Chromium(III) Decreases the Fidelity of Human DNA Polymerase β[†]

Jatinder Singh[‡] and Elizabeth T. Snow*

Nelson Institute of Environmental Medicine and Kaplan Comprehensive Cancer Center, New York University Medical Center, 57 Old Forge Road, Tuxedo, New York 10987

Received December 23, 1997; Revised Manuscript Received April 30, 1998

ABSTRACT: Certain particulate compounds of hexavalent chromium are well-known occupational and environmental human carcinogens. Hexavalent chromium primarily enters the cells and undergoes metabolic reduction; however, the ultimate trivalent oxidation state of chromium, Cr(III), predominates within the cell. DNA-bound Cr(III) has been previously shown to decrease the fidelity of replication in the M13 phage mutation assay. This study was done to understand how Cr(III), in the presence of physiological concentrations of magnesium, affects the kinetic parameters of steady-state DNA synthesis in vitro across site-specific O^6-methylguanine (m^6dG) residues by DNA polymerase β (pol β). Cr(III) binds to the short oligomer templates in a dose-dependent manner and stimulates the activity of pol β. Cr(III) stimulates the mutagenic incorporation of dTTP opposite m^6dG more than the nonmutagenic incorporation of dCTP, and thereby Cr(III) further decreases the fidelity of DNA synthesis across m^6dG by pol β. In contrast, Cr(III) does not affect the fidelity of DNA synthesis across the normal template base, dG. Both the enhanced activity and the mutagenic lesion bypass in the presence of Cr(III) may be associated with Cr(III)-dependent stimulation of pol β binding to DNA as reported here. This study shows some of the mechanisms by which mutagenic chromium affects DNA synthesis.

Chromium is a well-known genotoxic and carcinogenic metal (*1*−*5*). Chromium enters cells predominantly in the hexavalent state, and is metabolically reduced by intracellular reducing agents, primarily ascorbic acid and glutathione, to the ultimate, stable Cr(III)[1] form. Most of the chromium in living cells exists as Cr(III). There is no known mechanism by which Cr(III) can be excreted from cells; thus, it may accumulate with prolonged exposure. Cr(III), but not Cr(VI), forms complexes with DNA, dNTP's, and proteins such as glutathione (*6*). Cr(III) decreases the fidelity and increases the processivity (the number of dNTP's incorporated per single polymerase binding event) of DNA polymerases during DNA synthesis in vitro (*7*−*11*). When Cr(III)-treated M13 bacteriophage DNA is transfected into *E. coli*, there is a dose-dependent increase in the mutation frequency (*12*). Cr−histidine and Cr−glutathione adducts are also mutagenic in human cells (*13*) and bacteria (*14*). These studies indicate that Cr(III) may play a role in the mutagenesis and carcinogenesis of chromium in vivo. This observation is verified by other studies that show that Cr(III) is mutagenic in mammalian and bacterial cells when its uptake is facilitated by phagocytosis or by complexing with organic ligands (*2*).

In the present study, we have investigated the effect of Cr(III) on the fidelity of a model DNA polymerase, pol β, during DNA synthesis across a DNA lesion, m^6dG, site-specifically located in a short oligomeric template. Pol β is an ideal polymerase to study the effects of metals on DNA synthesis for several reasons. It has a simple, single-subunit structure, with no confounding 5′-3′ exonuclease activity [reviewed by Wilson (1990)], and crystallographic studies of pol β complexed with DNA, dNTP's, and metals [including Cr(III)] have been reported (*15*−*17*). m^6dG is a cytotoxic, mutagenic, and carcinogenic DNA lesion that results from exposure to methylating agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (*18*−*20*). We have previously reported the mechanisms of DNA synthesis across m^6dG, and the preferential incorporation of the mutagenic substrate, dTTP, by pol β (*21*). The steady-state approach was used to individually look at the sequential steps of nucleotide insertion opposite the lesion and postlesion extension, by varying the concentrations of the nucleotide substrates, dCTP and dTTP.

We report here that at low, physiologically relevant, submicromolar concentrations, Cr(III) stimulates the activity as well as the DNA-binding affinity of pol β. Cr(III) further decreases the fidelity of pol β during DNA synthesis across m^6dG in vitro and stimulates the mutagenic bypass of the lesion.

MATERIALS AND METHODS

Purified recombinant human DNA polymerase β was a kind gift from Dr. Samuel Wilson, NIEHS, Research Triangle Park, NC (formerly at the Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, TX). The oligonucleotide primer-templates used for this

[†] This research was supported by NYUMC/NIEHS Center Grant ES00260, NYU Medical Center Kaplan Cancer Center Grant CA13343, and NIH Grant ES06498 to E.T.S.

* To whom correspondence should be addressed. Telephone: 914-351-2119. Fax: 914-351-3317. E-mail: snow@charlotte.med.nyu.edu.

[‡] Present address: Department of Pharmacology, The George Washington University Medical Center, Washington, DC.

[1] Abbreviations: Cr(III), chromium(III); pol β, DNA polymerase β; m^6dG, O^6-methylguanine; $K_{M(app)}$, apparent substrate concentration giving half-maximal velocity; $V_{max(rel)}$, relative maximal velocity; *f*, efficiency of the reaction [$V_{max(rel)}/K_{M(app)}$]; *S*/*V*, ratio of substrate concentration to reaction velocity; dNTP, deoxyribonucleotide triphosphate.

S0006-2960(97)03155-3 CCC: $15.00
Published on Web 06/12/1998

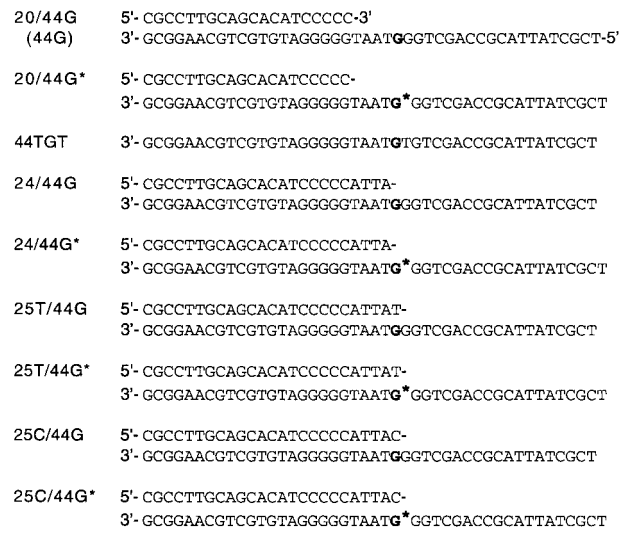

FIGURE 1: Deoxyoligonucleotide primer-templates used in this study. G* indicates the presence of m^6G.

study are shown in Figure 1. Other materials and methods used for this study have been previously published in detail (*21*). Additional details pertaining to the Cr(III) assays are given here.

