC → TT Mutations in Double-Stranded DNA. 2. Kinetics of Cytosine Deamination[†,‡]

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ABSTRACT: Deamination of cytosine to uracil in double-stranded DNA (ds DNA) by sodium bisulfite has been monitored with a sensitive genetic assay. In this system, reversion of a mutant in the *lacZ* α gene coding sequence of bacteriophage M13mp2 C141 was detected by employing an *ung*⁻ bacterial strain defective in the enzyme uracil glycosylase. Within the 4-base target, it is possible to measure the rates of induction of C → T, C → A, C → G, and CC → TT mutations in DNA that has been incubated at physiological temperature and pH and then transfected into *ung*⁺ and *ung*⁻ *E. coli* cells, respectively, for amplification and detection of the mutation. For concentrations of bisulfite from 1 to 50 mM, the reversion frequency in *ung*⁻ cells increased linearly with time of incubation. The most interesting features of the bisulfite reaction were as follow: (1) Mutations were reduced 5-fold in *ung*⁺ cells, indicating *ung* is involved in repair of bisulfite-treated transforming DNA. (2) Sequencing of 157 revertants revealed that C → T and tandem CC → TT transition mutations comprised 100% of the mutations scored. (3) A unique finding was that, at the highest concentrations and longest incubation times, *almost every mutant obtained in ds DNA exposed to bisulfite was found to be a CC → TT tandem double mutation.* (4) The high frequency of tandem double mutants is inconsistent with two random, independent mutational events and, coupled with the observed *ung* dependence, lends support to the concept of catalytic deamination, wherein bisulfite induces deamination in contiguous cytosines by a concerted mechanism. (5) Equations were derived for calculating the rate constant per site based on the data from DNA sequencing. Mutational rate constants at four cytosines in the ds DNA target at 37 °C in 10 mM sodium bisulfite, 10 mM Hepes, pH 7.4 varied from 0.06×10^{-10} s^{-1} to 0.35×10^{-10} s^{-1} and showed a sequence context effect. A cytosine bordered on both sides by cytosine residues exhibited a mutation rate which was twice as great as a cytosine having only one nearest-neighbor cytosine. (6) In a tandem double CC → TT mutation, there was a 4–5 order of magnitude increase in mutational rate constant of the second genetic event. These findings show that tandem double mutations, which have traditionally been ascribed to UV damage, can also be caused by chemical damage.

The use of sodium bisulfite to induce deamination of cytosine derivatives was described by Shapiro et al. (1970, 1973a) and Hayatsu et al. (1970). Subsequently, Shapiro et al. (1973b, 1977) reported that double-stranded (ds)[1] DNA at pH 5.4 and 37 °C reacted with bisulfite at less than 2% the rate of single-stranded (ss) DNA, leading to the conclusion that the mutagenic properties of bisulfite were most probably due to its reaction with ss DNA. By restriction enzyme analysis, Gough et al. (1986) showed that cytosines in potential cruciform structures in highly supercoiled DNA were sensitive to bisulfite, again suggesting that bisulfite has a preferential bias for regions of single-stranded DNA. In RNA, as well, the reaction is very sensitive to ordered structure, with only the unstacked and accessible cytosine residues reacting, making bisulfite a method of choice to alter sequence (reviewed by Bhanot & Chambers, 1977). Until now, the rate of bisulfite-induced deamination of cytosine in double-stranded DNA has never been measured under physiologically relevant conditions (37 °C, pH 7.4), even though sodium bisulfite is a mutagen at acid pH (see review by Pagano & Zeiger, 1987).

An estimate for the rate constant of bisulfite-induced deamination in ds DNA was obtained by extrapolating data obtained at >55 °C with cytidine nucleoside to lower temperatures (Slae & Shapiro, 1978). The extrapolated rate (4×10^{-12} s^{-1} at 37 °C, pH 7.4 in 0.0001 M sodium bisulfite) is so small that standard chemical methods of analysis have not had the sensitivity to detect the low level of deamination expected, and thus a more sensitive type of assay was needed to directly examine deamination in double-stranded DNA.

We report here a sensitive genetic method that allows us to obtain the rates of bisulfite-induced deamination in ds DNA under physiological conditions and to examine the products of the reaction. The assay described by Frederico et al. (1990) makes it possible to accurately assess the site-specific rate constant of spontaneous hydrolytic deamination of cytosine in both ss and ds DNA and under conditions of temperature and ionic strength that closely approximate those that exist in vivo. The detection system is based on reversion of a mutant of bacteriophage M13mp2C141 from a colorless to a blue plaque phenotype, resulting from C → T or CC → TT transitions within the *lacZ* α gene coding sequence. This approach is highly sensitive and specific. It permits detection of one deamination event, *at a single site* in a 7196 base pair M13mp2 DNA molecule, from a pool of more than 10^5 M13mp2 DNA molecules.

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[1] Abbreviations: ds, double-stranded; ss, single-stranded; SM, single mutants; TDM, tandem double mutants; TM, total mutants; buffer A, 10 mM Hepes–NaOH, pH 7.4; buffer B, 100 mM KCl, 50 mM Hepes–KOH, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4.

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Here we show that an inordinately large proportion of CC → TT tandem double mutants were found to be induced by bisulfite in a 4-base target site. Further study indicated that induction of CC → TT tandem double mutants by bisulfite was time and bisulfite concentration dependent. These findings show that tandem mutations in ds DNA, which traditionally have been ascribed to UV damage, can also be caused by chemical damage.

MATERIALS AND METHODS

Materials

RNase A and RNase T1 were from United States Biochemical Corp. MOPS was from Sigma Chemical Corp. All other materials used in this assay were the same as described in Chen and Shaw (1993a).

