Genotoxicity and Mutagenicity of Chromium(VI)/Ascorbate-Generated DNA Adducts in Human and Bacterial Cells†

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ABSTRACT: Reduction of carcinogenic Cr(VI) by vitamin C generates ascorbate−Cr(III)−DNA cross-links, binary Cr(III)−DNA adducts, and can potentially cause oxidative DNA damage by intermediate reaction products. Here, we examined the mutational spectrum and the importance of different forms of DNA damage in genotoxicity and mutagenicity of Cr(VI) activated by physiological concentrations of ascorbate. Reduction of Cr(VI) led to a dose-dependent formation of both mutagenic and replication-blocking DNA lesions as detected by propagation of the pSP189 plasmids in human fibroblasts. Disruption of Cr−DNA binding abolished mutagenic responses and normalized the yield of replicated plasmids, indicating that Cr−DNA adducts were responsible for both mutagenicity and genotoxicity of Cr(VI). The absence of DNA breaks and abasic sites confirmed the lack of a significant production of hydroxyl radicals and Cr(V)−peroxo complexes in Cr(VI)−ascorbate reactions. Ascorbate−Cr(III)−DNA cross-links were much more mutagenic than smaller Cr(III)−DNA adducts and accounted for more than 90% of Cr(VI) mutagenicity. Ternary adducts were also several times more potent in the inhibition of replication than binary complexes. The Cr(VI)-induced mutational spectrum consisted of an approximately equal number of deletions and G/C-targeted point mutations (51% G/C → T/A and 30% G/C → A/T). In *Escherichia coli* cells, Cr(VI)-induced DNA adducts were only highly genotoxic but not mutagenic under either normal or SOS-induced conditions. Lower toxicity and high mutagenicity of ascorbate−Cr(III)−DNA adducts in human cells may result from the recruitment of an error-prone bypass DNA polymerase(s) to the stalled replication forks. Our results suggest that phosphotriester-type DNA adducts could play a more important role in human than bacterial mutagenesis.

Several million industrial workers worldwide are exposed to chromium and its compounds, and approximately 70 professional groups experience exposure to toxic hexavalent chromium (*1*, *2*). There are also concerns about Cr(VI) exposure by the general population through consumption of Cr(VI)-contaminated drinking water (*3*) and by other routes (*4*, *5*). Cr(VI) is a recognized human and animal carcinogen that by itself is not reactive and requires reductive activation to cause DNA damage (*6*). The end-product of Cr(VI) reduction is always trivalent Cr. Direct reduction by low molecular weight thiols and ascorbic acid (vitamin C) are believed to be a predominant mechanism for activation of Cr(VI) in cells (*6*−*8*). Certain enzymatic systems can also be involved in the intracellular metabolism of Cr(VI) (*9*). Experimental data strongly indicate that ascorbate is probably the most important reducing agent for Cr(VI) in the major target tissues. (I) Ascorbate has the highest rate of Cr(VI) reduction in vitro among all known reducing agents (*7*, *8*, *10*). (II) Intratracheal administration of Cr(VI) results in the depletion of ascorbate from the lung tissue (*11*). (III) Lung homogenates and buffer solutions containing equivalent concentrations of ascorbate have similar rates of Cr(VI) reduction (*11*). (IV) Finally, the pretreatments of cytosolic preparations of lung, liver, and kidney tissues with ascorbate oxidase led to a strong (80−95%) inhibition of Cr(VI) reduction (*12*, *13*).

Reduction of Cr(VI) to Cr(III) by ascorbate is associated with the production of organic radicals and intermediate Cr(V) and Cr(IV) forms (*14*, *15*). The relative yield of these intermediate products is strongly influenced by the ratio of ascorbate to Cr(VI) (*15*). For example, the presence of Cr(V) was only detectable in Cr(VI) reduction reactions containing equimolar or lower amounts of ascorbate (*15*). The final product of the reduction process, Cr(III), was found to be responsible for the formation of binary Cr(III)−DNA and ternary ascorbate−Cr(III)−DNA adducts (*10*), as well as interstrand DNA cross-links (*16*). The DNA phosphate backbone is the primary site of adduction for Cr(III) complexes formed in Cr(VI)−ascorbate (*10*) and other reactions (*17*−*20*). Reductive metabolism of Cr(VI) has also been reported to lead to the formation of oxidative DNA lesions (*21*−*23*). Both Cr−DNA adducts (*18*, *19*) and oxidative DNA lesions (*24*, *25*) are mutagenic; however, their relative significance in the mutagenic responses generated by Cr(VI)−ascorbate reactions remained unknown. The genotoxic (replication-blocking) potential of individual Cr−DNA adducts or Cr(VI)/ascorbate-induced DNA modifications have never been examined in intact cells. The objectives of this study were (1) to determine the mutational spectrum and the role of oxidative damage and individual Cr−DNA adducts in the mutagenicity of Cr(VI) activated by ascorbate

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at biologically relevant concentrations; (II) to characterize the genotoxic potential of Cr(VI)-induced DNA lesions in intact cells; and (III) to examine the differences in mutagenic and genotoxic responses to Cr−DNA adducts between human and bacterial cells.

EXPERIMENTAL PROCEDURES

Materials. L-ascorbic acid was from Sigma, Na_2CrO_4 and $[Cr(H_2O)_4Cl_2]Cl\cdot 2H_2O$ (99.995% pure) were from Aldrich, and 1,2-diamino-4,5-dimethoxybenzene dihydrochloride and 2′,7′-dichlorofluoroscin diacetate were obtained from Molecular Probes. [^{51}Cr]chromate and [^{51}Cr]chromium(III) chloride were from Amersham. All reagents excluding Cr(III) salts were additionally purified by Chelex-100 chromatography (*26*). The pSP189 plasmid was generously provided by M. Seidman. SV40-immortalized human HF/SV fibroblasts (provided by Dr. H. Ozer) were propagated in 90% DMEM/10% serum.