Measurement of Binding of Cr(III) to 44TGG Template DNA. Cr(III) treatment of the 44G template was done by the method of Snow and Xu (*7*); 50 pmol of 44G DNA was incubated for 30 min at 37 °C with 0−50 μM $^{51}CrCl_3 \cdot 6H_2O$ (Sigma, St. Louis, MO) in a 50 μL reaction containing 200 μM Tris and 20 μM EDTA (pH 8). This mixture was then passed through a Sephadex G-25 column to remove unbound Cr(III). The number of Cr(III) ions bound per template were calculated after correcting for the loss of DNA during filtration.

The assays involving Cr(III) were done in the presence of Mg(II) and physiologically relevant concentrations of Cr(III). Cr(III) alone was not able to activate pol β (data not shown). To study the effects of Cr(III) on DNA synthesis, templates were treated with $CrCl_3$, as described above. The Cr(III)-treated templates were then hybridized to different primers and replicated by pol β. It was observed that the effect of Cr(III) was similar whether the DNA was pretreated with Cr(III) or when the final effective concentration (10% of the initial concentration) of Cr(III) was directly incubated with DNA for 30 min at room temperature before the replication/binding reactions. Thus, in most experiments Cr(III) was added directly to the replication/binding reactions at a final concentration of 0.1−0.4 μM. This was the concentration range of Cr(III) that had the greatest stimulatory effect on the activity of pol β. The binding of pol β to DNA was also greatly stimulated by 0.1−0.4 μM Cr(III), suggesting that this was the appropriate concentration of Cr(III) for the binding and DNA synthesis experiments. In all cases, the kinetics of insertion and bypass with and without Cr(III) for each set of primer-templates were determined in the same set of experiments. This allows for a direct comparison of the results ± Cr(III) under otherwise identical conditions.

Binding of Pol β to m^6dG-Modified and Normal DNA in the Presence of Cr(III). This assay (shown in Figure 2A) was based on the enzyme-trapping assay by Menichini et al. (*22*) and was designed to provide a convenient index of the relative binding of pol β to 24/44G* (prelesion complex) and the normal 24mer-44TGG DNA in the presence of Cr(III). In this two-step assay, pol β (2 pmol) and unlabeled 24/44G* or 24/44G DNA (10 pmol) were incubated together on ice for 30 min with different concentrations of Cr(III) (added directly to the reaction) to allow equilibrium binding. Next, 10 pmol of the labeled trap/competitor (^{32}P-20/44G) (added to scavenge unbound pol β) and all four dNTP's (50 μM each) were added, and DNA synthesis was allowed to occur for 0.5 min at 37 °C. The trap DNA was added in the same concentration as the unlabeled target DNA so that there was no appreciable shift in the binding equilibrium to favor dissociation of pol β from the unlabeled DNA. Extension of the 20mer trap primer is due to the fraction of pol β molecules that are not specifically bound to 24/44G* or 24/44G. The reaction products were denatured by heating at 100 °C for 5 min and resolved by 16% denaturing polyacrylamide gel electrophoresis. This was followed by autoradiography and densitometric quantitation of the bands corresponding to the 20mer trap primer and its extension products (the unlabeled 24mer primer of the target DNA does not show on the autoradiograph). The percent extension of the 20mer trap primer was determined as

$$\frac{100 \times \text{total IOD of the extension products}}{\text{total lane IOD}}$$

This is an inverse index of the binding affinity of pol β for the modified and normal templates. The extension of the trap primer alone in the presence of Cr(III) was also determined.

DNA Synthesis Assays with Cr(III). The effect of 0.1 μM Cr(III) on the different steps of running-start DNA synthesis across m^6dG (prelesion block, insertion opposite the lesion, and postlesion extension) by pol β (0.2 unit) was determined in the presence of all four normal dNTP's (50 μM each). The primer-template was 20/44G* (10 pmol), and the reaction time was 5 min at 37 °C. Steady-state kinetic assays of nucleotide insertion and base pair extension were performed as described previously (*21*), but in the presence of Cr(III) at a final concentration of 0.4 μM. Briefly, the concentration of either dCTP or dTTP was varied in separate running-start nucleotide insertion reactions with normal or modified template DNA. Standing-start base pair extension studies were done with 25mers ending with dC or dT hybridized with normal or modified primers, and the concentration of the next correct nucleotide, dCTP, was varied. The primer-template combinations used are shown in Figure 1. The radiolabeled primers and the extension products were analyzed by 16% polyacrylamide gel electrophoresis, autoradiography, and densitometry.

During the steady state, the concentration of the polymerase−DNA (enzyme−substrate) complex is maintained at a steady level by having a large excess of the DNA substrate. The rule that less than 20% of the original primer be extended during steady-state conditions (*23*) was adhered to during all DNA synthesis assays. This approach minimizes any changes in the off-rate (polymerase−DNA dissociation constant) which can confound the comparison of band intensity ratios in fidelity measurements (*24*).