Methods

Preparation of Double-Stranded DNA. Double-stranded DNA substrates were prepared by an improved method of Birnboim and Doly (1979). Briefly, bacteriophage M13-mp2C141 was plated on minimal medium plates using *E. coli* NR9099 as a host strain. A single plaque was added to 1 L of 2 × YT medium (containing 16 g of Bacto-Tryptone, 10 g of yeast extract, and 5 g of NaCl, pH 7.4 per liter) with 10 mL of an overnight culture of *E. coli* NR9099. M13mp2-infected cells were grown overnight at 37 °C with vigorous shaking, and cells were harvested by centrifugation at 4000*g* for 30 min at 0 °C in a Sorvall centrifuge with a GS3 rotor without application of the brake for deceleration. The pellet was resuspended in 0.9% NaCl and was centrifuged at 5000 rpm for 15 min. The supernatant was discarded, and the pellets were resuspended in 70 mL of GET buffer (50 mM glucose, 10 mM EDTA, and 25 mM Tris, pH 8.0) and 10 mL of GET containing 100 mg of lysozyme (10 mg/mL). After 30 min on ice, 80 mL of alkali–SDS (0.2 N NaOH and 1% sodium dodecyl sulfate) was added. After 10 min on ice, 60 mL of high salt solution (3 M KOAc, 1.8 M formic acid) was added. The mixture was kept on ice for 30 min and centrifuged at 8000 rpm, 4 °C for 15 min. Then, 100 mL of the supernatant was transferred to a sterile centrifuge bottle, and 200 mL of 95% ethanol was added to the supernatant; then the replicative form (RF) was precipitated at −20 °C overnight. After centrifuging at 5000 rpm, 4 °C for 25 min, the supernatant was removed, and the pellet was resuspended in 10 mL of acetate/MOPS (100 mM NaOAc, 50 mM MOPS). Following overnight precipitation by alcohol and centrifugation as above, the pellet was resuspended in 3 mL of deionized water and 3 mL of LiCl–MOPS (5 M LiCl, 50 mM MOPS, pH 8.0) and placed on ice for 15 min. After centrifugation at 5000 rpm for 20 min, the supernatant was precipitated again with 12 mL of 95% ethanol. After the ethanol was poured off, 3 mL of TE buffer (10 mM Tris, 5 mM EDTA, pH 8.0), 3 μL of RNase A solution (10 000 μg/μL made by mixing 50 mg of RNase A, 25 μL of 1 M Tris-HCl, 5 mL of deionized water, heating at 80 °C for 10 min, and storing at −20 °C until use), and 30 μL of RNase T1 (made by mixing 578 μL of RNase T1, 25 μL of 1 M Tris-HCl, and 4.4 mL of deionized water, heating for 10 min, and storing at −20 °C until use) were added to the pellet and then incubated at 37 °C for 30 min. The solution was extracted twice with phenol to separate ds DNA from protein and then extracted once with chloroform–isoamyl (24:1) to remove the remaining phenol. Then, 300 μL of 3 M NaOAc and 6 mL of 95% alcohol were added, and after 30 min at −20 °C the solution was spun at 5000 rpm at 4 °C for 30 min. The pellet containing ds DNA was resuspended in 1 mL of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4). This procedure typically yields 13 mg of ds C141DNA ($A_{260}/A_{280} > 1.7$). The concentration of ds DNA was about 13 μg/μL.

Incubation of DNA, Transfection, and Plating. Conditions of incubation with bisulfite were the same as described by Chen and Shaw (1993a) for ss DNA with the following exceptions: (i) The longest incubation time was increased to 54 days, and many more plaques were scored. Since deamination with bisulfite is about 10-fold slower in ds DNA relative to ss DNA, the low reversion frequency and spontaneous deamination rate constant in ds DNA required more plating to obtain a sufficient number of revertants for statistical analysis and sequencing. Platings were done at 10 000 plaques per 9-cm-diameter Petrie dish in order to minimize camouflage of the wild-type phenotype that can occur with higher plating densities. (ii) The efficiency of electroporation of ds DNA into *E. coli* NR9404 *ung*⁻ and MC1061 *ung*⁺ cell strains averaged $\leq 20 \times 10^3$ plaques/ng of DNA, which is 4–10-fold greater than that for ss DNA and required that samples be diluted from 20 ng/μL to 2–10 ng/μL just prior to electroporation. The protocols for transfection and plating followed Chen and Shaw (1993a) including, as before, desalting of the DNA by centrifuging at 1600 rpm through a 1-mL Sephadex G-50 column prior to transfection. The reversion frequency is defined as the number of blue revertants divided by the total number of (blue plus white) plaques, and in *ung*⁻ strains it corresponds to the fraction of cytosines that have been converted to uracil.

DNA Sequencing. C141 revertant plaques appear as dark blue plaques against a colorless background after incubation. For each DNA sample, at least 10 revertant plaques were picked from the plates, diluted in 50 mM sodium borate, and replated to eliminate false positives. Out of every 100 plaques chosen, 95 were confirmed to be wild-type blue. To determine the specific base change responsible for reversion, DNA sequencing of randomly selected plaques from each experiment was performed using the chain terminator method (Sanger et al., 1977).

DNA extraction was simplified as follows for DNA sequencing. A randomly selected plaque was placed in 6 mL of 2 × YT, grown overnight at 37 °C, and then centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was transferred to a 15-mL corex tube and 1.5 mL of 15% PEG, 2.5 M NaCl solution was added to it, and the resulting mixture was refrigerated for 2 h. After centrifugation at 4000 rpm for 20 min at 4 °C, the pellet was resuspended in 600 μL of PEB (0.1 M Tris, 1 mM EDTA, and 300 mM NaCl, pH 8.0) and was then added to a mixture of 500 μL of phenol, 100 μL of chloroform/isoamyl alcohol (24:1). The mixture was kept at room temperature for 10 min and then spun at 8000 rpm for 15 min. The aqueous phase was transferred to 1 mL of 95% alcohol, 50 μL of 3 M NaOAc and placed in a freezer for 30 min. After centrifugation at 9000 rpm for 30 min, the supernatant was discarded and the pellet was resuspended in 10 μL of cold TE buffer. The OD of each sample was measured at 260 and 280 nm. Twenty to thirty micrograms of DNA can be obtained from each preparation; 500–1000 ng of DNA was used for sequencing each sample.

RESULTS

The Target for Cytosine Deamination. Bisulfite acting as a mutagen is expected to deaminate cytosine bases in DNA to uracil. In our assay, the four cytosine residues in the 33rd and 34th codons (^{138}TTC CCC^{143}) of the M13mp2C141 DNA mutant are the targets for scoring cytosine deamination.

