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Chromium(III) Bound to DNA Templates Promotes Increased Polymerase Processivity and Decreased Fidelity during Replication in Vitro†

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ABSTRACT: Carcinogenic chromium [Cr(VI)] compounds are reduced intracellularly to DNA- and protein-reactive chromium(III) species. However, the role of Cr(III) ions in chromium-induced genotoxicity remains unclear. We have investigated the effects of chromium(III) binding on DNA replication and polymerase processivity in vitro. Chromium ions bind slowly and in a dose-dependent manner to DNA. Micromolar concentrations of free chromium inhibit DNA replication, but if the unbound chromium is removed by gel filtration, the rate of DNA replication by polymerase I (Klenow fragment) on the chromium-bound template is increased greater than 6-fold relative to the control. This increase is paralleled by as much as a 4-fold increase in processivity and a 2-fold decrease in replication fidelity. These effects are optimum when very low concentrations of chromium ions are bound to the DNA [3-4 Cr(III) ions per 1000 nucleotide phosphates]. Increased concentrations of chromium lead to the production of DNA-DNA cross-links and inhibition of polymerase activity. These results suggest that low levels of DNA-bound chromium(III) ions may contribute to chromium mutagenesis and carcinogenesis by altering the kinetics and fidelity of DNA replication.

Chromate is one of the best documented human and animal carcinogens. Occupational exposure to chromium compounds has been widespread, and many studies of the biological effects of chromium have been conducted (Stern, 1982; Bianchi et al., 1983; De Floro & Wetterhahn, 1989). However, because of its complex intracellular metabolism, molecular mechanisms of chromium-induced genotoxicity are not thorougly understood.

Chromium exists in a number of oxidation states, of which only Cr(VI) and Cr(III) are environmentally stable (Goyer, 1986). While chromium(VI), as chromate, is biologically active, because it is taken up by cells, it is rapidly reduced through relatively unstable Cr(V) and Cr(IV) intermediates to kinetically stable Cr(III) species (Arslan et al., 1987). Because Cr(VI) is cellularly reduced, numerous studies have attempted to determine which intracellular form of chromium is the ultimate carcinogenic and/or mutagenic species (Lofroth, 1978; Macrae et al., 1979; Whiting et al., 1979). While the

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consensus is that Cr(VI) compounds are biologically efficacious, studies of Cr(III) compounds yield conflicting results. This is in part because chromium(III) species are not actively taken up by cells (Leonard & Lawerys, 1980). However, Cr(III) is the final stable intracellular valence state of carcinogenic and mutagenic Cr(VI) (De Floro & Wetterhahn, 1989). In biological materials, as much as 90% of the cellular chromium is present as the trivalent species (Schroeder et al., 1962); indeed, Cr(III) forms a variety of ligand complexes with both nucleic acids and proteins (Tamino et al., 1981). It has even been suggested that trace quantities of chromium may be essential for glucose metabolism (Guthrie, 1982).

Chromate is mutagenic in a number of bacterial systems (Nishioka, 1975; Kanematsu et al., 1980; Tindall et al., 1978). When tested in the Ames histidine reversion assay using *Salmonella typhimurium*, Cr(VI) is most potent in producing base pair substitutions in the A·T-specific strains TA104 and TA102 (De Floro & Wetterhahn, 1989). These strains are especially susceptible to oxidative damage, and it is very likely that a major component of chromate genotoxicity is mediated by active oxygen species produced during intracellular chromate reduction (Standeven & Wetterhahn, 1989). Both mutagenicity and carcinogenicity of Cr(VI) are also well established in mammalian cell cultures and in vivo (Bianchi et al., 1983; Sen & Costa, 1986; Sen et al., 1987; Sugiyama et al., 1986). Chromium(VI) compounds induce chromosomal aberrations and increase the incidence of sister chromatid exchanges, as well as DNA–protein cross-links and DNA strand breaks in cultured mammalian cells (Sen & Costa, 1986; Sen et al., 1987; Sugiyama et al., 1986). In animal experiments, the administration of Cr(VI) compounds results in tumor formation at the site of injection or implantation (Bianchi et al., 1983).