Formation of Cr−DNA Adducts in the Reactions with Cr(VI). A standard reaction contained 25 mM MOPS buffer (pH 7.0), 1 mM Asc[1], 2 μg of pSP189 DNA, and 0−200 μM Na_2CrO_4 in the final volume of 50 μL. A set of reactions was also performed in 25 mM sodium phosphate buffer (pH 7.0) or in 25 mM MOPS buffer supplemented with 5 mM EDTA. Samples were incubated at 37 °C for 30 min, and Cr-modified DNA was purified by size-exclusion chromatography and ethanol precipitation (*19*). DNA was dissolved in water and then used for cell transfections or determination of DNA adducts. Total Cr−DNA adducts were quantified by inclusion of trace amounts of [^{51}Cr]chromic chloride. The amount of Asc−Cr(III)−DNA adducts was quantified using HPLC detection of a fluorescent derivative of dehydroascorbic acid with 1,2-diamino-4,5-dimethoxybenzene dihydrochloride (*10*).

Formation of Cr(III)−DNA and Ascorbate−Cr(III)−DNA Adducts. $K[Cr(asc)_2]\cdot 7H_2O$ was synthesized and purified as described elsewhere (*27*). Freshly dissolved $[Cr(H_2O)_4Cl_2]Cl\cdot 2H_2O$ or $K[Cr(asc)_2]\cdot 7H_2O$ was reacted with 2 μg of pSP189 DNA in 25 mM MES buffer (pH 6.0) for 0.5 or 2 h at 37 °C to form Cr(III)−DNA adducts and Asc−Cr(III)−DNA adducts, respectively (*10*). DNA was purified by Bio-Gel P-30 chromatography and ethanol precipitation.

Shuttle-Vector Mutagenesis in Human Fibroblasts. Mutagenic potential of Cr−DNA adducts was assessed using the pSP189 shuttle-vector containing the *supF* gene as a mutagenic target (*28*). Replication of Cr-damaged and control plasmids was performed in HF/SV human fibroblasts. *SupF* mutants was selected in the *Escherichia coli* MBL50 strain that contains *araC* and *lacZ* amber mutations suppressed by the *supF* tRNA (*29*). Amber mutation in the *araC* gene allows selection of the *supF* mutants in the presence of L-arabinose, whereas amber mutation in the *lacZ* gene permits white/blue color screening. The details of cell transfections and mutant selection have been described previously (*19*). In brief, Cr-treated or control plasmids were transfected into the cells and allowed to replicate for 48 h. The pSP189 plasmids were recovered from cells by a Miniprep kit from Qiagen. The presence of a strong alkali treatment step in this protocol causes dissociation of Cr from DNA (*30*), which allowed us to omit the digestion with DpnI. Control experiments showed that the presence or absence of DpnI digestion had no significant effect on the mutation frequency or the yield of bacterial transformants. Replicated progeny of pSP189 DNA was introduced into the *E. coli* MBL50 strain by electroporation. Transformation efficiency was determined on the minimal plates containing 30 μg/mL ampicillin and 0.5 μg/mL chloramphenicol. Mutant selection was performed on plates additionally containing 2 mg/mL L-arabinose. Mutation frequency was calculated by dividing the number of arabinose resistant colonies by the number of ampicillin resistant colonies.

Sequencing. Mutant colonies were picked from samples treated with 50 μM Cr(VI). DNA damage with this concentration of Cr(VI) typically resulted in the mutation frequencies more than 20 times higher than background values. Plasmid DNA was isolated from 2 mL of overnight culture using a kit from Qiagen. The sequencing primer corresponding to the positions 4889−4908 of the pSP189 plasmid was 5′-GGCGACACGGAAATGTTGAA-3′.

Plasmid Mutagenesis Assay in E. coli. In the first set of the experiments, Cr(VI)-modified pSP189 DNA was electroporated into the *E. coli* MBL50 strain, and the number of surviving clones (ampicillin resistance) and the mutation frequency (combined ampicillin and arabinose resistance) were determined as above. To induce SOS system (*31*), bacteria were suspended in a small volume of 10 mM $MgSO_4$ and irradiated with 0−20 J/m^2 UV light using a 254 nm germicidal lamp (Spectroline XX-15G). UV fluency was measured with a UVX-25 radiometer (Ultra Violet Products, CA). Cells were allowed to express SOS proteins for 40 min at 37 °C in SOB medium, washed, and then used for electrotransfection of Cr-containing plasmids. To enhance detection sensitivity of mutants under conditions of low plasmid survival, we employed a two-step mutagenesis procedure. SOS-induced and control bacteria were transfected with adducted pSP189 plasmids, and cells with completely replicated plasmids were allowed to expand overnight in LB medium containing ampicillin. Replicated plasmids were isolated, equal amounts of DNA were transfected again in electrocompetent MBL50 cells, and the standard survival/mutagenesis protocol was followed.

Other Procedures. Kinetics of Cr(VI) reduction was followed by the decrease in chromate absorbance at 372 nm (*19*). DNA-breaking activity of Cr(VI)−Asc mixtures was assessed by the plasmid relaxation assay using supercoiled preparations of pBR322 DNA (*26*). The formation of intermediate reaction products was measured by fluorescence of the oxidant-sensitive dye, 2′,7′-dichlorofluoroscin diacetate (DCFH) (*8*, *32*).

RESULTS

Kinetics of Cr(VI) Reduction. Figure 1 shows that the disappearance of Cr(VI) in the presence of 1 mM ascorbate is very fast ($t_{1/2}$ = 1 min), and essentially all Cr(VI) was reduced during the first 5 min. The initial rate of reduction by ascorbate was approximately 13 and 61 times faster than that by cysteine ($t_{1/2}$ = 13.3 min) and glutathione ($t_{1/2}$ = 60.7 min), respectively. Ascorbate and glutathione are frequently found in approximately equimolar concentrations (*33*), whereas the glutathione-to-cysteine ratio is about 8:1 in cells

[1] Abbreviations: Asc, ascorbic acid; bp, base pairs.

