Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3904–3907, May 1993
Genetics

Tandem double CC → TT mutations are produced by reactive oxygen species

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Communicated by Irwin Fridovich, January 8, 1993

ABSTRACT **Oxidative damage to DNA is mutagenic and thus may play a role in carcinogenesis. Because of the large number of different DNA lesions formed by oxidative species, no genetic alteration so far identified is exclusively associated with oxygen damage. Tandem double CC → TT mutations are known to occur via UV damage to DNA and are thought to be a specific indicator of UV exposure. Using a sensitive reversion assay that can detect both single and double mutations within the same codon of the M13-encoded *lacZα* gene, we show that treatments that produce reactive oxygen species can also produce tandem double CC → TT mutations. The frequency at which these mutations occur is less than that for single base mutations by a factor of approximately 30. The induction of these mutations is inhibited by treatment that scavenges hydroxyl radicals. This unique mutation provides a marker of oxygen free radical-induced mutagenesis in cells that are not exposed to UV-irradiation and an indicator for assessing the involvement of oxidative damage to DNA in aging and tumor progression.**

Oxygen free radicals have been implicated in a number of degenerative diseases including aging and cancer (1–4). These reactive species arise through normal cellular processes, inflammatory events, ischemia, and xenobiotic metabolism (5, 6). The ability of oxygen radicals to damage DNA is well documented (7, 8). This damage has been hypothesized to be a factor in the initiation and/or promotion of malignancies, but as yet there is no direct proof of this. A major problem is that the multitude of lesions that oxygen radicals produce in DNA makes it difficult to assign a particular mutation to a given oxidative lesion. In contrast, many chemical carcinogens produce a limited number of DNA lesions and base changes, thus allowing a correlation between the mutational spectrum found in commonly mutated genes of tumors and the proposed etiologic agent (9, 10).

This use of molecular epidemiology was recently extended in studies that showed a significant proportion of individuals with squamous cell skin carcinoma had tandem double CC → TT mutations in the p53 genes of these tumors (11). This unique mutation has been thought to be a specific indicator of UV-irradiation and provided a means of substantiating the involvement of sunlight in the origin of the skin carcinomas. Recently however, in studies using metal ions or human neutrophils as sources of oxygen radicals (12, 13), we have also observed tandem double CC → TT mutations, suggesting that reactive oxygen species and UV light may produce a common intermediate in DNA that leads to a tandem double mutation.

To determine the potential for reactive oxygen species to produce tandem double CC → TT mutations, we have adapted a reversion assay (14) that is specific for damage to cytosine residues and can detect both single and tandem double mutations at the same locus. We have used this assay to measure the frequency of tandem double CC → TT mutations produced by Fe^{2+} or a combination of Cu^{1+} or Cu^{2+} plus H_2O_2, to demonstrate the inhibition of mutagenesis by a hydroxyl radical scavenger and to compare oxygen radical-induced mutagenesis with that produced by UV light. The data suggest that tandem double CC → TT mutations might provide a marker to assess the involvement of oxidative damage to DNA during aging or tumor progression.

MATERIALS AND METHODS

***Escherichia coli* Strains.** *E. coli* strain MC1061 [*hsdR, mcrB, araD*, 139Δ(*ara-leu*), 7679D, *lacX74, galU, galK, rpsL, thi*] was the host strain used for all transfection experiments. Cells were plated on the indicator strain *E. coli* CSH50 [Δ(*pro-lac*) *thi, ara*/F′ *traD36, proAB, lacI*^{q}ZΔM15). The UV dose used to induce the SOS response in *E. coli* MC1061 was 46 J/m^2.

Treatment of DNA. The construction and sequence of M13G*1 DNA have been described (14). Single-stranded DNA was treated with Cu or Fe as described (12, 15) or irradiated with 254-nm light in 5-μl drops in an uncovered plastic Petri dish.