Many experiments attempting to determine the carcinogenic and mutagenic potential of Cr(III) compounds in vivo have yielded negative or ambiguous results (Bianchi et al., 1983). The general lack of mutagenic activity of Cr(III) in vivo is primarily due to its inability to pass through the cell membrane (Leonard & Lawerys, 1980). Nevertheless, Cr(III) compounds that are taken up by cells can be mutagenic (Warren et al., 1981; Elias et al., 1986; Sugden et al., 1990), and $CrCl_3$ increases mutagenesis when chromium-treated phage DNA is transfected into *Escherichia coli* (Sirover & Loeb, 1977; Tkeshelashvilli et al., 1980; Schaaper et al., 1987; Snow & Xu, 1989; Snow, 1991). Chromium(III) also induces at least a 2-fold increase in misincorporation by DNA polymerase during DNA replication in vitro, using either synthetic polynucleotides (Sirover & Loeb, 1977) or phage *φX174* DNA templates (Tkeshelashvili et al., 1980). Chromium(III) ions bound to nuclear DNA increase RNA synthesis in vitro by increasing nonspecific initiation (Okada et al., 1981, 1983; Ohba et al., 1986). This suggests that Cr(III) might influence the regulation of gene transcription (Okada et al., 1981).

In the present studies, we have investigated Cr(III) binding to DNA and the mechanism of Cr(III)-induced alterations in DNA polymerase activity in order to better understand the possible role of Cr(III) in chromate-induced carcinogenesis in vivo. M13mp2 bacteriophage DNA was used to study the genetic effects of chromium in vitro by measuring the effects of Cr(III) on polymerase–template and DNA–DNA interactions. The results of these experiments indicate that $CrCl_3$ influences the rate of nucleotide incorporation during in vitro DNA replication by altering DNA polymerase–template interactions and dramatically increasing DNA polymerase processivity. In addition, Cr(III) when bound to the DNA template reduces DNA polymerase fidelity during DNA replication in vitro and promotes increased mutagenesis when the Cr-bound DNA is transfected into *E. coli* (Snow & Xu, 1989; Snow, 1991). It is postulated that Cr(III) may act synergistically to enhance the mutagenic potential of DNA lesions produced during the intracellular reduction of Cr(VI) in vivo.

Experimental Procedures

Materials. M13mp2 single-stranded DNA was prepared using standard procedures (Maniatis et al., 1982). Unlabeled deoxynucleotides were obtained from Sigma Chemical Co. (St. Louis, MO); ^{32}P-labeled nucleotides and $^{51}CrCl_3$ (20.5 mCi/mmol) were from New England Nuclear (Boston, MA). The M13 sequencing primers were synthesized by Dr. Bernard Goldschmidt, Department of Environmental Medicine, New York University Medical Center. The 15-mer sequencing primer was synthesized by the Howard Hughes Medical Institute at the University of Washington (Seattle, WA). The T7 Sequenase kit was obtained from the United States Biochemical Corp. (Cleveland, OH). Polymerase I–Klenow fragment (pol I–KF) and Sephadex G-50 spin columns were both obtained from Boehringer Mannheim (Indianapolis, IN). Spin columns were also prepared as described in Maniatis et al. (1982). All other chemicals were of molecular biology or DNA grade, and all water was purified through a Milli-Q purification system. All $CrCl_3$ solutions were made fresh daily, except as noted, and diluted as required immediately before use.

Measurement of Cr(III) Bound to M13mp2 DNA. M13mp2 single-stranded DNA was treated with $^{51}CrCl_3$ (0.4–50 μM) at 37 °C for a fixed amount of time (as indicated) in a 50-μL reaction mixture containing 2 μg of DNA, 0.2 mM Tris-HCl (pH 8.0), and 20 μM EDTA. After incubation, the reaction mixtures were filtered through Sephadex G-50 spin columns (1200 rpm, 3 min) to remove the unbound $^{51}Cr(III)$ ions. The amount of Cr(III) remaining bound to the DNA was determined by counting the samples in a NaI(Tl) scintillation detector coupled to a multichannel analyzer. [^3H]TdR-labeled DNA was used in a control reaction to measure DNA recovery.