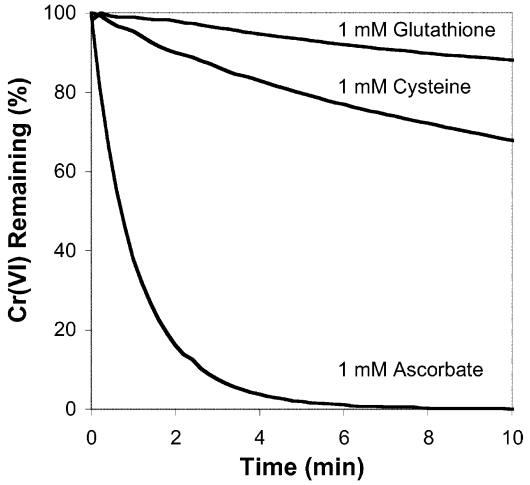

FIGURE 1: Reduction kinetics of chromate in the presence of 1 mM ascorbate, cysteine, or glutathione. Rate of chromate reduction was monitored at 372 nm. Reactions were carried out at 37 °C with 50 μM chromate in 25 mM MOPS (pH 7.0).

from unexposed individuals and approximately 3:1 in lymphocytes from Cr(VI)-exposed welders (*8*). Assuming the independence of each reduction reaction and using the initial reaction rates, we calculated that ascorbate should metabolize 97.5% of all intracellular Cr(VI) in unexposed people. Cysteine and glutathione would reduce 0.9 and 1.5% fractions, respectively. The corresponding values for welders, the largest Cr(VI)-exposed occupational group, are 96, 2.4, and 1.5%. Our estimates are very close to the reported contribution of ascorbate to the metabolism of Cr(VI) in the rat lung tissue (more than 95%) (*13*). A good concordance with biological systems and the lack of a significant buffer effect on the reduction of Cr(VI) (see below) indicate that our in vitro conditions provide a reasonable approximation for the reduction conditions in the lung tissue.

Role of Cr(III) in the Induction of Mutations and Replication Blockage by Cr(VI)−Ascorbate Reactions. Reduction of Cr(VI) in MOPS buffer resulted in a dose-dependent formation of Cr−DNA adducts (Figure 2A). Addition of Cr(III)-chelating agents EDTA or phosphate to Cr(VI)−ascorbate reactions completely blocked Cr−DNA binding. Reductive conversion of Cr(VI) to Cr(III) resulted in the production of mutagenic DNA damage as evidenced by a strong, dose-dependent increase in the frequency of the *supF* mutants following replication of Cr(VI)-treated pSP189 plasmids in human fibroblasts (Figure 2B, MOPS samples). Blocking of Cr(III)−DNA binding in reactions containing EDTA or inorganic phosphate eliminated mutagenic responses at all Cr(VI) concentrations, suggesting a critical role of Cr(III)-containing DNA adducts in the induction of mutagenesis by Cr(VI). Control reactions showed that EDTA and phosphate did not significantly change the rates of Cr(VI) reduction ($t_{1/2} = 0.8 \pm 0.1$, 1.2 ± 0.3, and 1 ± 0.2 min for phosphate, EDTA, and MOPS-based reactions, respectively). Reduction reactions containing either MOPS or phosphate buffer also generated similar amounts of DCFH-oxidizing species (Figure 3A), indicating that phosphate did not alter the formation of intermediate reaction products, such as Cr(V) and organic radicals (*8, 32*). It should be noted that DCFH is more susceptible to oxidation than DNA (*19*), and therefore, increased DCFH fluorescence is neither indicative nor dismissive of the presence of DNA-damaging oxidants.

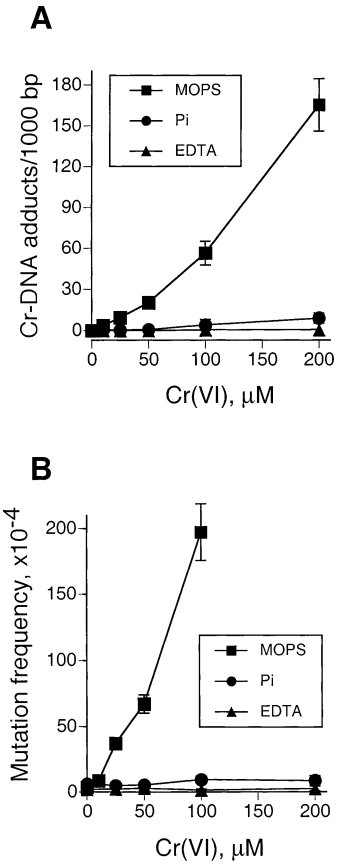

FIGURE 2: Formation of Cr−DNA adducts and mutagenesis in the pSP189 plasmids. A standard reaction contained 25 mM buffer (pH 7.0), 1 mM ascorbate, 2 μg of pSP189 DNA, and indicated concentrations of Cr(VI). Unreacted Cr was removed by Bio-Gel P-30 chromatography and ethanol precipitation. Cr-modified plasmids were replicated in human fibroblasts for 48 h, replicated progeny was isolated, and the presence of the *supF* mutants and the yield of replicated plasmids were scored in the *E. coli* MBL50 strain. MOPS—the reactions contained MOPS buffer; EDTA—MOPS buffer was supplemented with 5 mM EDTA; Pi—reaction contained 25 mM sodium phosphate. Shown are means ± SD from six to nine independent experiments. (A) Formation of Cr−DNA adducts in Cr(VI)−ascorbate reactions. (B) Induction of mutations following replication of Cr(VI)-treated pSP189 plasmids in human fibroblasts.

