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Cr(III)-mediated crosslinks of glutathione or amino acids to the DNA phosphate backbone are mutagenic in human cells

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ABSTRACT

Carcinogenic Cr(VI) compounds were previously found to induce amino acid/glutathione–Cr(III)–DNA crosslinks with the site of adduction on the phosphate backbone. Utilizing the pSP189 shuttle vector plasmid we found that these ternary DNA adducts were mutagenic in human fibroblasts. The Cr(III)–glutathione adduct was the most potent in this assay, followed by Cr(III)–His and Cr(III)–Cys adducts. Binary Cr(III)–DNA complexes were only weakly mutagenic, inducing a significant response only at a 10 times higher number of adducts compared with Cr(III)–glutathione. Single base substitutions at the G:C base pairs were the predominant type of mutations for all Cr(III) adducts. Cr(III), Cr(III)–Cys and Cr(III)–His adducts induced G:C→A:T transitions and G:C→T:A transversions with almost equal frequency, whereas the Cr(III)–glutathione mutational spectrum was dominated by G:C→T:A transversions. Adduct-induced mutations were targeted toward G:C base pairs with either A or G in the 3′ position to the mutated G, while spontaneous mutations occurred mostly at G:C base pairs with a 3′ A. No correlation was found between the sites of DNA adduction and positions of base substitution, as adducts were formed randomly on DNA with no base specificity. The observed mutagenicity of Cr(III)-induced phosphotriesters demonstrates the importance of a Cr(III)-dependent pathway in Cr(VI) carcinogenicity.

INTRODUCTION

A large number of epidemiological studies have firmly established hexavalent Cr compounds as carcinogenic to humans (1,2). Human exposure to Cr and its compounds is widespread, with an estimate of occupationally exposed humans being ~300 000 in the US alone (3). Workers in ~80 professional categories have a potential exposure to Cr. The ability of Cr(VI) compounds to induce tumors has been confirmed in many animal studies (1). Cr(VI) has also been found to be genotoxic to mammalian cells based on induction of chromosomal damage (4), cell transformation (5) and mutagenicity assays (6). Carcinogenicity of Cr(VI) is widely believed to result from induction of DNA damage, however, the nature of the DNA lesions underlying Cr(VI) genotoxicity remains to be established.

Under physiological conditions Cr(VI) predominantly exists as the chromate anion, CrO_4^{2-}, which is by itself unreactive toward DNA. After entering cells through non-specific anion channels Cr(VI) undergoes non-enzymatic reductive metabolism yielding stable Cr(III) (7). *In vitro* experiments have shown that Cr(VI) reduction is accompanied by production of intermediate Cr(IV) and Cr(V) forms, as well as several reactive by-products (8–10). The reductive metabolism of Cr(VI) to Cr(III) can cause several forms of DNA damage, resulting from either DNA adduction by Cr(III) or from oxidative damage by reactive intermediates. Exposure of cells to Cr(VI) results in binding of Cr(III) to DNA, yielding binary Cr(III)–DNA adducts as well as various DNA crosslinks (11–13). Several *in vitro* studies have shown that Cr(VI) reduction can also lead to oxidative DNA lesions, such as DNA strand breaks and abasic sites (14–16). Induction of oxidative damage, however, remains controversial, as formation of oxidative lesions was attributed by some investigators to the presence of adventitious iron (17). Relative contributions of these different types of DNA damage to Cr(VI) mutagenicity have not yet been elucidated. While mutagenicity of oxidative forms of DNA damage has been established (18–20), the mutagenic potential of Cr(III)-mediated DNA adducts remains to be determined. Comparison of mutational spectra induced by Cr(VI), hydrogen peroxide and X-rays in exon 3 of the *hprt* gene from human lymphoblastoid TK6 cells revealed little similarity between Cr(VI) and the oxygen radical-producing agents, pointing to the potential significance of non-oxidative mechanisms in chromate mutagenicity (21).

We have recently found that chromate exposure of cultured cells leads to formation of a new type of Cr(III)–DNA adducts, Cr(III)-mediated crosslinks of amino acids and glutathione with DNA (12). The total number of these ternary adducts has been calculated to constitute up to 50% of all Cr–DNA adducts and, therefore, they represent the major type of DNA lesions induced

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by carcinogenic chromate. The adducts are comprised of amino acid or glutathione bridged to DNA by Cr(III), with the primary site of attachment to DNA being the phosphate group (22).

In the present study we have examined the mutagenic potential of major Cr(III)–DNA adducts formed either by Cr(III) alone or by Cr(III) complexes with cysteine, histidine or glutathione. In addition, the sites of adduct formation along the target *supF* gene sequence were mapped and compared with mutational positions.

MATERIALS AND METHODS

Cells and plasmid used in mutagenesis experiments

An SV40-based pSP189 plasmid containing the *supF* gene as a mutagenic target was used in shuttle vector mutagenesis experiments (23). The plasmid was courtesy of Dr M.Seidman. Replication of the adducted and normal plasmids was performed in normal human fibroblasts immortalized with SV40 virus (a gift from Dr H.Ozer). Mutant selections were performed using *Escherichia coli* strain MBL50 (courtesy of Dr C.Pueyo). This indicator *E.coli* strain has amber mutations in the *araC* gene that allow selection of the colonies with a mutated *supF* gene on selective medium with L-arabinose (24).