Statistics. Standard errors associated with the kinetic parameters of DNA synthesis (K_M and V_{max}) were generated

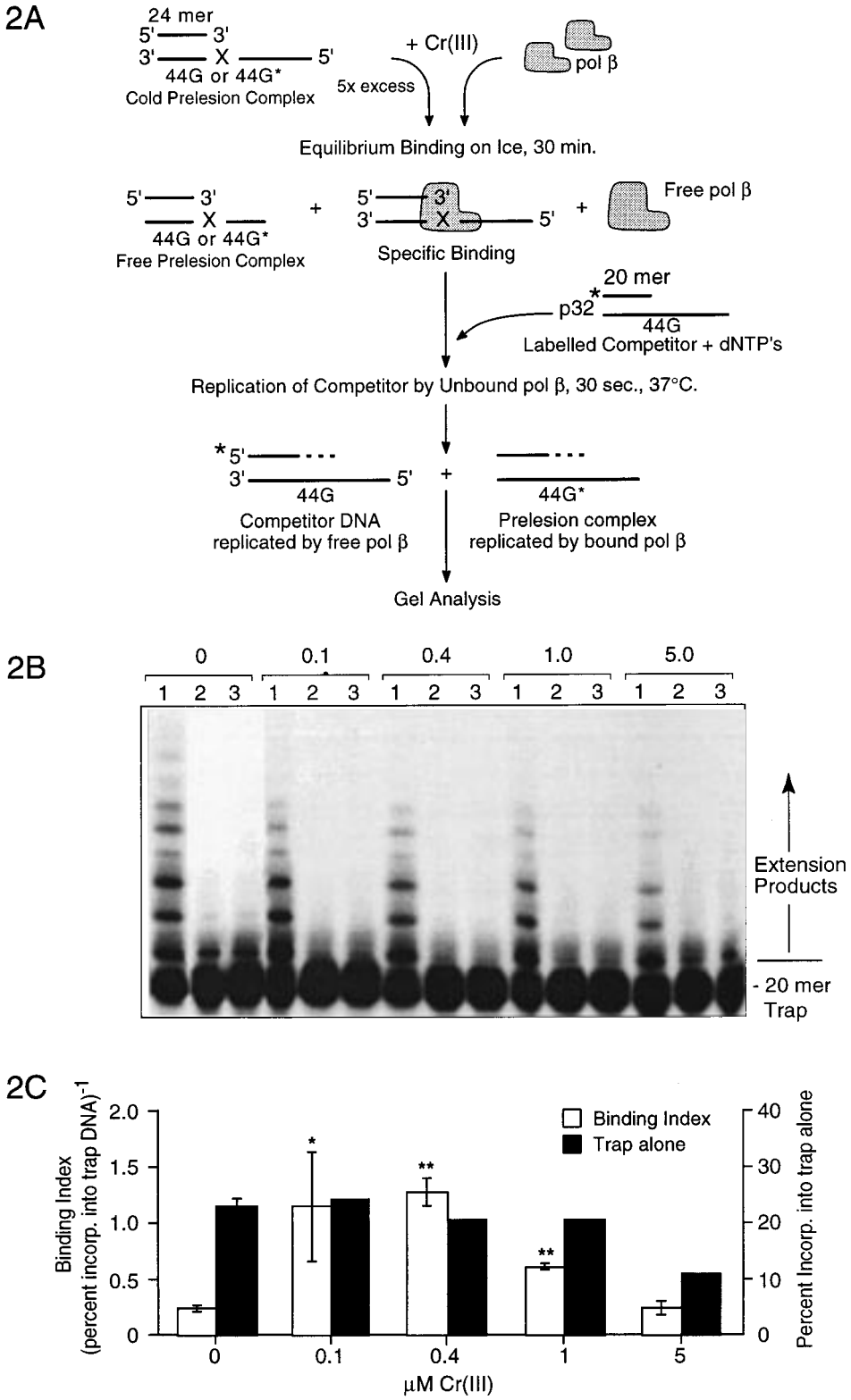

FIGURE 2: Effect of Cr(III) on the binding of pol β to 24/44G* and 24/44G DNA. (A) Binding assay: The unlabeled target DNA (24/44G* or 24/44G) was incubated with pol β on ice for 30 min in the presence of different concentrations of Cr(III). Next, the labeled competitor (trap) DNA, ^{32}P-20/44G, and dNTP's were added, and DNA synthesis was allowed to occur for 0.5 min at 37 °C. Extension of the ^{32}P-20mer was quantitated by gel autoradiography and scanning densitometry, and the ratio of the extended to the nonextended 20mer was determined. Copying of the labeled trap DNA is due to the fraction of pol β molecules not bound to the unlabeled target DNA and is therefore an inverse index of the binding affinity of pol β for the unlabeled DNA. (B) Gel autoradiogram of the binding assay: At each concentration of Cr(III), lane 1 shows the replication of the 20/44G competitor alone, and lanes 2 and 3 show replication of the 20/44G competitor DNA by the fraction of pol β not bound to either 24/44G or 24/44G* target DNA, respectively. (C) Graphical presentation of the effect of Cr(III) on the average binding index for 24/44G* and 24/44G DNA (±the range), measured as the inverse of the amount of DNA synthesis on the trap DNA alone (left axis), and the effect of Cr(III) on synthesis of the trap DNA alone (right axis). One asterisk indicates marginal significance ($p = 0.1$), and two asterisks indicate a significant difference in the binding index ($p < 0.01$) compared to the control with no Cr(III).

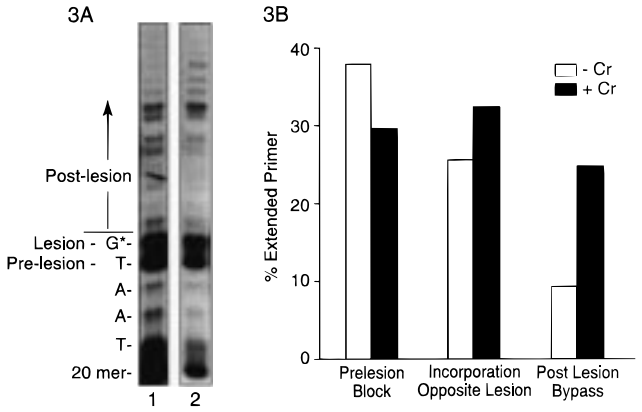

FIGURE 3: Effect of Cr(III) on DNA synthesis across m^{6}dG by pol β in single-stranded DNA. (A) Gel autoradiogram: Lanes 1 and 2 of the gel autoradiogram show the replication of 20/44G* (10 pmol) in the absence and presence of 0.1 μM Cr(III), respectively. The reaction contained 0.2 unit of pol β and 50 μM of each of the four dNTP's. Reaction time was 5 min. (B) Graphical presentation of the data shown in (A): The bars indicate the percentage of extended primer at the prelesion, lesion, and postlesion sites, as noted. The percent of total primer extension (i.e., the activity of pol β) in the absence and presence of Cr(III) was 52.2% and 78.3%, respectively.

by the K_{cat} enzyme kinetics software (Biometallics Inc., Princeton, NJ). This program fits the velocity versus substrate concentration data directly to the Michaelis−Menten equation using a robust weighted nonlinear regression method (*25*). The standard errors associated with efficiencies of insertion and extension (f_1 and f_2, respectively) and overall efficiency ($f_1 \times f_2$) values were calculated using standard formulas for the variance of ratios and products (*26*, *27*). Statistical comparisons were done by Student's *t*-test, wherever appropriate.