The potential formation of DNA breakage and abasic sites during Asc-driven reduction of Cr(VI) was examined using a pBR322-based plasmid relaxation assay (Figure 3B,C). The pSP189 DNA is typically isolated as the mixture of monomeric and multimeric molecules, which makes it somewhat more difficult to detect low levels of strand breakage (*10, 19*). To further increase the detection sensitivity, the concentration of pBR322 DNA was decreased 4-fold relative to the standard reactions with pSP189 plasmids. Nevertheless, we did not observe any detectable increase in the level of strand breaks or abasic sites following reduction of Cr(VI) by ascorbate (Figure 3B,C). On the basis of the detection limit of plasmid breakage assay (*8*) and the amount of DNA-bound Cr at 100 μM Cr(VI), we calculated that the yield of Cr−DNA adducts exceeded that of strand breaks and abasic sites by more than 8000-fold. A slower electrophoretic mobility and diminished ethidium bromide fluorescence of plasmids treated in MOPS but not phosphate buffer are caused by the presence of Cr−DNA adducts(*19*).

Another test for the role of oxidative and Cr(III)-dependent mechanisms in Cr(VI)-induced mutagenicity can be per-

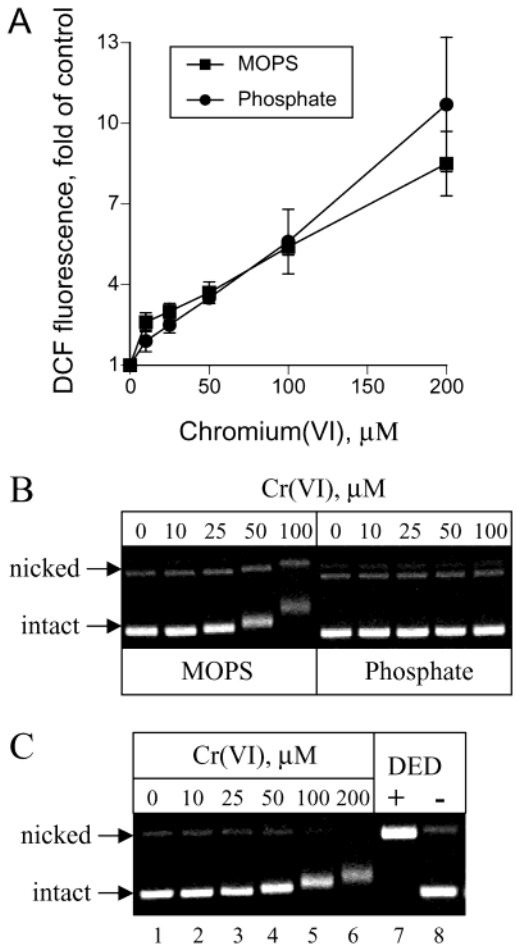

FIGURE 3: Oxidation of DCFH and the presence of the sugar−phosphate damage in plasmid DNA following Cr(VI) reduction with 1 mM ascorbate. Reactions were allowed to proceed for 30 min at 37 °C in 25 mM MOPS (pH 7.0) or 25 mM phosphate buffer (pH 7.0). (A) Oxidation of DCFH as monitored by the formation of its fluorescent oxidized product, DCF. Shown are means ± SD of three to six independent measurements. (B) Strand breakage detection in Cr(VI)-treated pBR322 DNA. DNA samples were loaded directly on 1% agarose gel at the end of the reaction. (C) Detection of abasic sites in DNA treated with Cr(VI)-ascorbate in MOPS buffer. Cr(VI)-treated DNA samples (lanes 1−6) were incubated with 100 mM *N*,*N*′-dimethylethylenediamine (DED), which converts abasic sites into strand breaks (*26*). Lane 7 (positive control)—acid/heat treated DNA after incubation with DED; lane 8—acid/heat treated DNA without DED incubation.

formed by analyzing residual mutagenic responses after the stripping of Cr(III) from DNA. Figure 4A shows that overnight incubation of Cr(VI)-modified DNA with 50 mM phosphate dissociated the majority of Asc−Cr(III)−DNA and Cr(III)−DNA adducts (86 and 77% loss of DNA-bound Asc and Cr, respectively). The observed loss of Cr−DNA adducts was accompanied by an 84% decrease in the mutagenic responses, confirming the primary role of Cr(III) in the mutagenic lesions generated by Cr(VI)−Asc reactions. Cr−DNA adducts were also responsible for the decreased template activity of Cr(VI)-treated plasmids since blocking of Cr(III)−DNA binding by EDTA or phosphate, as well as reversal of Cr(III)−DNA adducts essentially restored unobstructed replication of the plasmids (normal yield of bacterial transformants) (Figure 4B). A slightly increased plasmid survival in the phosphate-containing samples (111.5 ± 13%) was not statistically different from control, and it was probably caused by the experimental variability.

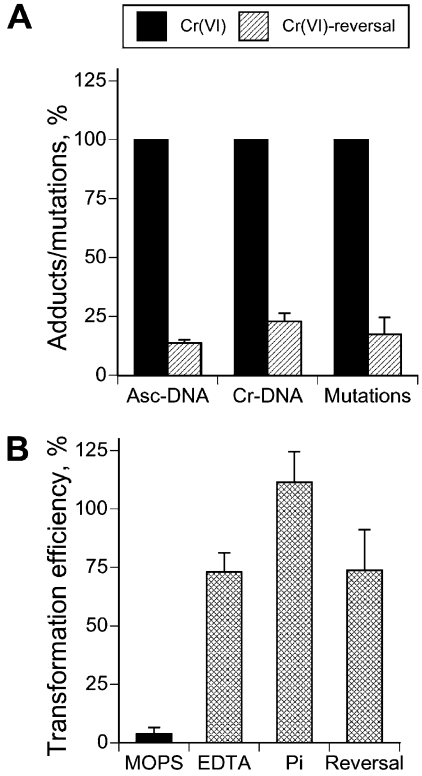

FIGURE 4: Mutagenesis and replication activity after dissociation of Cr(III) from Cr(VI)-treated DNA. Cr−DNA adducts were dissociated by incubation with 50 mM phosphate (pH 7.0) at 37 °C for 24 h. DNA was passed through P-30 columns, precipitated with ethanol, and then used for determination of adducts and transfections. Shown are means ± SD of four to six independent experiments. (A) Mutagenic responses in Cr(VI)-treated plasmids before (CrVI) and after dissociation of Cr(III)−DNA adducts (CrVI reversal). (B) Replication activity of plasmids treated with Cr(VI) in the presence of MOPS buffer, EDTA (MOPS + 5 mM EDTA), Pi (25 mM phosphate), or in MOPS buffer followed by removal of Cr adducts by phosphate postincubation (reversal samples). The number of bacterial transformants reflects the yield of replicated plasmids.