Table 1: Reversion Frequencies in *ung*⁻ Cells of Double-Stranded M13mp2 C141 DNA Incubated at 37 °C, pH 7.4

incubation time (days)	$[HSO_3^-]$ mM[a]	no. of revertants	total plaques (10^3)	reversion frequency[b] (10^{-5})	efficiency[c] (10^3 plaques/ng of DNA)	k' target[d] (10^{-10} s^{-1})
0	0 (buffer A)	19	3900	0.49	19	–
3	0 (buffer A)	15	3000	0.50	19	(0.004)[e]
	1.0	9	1050	0.86	17	0.14
	2.5	15	1300	1.2	16	0.27
	5.0	10	600	1.7	15	0.46
	10	13	700	1.9	14	0.54
	50	11	470	2.3	12	0.69
9	0 (buffer A)	8	950	0.84	19	(0.04)[e]
	1.0	9	390	2.3	19	0.19
	2.5	12	300	4.0	15	0.41
	5.0	17	380	4.5	13	0.47
	10	14	250	5.6	12	0.61
	50	15	190	7.9	9.4	0.91
27	0 (buffer A)	10	750	1.3	15	(0.03)[e]
	1.0	10	260	3.8	13	0.11
	2.5	13	170	7.6	8.3	0.27
	5.0	14	120	12	7.2	0.46
	10	16	120	13	6.0	0.50
	50	20	110	18	5.5	0.72
54	0 (buffer A)	12	580	2.1	14	(0.03)[e]
	1.0	19	220	8.6	11	0.14
	2.5	20	160	13	7.9	0.23
	5.0	13	82	16	7.2	0.30
	10	16	87	18	6.7	0.34
	50	21	96	22	5.6	0.43
0	0 (buffer B)	18	4310	0.4	21	–
9	0 (buffer B)	14	1300	1.1	13	0.09
	10	32	625	5.1	12	0.51
54	0 (buffer B)	6	288	2.1	6	0.04
	10	20	137	15	4	0.28

[a] ds M13mp2 C141 DNA (20 ng/μL) was incubated in buffer A (10 mM Hepes–NaOH buffer, pH 7.4) or buffer B (100 mM KCl, 50 mM Hepes–KOH, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4), with and without bisulfite, in glass capillary tubes wrapped in aluminum foil. Prior to transfection into NR9404 (*ung*⁻) cells, each sample was desalted thru an individual Sephadex G-50 column preequilibrated with 10 mM Hepes, pH 7.4. One microliter sample (2–10 ng of DNA/μL) was used for each transfection, and the transfection mixture (about 2 mL) was distributed onto 10 medium plates. [b] After incubation of ds C141 DNA at the indicated temperatures, reversion frequencies were determined by transfection into *ung*⁻ cells as described under Methods. Each reversion frequency represents the average value of three or more separate transfection experiments. Because the number of plaques in each experiment could differ, the reversion frequency in this table is close to, but does not necessarily correspond to, that which can be obtained from dividing the total revertants by the total plaques. The first row of each time point gives the background reversion frequency (i.e., no bisulfite) for the corresponding incubation time. [c] The efficiency is defined as the total plaques obtained from a nanogram of DNA for a sample, averaged here for three or more transfection experiments. Efficiency here is the mean average efficiency of the separate transfections. [d] k' target is the pseudo-first-order rate constant for the entire target obtained from eq 1 and reflects the summation of deamination at the four target cytosines. [e] The values in parentheses are the background rate constants, obtained by subtracting the reversion frequency (of DNA incubated in buffer A, no bisulfite) at 0 days incubation time from the background reversion frequency at the designated incubation time, all divided by t.

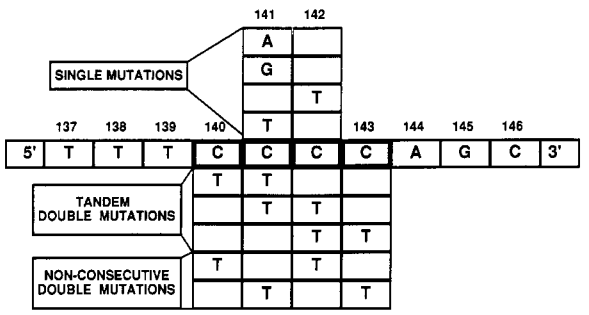

FIGURE 1: Types of mutations scored (or able to be scored) in this study with the M13mp2 C141 target. M13mp2 C141 DNA (clear plaque phenotype) can revert to a blue plaque phenotype by a number of mutations at cytosine sites in the 140–143 region of the *lacZα* gene. Only known, dark blue phenotypes are shown in this table. Transversion mutations (C → A or C → G) were not observed in the experiments here. Noncontiguous double mutations (at sites 140 and 142, or at sites 141 and 143) as well as triple mutations (CCC → TTT at sites C141, C142, C143) with dark blue phenotypes could have been scored in this study, but none were found.

Figure 1 summarizes the types of mutations scorable in this study. Single mutational events can be scored phenotypically only at sites 141 or 142. However, tandem-double-mutational events also can be scored at sites (140, 141), (141, 142), or (142, 143) should they occur. On the basis of codon usage during translation, nonconsecutive CC → TT double mutations at sites (140, 142) and (141, 143) should also be scorable, although we have not seen any in this study.

If the mutational event occurs via a uracil intermediate, the uracil cannot be removed if the DNA is transfected into a NR9404 *ung*⁻ strain which is defective in repair of uracil. The uracil will code like thymine during the first round of DNA replication in vivo and eventually generate, within the infected cell, progeny that contain either a single C → T transition at position 141 or 142 (due to ^{141}TCC^{143} or ^{141}CTC^{143}) or a tandem double CC → TT transition [at two adjacent positions among the cytosines at 140, 141, 142, and 143] due to ^{140}TTCC^{143}, ^{140}CTTC^{143}, or ^{140}CCTT^{143} (see Figure 1). All these mutations result in a functional protein and produce a blue plaque phenotype. Thus, bisulfite-induced deamination can be scored as blue plaques in a field of colorless plaques. Molecules that have deaminated or mutated elsewhere, but not at the four detectable sites, will give colorless plaques. The assay is specific for deamination, since 100% of the blue plaques (i.e., revertants) scored in this study were confirmed to be C → T or CC → TT transition mutations.

Time Dependence of Bisulfite-Induced Deamination in ds DNA. M13mp2C141 DNA was incubated at 37 °C in 10 mM Hepes, pH 7.4 for varying times and with varying

concentrations of bisulfite. After the specified time, the bisulfite was removed with Sephadex G-50, and the DNA was used for transfection into *ung*⁻ cells. When the fraction of cytosine converted to thymine (equivalent here to the reversion frequency, E' in eq 1) in *ung*⁻ cells was plotted as a function of time for various concentrations of bisulfite (see supplementary material), the graphs for each concentration of bisulfite yielded a straight line, each having a correlation coefficient greater than 0.95. For all concentrations of bisulfite examined (1–50 mM) at pH 7.4, the reversion frequency increased linearly with time. After 9 days of incubation in 10 mM bisulfite, the reversion frequency was 7-fold above the background level. Thus, the presence of bisulfite increases the probability of mutation linearly over time, as expected for a pseudo-first-order reaction.