Scoring Mutants and Analysis of DNA Sequences. Transfection protocols were as described (12, 15), except that aliquots of the transfection mixtures were plated to yield plaque densities of no more than 3000 plaques per plate. Mutation frequency was determined by dividing the number of blue plaques within a given experimental group by the total combined number of white and blue plaques. Statistical analysis was conducted according to Birnbaum as described by Kastenbaum and Bowman (16). After isolation and replating of mutant plaques to ensure genetic purity of the samples, the DNA was either sequenced or the plaques themselves were probed with oligonucleotides specific for either possible CC → TT double mutation. Two oligonucleotides were used for hybridization analysis, either 5′-GCCAGCTAAGAAATGGG-3′ (CTT probe) or 5′-GCCAGCTGAAAAATGGG-3′ (TTC probe). After binding of the plaques to nylon filters and incubation with ^{32}P-labeled oligonucleotides, the filters were washed at 50°C as described (17, 18). Purified plaques representing CCC → TTC or CCC → CTT double mutations were included on each filter as an internal control. In control experiments it was determined that the oligonucleotide probes did not bind to samples containing single base mutations after the 50°C wash. Tandem double mutations identified by oligonucleotide hybridization were confirmed by sequence analysis.

RESULTS

Principles of the Mutation Assay. The M13G*1 reversion assay has previously been used to detect single mutations produced by misincorporation across from single 8-hydroxyguanine or N^2,3-ethenoguanine lesions inserted at a specific

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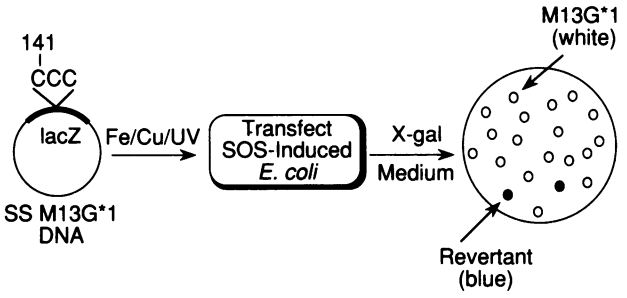

FIG. 1. Principles of the M13G*1 reversion assay. The CCC codon at positions 141–143 of the *lacZα* gene is the target for mutagenesis. Any single base substitution at the first two positions of this codon or any tandem double CC → TT mutation causes a reversion in plaque phenotype from white to dark blue. X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

site in a double-stranded DNA template (14, 19). Essentially, the M13G*1 construct has an altered codon (GCC → CCC) within the *lacZα* gene that yields white plaques rather than the dark blue of the parental M13mp2 DNA (Fig. 1). Any single base substitution at the first position of this altered codon causes a reversion to wild-type dark-blue phenotype. In a similar system, Frederico *et al.* (20) showed that single C → T transitions at the second position of this codon are also scorable but that mutations at the third position of this codon are phenotypically silent. Our analysis of the amino acid substitutions that result in reversion to wild type indicated that certain tandem double mutations could also be scored, including CCC → TTC and CCC → CTT mutations. Thus, this DNA construct allows detection of both single and double C → T mutations within the same codon of the *lacZα* gene.

Mutagenesis Due to Oxidative DNA Damage. In previous studies we have shown that Fe and Cu ions are mutagenic and that the mutagenicity is dependent upon the generation of oxygen-free radicals or other reactive oxygen species (12, 15). These metal ions also induce mutations in the M13G*1 reversion assay as summarized in Table 1. Fe^{2+} or a combination of Cu^{1+} or Cu^{2+} with H_2O_2 produced an average 7-fold increase in mutation frequency at the CCC locus of M13G*1. Each of these treatments also decreased the biological activity of the DNA by ≈90%. The addition of the hydroxyl radical scavenger mannitol to the Fe^{2+}-catalyzed reactions produced both a doubling in survival and a halving in overall mutation frequency. In parallel experiments, single-stranded M13G*1 DNA was irradiated with 254-nm light and assayed for mutagenesis. This treatment produced a 9-fold increase in mutation frequency. In contrast to the results with Fe^{2+}, the presence of mannitol did not decrease mutation frequency for UV-irradiated DNA. However, it did increase the biological activity of the DNA relative to that without mannitol.

DNA Sequence Analysis of Single Base Substitutions. The nucleotide sequence of ≈200 of the revertants produced by exposure to metal ions or UV light was analyzed by sequence analysis (Table 2). It is interesting to note that all possible single base substitutions were detected at the first two positions of the CCC codon (Fig. 2). However, C → T transitions were by far the most prevalent mutation produced by either oxygen damage or UV-irradiation. The high frequency of C → T transitions appears to be characteristic of mutagenesis by oxygen-reactive species (12, 15). This bias toward C → T substitutions has also been reported in the *cI* gene of λ phage after exposure to γ-irradiation (21). Approximately twice as many single C → T mutations occurred at the first position of the codon as at the second for both oxygen and UV treatment. Whether this is due to a difference in the degree to which each base is modified or a bias toward insertion of a serine (TCC) rather than a leucine (CTC) at this position is not known.