RESULTS

Binding of Cr(III) to the 44G DNA Template. Binding of radioactive ^{51}Cr(III) to the 44G template DNA was determined as a function of the initial concentration of $^{51}CrCl_3$. The binding of Cr(III) to the DNA occurred in a linear, dose-dependent manner. We have previously reported a similar dose-dependent binding of Cr(III) to M13 DNA (*7*). After removal of the nonbound Cr(III), the final concentration of Cr(III) was approximately 10% of the initial concentration. At the optimal (stimulatory) concentration of 0.4 μM, there is an average of less than one Cr(III) ion bound per molecule of template DNA.

Effect of Cr(III) on the Binding of Pol β to the Prelesion Complex. This assay was performed to determine whether Cr(III) (added to the reactions directly) alters the binding of pol β to the prelesion complex (24/44G*) DNA or its normal counterpart (24/44G). The assay is shown in Figure 2A. Figure 2B depicts a gel autoradiogram showing the DNA synthesis of the 20/44G competitor alone (lane 1), in the presence of 24mer-44TGG (lane 2), or in the presence of 24mer-44TXG (lane 3), plus various concentrations of Cr(III), as noted. Figure 2C shows that submicromolar concentrations of Cr(III) (0.1 and 0.4 μM) stimulated the binding of pol β to both the m^{6}dG-containing and normal DNA primer-templates 5−6-fold relative to the control [0 μM Cr(III)]. At higher concentrations of Cr(III) (1−5 μM), the binding progressively decreased to control levels.

Effect of Cr(III) on the Activity of Pol β. The effect of Cr(III) on the activity of pol β was determined in three independent primer extension assays (a representative assay is shown in Figure 3) and in an additional assay that measured the incorporation of radioactive nucleotides into activated calf thymus DNA (not shown). All four assays showed a distinct stimulatory effect of submicromolar Cr(III). In the three primer extension assays, the average activity of pol β, as measured by the percent primer extension (±the standard deviation), was 59.1% (±8.1%) in the absence of Cr(III) and 78.3 (±0.5%) in the presence of 0.1 or 0.4 μM Cr(III). This stimulatory effect of Cr(III) was statistically significant ($0.01 > p > 0.005$). Higher micromolar concentrations inhibited DNA synthesis in a dose-dependent manner (data not shown). Submicromolar final concentrations of Cr(III) were previously shown to stimulate the activity of pol β on the M13 phage single-stranded DNA template (*7*).

Effect of Cr(III) on the Replication Across m^{6}dG by Pol β in Single-Stranded DNA in the Presence of All dNTP's. Figure 3A presents a gel autoradiogram showing the effect of 0.1 μM Cr(III) on extension of the 20mer primer on the 44G* template by pol β. Lanes 1 and 2 show extension of the 20mer in the absence and presence of 0.1 μM Cr(III), respectively. Figure 3B is a graphical representation of the effect of Cr(III) on the different steps of replication across m^{6}dG by pol β. The columns represent the percent of extended primer at the prelesion (1), lesion (2), and postlesion (3) sites. Cr(III) decreased prelesion blockage and stimulated both nucleotide insertion opposite the lesion and postlesion extension. These results indicate that Cr(III) stimulates the ability of pol β to bypass m^{6}dG in a single-stranded template. This was also observed in the steady-state kinetic assays, indicating good correlation between the different experimental approaches used to understand the effects of Cr(III) on DNA replication.

Effect of Cr(III) on the Steady-State Kinetics of DNA Synthesis Across dG and m^{6}dG by Pol β. This was determined in separate nucleotide insertion and base-pair extension assays by varying the concentrations of the individual nucleotide substrates, dCTP and dTTP. The

Table 1: Effect of 0.4 μM Cr(III) on Running-Start Insertion Opposite dA and dG by Pol β[a]

dNMP → base	primer-template	Cr	$K_{M(app)}$ (μM)	$V_{max(rel)}$ [rlOD min^{-1}]	f_1 insertion	relative f_1
dTMP → dA	20/44G	−	2.5 (0.4)	8.0 (0.6)	3.2 (0.6)	1.0
		+	3.7 (2.2)	9.1 (2.7)	2.5 (1.6)	0.8
dTMP → dG	20/44G	−	2.9 (0.5)	3.9 (0.3)	1.3 (0.3)	1.0
		+	1.0 (0.1)	2.4 (0.1)	2.4 (0.2)	1.8
dTMP → dG	23/44TGT	−	840 (144)	9.3 (0.6)	1.1×10^{-2} (0.2×10^{-2})	1.0
		+	846 (109)	11.0 (0.6)	1.3×10^{-2} (0.2×10^{-2})	1.2

[a] $f_1 = V_{max}/K_M$. Numbers in parentheses indicate the standard errors.