Dose Dependence and Rate Constants of Deamination. Pseudo-first-order rate constants k' for the whole 4-base target can be calculated at designated time periods for each set of bisulfite concentrations in Table 1. The best fit (whole target) pseudo-first-order rate constants (k') were 0.03, 0.09, 0.25, 0.45, 0.48, and 0.5×10^{-10} s^{-1} for 0.0, 1.0, 2.5, 5.0, 10, and 50 mM bisulfite, respectively. Clearly, the calculated reversion frequencies and corresponding mutational rate constants k_1' increase with increasing concentration of bisulfite. Yet, they are not directly proportional to the bisulfite concentration because (1) deamination with bisulfite is a multistep reaction involving multiple pathways, as reviewed by Shapiro (1977, 1983) and as observed for *ss DNA* by Chen and Shaw (1993a) and (2) bisulfite catalyzes the formation in ds DNA of not only single C → T mutants, but also tandem double CC → TT mutants in a concerted manner (see below). As expected, however, the data do show a dose-dependent relationship between deamination and bisulfite concentration.

The reversion frequency and pseudo-first-order rate constants, k', in Table 1 are given for the whole (4-base) ds C141 DNA target, i.e., they represent the total of the respective values from both single mutants and double mutants (as described below), so their values correspond to the sum of the seven (out of nine) known ways (depicted in Figure 1) that the target can revert. To obtain the values per site, it is necessary to derive equations (see below).

Sequencing Reveals That Bisulfite Induces a High Proportion of Tandem Double Mutants. A total of 157 blue revertants were sequenced by randomly choosing 6–11 revertants from each of the experiments in Table 1. Sequencing revealed the following:

(1) Only C → T and CC → TT mutants in the 4-base target were found (Supplement Table 1, supplementary material). The mutants were of five types: C → T mutants were found at sites 141 and 142, and tandem double CC → TT mutants were found at sites (140, 141), (141, 142), and (142, 143). Neither C → A or C → G transversions nor any noncontiguous (140, 142) or (141, 143) double mutants were found.

(2) Tandem *double* mutants (80 in all) comprised about half of all the accumulated (157 total) mutations (Supplement Table 1, supplementary material). This large proportion of double mutants was unexpected assuming single-hit kinetics, since the probability of finding a double mutation should be the product of the probabilities (or reversion frequencies) of the individual reactions if the mutations occur randomly and independently. In fact, if a single C → T mutations occurs with a reversion frequency of 10^{-5}, then a tandem double CC → TT mutation should occur with a reversion frequency of 10^{-10}, if the events occur independently. Such a frequency would be too small for us to detect. The sequencing results

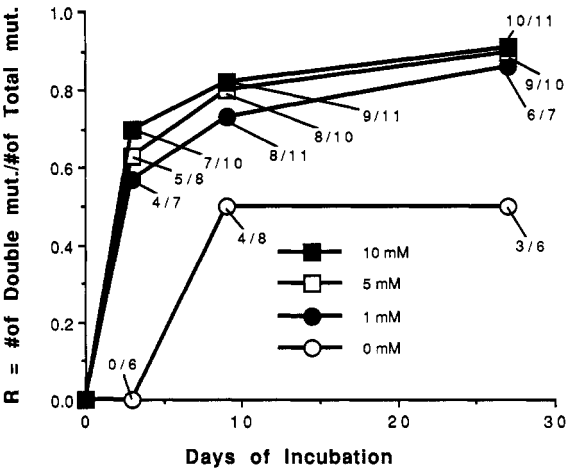

FIGURE 2: Dose and time dependence of tandem double mutants. Double-stranded M13mp2 C141 DNA was treated with different concentrations of bisulfite in buffer A. The ratio R of tandem CC → TT double mutants found (numerator) to total revertants sequenced (denominator) is plotted *vs* sample incubation time. The fraction indicated for each point is the precise number of TDM found divided by the total mutants sequenced for each set of conditions (as in Supplement Table 2 in the supplementary material).

revealed that bisulfite induces an extremely high proportion of tandem CC → TT double mutants, suggesting involvement of some type of concerted production of tandem double mutants from single mutants.

(3) The ratio of tandem double mutants (TDM) to total mutants increased both with time of incubation and with concentration of bisulfite, as seen in Supplement Table 2 (supplementary material) and Figure 2. Here we define a quantity, R, as the ratio of double mutants to total mutants. At the shortest incubation time, no double mutants were formed and R was equal to zero. This was true both in the presence and the absence of bisulfite. However, at the longest incubation times *with bisulfite present*, almost every mutant was a tandem CC → TT double mutant and R approached the value of 1. A detailed mathematical description of this behavior is presented later.

Physiological Relevance. Changing the buffer composition did not affect the overall results. Similar results were seen (Table 1) both with a low ionic strength ($\mu = 0.04$) buffer A and a more physiologically relevant, $KCl/MgCl_2$-containing buffer B of high ionic strength ($\mu = 0.22$). The relative proportion of double mutants with and without bisulfite was nearly the same in the two buffers (see Supplement Table 2, supplementary material), as were the reversion frequencies (Table 1). Thus, variations in the salt and ionic strength during incubation had little effect on the results and suggest that the mutations scored here are probably relevant to mutations that occur in vivo.

Confirmation of Deamination by Bisulfite. The above sequencing experiments showed that bisulfite was inducing C → T transition mutations, i.e., the type of mutation expected from deamination of cytosine. A second approach was used to determine whether or not the observed revertants were caused by deamination. DNA samples incubated with 10 mM bisulfite were used to transfect both *ung*⁻ and *ung*⁺ host cells (see Table 2). The reversion frequency was lowered over 5-fold in *ung*⁺ vs *ung*⁻ strains, indicating that the majority of the revertants were eliminated by cells having a functional uracil glycosylase enzyme. This decrease is typical for deamination damage and was observed in buffers with and without bisulfite and for ds DNA incubated for both 4 and 27 days. Bisulfite-induced rate constants for the entire target

Table 2: *Ung* Dependence of Bisulfite Mutagenesis

cell strain	$[HSO_3^-]$ (mM)	incubation time (days)	plaques scored: blue	plaques scored: total (10^5)	reversion frequency (10^{-5})	target rate constant k_1' [a] (10^{-10} s^{-1})
NR9404 (*ung*$^-$)	0	0	19	39	0.49	*b*
	10	0	11	22	0.50	–
	0	4	16	32	0.50	*b*
	10	4	13	7.0	1.9	0.41
	0	27	10	7.5	1.3	*b*
	10	27	16	1.2	13	0.51
						av k_1' = 0.46
MC1061 (*ung*$^+$)	0	0	8	400	0.02	*b*
	10	0	9	300	0.03	–
	0	4	9	301	0.03	*b*
	10	4	10	30	0.33	0.09
	0	27	12	30	0.4	*b*
	10	27	9	3.5	2.6	0.09
						av k_1' = 0.09

[a] All samples were 8×10^{-9} mM M13mp2 C141 ds DNA and 10 mM HSO_3^- in buffer A, 37 °C. The calculation of rate constants for the entire target, k', is the same as in Table 1. [b] The background experiments were performed using the same DNA, under the same experimental conditions, but with no bisulfite.

were found to be 0.5×10^{-10} s^{-1} upon transfection into an *ung*$^-$ cell strain and 0.09×10^{-10} s^{-1} in an *ung*$^+$ strain (Table 2) compared to a background rate constant of 0.03×10^{-10} s^{-1} in buffer A alone (Table 1). The reduction in reversion frequency upon transfection of treated DNA into *ung*$^+$ cells (Table 2) indicates that the majority of mutations are occurring via a uracil intermediate. This result is consistent with the deamination pathway proposed by Shapiro (1983) wherein cytosine undergoes catalytic deamination by bisulfite, generating uracil and regenerating bisulfite (see also Chen and Shaw, 1993a).