Preparation of M13mp2 Primer-Templates. M13 17-mer sequencing primer was 5′ end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase as described in Maniatis et al. (1982). M13mp2 single-stranded DNA was hybridized with a 2-fold molar excess of either radiolabeled or cold primer in a 100-μL reaction mixture containing 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM NaCl, and 10 mM dithiothreitol at 55 °C for 10 min and then slowly cooled to room temperature. Filtration through a Sephadex G-50 spin column was used to remove excess [γ-^{32}P]ATP, $CrCl_3$, and unbound primer.

DNA Replication in Vitro. For reactions in the presence of $CrCl_3$, 20-μL reaction mixtures were prepared containing 5 μg/mL DNA template (M13mp2/17-mer hybrid), 0.1 unit of pol I–KF, and $CrCl_3$ (diluted in H_2O to an appropriate concentration). The chromium stock solution was prepared in distilled H_2O immediately before use, and the reaction was initiated by the addition of 2 μL of a 10× reaction buffer to give a final concentration of 40 mM Tris-HCl (pH 7.4), 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, and 50 μM each dNTP (dATP, dCTP, dGTP, and dTTP) and incubated at 37 °C for 15 min. The total concentration of $CrCl_3$ used in these assays was 0.4–50 μM. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM, and the amount of nucleotide incorporation was calculated from the

10% (w/v) trichloroacetic acid precipitable counts collected on GF/C filters. Each determination was performed in triplicate, and the results are presented as the average (±standard error).

DNA replication on chromium-treated templates was determined similarly. However, the template was first treated with chromium for 30 min at 37 °C, and the excess chromium was removed by gel exclusion using a 1-mL Sephadex G-50 spin column. The treated DNA was then hybridized with 2-fold excess primer. In one experiment, the end-labeled primer was hybridized with the DNA template prior to treatment with $CrCl_3$, and the excess chromium and unhybridized primers were subsequently removed by gel exclusion. Replication was carried out using 0.2 μg of the modified DNA template and 0.04 unit of pol I–KF in a 20-μL reaction, and nucleotide incorporation was determined as described above.

Primer Extension Assays. Primer extension was carried out as above using a 5′ end-labeled primer and 0.02 unit of pol I–KF. The extension reactions were initiated by the addition of dNTPs and 5 mM $MgCl_2$ and incubated at 37 °C for different lengths of time. The reactions were stopped by the addition of 0.5 M EDTA to yield a final concentration of 10 mM. Aliquots were transferred to 1.5-mL microtubes and mixed with loading buffer (99% deionized formamide, 10 mM Na_2EDTA, 10 mM NaOH, and 0.3% each of xylene cyanol FF and bromphenol blue) and then heated to 96 °C for 3–4 min and rapidly chilled on ice before being loaded on an 8% (w/v) polyacrylamide–5 M urea gel which has been prerun for 15–30 min. Gel electrophoresis was carried out at 1600–1800 V until the dye front was within 2 in. of the bottom. The gel was then transferred to 3M Whatman filter paper and dried for 30 min at 70 °C. The dried gel was placed with X-ray film (Kodak XAR-2) and exposed for 1–7 days at −70 °C with an intensifying screen.

Polymerase Processivity. The processivity of pol I–KF on Cr(III)-treated templates was assayed by limiting polymerase (Bambara et al., 1978). Synthesis was initiated by addition of the reaction buffer, including dNTPs and $MgCl_2$, as described above. The reaction was carried out at 37 °C for 1–5 min and stopped by the addition of 10 mM EDTA (final concentration). The results were assayed on 8% polyacrylamide–5 M urea sequencing gels or 0.8% agarose gels, as described below. After autoradiography, the gels were scanned on an LKB Ultroscan XL laser densitometer, and the relative areas under the peaks were determined.

DNA–DNA Cross-Linking Analysis. After primer extension using an end-labeled primer in the presence of competitor DNA, 10-μL aliquots of the reaction were heated to 96 °C for 3–4 min to denature the DNA. Denatured DNA samples were then mixed with 0.5 volume of agarose gel loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, and 15% (w/v) Ficoll type 400], loaded on an 0.8% (w/v) agarose gel, and electrophoresed for 2 h at 100 V. Undenatured samples were analyzed in the same manner to determine the total amount of radioactivity present in the primer pool. The gels were dried at 45 °C overnight and then autoradiographed and scanned as above. In order to determine the relative radioactivity in the primer peaks before and after denaturation, both 16-h (overnight) and 5-day exposures were scanned.