Adduct formation

Cr(III)–DNA adducts were formed according to a previously published procedure with some modifications (22). Cr(III)–amino acid complexes were formed by incubation of 0.5 mM $CrCl_3 \cdot 6H_2O$ and 5 mM cysteine or histidine in 10 mM MES buffer, pH 6.1, at 50°C for 5 h. Cr(III)–glutathione complexes were formed at 1 mM $CrCl_3 \cdot 6H_2O$ and 10 mM glutathione in the same buffer for 5 h at 50°C. DNA (1 µg) was reacted in 100 µl mixture containing 10 mM MES buffer, pH 6.1, and different dilutions of the Cr(III)–amino acid/glutathione complexes. The reaction was allowed to proceed for 2 h at 37°C, followed by Sephadex G-50 chromatography. Cr(III) treatment of DNA was performed for 30 min at 37°C with freshly dissolved $CrCl_3 \cdot 6H_2O$. DNA adducts were quantitated by the amount of DNA-bound Cr by the inclusion of radioactive $^{51}CrCl_3$ (10 µCi/ml) in a parallel set of tubes. The effect of Mg^{2+} ions on DNA adduction and mutagenesis was studied by preincubation of pSP189 plasmid DNA with 10 mM $MgCl_2$ for 10 min at 37°C prior to addition of Cr(III)–glutathione complexes.

DNA transfection into human fibroblasts and recovery of plasmids

Amino acid/glutathione–Cr-treated or untreated plasmids were transfected into the cells using LipofectAMINE (Life Technologies) according to the manufacturer's recommendations. After 48 h plasmids were recovered by Minipreps from Promega and digested with *Dpn*I restriction enzyme to eliminate the unreplicated plasmids, which contained the bacterial methylation pattern. DNA was ethanol precipitated and dissolved in distilled water.

Selection of *supF* mutants

The *Dpn*I-treated plasmids were introduced into the indicator bacteria *E.coli* MBL50 by electroporation according to Hanahan *et al.* (25). To determine the total number of transformants, dilutions from each tube were plated on minimal agar plates containing 30 µg/ml ampicillin, 0.5 µg/ml chloramphenicol. Mutant selection was performed on plates additionally containing 2 g/l L-arabinose. Mutation frequency was calculated as the ratio of ampicillin/arabinose-resistant to ampicillin-resistant colonies in each sample.

Sequencing of mutants

The mutant *E.coli* colonies were inoculated into 2 ml LB medium containing 50 µg/ml ampicillin and the cultures incubated at 37°C overnight. Plasmids were recovered with Minipreps and 0.5–1 µg DNA was used in the sequencing mixture. The *supF* gene was sequenced using a PCR-based protocol (AmpliCycle Sequencing Kit; Perkin Elmer). The sequencing oligo was 5′-GGCGACACGGAAATGTTGAA, which corresponds to positions 4889–4908 of the pSP189 plasmid. Aliquots of the sequencing reaction were analyzed in 8% polyacrilamide, 8 M urea gels followed by autoradiography.

Exonucleolytic digestion of DNA with T4 DNA polymerase

The pSP189 plasmid was digested with *Eco*RI enzyme that cuts once 27 bp upstream of the coding sequence of the *supF* gene. The resulting DNA was 5′-labeled by reaction with [γ-^{32}P]ATP and T4 DNA polynucleotide kinase, then digested with *Bam*HI to produce a fragment of 131 bp. The 131 bp fragment was purified on a 1% low melting agarose gel and treated with amino acid/glutathione–Cr complexes as described above. Approximately 100 000 c.p.m. were used for each sample. After treatment DNA was ethanol precipitated and dissolved in distilled water. The exonuclease reaction was performed according to Panigraphi and Walker (26). The reaction was performed with 4.5 U T4 DNA polymerase in the absence of dNTPs for 1 h at 37°C and was stopped by addition of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.02% xylene cyanol). Products of the exonuclease digestion reactions were separated in 8% polyacrylamide, 8 M urea gels with a glycerol-tolerant gel buffer (70 mM Tris, 25 mM taurine, 0.5 mM EDTA). Results were visualized by autoradiography.

Statistical analysis

The χ^2 and Fisher's exact tests were used to determine statistical significance of types of mutations among different treatments. Mutational spectra were compared using a statistical software based on the Adams and Skopek algorithm (27).

RESULTS

Adduct formation

We have previously shown that reaction of DNA with the preincubated mixtures of a 10-fold molar excess of amino acid or glutathione with Cr(III) results in formation of ternary DNA adducts with an equimolar ratio of amino acid to Cr(III) (22). Kinetic studies of DNA binding showed that the reaction was complete after 2 h incubation of the preformed complexes with DNA (22). Cr(III) adduction to DNA was most efficient when the $CrCl_3$ solution was freshly prepared and the reaction was completed after a 30 min incubation. The number of adducts formed on DNA was dose-dependent with respect to the concentration of the amino acid/glutathione–Cr(III) complexes used and the relationship was linear in the dose range studied (Fig. 1). DNA strand breakage was

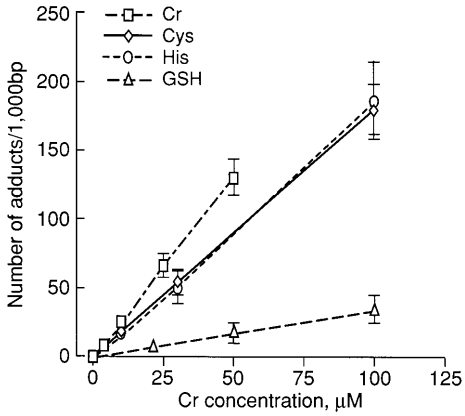

Figure 1. Formation of DNA adducts as a function of Cr(III) concentration. DNA was reacted with $CrCl_3$ for 30 min at 37°C or 2 h with pre-formed amino acid/glutathione–Cr(III) complexes. Adducts were measured using radioactive $^{51}CrCl_3$. Results are expressed as the number of adducts per 1000 bp. Shown are means ± SD.