Genotoxic and Mutagenic Potentials of Cr(III)−DNA and Ascorbate−Cr(III)−DNA Adducts in Human Cells. Reduction of Cr(VI) by ascorbate leads to the production of ternary Asc−Cr(III)−DNA and binary Cr(III)−DNA adducts (*10*). To assess the relative importance of these adducts in Cr(VI)-induced mutagenesis, we first determined mutational responses in plasmid samples modified with Cr(VI) at different phases of the reduction reaction (Figure 5). We have previously found that essentially all ternary adducts were formed during Cr(VI) reduction (first 5 min), while addition of DNA after the completion of the reduction process (5−30 min) resulted in a greatly diminished Asc−DNA binding (*10*). In contrast to Asc−DNA cross-linking, overall Cr−DNA binding is still quite extensive during the post-reduction incubations (Figure 5A). Therefore, a comparison of mutational responses in samples modified during and after Cr(VI) reduction can provide clues to the importance of ternary adducts in Cr(VI)-induced mutagenesis. Figure 5B shows that replication of plasmids containing approximately 25% Asc−Cr−DNA cross-links and 75% binary adducts (0−5 min samples) (*10*) induced much stronger mutagenic responses than binary Cr(III)−DNA adducts alone (5−30 min samples). This result was indicative of a high mutagenic potential of Asc-containing adducts. To examine the biologi-

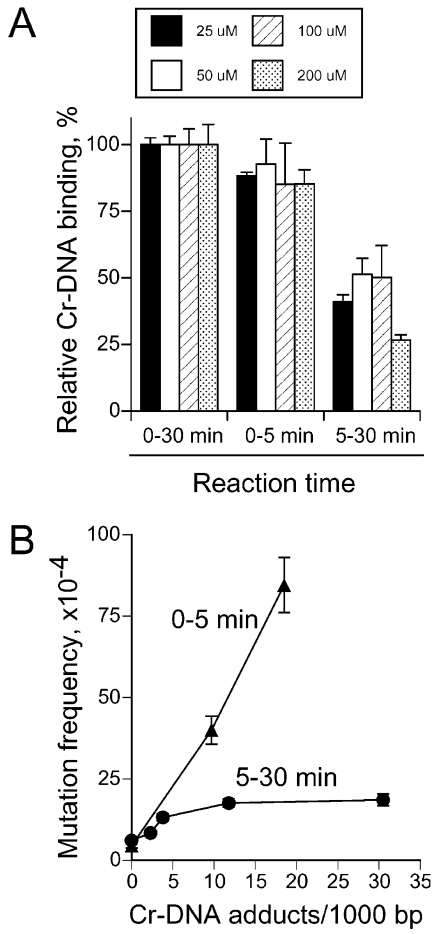

FIGURE 5: Cr−DNA binding and formation of mutagenic DNA lesions during different phases of Cr(VI) reduction. Plasmid DNA was modified with Cr(VI)−ascorbate for 30 min (0−30 min samples), for the first 5 min of Cr(VI) reduction (0−5 min samples), or DNA was added after the completion of Cr(VI) reduction (5−30 min samples). (A) Cr−DNA binding. (B) *SupF* mutagenesis. Shown are means ± SD from four independent transfections.

cal importance of Cr−DNA adducts more directly, binary and ternary adducts were formed separately, and their mutagenic potencies were determined following replication in human cells (Figure 6). We found that the Asc−Cr(III)−DNA adduct was 31 times more mutagenic relative to a smaller binary Cr(III)−DNA adduct, as judged by the slope of the dose−response curves. Calculated mutagenic responses for Cr(VI), based on the relative mutagenic potency of binary and ternary adducts and their yield, were very close to the actual results, indicating that our synthetic procedures produced DNA adducts with biological properties similar to those formed during reduction of Cr(VI).

The Asc−Cr(III)−DNA cross-link was also a more potent replication-blocking lesion than the Cr(III)−DNA adduct as evidenced by approximately 7 times lower yield of bacterial transformants per adduct (Figure 6B). The plasmid survival curve for Cr(VI)-modified samples was very close to that of ternary adducts, indicating that Asc−Cr(III)−DNA adducts played a major role in replication blockage induced by Cr(VI) damage. The presence of Cr−DNA adducts had no or only a minimal effect on the transfection efficiency of the pSP189 plasmids, as determined in four co-transfection experiments with the pEGFP−N1 plasmid (25.6, 25.0, 17.8, and 17.8% transfection efficiency for 0, 10, 25, and 50 μM Cr(VI)-treated samples, respectively).

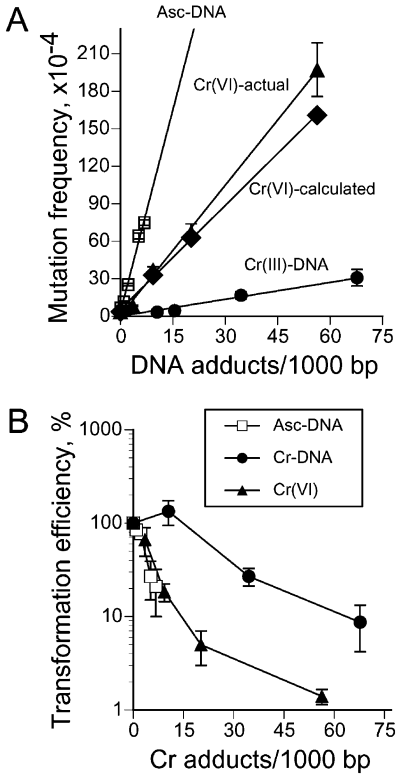

FIGURE 6: Mutagenic and replication-blocking potentials of Cr(III)−DNA and ascorbate−Cr(III)−DNA adducts. (A**)** Mutagenicity of Cr(III)−DNA and ascorbate−Cr(III)−DNA adducts. Cr(VI) actual—actual mutagenesis results from Figure 2A,B; Cr(VI) calculated—predicted Cr(VI) mutagenic response using mutagenic potentials of binary and ternary adducts (slopes) and the yield of these adducts. (B**)** Yield of replicated progeny following propagation of Cr-modified plasmids in human cells. Shown are means ± SD of six independent experiments.