Tandem Double Mutations Due to Oxidative DNA Damage. While single mutations occurred at a frequency of ≈1/2000, tandem double CC → TT mutations were less frequent by a factor of about 30. No tandem double mutations were observed among the 349,000 plaques obtained with DNA that was not exposed to agents that generate reactive oxygen species (Table 3). In contrast, damage to M13G*1 DNA by either Fe^{2+} or Cu/H_2O_2 resulted in the production of CC → TT substitutions. Fe^{2+} tended to produce mutations at the first two positions of the CCC codon while Cu/H_2O_2 did not show this specificity. The diminution of the Fe^{2+}-induced mutations by the addition of mannitol provides evidence that these mutations are dependent on the production of reactive oxygen species. UV-irradiation was ≈3-fold more efficient per lethal event in producing tandem double mutations than oxidative damage. Tandem double CC → TT mutations accounted for ≈3% of the total mutations produced by oxidative damage; ≈7% of the UV-induced mutations were tandem doubles. The lack of inhibition of UV-induced mutagenesis by mannitol indicates that the UV-induced mutagenesis is not mediated by the generation of hydroxyl radicals.

DISCUSSION

Tandem double CC → TT mutations have been thought to be a specific indicator of UV damage to DNA and have been detected after UV-irradiation in bacteria (22–24), yeast (25), and primate cells (26). This mutation has also been identified in the p53 gene of squamous cell skin carcinomas of human subjects (11), presumably as a result of UV exposure. We are unaware of any report of this mutation occurring because of misincorporation by DNA polymerases or after exposure of DNA to chemical carcinogens. In fact, no tandem double CC → TT mutations have been observed in the *lacZα* gene in at least 10,000 sequenced mutants arising from DNA polymerase errors, the insertion of site-specific chemical adducts, or other types of DNA damage. Recently, however, tandem double CC → TT mutations have been detected in forward mutation assays when using either Cu ions (12) or human neutrophils as a source of reactive oxygen species (13). The goal of this study was to determine whether oxygen free radicals were responsible for inducing this mutation and

Table 1. Mutation frequency and survival of M13G*1 DNA after oxidative or UV damage

Treatment	Plaques scored		Mutation frequency	
	Total	Mutants	× 10^{-4}	% survival
Control	348,953	25	0.7	100
Cu^{1+} (2.5 μM) + H_2O_2 (5 μM)	123,657	74	6.0	12
Cu^{2+} (1.0 μM) + H_2O_2 (2.5 μM)	296,866	150	5.1	10
Fe^{2+} (5 μM)	344,402	185	5.4	10
Fe^{2+} (5 μM) + mannitol (0.1 M)	431,382	119	2.8	22
UV (25 J/m²)	141,626	91	6.4	3
UV (25 J/m²) + mannitol (0.1 M)	247,044	195	7.9	9

Table 2. Analysis of DNA sequence changes in M13G*1 DNA after oxidative or UV damage

Sample	Codon from CCC mutation					
	ACC	GCC	TCC	CAC	CGC	CTC
Control	2	—	11	2	4	6
Cu†	4	4	23	2	2	15
Fe	9	2	33	2	2	13
UV	3	1	23	3	1	10
Total	18	7	90	9	9	44
% total	10	4	48	5	5	24

†Includes Cu^{1+} or Cu^{2+} in combination with H_2O_2.

whether the mechanism of its formation is similar to UV-induced double mutations. To do this we have utilized a reversion assay that can specifically detect mutations at cytosine residues in DNA and assayed for tandem double CC → TT substitutions by DNA sequencing or plaque hybridization.

