Effects of Chromium on DNA Replication In Vitro

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Chromium is an environmentally significant human carcinogen with complicated metabolism and an unknown mechanism of mutagenesis. Chromium(VI) is taken up by cells as the chromate anion and is reduced intracellularly via reactive intermediates to stable Cr(III) species. Chromium(III) forms tight complexes with biological ligands, such as DNA and proteins, which are slow to exchange. In vitro, Cr$_3$O$_7$$^{2-}$ primarily interacts with DNA to form outer shell charge complexes with the DNA phosphates. However, at micromolar concentrations, the Cr(III) binds to a number of saturable tight binding sites on single-stranded M13 DNA. Additional chromium interacts in a nonspecific manner with the DNA and can form intramolecular DNA cross-links. Although high concentrations of Cr(III) inhibit DNA replication, micromolar concentrations of Cr(III) can substitute for Mg$^{2+}$, weakly activate the Klenow fragment of *E. coli* DNA polymerase I (Pol I-KF), and act as an enhancer of nucleotide incorporation. Alterations in enzyme kinetics induced by Cr(III) increase DNA polymerase processivity and the rate of polymerase bypass of DNA lesions. This results in an increased rate of spontaneous mutagenesis during DNA replication both in vitro and in vivo. Our results indicate that chromium(III) may contribute to chromate-induced mutagenesis and may be a factor in the initiation of chromium carcinogenesis. — Environ Health Perspect 102(Suppl 3):41–44 (1994).

Key words: chromium(III), *Escherichia coli*, DNA polymerase, processivity, mutagenesis

Introduction

Metal compounds, as a class, are among the best documented of all human carcinogens (1), but the mechanisms of metal carcinogenesis are not fully understood. Although it is often assumed that carcinogenesis is (at least in part) the result of DNA damage and mutagenesis, metals have many genotoxic effects and the relative importance of these different effects are not known. Each metal—and in many cases different compounds of a single metal—exhibits a unique spectrum of genotoxic effects. Cr(VI) or chromate, the biologically active form of environmental chromium, is taken up by cells and reduced intracellularly to reactive Cr(V) and Cr(IV) species and then to stable Cr(III). In the process, chromium induces oxidative DNA damage, DNA strand breaks, DNA-DNA and DNA-protein cross-links, and mutations (2–4). The genetic consequences of the different types of chromium complexes and the relative importance of oxidative DNA damage are unknown. Although much of the published research on the mechanisms of chromium-induced genotoxicity has dealt with the complex intracellular metabolism of chromium and the DNA damage produced by Cr(VI) in whole cells (5,6), it is well established that only the reduced forms of chromium, such as Cr(V) and Cr(III), form complexes with DNA and proteins (6,7). Cr(III), for example, forms stable complexes with ligands on DNA, proteins, and small molecules such as glutathione. Since these complexes constitute the most persistent form of intracellular chromium their impact on chromium toxicology is of significance. The research outlined here describes the effects of Cr(III)–DNA complexes on DNA replication in vitro.

Materials and Methods

M13mp2 single-stranded DNA was prepared as previously reported (8) using standard procedures (9). Unlabeled deoxyribonucleotides were obtained from Sigma Chemical Company (St. Louis, MO). $^{32}$P-labeled deoxyribonucleotide triphosphates were from New England Biolabs (Beverly, MA) and the M13 sequencing primers were either purchased from New England BioLabs (Beverly, MA) or synthesized by Dr. Bernard Goldschmidt, Department of Environmental Medicine, NYU Medical Center. Polymerase I-Klenow fragment was obtained from Pharmacia, Inc., Piscataway, NJ, and polynucleotide kinase was from New England Biolabs (Beverly, MA). All other reagents and chemicals were of molecular biology or DNA grade. Chromium solutions were made daily and all water was purified through a Milli-Q water system from Millipore.

Single-stranded M13mp2 DNA and Cr(III)-treated DNA was prepared and hybridized with a 2-fold excess of 17-mer primer (~40 primers, New England Biolabs) as described previously (8). For measurement of nucleotide incorporation, the untreated and chromium-treated M13 DNA was primed and replicated in *vitro* using $^{32}$P-labeled dATP as a label and 0.02 to 0.2 units of DNA polymerase I-Klenow fragment (Pol I-KF). The reaction was stopped by the addition of disodium ethylenediaminetetraacetate (EDTA) and 10% trichloroacetic acid (TCA) (8).