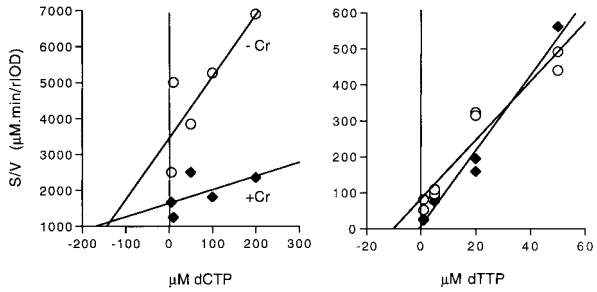

FIGURE 4: (A, left panel) Hanes−Woolf plot showing the effect of Cr(III) on the running-start insertion of dCMP opposite m^{6}dG. The reaction contained 0.4 μM Cr(III), pol β (0.2 unit), 23/44G* primer-template (10 pmol), and dATP at a fixed concentration of 10 μM. The concentration of dCTP was varied. Reaction time was 0.5 min. (B, right panel) Running-start insertion of dTMP opposite m^{6}dG. The reaction contained 0.4 μM Cr(III), pol β (0.3 unit), 23/44G* primer-template (10 pmol), and dCTP, dATP, and dGTP at a fixed concentration of 50 μM each. The concentration of dTTP was varied. Reaction time was 5 min.

results of these assays show that Cr(III) decreases the fidelity of pol β by exerting different effects on the mutagenic versus the nonmutagenic bypass of m^{6}dG.

The steady-state kinetic parameters of running-start insertion of both dCMP and dTMP opposite template dG and m^{6}dG in the TGG and TG*G sequence contexts were determined in the presence of 0 and 0.4 μM Cr(III). 44TGT was used to measure the insertion of dTMP opposite dG. The kinetic parameters of insertion of dCMP and dTMP opposite template dG are tabulated in Table 1. Cr(III) does not significantly change the $K_{M(app)}$, the $V_{max(rel)}$, or the efficiency of insertion of dTMP opposite dA or dG, but does decrease the $K_{M(app)}$ for insertion of dCMP opposite dG by 3-fold and increases the $V_{max(rel)}$ for insertion of dCMP opposite dG slightly (Table 1). The net result is that the efficiency of insertion of dCMP opposite template dG is increased almost 2-fold in the presence of Cr(III). Thus, Cr(III) neither alters the fidelity nor enhances mutagenesis at the normal base dG during the initial the insertion step.

Cr(III) has a more pronounced effect on the insertion of dCMP and dTMP opposite m^{6}dG. Figure 4A and Figure 4B (left and right panels, respectively) show Hanes−Woolf plots of the effect of 0.4 μM Cr(III) on the insertion of dCMP and dTMP opposite m^{6}dG, respectively. The kinetic parameters of nucleotide insertion opposite m^{6}dG are presented in Table 2. Cr(III) increases the $V_{max(rel)}$ for the insertion of dCMP opposite m^{6}dG by 3.8-fold while the $K_{M(app)}$ is slightly increased. The net effect is that Cr(III) increases the efficiency of insertion of dCMP opposite m^{6}dG by 2.7-fold. While Cr(III) stimulates this nonmutagenic event by increasing the $V_{max(rel)}$, it stimulates the mutagenic event, the insertion of dTMP opposite m^{6}dG, to a similar extent predominantly by decreasing the $K_{M(app)}$ (Table 2). Cr(III) stimulates the insertion of both the mutagenic and nonmutagenic substrates almost equally, so there is no significant mutagenic effect induced by Cr(III) at the insertion step during DNA synthesis across m^{6}dG by pol β.

Table 3: Effect of 0.4 μM Cr(III) on Standing-Start Extension from dC-dG and dT-dG Base Pairs by Pol β[a]

base pair	Cr	$K_{M(app)}$ (μM)	$V_{max(rel)}$ (rlOD min^{-1})	f_2 (extension)	f_2 relative
dC-dG	−	9.6 (2.3)	0.32 (0.02)	3.3×10^{-2} (0.8×10^{-2})	1.0
	+	43.5 (4.3)	1.56 (0.06)	3.6×10^{-2} (0.4×10^{-2})	1.1
dT-dG	−	135.6 (47.8)	0.029 (0.002)	2.1×10^{-4} (0.8×10^{-4})	1.0
	+	50.8 (15.6)	0.025 (0.002)	4.9×10^{-4} (1.6×10^{-4})	2.3

[a] These terminal base pairs were formed using the 25C/44G and 25T/44G primer-templates, respectively. $f_2 = V_{max}/K_M$ for extension. Numbers in parentheses indicate the standard errors.

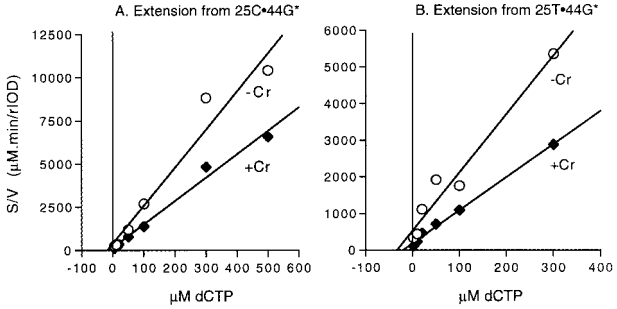

FIGURE 5: (A) Hanes−Woolf plot showing the effect of 0.4 μM Cr(III) on the standing-start extension from the dC-m^{6}dG terminal base pair. The primer template, 25C/44G* (8 pmol), was replicated by pol β (0.2 unit). The concentration of the next correct nucleotide substrate, dCTP, was varied. Reaction time was 2 min. (B) Standing-start extension from the dT-m^{6}dG terminal base pair. The primer template, 25T/44G* (8 pmol), was replicated by pol β (0.2 unit). The concentration of the next correct nucleotide substrate, dCTP, was varied. Reaction time was 2 min.

The effects of 0.4 μM Cr(III) on the steady-state kinetics of standing-start extension from the normal base pairs, dC-dG and dT-dG, are presented in Table 3. Cr(III) increases both the $K_{M(app)}$ and the $V_{max(rel)}$ for the standing-start extension from dC-dG such that the overall efficiency is unaffected (Table 3). However, while Cr(III) does not alter the efficiency of extension from the normal Watson−Crick base pair, dC-dG, the $K_{M(app)}$ for extension from the wobble (non-Watson−Crick) base pair, dT-dG, is decreased by 2.7-fold in the presence of Cr(III). The $V_{max(rel)}$, on the other hand, is not significantly affected (Table 3). Thus, Cr(III) is able to stimulate extension from the mutagenic dT-dG mispair by a factor of 2.3. While Cr(III) does not enhance the mutagenic insertion of dT opposite dG (Table 1), it does stimulate the mutagenic extension from the dT-dG base pair.