Fig. 3 is a compilation of oxidatively induced tandem double mutations observed in this laboratory in previous studies (12, 13) as well as the reversion assay described here. The ability of Fe, Cu, and phorbol ester-stimulated neutrophils to cause CC → TT mutations and the multiplicity of sites at which they occur suggest that CC → TT mutations are a general manifestation of oxygen damage to DNA. The common sequence motif among the sites at which CC → TT mutations have been detected is the presence of at least three consecutive cytosines. In fact, there is only one stretch of three consecutive cytosines within the target region of the *lacZα* gene in which this mutation has not been detected. In contrast, UV-induced tandem double mutations have been shown to occur at dicytosine sites or runs of three or more cytosines with equal efficiency (23). The dependence of CC → TT mutations on induction of SOS functions is not known, nor is it established that a similar error-prone response occurs in human cells. Previous studies have shown an enhancement of mutagenicity by oxygen radicals when using SOS-induced cells (15, 27). Because of the rarity of CC → TT mutations, we have elected to use SOS-induced cells to increase the sensitivity with which they can be detected.

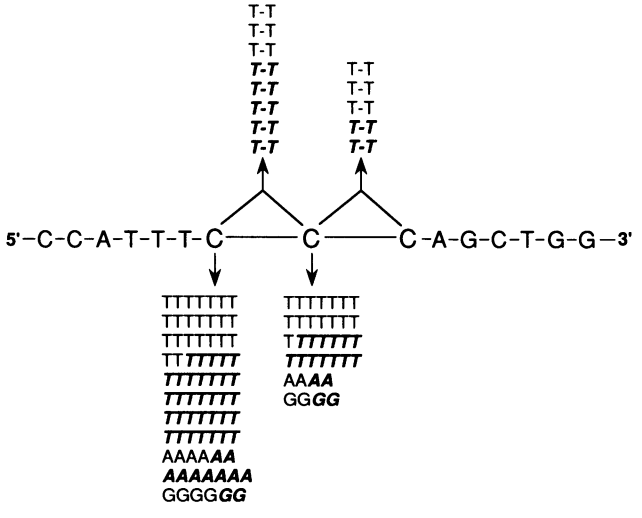

FIG. 2. Single and tandem double mutations at the CCC locus of M13G*1. The sequence shown is the viral strand of M13G*1 from position 135 to 149, where position 1 is the first transcribed base. Single base substitutions are shown below the sequence. Tandem double mutations are shown above the sequence. Single and double mutations produced by exposure to Fe^{2+} are shown in italicized bold type; those produced by Cu/H_2O_2 are shown in regular type.

Table 3. Tandem double mutations in M13G*1 DNA after oxidative or UV damage

Treatment	Plaques scored	Codon from CCC mutation		Total	Mutation frequency × 10^{-5}
		TTC	CTT		
Control	348,953	—	—	0	<0.3
Cu*	385,979	3	3	6†	1.6
Fe	344,402	4	1	5†	1.5
Fe + mannitol	431,382	1	1	2	0.5
UV	141,626	2	5	7‡	4.9
UV + mannitol	247,044	8	5	13‡	5.3

*Includes Cu^{1+} or Cu^{2+} in combination with H_2O_2.
†Significantly greater than control DNA ($P \leq 0.05$).
‡Significantly greater than control DNA ($P \leq 0.01$).

Oxygen radicals cause a large number of modifications to cytosine residues, and single C → T transitions are among the most common mutations produced by most (12, 15) but not all (27) reactive oxygen species. Chemical modifications produced by reactive oxygen at cytosine residues include addition to the 5,6 double bond of the pyrimidine ring, formation of glycols, hydantoins, deamination products, and species with rearrangements of the pyrimidine ring (7, 8). Based on the frequency of two independent hits, it is unlikely that the same adduct causes both the single and double substitutions. The frequency of CCC → TTC mutations produced by Cu/H_2O_2 is 1000-fold higher than would be expected from two random independent events (i.e., CCC → TCC plus CCC → CTC). Treatments designed to induce deamination of cytosine residues have been shown to cause tandem double CC → TT mutations (28). However, it should be noted that the tandem double mutations observed in that study were dependent on the use of host cells that do not possess a functional uracil glycosylase gene. All tandem double CC → TT mutations observed in the present study were produced in cells containing a functional uracil glycosylase. Moreover, we have evidence that the frequency of both single and double C → T mutations produced by reactive oxygen species is independent of uracil glycosylase activity (data not shown). Thus, it seems more likely that a single oxygen-radical-induced modification at adjacent cytosine residues is responsible for the CC → TT mutations and that this modification is different from those that elicit single C → T substitutions.