not induced during the adduct formation procedure, as detected by the plasmid relaxation assay (data not shown). The reactivities of the Cr(III) complexes towards DNA were different. Cr(III), Cr(III)–cysteine and Cr(III)–histidine complexes had similar binding activities that were significantly higher compared with that of Cr(III)–glutathione. This may indicate that glutathione can readily form inert complexes with Cr(III), such as complexes of two glutathione molecules and one Cr(III), that have been shown to be unreactive toward DNA (28). It is also quite likely that the presence of the bulky glutathione ligand in Cr(III) complexes decreases their DNA binding activity due to steric hindrance. A high level of Cr(III)–glutathione adducts found on DNA from chromate-exposed cells is apparently not related to more avid formation of these adducts and is attributed to preferential formation of DNA-reactive Cr(III)–glutathione complexes *in vivo* (12).

Assessment of sequence specificity in the formation of Cr(III) adducts on DNA by the exonuclease digestion test

Alkylation of the DNA phosphate group makes the resulting phosphotriester resistant to hydrolysis by all tested nucleases (29,30). Previous studies (22) demonstrated that Cr(III) adducts are bound to DNA through the phosphate backbone and we reasoned that the pattern of exonuclease digestion of Cr(III)-adducted DNA could be used as a method of mapping the distribution of adducts along the DNA sequence. We have chosen the *supF* gene from plasmid pSP189 as a target sequence for exonuclease digestion, the same gene that was used in the mutagenicity assay. The intent was to compare positions of the mutagenic events with the specificity of DNA adduction in the same sequence context. Exonuclease digestion was performed with T4 DNA polymerase in the absence of deoxynucleotides using the 131 bp DNA fragment. Figure 2 shows that treatment of the DNA with Cr(III), Cr(III)–cysteine, Cr(III)–histidine and Cr(III)–glutathione caused blockage of exonuclease activity, whereas untreated DNA was almost completely digested. The exonuclease stops occurred at nearly every nucleotide along the sequence and the pattern was almost identical for all adducts. Although the frequency of blockage was nearly equal at almost every nucleotide, several clusters of sites with slightly higher intensities were observed.

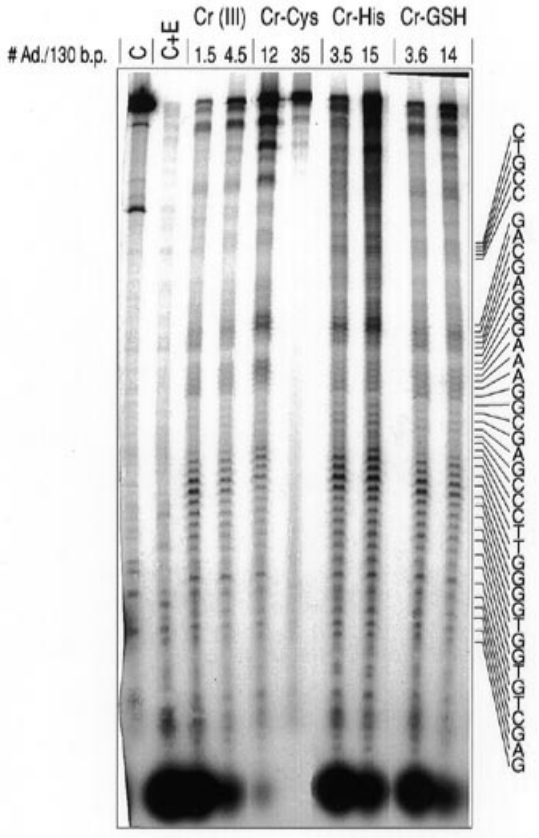

Figure 2. Exonuclease digestion of DNA treated with $CrCl_3$ or amino acid/glutathione–Cr(III) complexes. Adducted and control DNA was digested by the exonuclease activity of T4 DNA polymerase in the absence of NTPs. Lane C, undigested control DNA fragment; lane C+E, exonuclease digest of untreated DNA; lanes Cr(III), Cr–Cys, Cr–His and Cr–GSH show exonuclease digests of the DNA fragment treated with $CrCl_3$, Cr(III)–cysteine, Cr(III)–histidine and Cr(III)–glutathione respectively. The numbers shown on the top of each lane are the number of adducts per 131 bp DNA fragment.

However, these regions were not characterized by any specific base or sequence content and we conclude that binding of the adducts was not base or sequence specific.

Mutagenicity of Cr(III)–DNA adducts

Selection of *supF* mutants was performed in *E.coli* strain MBL50 using medium containing L-arabinose. Figure 3 shows mutation frequencies of the plasmid treated with Cr(III)–amino acid/glutathione complexes as a function of the number of adducts. The background mutation frequency (4.5×10^{-4}) was somewhat high, which might be related to the type of cell used for plasmid replication. Cr(III)–glutathione exhibited the highest mutagenic potential and a mutation frequency five times the background was obtained, with ~15 adducts/1000 bp. In contrast, Cr(III) was weakly mutagenic, giving a 2- to 3-fold increase over background at 70 adducts/1000 bp. Although we observed a further increase in mutation frequency (up to 5-fold), when the number of Cr(III) adducts reached over 130 Cr atoms/1000 bp it occurred at Cr(III) concentrations that induce plasmid aggregation and precipitation of DNA.