Most, but not all, the mutants in bisulfite-treated DNA (Table 2) are removed by *ung*$^+$ strains active in uracil glycosylase. Incomplete removal is typical of the assay (see Frederico et al., 1990, and Chen and Shaw, 1993a), where up to 10–20% of spontaneous mutations detected by this system are not repaired in *ung*$^+$ strains. The incomplete removal suggests that either (a) uracil glycosylase is not completely efficient or (b) an alternative and minor mechanism of mutation may be operative, e.g., formation of a miscoding lesion at the target site might lead to increased misincorporation by DNA polymerase of a thymine, which cannot be repaired by uracil glycosylase. In either case, the data in Table 2 shows that these make only minor contributions to the overall reversion frequency.

Since the majority of mutations at both 4 and 27 days of incubation were tandem double mutants, and since the reversion frequency dropped by 80% in *ung*$^+$ cells, it is clear that the uracil glycosylase-containing cells are able to repair or remove the majority of the tandem double mutants, implying that *ung* may be effective on U and UU bases in DNA. Thus at least one, and possibly both, of the thymine residues in CC → TT tandem mutants arose via a uracil intermediate.

Calculation of Rate Constant per Site of Bisulfite-Induced Deamination for ds DNA. When bisulfite is in excess, ds DNA should exhibit a pseudo-first-order rate constant (k') for cytosine deamination (Chen & Shaw, 1993a) which can be calculated from eq 1

$$k' = E'/t \quad (1)$$

where E' is the corrected reversion frequency for the entire target (obtained by calculating the reversion frequency equal to the number of revertants divided by the number of total plaques at time of incubation, t, and corrected by subtracting the background reversion frequency at the corresponding time). The corrected target reversion frequency E' differs in two respects from the site-specific reversion frequency F used in Chen and Shaw (1993a): E' is the corrected sum of the reversion frequencies of both single and double mutants, and it equals F from Chen and Shaw (1993a) only when single mutants occur with equal probability at sites C141 and C142, when no TDM occur, and when the background reversion frequency is subtracted. In the present study, single mutants were scored at site 141 or 142 and TDM were scored at sites (140, 141), (141, 142), or (142, 143); thus, the bisulfite-induced rate constant k' for ds DNA from eq 1 represents the total measurable mutation rate for the whole target (^{138}TTC ^{140}CCC). It is important to recognize that k' here is not exactly comparable to the rate constant derived for ss DNA (Chen & Shaw, 1993a), because that rate constant reflected deamination normalized for equal deamination at two sites. Here, the rate constant k' for ds DNA reflects the cumulative deamination at four cytosine sites, and k' contains a large contribution from tandem double mutations (TDM) as well as from single mutations (SM). The equations which relate the total reversion frequency to the rate constant *per site* for ds DNA can be derived as follows:

$$E' = E'_{SM} + E'_{TDM} \quad (2)$$

where E'_{SM} and E'_{TDM} are the corrected target reversion frequencies for single and double mutants, respectively. Upon substituting eq 2 into eq 1, we have

$$k' = (E'_{SM} + E'_{TDM})/t = k'_{SM} + k'_{TDM} \quad (3)$$

where k'_{SM} and k'_{TDM} are the rate constants for formation of single and tandem double mutants, respectively. R_1 and R_2, the fraction of single and double mutants, respectively, can be defined as follows:

$$R_1 = \text{no. SM}/(\text{no. SM} + \text{no. TDM}) \quad (4)$$

$$R_2 = \text{no. TDM}/(\text{no. SM} + \text{no. TDM}) \quad (5)$$

where no. SM is the number of revertants that are single mutations and no. TDM is the number of revertants that are tandem double mutations. R_1 and R_2 should have the following properties:

(a) R_1 and R_2 are functions of incubation time and the concentration of bisulfite used in the assay.

(b) $R_1 + R_2 = 1$ (6)

where $0 \leq (R_1 \text{ or } R_2) \leq 1$.

(c) Limit $R_1 = 1$ and limit $R_2 = 0$ when incubation time $t \rightarrow 0$.

(d) Limit $R_1 = 0$ and limit $R_2 = 1$ when incubation time $t \rightarrow \infty$. On the basis of these properties, a weighting function can be chosen:

$$R_1 = 1/(1 + \alpha t) \quad (7)$$

$$R_2 = \alpha t/(1 + \alpha t) \quad (8)$$

where t is the incubation time (days) and α is a regression constant that is dependent on the concentration of bisulfite.

The result of regression (using the sequencing data from Figure 1 and Supplement Table 2 in the supplementary

material) for α is 0.05 at 0 mM (buffer only), 0.32 at 1 mM, 0.45 at 5 mM, and 0.58 at 10 mM of bisulfite. Multiplying the two sides of eq 6 by k', we have:

$$k' = \frac{1}{(1+\alpha t)}k' + \frac{\alpha t}{(1+\alpha t)}k' \quad (9)$$

Upon comparing eq 3 with eq 9, we find:

$$k'_{SM} + k'_{TDM} = \frac{1}{(1+\alpha t)}k' + \frac{\alpha t}{(1+\alpha t)}k' \quad (10)$$

As $t \rightarrow 0$, only single mutants are observed (see Figure 2), so

$$k'_{SM} = \frac{1}{(1+\alpha t)}k' \quad (11)$$

In the limit of $t \rightarrow \infty$ (long enough), only double mutants are observed (see Figure 2), so

$$k'_{TDM} = \frac{\alpha t}{(1+\alpha t)}k' \quad (12)$$

The rate constants for SM (at sites 141 and 142) and TDM [at sites (140, 141), (141, 142), and (142, 143)] at any period of incubation time can be calculated from eqs 11 and 12, respectively.