Mutagenesis Using Cr-Treated Templates. M13mp2 single-stranded DNA (0.02 μg/μL) was either sham-treated or reacted with 5 μM $CrCl_3$ for 30 min at 37 °C in a final volume of 50 μL of H_2O. The samples were filtered through Sephadex G-50 spin columns (2000 rpm, 4 min) to remove unbound $CrCl_3$. Prior to in vitro replication, the Cr-treated DNA was hybridized with a 15-mer primer specific for the template sequence corresponding to amino acids 46–50 of the *lacZ* gene. DNA replication was carried out for 30 min at 37 °C, as described above. The reaction mixture was then filtered through an additional Sephadex G-50 spin column. Copied and uncopied chromium-treated DNA was used to transfect SOS-induced (75 J/m² UV) calcium-treated mismatch repair deficient *E. coli*, strain NR9064 (Schaaper et al., 1989), at a ratio of 15 ng of DNA per 0.2 mL of competent cells. Aliquots of the transfected cells were plated on minimal agar to give approximately 200–600 plaques per plate, as described previously (Snow & Xu, 1989). The plates were incubated overnight at 37 °C and then placed at room temperature for 16–24 h prior to being screened for survival and mutagenesis.

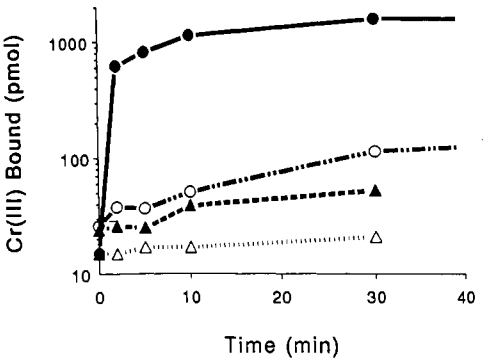

FIGURE 1: Time course of chromium(III) binding to DNA in vitro. A total of 2 μg of single-stranded M13mp2 DNA per reaction was incubated with 1 (△), 5 (▲), 10 (○), or 50 (●) μM $^{51}CrCl_3$ in a total volume of 50 μL of TE/50 (0.2 mM Tris-HCl, pH 8, and 20 μM EDTA) at 37 °C for up to 2 h, as noted. Nonbound chromium was then removed by gel filtration through a 1-mL Sephadex G-50 spin column at room temperature. DNA recovery was determined in a separate experiment using ^3H-labeled M13 DNA and was found to average 85 ± 3%.

RESULTS

Effect of Cr(III) on DNA Replication in Vitro with pol I–KF. In order to study the mutagenic mechanism(s) of Cr(III), the effect of $CrCl_3$ on DNA replication in vitro was first examined. In previous experiments (Snow & Xu, 1989; Snow, 1991), polymerization reactions were carried out in the presence of both template-bound Cr(III) and free Cr(III) ions. In subsequent experiments, in order to eliminate the effect of free Cr(III) ions, the unbound ions were removed by spin column before the polymerization reaction. Chromium binding to DNA is a relatively slow process, as shown in Figure 1, and a preincubation time of 30 min was chosen to ensure near-maximum binding. The mode of chromium binding under these conditions is not entirely clear. It appears that most of the binding after a 30-min reaction is electrostatic since 40% or more of the bound ions can be displaced by a high-salt wash (0.5 M NaCl) at 37 °C, while only 20% or less of the chromium is chelatable by 20 mM EDTA (a kinetically slow process) under the same conditions (Table I).

Both nucleotide incorporation and primer extension were then examined using the chromium-treated templates. Figure 2 shows the results of nucleotide incorporation in which DNA pretreated with as little as 0.4 μM $CrCl_3$ increased the nucleotide incorporation 2-fold, while pretreatment with 2 μM $CrCl_3$ produced a 6.3-fold increase in nucleotide incorporation. The amount of Cr(III) bound to the template under the conditions used for these experiments was determined by measuring the amount of radioactive ^{51}Cr bound to the DNA after gel filtration through Sephadex G-50. Thus, it can be

Table I: Stability of Cr(III) Bound to Single-Stranded DNA in Vitro[a]