Table 1: Characterization of Cr(VI)-Induced Mutations.

type of mutations	frequency (%)	base substitutions	frequency (%)
deletions	25[a] (40)[b]	transitions	
small (<15bp)	5 (20)	G:C → A:T	13 (30)
large (>15bp)	20 (80)	A:T → G:C	1 (2)
insertions	4 (6)	transversions	
point mutations (all)	28 (44)	G:C → T:A	22 (51)
single	19 (68)	G:C → C:G	4 (9)
tandem	2 (7)	A:T → T:A	2 (5)
multiple	7 (25)	A:T → C:G	1 (2)
complex mutants	6 (10)		

sequence (3′ base effect)	frequency (%)	sequence (5′ base effect)	frequency (%)
5′-G*A-3′ [c]	9 (23)	5′-AG*-3′	19 (49)
5′-G*G-3′	18 (46)	5′-GG*-3′	15 (38)
5′-G*T-3′	4 (10)	5′-TG*-3′	2 (5)
5′-G*C-3′	8 (21)	5′-CG*-3′	3 (8)
5′- G*Pu-3′	27 (69)	5′-PuG*-3′	34 (87)

[a] Number of mutants of the indicated type. [b] Percent of total plasmids. [c] G*—mutated G base; Pu—purine base.

Characterization of Cr(VI)-Induced Mutants. To determine the spectrum of Cr(VI)-induced mutations in human cells, we purified pSP189 DNA from arabinose-resistant colonies and sequenced the *supF* gene. Sibling mutants identified based on the presence of a unique 8-bp signature sequence in individual plasmids were excluded from the subsequent analyses. Two major types of mutations were point mutations

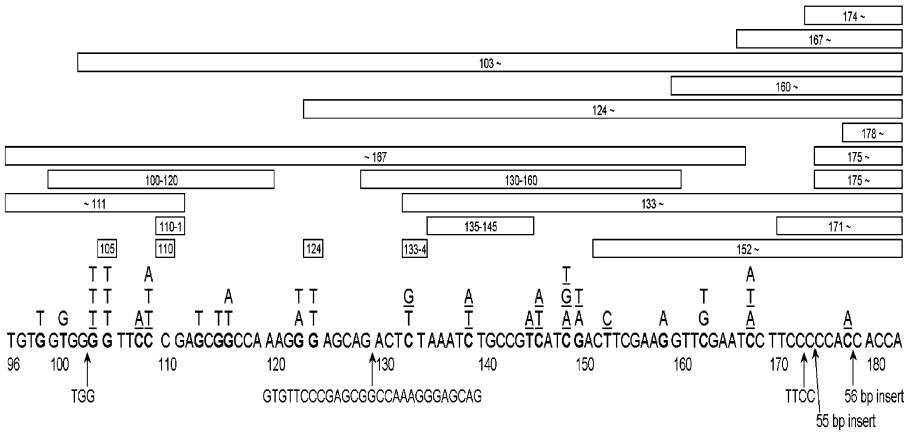

FIGURE 7: Mutational spectrum induced by Cr(VI) in the *supF* gene of pSP189 plasmid. Mutational changes were detected by sequencing. Underlined bases indicate substitutions originated from clones containing multiple mutations. Letters and ranges within boxes denote deletions of single bases and sequences, respectively. Arrowheads indicate sites of insertions.

(44%) and deletions (40%) (Table 1). The majority of point mutations involved substitutions at G:C pairs (90%) and primarily included G:C → T:A transversions (51%) and G:C → A:T transitions (30%). The percentage of G:C → C:G transversions was low (9%) and very similar to that for control plasmids (*18*). The frequency of the mutational events was the highest when G had a purine base in either the 3′ or 5′ position. Cr(VI)-induced mutations were broadly distributed along the *supF* gene (Figure 7). We also analyzed the mutational spectrum using our MutPlus computer program (http://www.cs.brown.edu/people/gq/mutplus_home.html) based on the SEM algorithm (*34*) and did not find any significant mutational hot-spots.

Cr−DNA Adducts Are Toxic But Not Mutagenic in E. coli. To examine the biological role of DNA backbone modifications by Cr(III) complexes in prokaryotic systems, we directly electroporated Cr(VI)-treated pSP189 plasmids into *E. coli* (Figure 8A). Cr−DNA adducts were extremely toxic in *E. coli* as evidenced by a very low number of bacterial transformants. At 20 Cr adducts/1000 bp DNA, plasmid survival in *E. coli* was about 3400-times lower than that in human fibroblasts (Figure 6B). Induction of SOS responses by UV exposure prior to electroporation of plasmids only modestly improved survival of Cr(VI)-treated plasmids (Figure 8B). We were unable to detect any increase in the mutation frequency at the *supF* gene following transfection of Cr-modified plasmids into bacteria and direct selection of mutants (not shown). A very low survival and the absence of error-prone polymerases in SOS-uninduced cells (*35*) could have accounted for the lack of detectable mutagenic responses. To overcome the problem of the low number of survivors, we used a two-step mutagenesis procedure. Following transfection of plasmids, bacteria were grown as a mass culture to allow expansion of cells with fully replicated plasmids. Replicated plasmids were isolated, and the equal amounts of DNA were electroporated into bacteria again to screen for the presence of *supF* mutants. This approach resulted in a very high yield of bacterial transformants without any significant differences between control and Cr-treated samples (not shown). However, we did not detect increased mutation frequency in Cr(VI)-treated samples in control or SOS-induced cells (Figure 8C). For comparison, replication of pSP189 plasmids containing 9 Cr adducts/1 kbp DNA in human cells led to approximately 11-fold