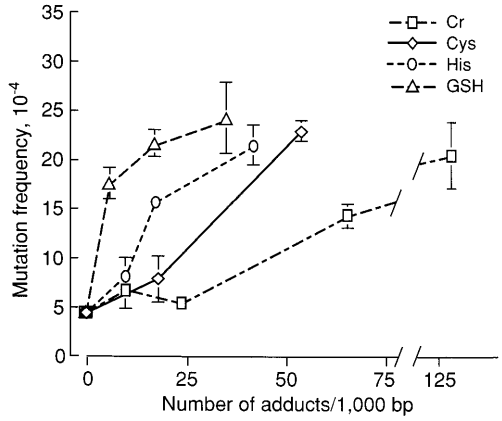

Figure 3. Mutation frequency generated in the *supF* gene induced by treatment of the pSP189 plasmid with amino acid/glutathione–Cr(III) complexes. Control and adducted pSP189 plasmids were transfected into human fibroblasts and allowed to replicate for 48 h. Plasmids were recovered, digested with *Dpn*I and electrotransfected into the indicator *E.coli* MBL50 strain. Selection of *supF* mutants was performed on arabinose-containing plates. Mutation frequency was calculated by dividing the number of antibiotic/arabinose-resistant colonies per number of antibiotic-resistant colonies.

To exclude a possibility that a mutagenic response in adducted plasmids was induced by DNA damage other than Cr(III) binding, we examined formation of glutathione–Cr(III)–DNA adducts and mutagenicity in plasmids treated in the presence of 10 mM $MgCl_2$ (Table 1). Mg^{2+} binds exclusively to the phosphate in DNA and at this concentration all phosphate sites are occupied by Mg^{2+} ions (31). As one would expect, blocking of the phosphate sites by Mg^{2+} prevented adduction of DNA by Cr(III)–glutathione and this was also accompanied by elimination of the mutagenic response in treated plasmids (Table 1). These results clearly demonstrate that under our experimental conditions induction of mutagenicity is dependent on binding of Cr(III) complexes to DNA.

Table 1. Inhibitory effect of Mg^{2+} ions on DNA adduct formation and mutagenicity by Cr(III)–GSH complexes

Experiment	No. DNA adducts per 1000 bp	Mutation frequency[a]
Cr(III)–GSH	34	21.4×10^{-4}
Cr(III)–GSH + $MgCl_2$	1	1.0×10^{-4}

[a]After subtraction of background mutation frequency.

Characterization of the *supF* mutants

Mutants were selected from several independent experiments (n = 3–5) which were performed at DNA adduct levels giving a mutation frequency of at least 5-fold over the spontaneous level. For Cr(III)–GSH this level was reached with ~15 adducts/1000 bp; for Cr(III)–histidine it was ~40 adducts; for Cr(III)–cysteine ~55 adducts; for Cr(III) ~130 adducts (Fig. 3). The types of mutations were analyzed by sequencing. The signature sequence of the plasmids was used to identify sibling mutants that were subsequently excluded from further analysis. The Cr(III)-treated plasmids with more than 100 adducts/1000 bp showed an unusually high number of sibling mutants, with some experiments producing only two or three independent mutants among 15 plasmids sequenced. This observation could be attributed to the fact that either transfection or replication by-pass was a rare event.

Single base substitutions were the predominant type of mutation in the spontaneous and Cr-induced mutants (Table 2). The deletions accounted for 13–25% of all mutations and insertions occurred in 0–4% of mutants. Among mutants with base substitutions 11–15% had multiple mutations and 0–8% had tandem mutations (Table 2). No statistically significant differences were observed in the types of mutations among all groups.

Table 2. Analysis of sequence alterations generated in the *supF* gene

	Spontaneous	$CrCl_3$	Cr–Cys	Cr–His	Cr–GSH
Deletions	12[a] (25%)[b]	8 (19%)	9 (20%)	6 (13%)	6 (14%)
Insertions	1 (2%)	0	2 (4%)	1 (2%)	1 (2%)
Point mutations	35 (73%)	35 (81%)	34 (76%)	38 (85%)	35 (83%)
Single	29 (83%)[c]	30 (86%)	29 (85%)	31 (82%)	31 (89%)
Tandem	1 (3%)	0	0	3 (8%)	0
Multiple	5 (14%)	5 (14%)	5 (15%)[d]	4 (11%)	4 (11%)

[a]Number of mutants sequenced of the indicated type.
[b]Percent of total number of mutants sequenced.
[c]Percent of total number of plasmids with point mutations sequenced.
[d]Includes two mutants containing both tandem and multiple mutations.

The types of single base substitutions

The majority of the point mutations in treated and control plasmids were G:C base pair substitutions (Table 3). Most G:C substitutions were G:C→T:A transversions or G:C→A:T transitions, while the G:C→C:G base change was observed significantly less frequently, with an especially low occurrence in Cr(III)–GSH and Cr(III)–histidine treatments (<4%). In control plasmids and in plasmids treated with Cr(III)–cysteine and Cr(III)–histidine G:C→T:A transversions were observed only slightly more frequently compared with G:C→A:T transitions, whereas a 3-fold difference was observed for Cr(III) treatment and a 4-fold difference was found for Cr(III)–GSH. The statistical analysis of types of G:C base pair mutations by the χ^2 test showed that only the differences between control and Cr(III)–GSH treatments were statistically significant ($P < 0.05$). Thus the relative proportion of different mutation types was found to be similar in control and all Cr-treated samples except for Cr(III)–GSH adducts. However, the average mutation frequency induced by different Cr treatments used for the sequence analyses was at least five times the spontaneous level, suggesting that the majority of induced mutations were probably derived from amino acid/glutathione treatment of the plasmids.