A primary candidate for an alteration in DNA that could produce the double mutation is the crosslinking of two adjacent cytosines. There is evidence that exposure of dinucleotides to oxidative damage mediated by Cu/H_2O_2 results in a crosslink between the two bases, although cytosine crosslinks were not assayed (29). The finding that CC → TT substitutions are observed after damage to DNA by both reactive oxygen species and UV-irradiation suggests that both types of agents may produce a similar intermediate, leading to a stable mutagenic adduct. However, the ability of the hydroxyl radical scavenger mannitol to decrease the frequency of oxidatively induced tandem double mutations but not those induced by UV light argues against the production of classical UV-induced cyclobutane pyrimidine dimers and (6–4) pyrimidine–pyrimidone photoproducts by reactive oxygen species. A more likely modification is suggested by studies on the effect of hydroxyl radicals generated by γ-irradiation of cytosine (30). Fifty percent of the products detected in N_2O-saturated aqueous solution were dimerized cytosines structurally distinct from UV-induced photoproducts. It remains to be established whether this particular modification is responsible for CC → TT mutations.

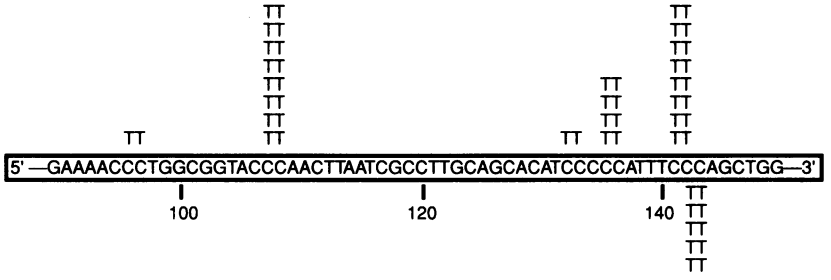

FIG. 3. Tandem double mutations in the *lacZα* gene. The sequence shown is the viral strand of M13 from position 90 to 149, where position 1 is the first transcribed base. The G → C change that produces M13G*1 is at position 141. The tandem double mutations include those observed in previous studies produced by Cu/H_2O_2 at positions 107 and 108 (12) and positions 131 and 132 (unpublished results); by phorbol ester-stimulated neutrophils at positions 96 and 97 and 135 and 136 (13); and by Fe and Cu/H_2O_2 in the present study at positions 141–143.

Carcinogenesis appears to be a multistep process involving the sequential accumulation of mutations that allows cells to gain a selective growth advantage and undergo clonal expansion (10). It is frequently hypothesized that many of these mutations are generated by cellular processes and in particular by oxygen free radicals (31). Seventy percent of human tumors have been shown to contain mutations in the p53 gene, and specific mutations in this gene have been used to identify environmental agents that are associated with the genesis of specific tumors (9, 10). To date there are only two reports of tandem double CC → TT mutations occurring in the p53 gene. They have been detected in skin carcinomas (11), where UV light is the presumed agent, and in a non-small-cell lung carcinoma (32), where UV light would not be expected to play a role. It is interesting to note that the CC → TT mutations observed in the skin carcinomas all occurred at dipyrimidine sites in the gene, whereas the CC → TT change observed in the p53 gene of the non-small-cell lung tumor occurred within a run of three consecutive cytosines similar to the mutations observed in this study. The lack of CC → TT mutations in the p53 gene obtained from most human tumors suggests that mutagenesis by oxygen free radicals is not a major contributor to the overall accumulation of mutations that characterizes tumor progression. However, it should be noted that while there are 14 tracts of three or more consecutive cytosines within the region of the p53 gene most commonly mutated in tumors (9), it is not likely that mutations at each of these sites would give rise to a dysfunctional p53 gene product or one that would lead to a selective growth advantage. In fact, of the 22 codons encompassed in the tracts of three or more consecutive cytosines, only 7 have been shown to be altered in tumors, and only 2 of these changes could be due to tandem double CC → TT mutations (9). Thus, the paucity of tandem double CC → TT mutations in the p53 gene is consistent with the rarity of this mutation and the relatively low number of target sites compared with single base substitutions. In cells not exposed to UV the presence of this rare mutation would provide strong evidence for the involvement of oxidative damage.

These studies were supported by National Institutes of Health Grants CA08855 (to T.M.R.) and CA39903 and AG01751 (to L.A.L.).

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