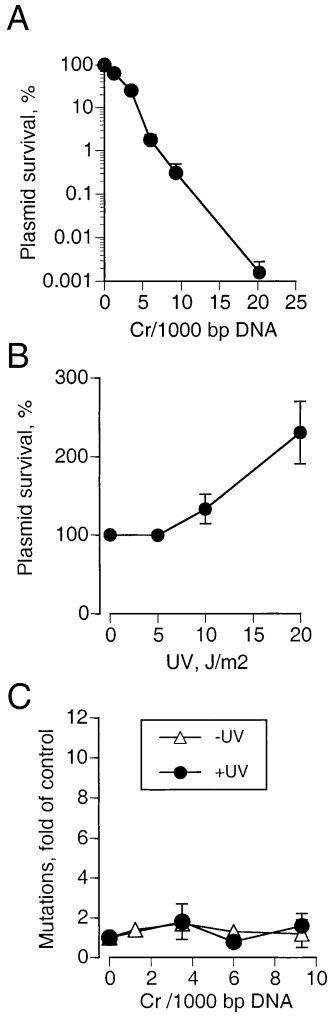

FIGURE 8: Genotoxicity and mutagenicity of Cr(VI)-induced DNA adducts in *E. coli*. The pSP189 DNA was modified with Cr(VI)−ascorbate for 30 min under the standard reaction conditions. Shown are means ± SD from eight experiments. (A**)** Survival of Cr(VI)-modified plasmids in control *E. coli*. (B) Plasmid survival in SOS-induced bacteria. DNA was modified with 10 μM Cr(VI)/1 mM ascorbate. Plasmid survival was normalized to that of UV-treated controls. (C**)** *SupF* mutagenesis in SOS-induced (+UV) and untreated (−UV) *E. coli*. SOS response was induced by exposure to 20 J/m^2 UV light (18.9% cell survival).

induction in mutations despite 20 times higher background frequency (4×10^{-4} and 2×10^{-5} for human and bacterial

cells, respectively). A very high toxicity and the absence of mutagenic responses in bacterial cells were also observed for Cr−DNA adducts formed during reduction of Cr(VI) by 2 mM cysteine (not shown). Similarly to ascorbate reactions, Cr−DNA adducts generated during reduction of Cr(VI) by cysteine are mutagenic in human fibroblasts (*19*).

DISCUSSION

Nature of Mutagenic DNA Damage Generated by Cr(VI)−Ascorbate Reactions. Our results showed that Asc-driven reduction of Cr(VI) resulted in the formation of DNA lesions that were mutagenic during replication in human fibroblasts. The predominant mechanism for the formation of these lesions is nonoxidative and involves binding of Cr(III) to DNA. This conclusion is supported by the lack of mutagenic responses when DNA was treated with Cr(VI)−ascorbate in the presence of Cr(III)-chelating agents and the fact that dissociation of Cr(III) from the Cr(VI)-treated pSP189 plasmids by phosphate resulted in a dramatic suppression of mutagenesis. Insignificant formation of potent DNA oxidizing species, such as hydroxyl radicals and Cr(V)−peroxo species, was further supported by the lack of abasic sites and strand breaks in Cr(VI)-modified DNA. Cr(V)−peroxo complexes have been reported to cause almost exclusively oxidative damage to the DNA backbone (*21*, *22*), while hydroxyl radicals attack both DNA bases and the sugar−phosphate backbone *(36)*. Replication of pSP189 plasmids containing either Cr(III)−DNA or Asc−Cr(III)−DNA adducts revealed that the ternary adduct had a much greater mutagenic potential. The As−Cr(III)−DNA cross-link appears to be more mutagenic than histidine/cysteine adducts and possibly, even the glutathione adduct (*18*, *19*). We estimated that Asc−Cr(III)−DNA adducts accounted for 92−96% mutagenicity induced by ascorbate-dependent reduction of Cr(VI) under our in vitro conditions.

It has been previously reported that Cr(VI)−ascorbate reactions led to the formation of DNA strand breaks and abasic sites but no base oxidation products (*21*, *22*). Oxidative damage to the DNA backbone was the most extensive in the reactions with high concentrations of Cr(VI) (low Asc:Cr ratio), which is expected to lead to the appreciable formation of the Cr(V) intermediate (*14*, *15*). Ascorbate is a two-electron reducing agent, and the production of Cr(V) is likely to occur through disproportionation or comproportionation of the first Cr intermediate, Cr(IV), under conditions of limited amounts of the reducer (2CrIV → CrV + CrIII, CrVI + CrIV → 2CrV). The absence of oxidized base products (GC−MS technique) and elimination of DNA backbone damage in the presence of catalase or in deaerated reaction mixtures led to the suggestion that DNA oxidizing species were Cr(V)−peroxo complexes, not hydroxyl radicals (*21*, *22*). The presence of Cr(V)−peroxo species was confirmed directly, and it was shown that these complexes arose from the reaction of Cr(V) with H_2O_2 that was produced during Fe-catalyzed autoxidation of ascorbic acid (*37*). We attribute the lack of oxidative DNA damage in our experiments to the facts that we rigorously purified all reagents and used 5:1 and higher ratios of ascorbate to Cr(VI), conditions that do not lead to a detectable formation of Cr(V) intermediate (*14*, *15*). Under most human exposure conditions, intracellular ascorbate concentrations (1−3 mM) (*33*) are expected to be much higher than those of gradually entering Cr(VI) (micromolar doses), and the formation of the Cr(V) intermediate should be very low. Delivery of a very high dose of Cr(VI), such as by phagocytosis of Cr(VI) particles, or simultaneous heavy exposure to other redox-active metals could overwhelm intracellular reducing capacity and cause significant oxidative DNA damage by Cr(V)−peroxo complexes (*22*) or by hydroxyl radicals generated in Fenton-like reactions of Cr(V/IV) with hydrogen peroxide (*38*, *39*). Ascorbate is a potent antioxidant (*33*), and therefore, its presence in Cr(VI)-exposed human cells should suppress oxidative DNA damage both through the diminished formation of Cr(V) and by the scavenging of reactive oxygen species. Many cultured human cells lack detectable amounts of intracellular ascorbate (*10*), which should make them more prone to oxidative damage by Cr(VI) than cells in vivo.