In order to obtain the rate constant *per site*, we can derive a relationship between the target and deamination sites, as follows: within the subset of scorable single mutants, η_1 and η_2 are the probabilities of scoring a single mutant at either site 141 and 142, respectively. Within the subset of double mutants, ξ_1, ξ_2, and ξ_3 are the probabilities of scoring tandem double mutants at sites (140, 141), (141, 142), and (142, 143), respectively. Since cytosine deamination at site 140 is detectable only when a double mutant (140, 141) occurs, its probability is ξ_1. Thus, the rate constant for finding tandem double mutants at site 140 should be

$$k'_{TDM(140)} = \xi_1 \frac{\alpha t}{(1+\alpha t)}k' \quad (13)$$

Cytosine deamination at site 141 can be detected in three ways, i.e., as a single mutant (141) which has probability η_1, as tandem double mutants (140, 141) and (141, 142) which have the probabilities ξ_1 and ξ_2, and as noncontiguous mutants (none were found). So, the rate constant for site 141 should be the sum of the rate constants from single mutants and rate constants from double mutants.

$$k'_{(SM+TDM)(141)} = \eta_1 \frac{1}{(1+\alpha t)}k' + (\xi_1 + \xi_2)\frac{\alpha t}{(1+\alpha t)}k' \quad (14)$$

On the same basis, we can obtain rate constants for site 142 and site 143

$$k'_{(SM+TDM)(142)} = \eta_2 \frac{1}{(1+\alpha t)}k' + (\xi_2 + \xi_3)\frac{\alpha t}{(1+\alpha t)}k' \quad (15)$$

and

$$k'_{TDM(143)} = \xi_3 \frac{\alpha t}{(1+\alpha t)}k' \quad (16)$$

The values found by sequencing in our assay are given as follows: $\eta_1 = 58\%$; $\eta_2 = 42\%$; $\xi_1 = 31\%$; $\xi_2 = 52\%$; $\xi_3 = 18\%$ (see Supplement Table 1 in the supplementary material). The general equation for the site-specific rate constant is as follows:

$$k'_{(\text{per site})} = [\sum_{\substack{i=1,2 \\ j=1-3}} (\eta_i R_1 + \xi_j R_2)]k' \quad (17)$$

for site 140, $\eta_1 = 0$ and $j = 1$

for site 141, $i = 1$ and $j = 1, 2$

for site 142, $i = 2$ and $j = 2, 3$

for site 143, $\eta_1 = 0$ and $j = 3$

In summary, in order to derive all equations here it has been assumed that the individual reversion frequencies are additive.

The corrected whole target rate constant, k', can be obtained directly from the results of transfections in Table 1 by plotting the reversion frequency *vs* incubation time (see Supplement Figure 1, supplementary material) for specified concentrations. Each slope represents the uncorrected target rate constants for that concentration. Subtracting the slope of the background (no HSO_3^-) from each uncorrected slope gives the corresponding corrected target rate constants as follows:

$[HSO_3^-]$ (mM)	0	1.0	2.5	5.0	10	50
slope [(10^{-12} s^{-1})]	3	9	25	45	48	50
rate constant k'(corrected)	–	6	22	42	45	47

Site-Specific Rate Constants. Equations 13–16 were used to calculate the rate constant per site for bisulfite-induced deamination in ds DNA, and the results are shown in Table 3. If the equations are representative of the experimental results, the calculated rate constants should be relatively constant over the entire period of incubation time. As seen in Table 3, the site-specific rate constants at any one site in the target for any one bisulfite concentration are found to be relatively constant over the time of incubation, supporting our analysis.

Four trends are apparent. (1) There is a noticeable dose dependence, with the rate constants at the individual sites increasing with bisulfite concentration. (2) The site-specific

Table 3: The Bisulfite-Induced Deamination Rate Constants *per Site* for ds C141 DNA in 10 mM Hepes–NaOH, pH 7.4, 37 °C[a]

$[HSO_3^-]$ mM	incubation time (days)	calculated rate constant (10^{-12} s^{-1}) at each site[a] 140	141	142	143
0 (buffer only)	3	–[b]	1.8	1.4	–[b]
	9	0.3	2.0	1.5	0.2
	27	0.5	2.2	1.7	0.3
		$\langle k' \rangle = 0.4$[c]	2.0	1.5	0.3
1	3	0.9	4.2	3.3	0.5
	9	1.4	4.5	3.7	0.8
	27	1.7	4.8	4.0	1.0
		$\langle k' \rangle = 1.3$	4.5	3.7	0.8
5	3	7.4	30	28	4.3
	9	10	32	27	6.0
	27	12	34	28	7.0
		$\langle k' \rangle = 9.8$	30	28	5.8
10	3	10	33	27	5.2
	9	12	35	29	6.8
	27	13	36	30	7.6
		$\langle k' \rangle = 12$	35	29	6.5

[a] Double-stranded C141 DNA was incubated in buffer A at 37 °C. Deamination rate constants at each site in the target were calculated using eqs 13–16 from the total reversion frequency measured for each sample (in Table 1) and the ratio of double mutants to total mutants (as determined from the sequence of each mutant). [b] No mutants were observed this site. [c] $\langle k' \rangle$ is the time averaged site-specific rate constant.

Table 4: Comparison of Site-Specific Deamination Rate Constants at Site 141 in ss DNA and ds DNA

	site-specific rate constants at site C141	
$[HSO_3^-]$ (mM)	ss DNA[a] (10^{-10} s^{-1})	ds DNA[b] (10^{-10} s^{-1})
0 (buffer only)	0.60	0.02
1	0.78	0.04
5	2.6	0.30
10	3.6	0.35

[a] From Chen and Shaw (1993a) in buffer A at 37 °C. [b] From this study (Supplement Table 2, supplementary material) in buffer A at 37 °C.

rate constants at sites 141 and 142 are 3–7-fold greater than those at sites 140 and 143, both with and without bisulfite. The sequence surrounding the 4-base CCCC target site is 5′ ^{138}TTCCCCAGC^{146} 3′. The two cytosines (141 and 142) in the middle of the run, each bounded by a cytosine on either side, are over three times as likely to deaminate as cytosines (140 and 143) having only one cytosine nearest neighbor. The analysis of site-specific rate constants presented above takes into consideration the fact that C → T mutations at C140 and C143, which are "silent" positions in the genetic code, can only be scored when they occur as part of a double mutation. (3) There is also a tendency toward increased deamination at the 5′-end of the run of contiguous cytosines (which is flanked by a thymine) as compared to the 3′-cytosine end (which is flanked by an adenine). (4) By comparing deamination in ds DNA here (Table 4) with that in ss DNA (Chen & Shaw, 1993a), the bisulfite-induced site-specific rate constant at site 141 in ds DNA is found to be 10% (or less) of that in ss DNA.