Figure 5A and Figure 5B show Hanes−Woolf plots of the effect of Cr(III) on the standing start extension from dC-m^{6}dG and dT-m^{6}dG base pairs, respectively. Again, Cr(III) does not greatly affect the extension from the nonmutagenic base pair, dC-m^{6}dG, although it stimulates the

Table 2: Effect of 0.4 μM Cr(III) on Running-Start Insertion of dCMP and dTMP Opposite m^{6}dG by Pol β[a]

dNTP → base	primer-template	Cr	$K_{M(app)}$ (μM)	$V_{max(rel)}$ [rlOD min^{-1}]	f_1 insertion	relative f_1
dCTP	23/44G*	−	173 (32)	0.05 (0.01)	2.9×10^{-4} (0.6×10^{-4})	1.0
		+	240 (18)	0.19 (0.01)	7.9×10^{-4} (0.7×10^{-4})	2.7
dTTP	20/44G*	−	7.6 (0.9)	0.12 (0.01)	1.6×10^{-2} (0.2×10^{-2})	1.0
		+	1.8 (0.6)	0.09 (0.01)	5.0×10^{-2} (1.7×10^{-2})	3.1

[a] $f_1 = V_{max}/K_M$ for insertion. Numbers in parentheses indicate the standard errors.

Table 4: Effect of 0.4 μM Cr(III) on Standing-Start Extension from dC-m^{6}dG and dT-m^{6}dG by Pol β[a]

base pair	Cr	$K_{M(app)}$ (μM)	$V_{max(rel)}$ (rlOD min^{-1})	f_2 extension	relative f_2
dC-m^{6}dG	−	4.8 (1.9)	0.042 (0.003)	8.8×10^{-3} (3.5×10^{-3})	1.0
	+	6.0 (2.5)	0.071 (0.003)	1.2×10^{-2} (0.5×10^{-2})	1.4
dT-m^{6}dG	−	43.5 (20.3)	0.066 (0.010)	1.5×10^{-3} (0.7×10^{-3})	1.0
	+	13.6 (4.0)	0.10 (0.01)	7.4×10^{-3} (2.3×10^{-3})	4.9

[a] These terminal base pairs were formed using the 25C/44G* and 25T/44G* primer-templates, respectively. $f_2 = V_{max}/K_M$. Numbers in parentheses indicate the standard errors.

Table 5: Effect of Cr(III) on the Overall Incorporation of dCMP and dTMP Opposite dG by Pol β[a]

base pair	Cr	f_1 insertion	f_2 extension	$f_1 \times f_2$ overall	relative $f_1 \times f_2$
dC-dG	−	1.3 (0.3)	3.3×10^{-2} (0.8×10^{-2})	0.044 (0.012)	1.0
	+	2.4 (0.2)	3.6×10^{-2} (0.4×10^{-2})	0.086 (0.009)	2.0
dT-dG	−	1.1×10^{-2} (0.2×10^{-2})	2.1×10^{-4} (0.8×10^{-4})	2.3×10^{-6} (0.9×10^{-6})	1.0
	+	1.3×10^{-2} (0.2×10^{-2})	4.9×10^{-4} (1.6×10^{-4})	6.4×10^{-6} (2.2×10^{-6})	2.8

[a] Numbers in parentheses indicate the standard errors.

extension from the mutagenic base pair, dT-m^{6}dG (Table 4). This effect is due predominantly to a 3.2-fold decrease in the $K_{M(app)}$ for insertion of the next correct nucleotide, dCTP.

Thus, while Cr(III) does not greatly decrease the fidelity at the insertion step, it causes a more pronounced decrease in the fidelity at the extension step during DNA synthesis across m^{6}dG by pol β. This is consistent with our earlier studies showing that the kinetic discrimination mechanisms of pol β are more relaxed during the extension step than the insertion step (*21*).

The overall efficiency of synthesis across a given template base is the product of the efficiency of insertion and extension. Cr(III) stimulates replication across the normal template base, dG, by pol β by increasing the efficiency of incorporation of both dCMP and dTMP (Table 5), but does not significantly affect the fidelity. In contrast to the limited effects during DNA synthesis across dG, Cr(III) stimulates the synthesis across m^{6}dG more robustly. The nonmutagenic incorporation of dCMP opposite m^{6}dG is stimulated by a factor of 3.8 (Table 6), but more notably, the mutagenic incorporation of dTMP is stimulated by a factor of 15.4. In summary, submicromolar concentrations of Cr(III) decrease the fidelity of pol β at m^{6}dG by mediating a 4-fold preference for mutagenic bypass.

For a distributive enzyme such as pol β, the effects of chromium on DNA synthesis are concurrent with the effects on the binding affinity (i.e., changes in the off-rate or binding constant) of pol β to the DNA template. The observed effects on the fidelity are likely to be a manifestation of both decreased kinetic discrimination and increased binding affinity. However, calculation of the ratio of efficiencies [$f_{(rel)}$] of the correct (nonmutagenic insertion of, or extension from, dC) versus incorrect steps (mutagenic insertion of, or extension from, dT) during synthesis across dG and m^{6}dG allows for the determination of the effects of Cr(III) on the fidelity of DNA synthesis independent of the effects on binding [the effect of Cr(III) on the off-rate cancels out]. The $f_{(rel)}$ values are tabulated in Table 7. $f_{(rel)}$ values greater than 1.0 signify decreased fidelity. It can be seen from Table 7 that Cr(III) decreases the fidelity at the base pair extension step during synthesis across both dG and m^{6}dG. Cr(III) decreases the fidelity at m^{6}dG almost 4-fold while the overall synthesis across dG is not greatly affected.