Replication-Blocking Lesions and Mechanisms of Mutagenesis by Cr−DNA Adducts. Cr(III)−DNA binding was also found to be responsible for the decreased template activity of Cr(VI)-treated plasmids during their propagation in human cells. This conclusion was based on the findings that plasmids lacking Cr(III)-containing DNA adducts either as a result of the presence of Cr(III) chelating agents in Cr(VI) reduction reactions or following dissociation of Cr(III) from Cr(VI)-modified DNA produced practically normal yields of replicated progeny. Asc−Cr−DNA adducts were determined to be approximately 7 times more potent in inhibiting DNA replication relative to the Cr(III)−DNA complex. A high percentage of deletion mutants in plasmids treated with Cr(VI)−ascorbate was consistent with a strong replication-obstructing potential of Asc−Cr(III)−DNA adducts. The contribution of Cr(III)−DNA adducts to the formation of deletion mutants should be very small since these adducts primarily induce point mutations (*18*), and they are responsible for less than 10% of all Cr(VI)-induced mutants. We estimated that 70% of the decrease in replication activity of Cr(VI)-treated templates could be attributed to the formation of ternary Asc−Cr(III)−DNA adducts. Binary Cr−DNA adducts accounted for the remaining 30%.

It has recently been reported that Cr−DNA binding was responsible for the decreased PCR amplification of DNA templates treated with Cr(VI) in the presence of low concentrations of Asc (*16*). Experimental conditions favoring the formation of interstrand DNA cross-links resulted in the most significant inhibition of PCR reaction, which led to the conclusion that these adducts were a major form of polymerase-arresting DNA lesions. The dose−response curves for the replication inhibition by binary Cr(III)−DNA adducts in vitro (*16*) and in human cells (this paper) are similar, suggesting that replication-blocking lesions are probably the same in both systems. The inability to unwind DNA beyond the interstrand cross-link should cause a permanent replication arrest at this site until this lesion is removed by a recombination-dependent repair process (*40*). Consequently, interstrand DNA cross-links are not expected to cause significant mutagenic responses (Figure 9*)*. However, processing of DNA cross-links by a minor recombination-independent repair mechanism can generate mutagenic changes (*41*). Abundant monofunctional Cr(III)−DNA adducts appear to present a minimal impediment to replication (*16*), which obviates the need for bypass polymerases and prevents the induction of high levels of mutagenesis by the

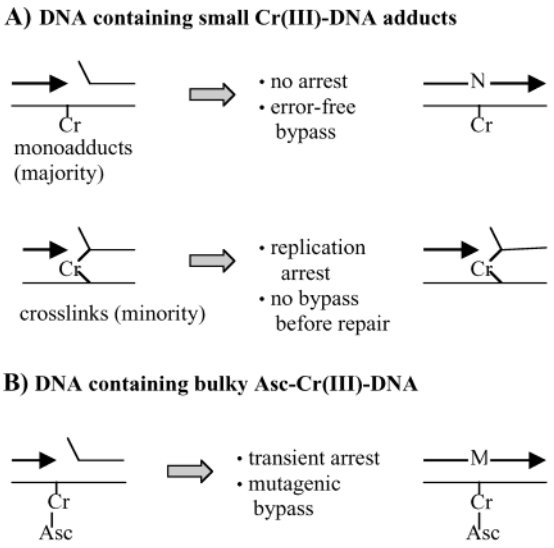

FIGURE 9: Proposed models for replicative bypass of Cr(VI)-induced DNA adducts in human cells. N—insertion of correct nucleotide; M—misincorporation.

error-prone translesion synthesis (*42*). A low-level mutagenesis observed in Cr(III)-treated plasmids could reflect a slightly decreased fidelity of replication on these templates (*43*) or rare replication arrests associated with the activation of translesion synthesis mechanisms. The absence of any detectable mutagenesis in *E. coli* supports the latter possibility.

Asc−Cr(III)−DNA adducts caused both mutations and decreased yield of replicated progeny during propagation of pSP189 plasmids in human cells. In *E. coli*, Cr−DNA adducts induced severe replication blockage but no detectable mutagenesis. The induction of SOS system, which upregulates error-prone Pol II (*polB*), Pol IV (*dinB*), and Pol V (*umuDC*) bypass polymerases (*42*), failed to activate mutagenic translesion synthesis in Cr(VI)-modified plasmids. SOS-induced cells had a modestly increased plasmid survival, probably reflecting enhanced error-free repair (*35*, *44*). Therefore, it appears that only human cells possess a bypass polymerase/process capable of mutagenic replication across Cr−DNA adducts, which was associated with a greater survival of Cr-modified plasmids. The presence of a significant number of multiple mutations in Cr(VI)-induced mutational spectrum (Table 1) supports the involvement of an error-prone polymerase in replication of Asc-adducted templates in human cells.

The specific characteristics of Asc−Cr−DNA adducts responsible for the inhibition of replication in cells may include their size and/or helix-distorting effects induced by the ascorbate ligand in the *R*p isomers of ternary Cr−DNA adducts (*19*). We favor the latter possibility, which would also be consistent with the observed targeting of mutational changes to G/C pairs. Interestingly, DNA templates treated with Cr(VI)−ascorbate under conditions that were expected to promote increased formation of Asc adducts showed only a marginally reduced PCR amplification (*16*). Our findings indicate that intact replication complexes of human and bacterial cells are much more sensitive to bulky Asc−Cr(III)−DNA adducts than purified Taq polymerase.

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