DISCUSSION

Tandem Double CC → TT Mutations Are a Hallmark of Bisulfite-Induced Mutations in ds DNA. Treatment of ds DNA with 1–50 mM sodium bisulfite at pH 7.4 and 37 °C gives rise exclusively (100%) to transition mutations at cytosine residues. The fraction of mutants (reversion frequency) increases with the time of incubation and the concentration of bisulfite used, as expected for a chemically-induced deamination reaction. A distinctive feature of this study with bisulfite is that a significant proportion of the mutations were tandem double CC → TT substitution mutants, in addition to the expected single C → T mutants. The proportion of tandem double mutants increased with higher concentrations of bisulfite and with greater incubation time. Interestingly, at the highest concentrations and longest incubation times, *almost every mutant scored was found to contain a CC → TT double mutation* (see Figure 2). Further, although noncontiguous double mutants in the same codon could have been detected, none were observed. Thus, tandem double mutants are a hallmark of bisulfite-induced mutation.

Traditionally, tandem double CC → TT mutants have been taken to be signatures of UV, and not chemical, damage (Miller, 1983; Horsfall et al., 1990; Brash et al., 1991). However, tandem double mutants have been scored with unexplained high frequency in the spontaneous (de Jong et al., 1988) and cisplatin (deBoer & Glickman, 1989) spectrum of the APRT locus in CHO cells and in the spontaneous and echinomycin-treated spectrum of M13mp2 in vitro (Moyer & Shaw, 1992; Moyer et al., 1993). Recently, tandem mutations produced at a frequency from 1 to 3% were postulated to be a manifestation of metal-induced (e.g., iron, copper) DNA damage by oxygen radicals (Tkeshelashvili et al., 1991; Reid & Loeb, 1992, 1993). The results in the present work demonstrate that, in the absence of UV light and by treating DNA with chemicals such as bisulfite, tandem double CC → TT mutations can be induced at frequencies much higher than those in the above studies.

Bisulfite is well-known to undergo autooxidation producing both sulfur- and oxygen-centered free radicals, including the bisulfite radical, $^{\bullet}SO_3^-$, which has been proposed to play an important role in bisulfite mutagenesis (Pagano et al., 1990). It remains to be determined whether or not such radicals are involved in producing the TDM observed here.

Mechanism of CC → TT Tandem Mutations Involves a Concerted Deamination at Contiguous Cytosines and Is Enhanced by DNA Secondary Structure. Some insight into the mechanism of producing tandem CC → TT mutations can be gained by analyzing their sequence dependence, frequency, *ung* dependence, and dependence on secondary structure.

(1) Double mutants are observed here in runs of cytosines and at contiguous, but not at noncontiguous, cytosines. Thus, there is a nearest neighbor effect, wherein the presence of one mutation increases the probability of a mutation in a flanking cytosine. There is also a tendency toward more tandem double mutants at the 5′-end of the 4-base run of cytosines.

(2) The frequency of occurrence of tandem mutants in our studies is so large that the double mutations could not be caused by completely independent mutational events. The reversion frequencies for single C → T mutations (obtained by multiplying data in Table 1 by the *R* values in Figure 2) hover close to 1×10^{-5} and the product of two single, independent, random events would be 10^{-10}, which is actually 10^4–10^5-fold less than the $(2–20) \times 10^{-5}$ reversion frequencies observed here for double mutants. The rate enhancement is consistent with some type of concerted mechanism for deamination, wherein one damage event (i.e., deamination) facilitates a second event at a neighboring cytosine. Bisulfite greatly catalyzes a second, concerted deamination event, with a rate that is 4–5 orders of magnitude greater than that for a single event.

(3) Uracil is involved. In *ung*$^+$ cells the frequency of tandem double mutants is reduced almost to background levels (Table 2), indicating that *most, if not all, the CC → TT mutations involve a uracil intermediate.* Thus, we suggest that bisulfite-induced tandem double mutants arise via a *cooperative, concerted process* wherein the presence of one uracil (which arises from deamination of cytosine in ds DNA by bisulfite) makes it more likely for a second deamination event to occur.

(4) This second event is highly dependent on the secondary structure (i.e., double- *vs* single-stranded character) of the DNA. Double mutants are very rare in ss DNA treated with bisulfite (Chen & Shaw, 1993a,b). In ss DNA incubated and transfected under conditions similar to those used here, 0 out of 54 mutants sequenced were tandem double mutants (Chen & Shaw, 1993a). (More recently we have observed one tandem double mutant upon sequencing an additional 16 mutants from that same ss DNA study.) Thus, with bisulfite treatment and also with echinomycin treatment (Moyer et al., 1993), *tandem double mutants occur almost exclusively in double-stranded DNA targets*, indicating that the secondary structure of DNA is important for inducing tandem double mutants. Studies of the mechanism of bisulfite-induced tandem double mutants is continuing in our laboratory.

The dichotomy between single- and double-stranded DNA with respect to induction of tandem double mutations would seem to rule out replication errors per se as possible causes of tandem double mutants. A priori, there is no obvious reason why DNA polymerase would make more double base

substitutions upon replicating ds DNA *vs* ss DNA. In fact, two laboratories find no evidence for tandem double mutations in the *lacZα* gene after scoring tens of thousands of mutants arising from DNA polymerase errors (Reid & Loeb, 1993; T. A. Kunkel, personal communication). It is possible that mismatch or other repair might cause tandem mutations. However, if repair of ds DNA was causing double mutants, one would expect to see TDM at early incubation times as well as at longer incubation times. Since TDM are seen only at longer incubation times, when greater damage is sustained, a more likely explanation for TDM is that bisulfite itself is catalyzing a second mutational event which chemically (or genetically) triggers the tandem mutation formation.

We propose that tandem double mutations arise chemically by a concerted process, involving deamination to uracil of one and possibly two neighboring cytosines, where the first event catalyzes the second event. The high frequency of tandem double mutations implicates a cooperative process. Two pathways are possible: (a) bisulfite damage to a cytosine could make that cytosine deaminate which in turn would somehow cause a neighboring cytosine to deaminate or miscode and (b) bisulfite damage to a cytosine would induce deamination in a neighboring cytosine, which then accelerates deamination or miscoding in the first cytosine. Our experiments cannot distinguish between the two possibilities, but they do shed some light on the direction of propagation of the mutation along the DNA. Tandem mutational damage in our target occurred more at the 5′-end of a string of cytosines than at the 3′-end. We speculate that the duplex structure optimally juxtaposes the two cytosine bases allowing for attack by bisulfite to yield tandem double mutations. Further experiments are needed to investigate the cause of tandem double mutants.