Table 6: Effect of 0.4 μM Cr(III) on the Overall Incorporation of dCMP and dTMP Opposite m^{6}dG by Pol β[a]

base pair	Cr	f_1 insertion	f_2 extension	$f_1 \times f_2$ incorporation	relative $f_1 \times f_2$
dC-m^{6}dG	−	2.9×10^{-4} (0.6×10^{-4})	8.8×10^{-3} (3.5×10^{-3})	2.5×10^{-6} (1.1×10^{-6})	1.0
	+	7.9×10^{-4} (0.7×10^{-4})	1.2×10^{-2} (0.5×10^{-2})	9.5×10^{-6} (4.0×10^{-6})	3.8
dT-m^{6}dG	−	1.6×10^{-2} (0.2×10^{-2})	1.5×10^{-3} (0.7×10^{-3})	2.4×10^{-5} (1.2×10^{-5})	1.0
	+	5.0×10^{-2} (1.7×10^{-2})	7.4×10^{-3} (2.3×10^{-3})	3.7×10^{-4} (1.4×10^{-4})	15.4

[a] Numbers in parentheses indicate the standard errors.

DISCUSSION

Physiologically relevant (*28*), submicromolar concentrations of Cr(III) produce the following three interrelated effects: (1) stimulation of the activity of pol β; (2) stimulation of binding of pol β to DNA; and (3) decreased fidelity of replication across m^{6}dG by pol β. Higher micromolar concentrations of Cr(III) lacked these effects. It is notable that the stimulatory effect of Cr(III) on the binding of pol β to DNA was similar for both m^{6}dG-modified and normal DNA, but Cr(III) exerted greater stimulatory and mutagenic effects during DNA synthesis across m^{6}dG than across dG. Thus, while Cr(III) may not greatly alter normal genomic replication in vivo, it may increase the mutagenic potential of endogenously induced DNA lesions (e.g., due to oxidative or alkylation damage).

The steady-state kinetic analysis reported here shows that Cr(III) affects the discrimination by pol β by altering both K_M and V_{max}. We and others have previously reported that pol β itself utilizes differences in both K_M and V_{max} to discriminate between the correct and incorrect nucleotide substrates (*21*, *29*). A mechanistic interpretation may be that Cr(III) affects both the binding interactions of pol β with the incoming dNTP substrate (K_M effect) as well as the rate of the phosphodiester bond formation which comprises the catalytic step (V_{max} effect).

Cr(III) decreases the fidelity of replication across m^{6}dG and, to a lesser extent, across dG, primarily at the extension step. Pol β has relaxed kinetic discrimination at the extension step (*21*) and is able to replicate primer-templates with one or more terminal mispairs (*30*, *31*). Thus, these results indicate, for the first time, that the insertion and extension steps of replication by DNA polymerases are not equally susceptible to a Cr(III)-mediated decrease in kinetic discrimination. Pre-steady-state kinetic studies and structural studies of the insertion and extension complexes of Cr(III), Mg(II), pol β, primer-templates, and dNTP's may elucidate the molecular mechanisms of this differential effect.

Table 7: Effect of Chromium on the Fidelity of DNA Synthesis at dG and dG* by Pol β[a]

value	parameter	template base	$f_{(rel)}$ −Cr	$f_{(rel)}$ +Cr	$f_{(rel)}$ ratio −Cr/+Cr
$f_{1(rel)}G$	insertion of C versus T	dG	1.2×10^2	1.8×10^2	0.67
$f_{2(rel)}G$	extension of C-G versus T-G	dG	1.6×10^2	0.7×10^2	2.3
$[f_1 \times f_2]_{(rel)}G$	incorporation of C versus T	dG	1.9×10^4	1.3×10^4	1.5
$f_{1(rel)}G^*$	insertion of C versus T	dG*	1.8×10^{-2}	1.6×10^{-2}	1.1
$f_{2(rel)}G^*$	extension of C-G* versus T-G*	dG*	5.9	1.6	3.7
$[f_1 \times f_2]_{(rel)}G^*$	incorporation of C versus T	dG*	0.1	2.6×10^{-2}	3.8

[a] $f_{(rel)}$ is an index of fidelity, being the ratio of the efficiencies of the correct (nonmutagenic) versus incorrect (premutagenic) events. The $f_{(rel)}$ ratio is the factor by which fidelity is reduced by Cr, this being the ratio of fidelities for −Cr to +Cr at the given insertion or extension step.

Cr(III) may mediate its stimulatory and mutagenic effects by interfering with the interactions and functions of Mg(II), the physiological metal ion activator of DNA polymerases, at one or more of the several metal ion coordination sites in pol β. Pol β exhibits relatively weak interactions with singly primed templates and, under these conditions, is a predominantly distributive enzyme that dissociates from the primer-template after each nucleotide incorporation event. The activity of pol β on gapped primer-templates is much more processive (*32*) and as a consequence is likely to be less susceptible to the action of Cr(III). Cr(III) is more electropositive than Mg(II), and therefore could act as a stronger bridging metal and result in stronger interactions between pol β and the template. Enhanced template binding may then allow greater processivity and activity in the presence of Cr(III). Pol β apparently dissociates from the prelesion site when it encounters m^6dG, resulting in the observed prelesion block. By stimulating the binding of pol β to the prelesion complex, Cr(III) may decrease the abortive dissociation and enhance the bypass of m^6dG by pol β with reduced fidelity. Previous studies from this laboratory suggest that Cr(III) also enhances the binding of DNA polymerase I (Klenow fragment) to DNA, thereby increasing its processivity and decreasing its fidelity of replication (*7*, *8*, *10*, *11*).

There are two metal-binding sites that coordinate with the three conserved catalytic aspartate residues in the active site of pol β (*16*, *17*, *33*). Crystallographic analyses of Cr(III)−pol β DNA−dATP complexes showed no evidence of either Cr(III) bound to the catalytic metal ion sites or the occurrence of the nucleotidyl transfer (polymerase) reaction. Thus, in the absence of Mg(II), Cr(III) alone cannot act as a metal cofactor for pol β. Since Cr(III) could not be found in the metal binding site of the active site of pol β, the active site may be selective for divalent cations only. In agreement with these observations, the present studies suggest that Cr(III) alone, in the absence of Mg(II), cannot activate pol β (data not shown). However, the crystal studies showed that Cr(III) caused a spatial dislocation in the side chain position of aspartate 192, one of the three catalytic aspartate residues in the active site (*16*). This perturbation may enhance the rate of phosphodiester bond formation but decrease nucleotide selectivity, and thus be related to the observed Cr(III)-mediated stimulation of the activity and decrease in the fidelity of pol β. One of the metal-binding sites in the active site of pol β is required for β,γ-coordination with the phosphate groups of the incoming dNTP. The Cr(III) in the Cr(III)−DNA pol β−dATP complex was found to coordinate differently than Mg(II) with the triphosphate moiety of dATP (*16*). These differences in the positioning of the triphosphate moiety of the substrate nucleotide may also contribute to the observed increase in the catalytic rate of pol β in the presence of Cr(III). The structural studies of Pelletier et al. (*32*, *33*) suggest that Cr(III) may affect the activity and fidelity without actually substituting for Mg(II) in the metal binding sites in the active site of pol β.