Mutagenesis assays were performed as described previously (8) using an *E. coli* strain (NR9064) in which the *mutS* gene was inactivated by Tn10 transduction giving a mismatch repair-deficient phenotype (10). SOS induction was achieved as described previously (11) by irradiating the cells with a dose of 75 J/m$^2$ UVC prior to CaCl$_2$ treatment.

Preparation of ss Linear DNA Fragment by Asymmetric PCR

For analysis of bypass of oxidative DNA lesions, an asymmetric polymerase chain reaction (PCR) protocol was used to generate a 167-nucleotide DNA fragment from...
M13mp2 DNA. Each 100 µl PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 µM each dATP, dCTP, dGTP, dTTP, 0.01% gelatin, 100 pmole M13 17-mer reverse primer, 1 pmole 15-mer (aa 46-50, corresponding to the minus-strand sequence at amino acids 46-50 in the lacZ gene), 20 ng M13mp2 double-stranded DNA template and 2.5 units Taq polymerase (Perkin Elmer). The PCR reactions were continued for 30 cycles to optimize the amplification. The amplified fragments were identified by agarose gel electrophoresis and by hybridization of a radioactive labeled 15-base oligonucleotide probe prepared by [α³²P]ATP end-labeling of the aa46-50 primer.

Oxidative Damage of ssDNA Fragment with KmnO₄

An oxidatively modified DNA template was prepared by incubating 5 µg of the single-stranded PCR products prepared as described above with 500 µM KmnO₄ in a 25 µl final volume in the presence of 50 mM Tris-HCl, pH 7.4, for 30 min at 37°C. The oxidized DNA was purified by Sephadex G-50 spin columns and then treated with CrCl₃, 6H₂O and hybridized with 5'-end labeled 15-mer primers (46-50 aa) as described above. The bypass of oxidative lesions (primarily thymine glycols) by Klenow fragment (0.01 units/reaction) was measured by primer-extension at 37°C for 5 min under the same conditions as for nucleotide incorporation. The extended primers were examined by 8% polyacrylamide sequencing gel electrophoresis. The dried gels were autoradiographed and the films were scanned by densitometer to quantify the bypass of lesions.

Results and Discussion

Cr(III) Can Activate DNA Replication by pol I-KF on a Primed Single-Stranded DNA Template

It is generally recognized that the Mg²⁺ cation is the primary metal cofactor for DNA polymerase activity. It was, therefore, a surprise to find, in two separate experiments, that Cr⁺³ can also allow DNA replication in vitro by pol I-KF in the absence of Mg²⁺ (manuscript in preparation). At an optimum concentration of 5 µM, CrCl₃ catalyzed the incorporation of an average of 1.09 ± 0.58 pmole dAMP per min (± standard deviation) on a primed single-stranded M13mp2 template. This is equivalent to 23% of the rate of Mg²⁺-catalyzed nucleotide incorporation measured in the same set of experiments. The mechanism of interaction between Cr(III) and the polymerase is not known. However, it is possible that the Cr(III) forms a bidentate-β,γ-complex (12) with the dNTP at the dNTP binding site on the polymerase. This Cr(III)-complex may then facilitate the pyrophosphatase reaction by providing a better leaving group than a Mg-β,γ-complex. This hypothesis is supported by kinetic analysis of nucleotide incorporation by pol I-KF in the presence of Cr(III) which shows that 5 µM Cr(III) increases the Vₘₕ by 160% and increases the apparent Kₘ for dATP by a factor of 10 (13). At higher concentrations (10 µM and above), Cr(III) inhibits both the initiation and extension of replication by DNA polymerases.

Table I. Reversion mutagenesis of chromosome-treated M13mp2-C141 phage DNA transfected into mismatch repair-deficient NR9064 (mutS) E. coli host cells.

| CrCl₃ (µM) | Total plaques | Survival % control | No. mutants | Mutant Frequency (x 10⁻⁴) |
|-----------|---------------|--------------------|-------------|--------------------------|
| Expt. 1   | (SOS-)        |                    |             |                          |
| 0         | 65,125        | 100                | 10          | 1.3 ± 0.3                |
| 5         | 24,498        | 38 ± 1             | 10          | 4.0 ± 0.2                |
| 10        | 1,712         | 7 ± 8              | 16          | 94 ± 25                  |
| Expt. 2   | (Stored)      |                    |             |                          |
| 0         | 22,304        | 100                | 0           | <0.5                     |
| 5         | 22,529        | 101                | 0           | <0.4                     |
| 10        | 39,218        | 54                 | 0           | <0.3                     |
| Expt. 3   | (SOS⁺)        |                    |             |                          |
| 0         | 43,066        | 100                | 6           | 0.9 ± 0.1                |
| 5         | 15,040        | 25 ± 7             | 3           | 2.0 ± 10                 |
| 10        | 11,784        | 2 ± 1              | 1           | 0.8                      |

Expt. 1 = Freshly treated DNA transfected into non-SOS induced host cells. Expt. 2 = Pretreated DNA which was stored for 23 days before transfection into non-SOS induced host cells. Expt. 3 = Freshly treated DNA transfected into SOS induced host cells.