Our experiments differ in a number of respects from those of Reid and Loeb (1992, 1993) who concluded that tandem double mutants arise from metal-ion induced reactive oxygen species. Their studies were done using (1) *single-stranded* DNA in the presence of *metal ions*, (2) *ung*$^+$ cell strains, and (3) SOS induction. In contrast, our study here used *double-stranded* DNA in the presence of bisulfite and *ung*$^-$ cells without SOS induction. Additionally, we have presented the kinetics of deamination and evidence for a uracil intermediate. Our conditions produced a far greater proportion of tandem double mutants than Reid and Loeb (1992, 1993), who found a maximum of 6% tandem double mutants which they ascribed to metal-induced oxidative damage and polymerase bypass. In contrast, with bisulfite we observed up to 91% tandem double mutants in ds DNA, most of which we can ascribe to a uracil intermediate. The predominance of one type of mutation (e.g., tandem double CC → TT) in our experiments makes it possible to ascribe more directly the type of mutational process involved and to state that a chemical pathway for tandem double mutations is operative.

It is likely that different types of mechanisms are responsible for the tandem mutations in these two studies. By using SOS-induced cells, Reid and Loeb (1993) forced a polymerase to bypass a damaged site, and by using *ung*$^+$ cells they eliminated most (but probably not all) lesions that might contain a uracil. On the basis of a decrease in TDM from 3% to 1% with the addition of mannitol, they suggested that oxygen is involved in producing tandem double mutants. In our study, we observed a time-dependent increase of tandem double mutations until TDM constituted 91% of the total mutants and we have related them directly to the presence of uracil intermediates. *Ung* has previously been shown to be involved in repair of bisulfite-treated transforming DNA (Mejean et al., 1991; Chen & Shaw, 1993a). The ability to implicate uracil as an intermediate in bisulfite mutagenesis is an important feature of our studies.

Bisulfite as a Mutagen. Conclusions about the genetic effects of bisulfite in viruses, bacterial and eukaryotic cells have often been conflicting (as reviewed by Shapiro, 1977, and Pagano & Zeiger, 1987). Whereas a number of studies have failed to detect bisulfite mutagenesis, other studies reported both frameshift and base-pair substitutions at pH 7.0 (Pagano & Zeiger, 1987; Pagano et al., 1990) as well as dose-dependent increases in cell transformation (Tsutsui & Barrett, 1990), frequencies of chromosomal aberrations, sister chromosome exchange and micronuclei (Meng & Zhang, 1992). The differences among various bisulfite-induced mutagenesis studies probably result from the complicated chemistry of bisulfite (which can involve transamination and free radical damage as well as deamination), coupled with differences in the sensitivity and specificity of the assays, the treatment conditions, as well as the repair capacities of the organisms used.

In our studies, we have separated the damage process from the replication and repair processes. Only the DNA, and not the cells, came in contact with bisulfite, and the pH and length of treatment were carefully controlled. Bisulfite was removed from the samples immediately after incubation and thus was not available to react with proteins like polymerases in the cells. Thus, our system is much cleaner and simpler, making it easier to relate the cause and effect. Most differences between our results and those of others can readily be explained by differences in the treatment of DNA, i.e., pH, bisulfite concentration, duration of bisulfite treatment, absence/presence of free-radical scavengers, and in vitro *vs* in vivo treatment. For example, we know that under certain circumstances the reversion frequency at high bisulfite (1 M) concentration can actually be less than that at 10 mM, indicating that several mechanisms of damage or processing are operative and that it is important to carry out the experiments under well-controlled conditions.

Rates of Induced Deamination under Physiologically Relevant pH and Temperatures. It has been known for many years that the deamination reaction catalyzed by bisulfite in vitro is markedly specific for ss DNA at nonphysiologically relevant conditions. Bisulfite can deaminate cytidine nucleosides and cytosine bases in *single*-stranded DNA at high (>1 M) concentrations, acidic conditions and high temperatures (Shapiro et al., 1970; Hayatsu et al., 1970). It is also known that humans ingest high concentrations of bisulfite (e.g., 3.3 mM bisulfite in wine) and inhale SO_2 (which is a byproduct of natural organic decay and combustion of fossil fuels that can be readily converted to bisulfite in the body). Yet only recently has it been possible to determine rates by which mutations with bisulfite occur in ss DNA at physiologically relevant conditions and to show that they proceed via a uracil intermediate (Chen & Shaw, 1993a). When the actual rate constants for deamination were measured, 10 mM bisulfite was shown to deaminate cytosine in *single*-stranded DNA with a rate that was about 6-fold higher than background at 37 °C, pH 7.4 (Chen & Shaw, 1993a). In the present paper and in the same buffer, 10 mM bisulfite is shown to deaminate cytosine in *double*-stranded DNA with rates that are 16-fold higher than background at site 141 in M13mp2 (Table 4). Thus, bisulfite is proportionately more mutagenic to double-stranded DNA than to single-stranded DNA. (Note, however, that the overall rate of deamination at site 141 in bisulfite-

treated ds DNA is still 10–20-fold slower than that in bisulfite-treated ss DNA, but this is in contrast to a 30-fold slower difference without bisulfite, Table 4.) Thus, we have shown that bisulfite induces measurable amounts of deamination in ss DNA (Chen & Shaw, 1993a,b), as well as in ds DNA at physiologically relevant pH, bisulfite concentrations, and temperature.

Chemically-induced tandem double mutations may be a universal effect caused by a number of agents. We have observed tandem double CC → TT mutations in a number of systems, including spontaneous and echinomycin spectrums (Moyer et al., 1992, 1993). Copper and iron both induce tandem mutants (Reid & Loeb, 1992, 1993; G. Fang and B. R. Shaw, unpublished data); however, the frequencies are much less than with bisulfite. Induction of tandem double mutants is greater with bisulfite than with other reported agents, including UV. These results warrant further study of the mechanism by which chemically-induced tandem double mutants can be formed. The results may be of significance in cancer research and may shed light on differences and similarities between chemical- and UV-induced mutations.

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SUPPLEMENTARY MATERIAL AVAILABLE

Supplement Figure 1 showing the dose and time dependence of bisulfite-induced mutations in ds DNA, Supplement Table 1 giving the sequence specificity of bisulfite-treated ds DNA, and Supplement Table 2 giving the ratios of bisulfite-induced double mutants to total mutants upon transfection of ds DNA into *ung*⁻ cells (3 pages). Ordering information is given on any current masthead page.

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