Published results from this laboratory (*7*, *8*, *10*, *11*), as well as the present study, suggest that a Cr(III)-mediated decrease in the fidelity of DNA synthesis may contribute to the mutagenesis of chromium. Cr(III) has been previously shown to increase the processivity of DNA polymerase I Klenow fragment, resulting in increased mutagenic bypass of oxidative DNA lesions (*8*). The effects of Cr(III) on the binding and fidelity of pol β are also modest but discernible. Thus, while Cr(III) may not greatly alter normal genomic replication in vivo, it may increase the mutagenic potential of endogenously induced DNA lesions (e.g., due to oxidative and alkylation damage) and contribute to mutagenesis. Similar studies of the effects of low, physiologically relevant concentrations of Cr(III) on the fidelity of replicative DNA polymerases (in the multiprotein replication machinery, the synthesome) may indicate to what extent the Cr(III)-mediated decrease in replication fidelity contributes to chromium mutagenesis.

ACKNOWLEDGMENT

We thank Andrew Cohen for his technical assistance and Dr. Samuel H. Wilson for the kind gift of DNA polymerase β.

REFERENCES

1. Cohen, M., Kargacin, B., Klein, C., and Costa, M. (1993) *Crit. Rev. Toxicol. 23*, 255−281.
2. Klein, C. B. (1996) in *Toxicology of Metals* (Chang, L. W., Ed.) pp 205−220, CRC−Lewis Publishers, New York.
3. De Flora, S., Bagnasco, M., Serra, D., and Zanacchi, P. (1990) *Mutat. Res. 238*, 99−172.
4. Snow, E. T. (1992) *Pharmacol. Ther. 53*, 31−65.
5. Costa, M. (1997) *Crit. Rev. Toxicol. 27*, 431−442.
6. Wolf, T., Kasemann, R., and Ottenwalder, H. (1989) *Carcinogenesis 10*, 655−659.
7. Snow, E. T., and Xu, L.-S. (1991) *Biochemistry 30*, 11238−11245.
8. Snow, E. T., and Xu, L.-S. (1992) *Environ. Mol. Mutagen. 19*, 59a.
9. Snow, E. T., Xu, L. S., and Belman, S. (1993) *Proc. Am. Assoc. Cancer Res. 34*, 116.
10. Snow, E. T. (1994) *Environ. Health Perspect. 102*, 41−44.
11. Snow, E. T., and Xu, L.-S. (1989) *Biol. Trace Element Res. 21*, 61−71.
12. Snow, E. T. (1991) *Environ. Health Perspect. 92*, 75−81.
13. Voitkun, V., Zhitkovich, A., and Costa, M. (1998) *Nucleic Acids Res. 26*, 2024−2030.
14. Liu, S., and Dixon, K. (1996) *Environ. Mol. Mutagen. 28*, 71−79.

15. Pelletier, H., Sawaya, M., Wolfle, W., Wilson, S., and Kraut, J. (1996) *Biochemistry 35*, 12742−12761.
16. Pelletier, H., Sawaya, M., Wolfle, W., Wilson, S., and Kraut, J. (1996) *Biochemistry 35*, 12762−12777.
17. Pelletier, H., and Sawaya, M. (1996) *Biochemistry 35*, 12778−12787.
18. Swann, P. (1990) *Mutat. Res. 233*, 81−94.
19. Basu, A., and Essigmann, J. (1990) *Mutat. Res. 233*, 189−201.
20. Kyrtopoulos, S., Anderson, L., Chhabra, S., Souliotis, V., Pletsa, V., Valavanis, C., and Georgiadis, P. (1997) *Cancer Detect. Prevent. 21*, 391−405.
21. Singh, J., Su, L., and Snow, E. T. (1996) *J. Biol. Chem. 271*, 28391−28398.
22. Menichini, P., Mroczkowska, M., and Singer, B. (1994) *Mutat. Res. 307*, 53−59.
23. Goodman, M., Creighton, S., Bloom, L., and Petruska, J. (1993) *Crit. Rev. Biochem. Mol. Biol. 28*, 83−126.
24. Creighton, S., Bloom, L., and Goodman, M. (1995) *Methods Enzymol. 262*, 232−256.
25. Duggleby, R. G. (1981) *Anal. Biochem. 110*, 9−18.
26. Kendall, M. G., and Stuart, A. (1967) *The advanced theory of statistics*, 2nd ed., Griffin Publishers, London.
27. Armitage, P., and Berry, G. (1994) *Statistical Methods in Medical Research*, 3rd ed., Blackwell Scientific Publications, Oxford.
28. Gerhardsson, L., Brune, D., Nordberg, G., and Wester, P. (1988) *Sci. Total Environ. 74*, 97−110.
29. Boosalis, M., Mosbaugh, D., Hamatake, R., Sugino, A., Kunkel, T., and Goodman, M. (1989) *J. Biol. Chem. 264*, 11360−11366.
30. Korn, D., Fisher, P., and Wang, T. (1981) *Prog. Nucleic Acid Res. Mol. Biol. 126*, 63−81.
31. Wang, T., and Korn, D. (1982) *Biochemistry 21*, 1597−1608.
32. Pelletier, H., Sawaya, M. R., Wolfle, W., Wilson, S. H., and Kraut, J. (1996) *Biochemistry 35*, 12742−12761.
33. Pelletier, H., Sawaya, M., Kumar, A., Wilson, S., and Kraut, J. (1994) *Science 264*, 1891−1903.

BI9731551