Cr(III) Interacts with the DNA Template and Increases DNA Polymerase Processivity and the Rate of Nucleotide Incorporation

We have found that Cr(III) interacts with single-stranded M13 DNA in vitro in a biphasic manner (8). Scatchard analysis of the binding from 0 to 5 µM Cr(III) shows that the metal ions bind to approximately 120 tight binding sites per DNA molecule with a dissociation constant of approximately 4 µM (Snow et al., unpublished). Higher concentrations of Cr(III) bind non-specifically to the DNA. The chromium bound to the DNA either interacts with DNA polymerases or acts as a source of very low concentrations of free Cr(III) during replication. This low concentration of Cr(III) increases DNA polymerase processivity and decreases DNA replication fidelity (14).

Figure 1. Autoradiograph of primer extension on a 167-nucleotide single-stranded KmO₄-treated DNA template in the presence of increasing concentrations of Cr(III). The top panel shows primer extension on a KmO₄-treated template in the presence of increasing concentrations of Cr(III). The arrow at the top indicates the end of the template. The bottom panel shows the sequence of the full length M13 template run on the same gel as shown above. Note that beyond the initial pause sites typical for primer extension on this template (at the left side of the gel), all polymerase pause sites on the KmO₄-treated template are found adjacent to or immediately before template thymidine residues (the top lane of panel 2) and are therefore indicative of pausing at oxidized thymine lesions.
The Cr(III)-mediated increase in polymerase processivity has been demonstrated by both primer extension analysis (8) and a newly developed competition assay using two different sized DNA templates (13). These assays show that Cr(III) bound to the DNA decreases the initiation of replication while, at the same time, the number of nucleotides incorporated per initiated template (a measure of processivity) is increased from 200% to 470% (8,13). The results suggest that the rate of binding of the polymerase to the Cr(III)-treated template is decreased, but that once bound the polymerase can incorporate more nucleotides before dissociating from the template.

The effects of Cr(III) on DNA polymerase processivity are not limited to Pol I-KF; other DNA polymerases show increased rates of nucleotide incorporation and increased processivity in the presence of Cr(III). We have shown, for example, that calf thymus polymerase α and cloned human DNA polymerase β both show enhanced processivity in the presence of Cr(III) (14,15). The most dramatic effect observed during replication of single-stranded M13mp2 DNA by polymerase α in the presence of CrCl3 is the loss of normal polymerase pause sites (16). Although polymerase β is not very active on a single stranded DNA template when Mg2+ is used as the cofactor; both Mn(II) (17) and Cr(III) (data not shown) are able to increase the activity of pol β on single-stranded M13 DNA (14,16). Not all polymerases are activated by Cr(III), however. T4 phage DNA polymerase, a normally processive enzyme, is inhibited by as little as 0.5 μM Cr(III) and does not show any significant activation by this trivalent cation, even in the absence of Mg (data not shown).

**Mutagenic Effects of Cr(III)**

The effect of Cr(III) on the fidelity of eukaryotic enzymes is unknown; however, we have shown that the presence of Cr(III) during replication by E. coli DNA polymerases either in vitro or in vivo is mutagenic. A 2- to 4-fold increase in mutagenesis by Cr(III) was previously reported using the lacZα gene of M13mp2 as a mutagenic target (8,14,15). More recently we have found that, if a mutant M13 phage DNA (M13mp2-C141) is replicated in the presence of Cr(III) in a mismatch repair deficient E. coli host, reversion mutagenesis is increased up to 94-fold in non-SOS-induced cells, but not in SOS-induced cells (Table 1). The chromium-dependent increase in mutagenesis requires the use of freshly treated DNA. If the chromium is allowed to equilibrate between the DNA and the buffer by storing the treated DNA (from which the unbound Cr(III) was removed by gel filtration) for 23 days before transfection no mutagenesis is seen. Some residual damage or bound chromium in the 10 μM treated sample does, however, produce a 46% decrease in phase recovery (Table 1).