Multiple point mutations are frequently observed in SV40-based shuttle vector mutagenesis data and they were linked to the presence of single-strand DNA breaks and their repair by error-prone DNA polymerase (32). Mutation types and hotspots of multiple mutations were shown to differ from single point mutations in many cases, including UV exposure (33), aflatoxin B_1 (34) and glyoxal (35). When multiple mutations were excluded from our analysis significant changes in overall distribution of the mutations were observed only for Cr(III)–GSH treatment. After exclusion of multiple mutations, the G:C→T:A transversions in Cr(III)–GSH-induced mutants were found to occur in 27 mutants (87% of the single point mutations) and G:C→A:T occurred in only three mutants (10%).

Table 3. Types of base substitutions observed in plasmids with point mutations

	Spontaneous	$CrCl_3$	Cr–Cys	Cr–His	Cr–GSH
Transitions					
G:C–>A:T	16 (37%)	8 (20%)	15 (35%)	17 (38%)	8 (20%)
A:T–>G:C	0	0	1 (3%)	1 (2%)	0
Transversions					
G:C–>T:A	23(53%)	25 (61%)	21 (49%)	20 (44%)	31 (78%)
G:C–>C:G	3 (7%)	6 (15%)	4 (10%)	2 (4%)	1 (2%)
A:T–>T:A	0	2 (5%)	2 (5%)	4 (9%)	0
A:T–>C:G	1 (3%)	0	0	1 (2%)	0
Total	43	41	43	45	40
G:C–>T:A/G:C–>A:T	1.4	3.1	1.4	1.2	3.9

Mutational spectra

Base substitutions in both spontaneous and induced spectra were significantly spread out along the gene sequence, however, some hotspots and clustering were observed (Fig. 4). Control and Cr(III)–cysteine shared the same hotspot at position 163, which was not observed in other samples. In addition, mutations at position 129 have been frequently observed (≥4 times) in control and Cr(III)–GSH samples and mutations at position 139 were frequently found in Cr(III)–cysteine- and Cr(III)–GSH-treated plasmids. Mutations in the Cr(III) spectra were the most widely scattered along the gene, with no more than three occurrences at any position. Cr(III)–histidine and Cr(III)–GSH treatments produced mutation clustering in certain regions of the gene. For Cr(III)–histidine clustering occurred at positions 99–105 and 121–126, whereas for Cr(III)–GSH treatment it occurred at positions 108–116. However, the positions of the exonuclease arrest spots with relatively higher intensity did not coincide significantly with clustering of mutations. The pattern of these arrest spots was nearly identical among all Cr treatments, whereas mutational clustering was different for each adduct. Statistical analysis using a computer program for comparison of mutational spectra (27) showed that only differences between Cr(III)–histidine- and Cr(III)–glutathione-induced spectra were statistically significant at $P = 0.007$.

Table 4. Influence of adjacent 3′ base on G:C targeted single point mutations

	Control	$CrCl_3$	Cr–Cys	Cr–His	Cr–GSH
5′-GA-3′	26 (84%)	12 (44%)	15 (52%)	9 (30%)	13 (42%)
5′-GG-3′	3 (10%)	12 (44%)	12 (41%)	15 (50%)	15 (48%)
5′-GT-3′	2 (6%)	3 (11%)	1 (3%)	3 (10%)	1 (3%)
5′-GC-3′	0	0	1 (3%)	3 (10%)	2 (6%)
Total	31	27	29	30	31
5′-GA-3′/ 5′-GG-3′	8.67	1.00[a]	1.25[a]	0.60[a]	0.87[a]

[a]$P < 0.01$, compared with control (χ^2 test for GA, GG).

Multiple G/C-targeted mutations were distributed as follows: spontaneous mutants, nine mutations at 5′-GA-3′ sites, three at 5′-GG-3′ and one at 5′-GC-3′; Cr(III)-induced, 12 mutations at 5′-GA-3′ sites; Cr(III)–Cys-induced, nine mutations at 5′-GA-3′ sites and two at 5′-GG-3′; Cr(III)–His-induced, five mutations at 5′-GA-3′ sites, two at 5′-GG-3′ and two at 5′-GT-3′; Cr(III)–GSH-induced, eight mutations at 5′-GA-3′ sites and one at 5′-GG-3′.

Additional analysis of the spectra showed that mutations targeted to the G:C base pair were not randomly distributed with respect to the nature of the 3′ nucleotide adjacent to the site of mutation. In all samples >90% of the G:C mutations occurred at positions with a 3′ adjacent purine to the mutated G (Table 4). Spontaneous and induced mutation spectra were found to be different in preference for 3′ adjacent base to the mutated G:C base pairs. While the frequency of G:C base pair substitutions at 5′-GA-3′ and 5′-GG-3′ sites was almost the same in all amino acid/glutathione–Cr(III) mutants, spontaneous mutations occurred nine times as frequently at 5′-GA-3′ compared with 5′-GG-3′ sites (Table 4). No preferences were found for 5′ nucleotide in either spontaneous or Cr-induced mutants.

DISCUSSION

DNA crosslinks of selected amino acids and glutathione represent ~50% of all Cr adducts found at non-toxic levels of exposure to carcinogenic chromate (12). Ternary Cr(III)–DNA adducts containing glutathione, histidine or cysteine were found to be mutagenic after replication of adducted pSP189 plasmids in human fibroblasts, with the Cr(III)–glutathione crosslink being the most potent pro-mutagenic lesion. Binary Cr(III)–DNA adducts were weakly mutagenic in our mutagenesis system. The weak mutagenicity of binary Cr(III)–DNA adducts observed in our experimental system is in good agreement with a study by Snow and Xu (36), who reported only a 2- to 3-fold increase in mutagenic response utilizing a single-stranded M13 vector treated with Cr(III). In all spectra studied, base substitutions at G:C base pairs accounted for >95% of all point mutations. Among spontaneous mutants G:C→T:A transversions and G:C→A:T transitions were found at approximately the same frequency, whereas G:C→C:G substitutions were observed in only 7% of mutants. The same distribution of types of base substitutions was found for plasmids adducted with Cr(III)–amino acid complexes, whereas Cr(III)–glutathione induced predominantly (78%) G:C→T:A transversions. Although the distribution of types of mutation for most Cr treatments was not found to be significantly different from spontaneous mutants, the underlying mechanism could be different. Analysis of spontaneous mutations in a single-stranded shuttle vector revealed that the major mutational events were targeted to cytosines and that 30% of point mutations and 80% of C→T base substitutions were at a uracil (the product of cytosine deamination) paired with adenine (37). In contrast,

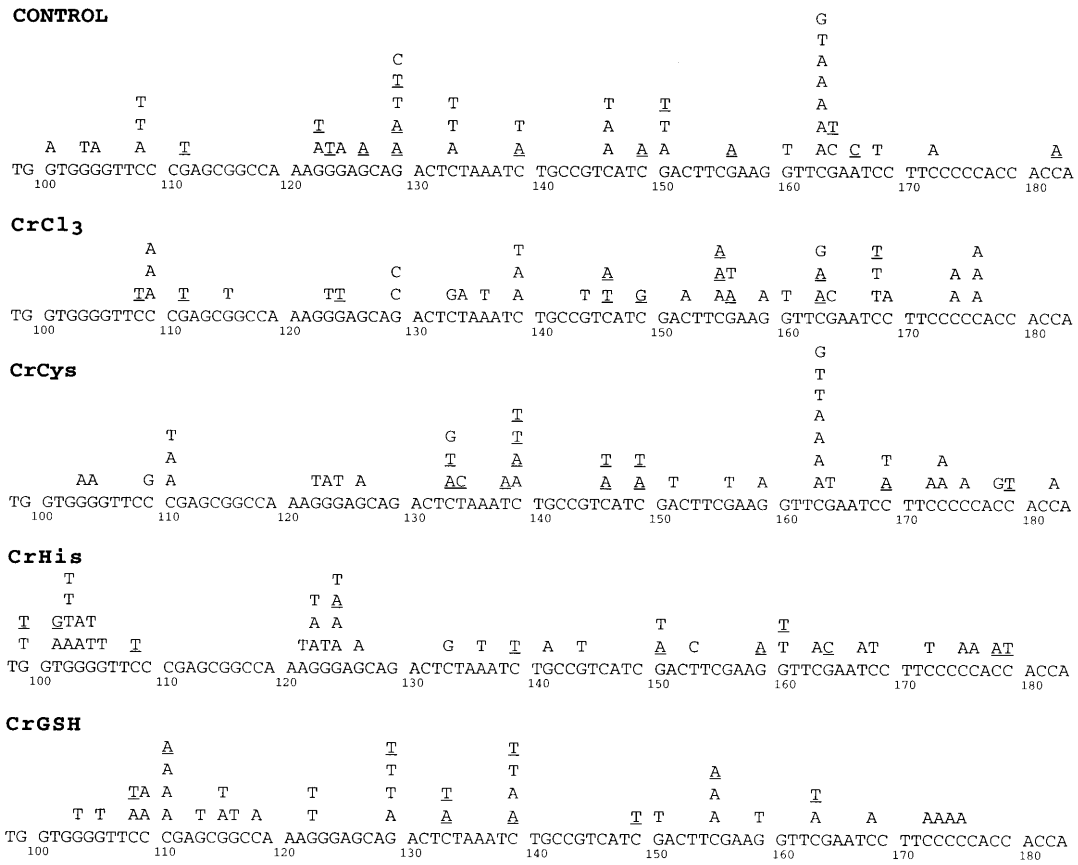

Figure 4. Mutation spectra generated in the *supF* gene by treatment of the pSP189 plasmid with amino acid/glutathione–Cr(III) complexes. Plasmids from individual mutant colonies were sequenced using a PCR-based procedure. Bases involved in multiple point mutations are underlined.

guanines are by far the most frequent sites for DNA adduct formation for the majority of carcinogens, which leads to generation of G:C base pair mutations.

Previous studies demonstrated that the binding site of Cr(III)–amino acid complexes to DNA is the phosphate group (22,38,39). Due to a lack of detailed information about the chemical structure of Cr(III)–DNA adducts, additional coordination to bases remains a possibility. The mutagenicity of adducts formed with phosphate groups may occur because of distortion in the conformation of the phosphodiester links that may interfere with the alignment of Watson–Crick base pairs. Recently, kinetic analysis of the base pairing of O^6-methylguanine showed that selection of the opposing base was determined not by the extent of hydrogen bonding or the strength of the bonding but rather by the extent to which base pairs supported the Watson–Crick conformation (40). DNA polymerase apparently catalyzes formation of a phosphodiester bond only when the 3′-OH of the primer strand and the α-phosphorus of the incoming dNTP are in the alignment found in Watson–Crick pairs (41). Thus, binding of bulky adducts such as amino acid/glutathione–Cr(III) complexes may present stereochemical problems for such an alignment and formation of phosphodiester links, on both the 3′- and 5′-side of the incoming base, may occur more easily when the adducted base is paired with a non-Watson–Crick base. Our results from the exonuclease digestion assay show that the adducts were distributed nearly uniformly along the DNA sequence, with no base or sequence specificity. In contrast, the mutations in all spectra occurred predominantly (>90%) at G:C base pairs. These results suggest that formation of Cr(III)–amino acid/glutathione adducts may distort the DNA conformation most profoundly at guanine residues, while only weakly affecting other nucleotides.