In contrast to the strong mutagenic response seen in mismatch repair-deficient cells, reversion mutagenesis by Cr(III) only reaches a maximum of 2- to 3-fold above background in a mismatch repair-proficient host (8). This suggests that most of the errors introduced during replication in the presence of Cr(III) can be repaired by the mismatch repair or are mitigated by SOS-associated processes. This may indicate that the RecA protein, a single-strand binding protein present at high concentration in the SOS-induced cells, can displace the Cr(III) from the DNA and thus mitigate the effects of the metal ion on replication. It can also be seen in Table 1 that 10 μM Cr(III) [a concentration which induces significant amounts of DNA crosslinking (8)] is significantly more mutagenic than 5 μM Cr(III) under non-SOS conditions, but not after SOS-induction. This implies that base substitution mutagenesis by Cr(III) may be promoted by DNA crosslinking and that the resulting mismatches may be repaired by either the mismatch repair system or by SOS-dependent processing.

Several of the Cr(III)-induced and spontaneous mutants were isolated and sequenced (Table 2). The results show that Cr(III) produces a significant increase in the frequency of C→A transversion mutations (at C141) relative to the spontaneous mutant spectrum. DNA replication in the presence of Cr(III) produced 31% C→A transversions, 19% C→G transitions, and 50% C→T transitions at target sites C141 and C142; whereas the spontaneous mutations at the same sites consisted of 9% C→A transitions; 27% C→G transitions, and 64% C→T transitions. Spontaneous mutagenesis in a mismatch repair-deficient host has been shown to consist primarily of an increased frequency of transition mutations (10); therefore, this increase in transitions induced by Cr(III) may indicate a significant alteration in the type of misincorporation that occurs during DNA replication in the presence of this metal ion.

**Cr(III) Increases Polymerase Bypass of DNA Damage**

The evidence for increased polymerase processivity, along with increased mutagenesis in the presence of Cr(III), led us to ask whether Cr(III) can increase polymerase bypass of other types of DNA damage. Since it has been shown that the intracellular reduction of chromium(III) produces oxidative base damage (17,18), we investigated the ability of Cr(III) to promote bypass of oxidative DNA lesions. A 167-nucleotide single-stranded fragment of M13mp2 DNA was prepared and oxidized with KMnO4 producing enough DNA damage (mostly thymidine glycols) to almost completely block replication at or before the first template thymidine (19). The KMnO4-treated DNA was further treated with 0 to 25 μM CrCl3 and then primed with an 32P-labeled primer. Replication of the treated primer-template was carried out with 0.01 unit of Pol I-KF and the products of replication were analyzed on an 8% polyacrylamide/7 M urea sequencing gel. In the absence of Cr(III), replication was limited and barely reached the first putative thymidine glycol (Figure 1). Fewer than 1% of the primers were extended beyond the first template thymine residue. After treatment of the oxidized DNA with 1 to 5 μM Cr(III), replication was greatly extended and increased bypass of the oxidized DNA base.

### Table 2. Sequence specificity of Cr(III)-induced reversion mutagenesis in M13mp2-C141.

| New base | C141 | Target base | C142 |
|----------|------|-------------|------|
| Spontaneous | A | 1 | 0 |
| | G | 3 | 0 |
| | T | 4 | 3 |
| Cr-induced | A | 5 | 0 |
| | G | 3 | 0 |
| | T | 1 | 7 |

The number of C→A, C→G, and C→T mutations at each of the target sites is given for a total of 11 spontaneous and 16 Cr-induced mutants.
damage was evident (Figure 1) (13). In the presence of up to 5 μM Cr(III), from 5% to 29% of the primers were extended beyond the first thymidine. Higher concentrations of Cr(III), in the range of 10 to 25 μM, led to the formation of DNA-DNA crosslinks (seen at the top of the gel, to the right of the figure) and decreased polymerase activity. We are currently investigating the fidelity and kinetics of nucleotide incorporation opposite the oxidative lesions to understand the mechanism by which Cr(III) mediates the bypass of polymerase blocking lesions.

In summary, micromolar concentrations of Cr(III) bound to the DNA template can increase DNA polymerase processivity and decrease DNA replication fidelity. These alterations in DNA function can result in greatly increased bypass of oxidative DNA lesions. Since several oxidative base lesions, such as 8-oxodeoxyguanine have been shown to be promutagenic (20), Cr(III) may serve to increase the mutagenic potential of these lesions and contribute to the genotoxic effects of chromium in vivo.

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