In addition to Cr(VI) compounds, adduction on phosphate oxygens is produced by a diverse group of alkylating agents that are potent carcinogens and mutagens (42,43). Mutagenicity of alkylating agents is generally attributed to formation of base adducts, particularly O^4-alkylthymine and O^6-alkylguanine, whereas formation of alkylphosphotriesters is presumed to have no mutagenic consequences, although it has not been experimentally addressed. Our results indicate that the pro-mutagenic potential of Cr(III) phosphotriesters could be related to the bulkiness of the adduct, as evidenced by the strong mutagenicity of large Cr(III)–glutathione adducts, the intermediate mutagenic potential of Cr(III)–histidine/cysteine and weak mutagenicity of Cr(III) alone. It is conceivable that a similar size effect may govern the mutagenicity of other alkylphosphotriesters. While formation of methylphosphotriesters may indeed have little pro-mutagenic effect, alkylation of the DNA phosphate with a bulky group in a specific sequence context may induce significant distortions in the DNA conformation, leading to base mispairing during replication.

The spectra of both induced and spontaneous mutations were quite widely spread along the target sequence, which was consistent

with random formation of Cr adducts on DNA. Some clustering of mutations occurred in Cr(III)–histidine- and Cr(III)–glutathione-induced spectra, however, most other mutations were found at particular sites with frequencies of no more than 7% of all point mutations. Mutations induced by Cr(III)–amino acid/glutathione treatment occurred predominantly at G:C pairs where a 3′ purine was adjacent to the mutated guanine. No preferences between G and A were found for induced mutations, whereas spontaneous mutations predominantly occurred at sites with 3′ adenine adjacent to the targeted guanine. The mutagenic preferences for sites with 3′ adjacent purines were also found with other agents, such as Cr(VI)–glutathione (20), glyoxal (35), chloroethyl cyclohexyl-nitrosourea (44) and X-rays (45). *In vitro* replication experiments using site-specifically modified oligonucleotides with different flanking sequences showed that the nucleotide immediately 3′ or 5′ of the adduct was a major determinant in replicational by-pass of O^4-methylthymine (46) and 1,N^6-etheno-2′-deoxyadenine adducts (47). Interestingly, in both cases inhibition of replication was most dramatic when adenines were adjacent to the adduct. It seems that in addition to the physico-chemical characteristics of a particular adduct, the local helix conformation and base stacking, determined by the local sequence context, might be important factors affecting the fidelity of replication.

The mutational spectra of Cr(VI) have been studied at the *hprt* locus in mammalian cells by two groups (21,48). The first report (48) showed that in Chinese hamster ovary cells chromate-induced mutations occurred predominantly at A:T base pairs, with a higher frequency of A:T→T:A and A:T→C:G base substitutions. In contrast, Chen and Thilly (21) reported that in human lymphoblastoid cells G:C base pairs were the most frequently mutated, with G:C→A:T and G:C→T:A changes. The reason for these differences in mutation spectra is unknown, although the method of mutant selection and/or type of cell could have been a significant factor. Comparison of the Cr(VI)-induced spectra obtained by exposure of cells to Cr(VI) with the spectra induced by known oxygen radical-producing agents (H_2O_2, Fe^{2+} and X-ray) showed little similarity (21), suggesting that mutagenesis by Cr(VI) *in vivo* may be largely due to Cr(III)-derived DNA adducts.

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REFERENCES

1 IARC (1990) Monograph on the evaluation of carcinogenic risk to humans. Chromium, Nickel and Welding. International Agency for Research on Cancer, Lyons, France, Vol. 49, pp. 49–256.
2 Langardt,S. (1990) *Am. J. Indust. Med.*, **17**, 189–215.
3 ATSDR (1992) Toxicological profile for chromium. Agency for Toxic Substances and Disease Registry, Atlanta, GA.
4 Sen,P., Conway,K. and Costa,M. (1987) *Cancer Res.*, **47**, 2142–2147.
5 Biedermann,K.A. and Landolph,J.K. (1990) *Cancer Res.*, **50**, 7835–7842.
6 DeFlora,S., Bagnasco,M., Serra,D. and Zanacchi,P. (1990) *Mutat. Res.*, **238**, 99–172.
7 Dillon,C.T., Lay,P.A., Cholewa,M., Legge,G.J.F., Bonin,A.M., Collins,T.J., Kostka,K.L. and She-McCarthy,G. (1997) *Chem. Res. Toxicol.*, **10**, 533–535.
8 Aiyar,J., Borges,K.M., Floyd,R.A. and Wetterhahn,K.E. (1989) *Toxicol. Environ. Chem.*, **22**, 135–148.
9 Stearns,D.M., Kennedy,L.J., Courtney,K.D., Giangrade,P.H., Phieffer,L.S. and Wetterhahn,K.E. (1995) *Biochemistry*, **34**, 910–919.
10 Stearns,D.M. and Wetterhahn,K.E. (1994) *Chem. Res. Toxicol.*, **7**, 219–230.
11 Costa,M. (1990) *J. Cell Biochem.*, **92**, 127–135.
12 Zhitkovich,A., Voitkun,V. and Costa,M. (1995) *Carcinogenesis*, **16**, 907–913.
13 Xu,J., Bubley,G.J., Detrick,B., Blankenship,L.J. and Patierno,S.R. (1996) *Carcinogenesis*, **17**, 1511–1517.
14 da Cruz Fresco,P.C. and Kortenkamp,A. (1994) *Carcinogenesis*, **15**, 1773–1778.
15 Casadevall,M. and Kortenkamp,A. (1995) *Carcinogenesis*, **16**, 805–809.
16 Sugiyama,M., Wang,X.W. and Costa,M. (1986) *Cancer Res.*, **46**, 4547–4551.
17 Aiyar,J., Berkovits,H.J., Floyd,R.A. and Wetterhahn,K.E. (1990) *Chem. Res. Toxicol.*, **3**, 595–603.
18 Cabral-Neto,J.B., Cabral,R.E., Margot,A., Le Page,F., Sarasin,A. and Gentil,A. (1994) *J. Mol. Biol.*, **240**, 416–420.
19 Dar,M.E. and Jorgensen,T.J. (1995) *Nucleic Acids Res.*, **23**, 3224–3230.
20 Liu,S. and Dixon,K. (1996) *Environ. Mol. Mutagen.*, **28**, 71–79.
21 Chen,J. and Thilly,W.G. (1994) *Mutat. Res.*, **323**, 21–27.
22 Zhitkovich,A., Voitkun,V. and Costa,M. (1996) *Biochemistry*, **35**, 7275–7282.
23 Parris,C.N. and Seidman,M.M. (1992) *Gene*, **117**, 1–5.
24 Ariza,R.R., Roldan-Arjona,T., Hera,C. and Pueyo,C. (1993) *Carcinogenesis*, **14**, 303–305.
25 Hanahan,D., Jessee,J. and Bloom,F.R. (1991) *Methods Enzymol.*, **204**, 83–88.
26 Panigraphi,G.B. and Walker,I.G. (1990) *Biochemistry*, **29**, 1222–2126.
27 Carielo,N.F., Piegorsch,W.W., Adams,W.T. and Skopek,T.R. (1994) *Carcinogenesis*, **15**, 2281–2285.
28 Hneihen,A.S., Stadeven,A.M. and Wetterhahn,K.E. (1993) *Carcinogenesis*, **14**, 1795–1803.
29 Miller,P.S., Fang,K.N., Kondo,N.S. and Ts'o,P.O.P. (1971) *J. Am. Chem. Soc.*, **93**, 6657–6665.
30 Saris,C.P., Damman,S.J., van den Ende,A.M.C., Westra,J.G. and den Engelse,L. (1995) *Carcinogenesis*, **16**, 1543–1548.
31 Sander,C. and Ts'o,P.O.P. (1971) *J. Mol. Biol.*, **55**, 1–21.
32 Seidman,M.M., Bredberg,A., Seethram,S. and Kraemer,K.H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4944–4948.
33 Madzak,C., Armier,J., Stary,A., Daya-Grosjean,L. and Sarasin,A. (1993) *Carcinogenesis*, **14**, 1255–1260.
34 Levy,D.D., Groopman,J.D., Lim,S.E., Seidman,M.M. and Kraemer,K.H. (1992) *Cancer Res.*, **52**, 5668–5673.
35 Murata-Kamiya,N., Kamiya,H., Kaji,H. and Kasai,H. (1997) *Nucleic Acids Res.*, **25**, 1897–1902.
36 Snow,E.T. and L.-S.,Xu (1991) *Biochemistry*, **30**, 11238–11245.
37 Fronza,G., Madzak,C., Campomenosi,P., Inga,A., Iannone,R., Abbondandolo,A. and Sarasin,A. (1994) *Mutat. Res.*, **308**, 117–125.
38 Vicens,M., Fiol,J.J., Terron,A., Moreno,V. and Goodgame,D.M.L. (1989) *Inorg. Chim. Acta*, **157**, 127–132.
39 Vincens,M., Prats,M., Fiol,J.J., Terron,A. and Moreno,V. (1989) *Inorg. Chim. Acta*, **158**, 59–68.
40 Swann,P.F. (1990) *Mutat. Res.*, **233,** 81–94.
41 Tan,H.B., Swann,P.F. and Chance,E.M. (1994) *Biochemistry*, **33**, 5335–5346.
42 Beranek,D.T. (1990) *Mutat. Res.*, **231**, 11–30.
43 Hall,J. and Montesano,R. (1990) *Mutat. Res.*, **233**, 247–252.
44 Inga,A., Iannone,R., Campomenosi,P., Molina,F., Menichini,P., Abbondandolo,A. and Fronza,G. (1995) *Cancer Res.*, **55**, 4658–4663.
45 Waters,L.C., Sikpi,M.O., Preston,R.J., Mitra,S. and Jaberaboansari,A. (1991) *Radiat. Res.*, **127**, 190–201.
46 Rao,S., Chenna,A., Slupsja,M. and Singer,B. (1996) *Mutat. Res.*, **356**, 179–185.
47 Litinski,V., Chenna,A., Sagi,J. and Singer,B. (1997) *Carcinogenesis*, **18**, 1609–1615.
48 Yang,J.L., Hseih,Y.C., Wu,C.W. and Lee,T.C. (1992) *Carcinogenesis*, **13**, 2053–2057.