$CrCl_3$ concn (μM)	control	cpm of bound ^{51}Cr (% of control) 500 mM NaCl	20 mM EDTA
2	14 ± 2	6 ± 0 (43)	11 ± 1 (79)
10	65 ± 9	26 ± 1 (45)	58 ± 7 (89)
50	125 ± 9	67 ± 2 (54)	103 ± 4 (82)
50 (−DNA)	6 ± 0	5 ± 0 (83)	12 ± 0 (200)

[a] Single-stranded M13mp2 DNA (2 μg per reaction) was incubated with various concentrations of $^{51}CrCl_3$ in 50 μL of 0.2 mM Tris-HCl, pH 8, and 20 μM EDTA for 30 min at 37 °C. Control samples then received a 5-μL aliquot of H_2O, and the other samples received 5 μL each of either 5 M NaCl (500 mM final concentration) or 0.2 M EDTA (20 mM final concentation), and incubation was continued for a further 15 min at 37 °C. The chromium-bound DNA was then separated from unbound chromium by exclusion chromatography using a 1-mL Sephadex G-50 spin column. The amount of ^{51}Cr bound to the DNA was determined using a NaI scintillation counter.

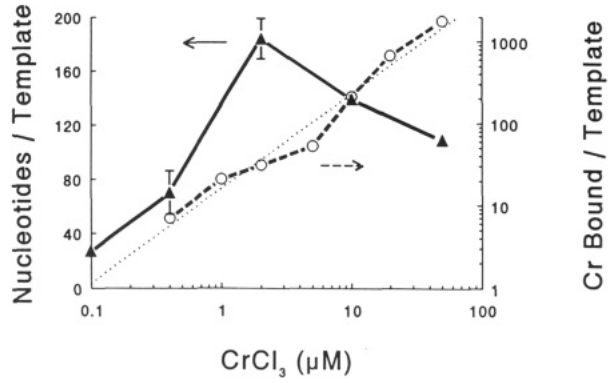

FIGURE 2: Nucleotide incorporation on a chromium-bound template and binding of $CrCl_3$ to single-stranded M13mp2 DNA. Single-stranded M13 DNA was treated for 30 min at 37 °C with 0–50 μM $CrCl_3$ in a total volume of 50 μL, and the unbound chromium was removed by gel exclusion chromatography, as described under Experimental Procedures. DNA replication was then carried out on 0.2 μg of treated template, and the TCA-precipitable nucleotide incorporation was measured (▲). In a set of parallel experiments, M13 DNA was treated with $^{51}CrCl_3$, and unbound $CrCl_3$ was removed as above. The amount of chromium bound to the DNA template after G-50 chromatography (○) was determined by using a NaI scintillation counter. Each determination was performed in triplicate, and each point represents the average (±the standard error).

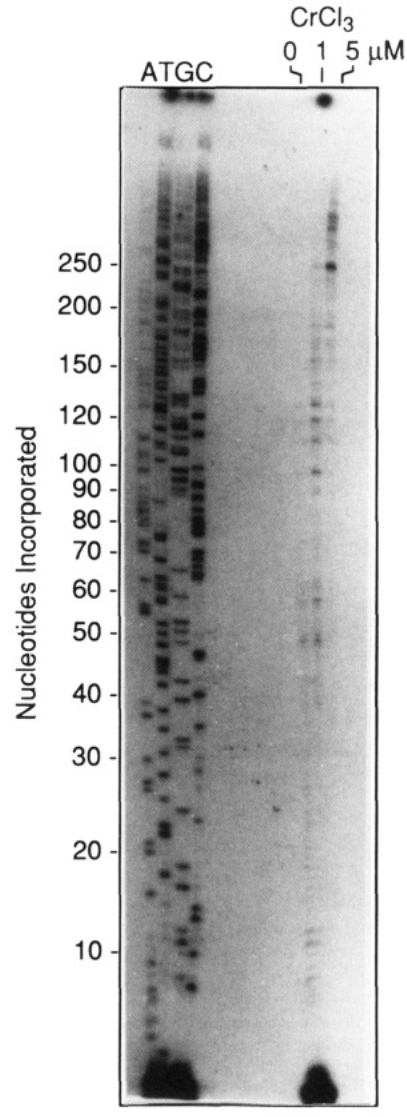

FIGURE 3: Chromium(III)-enhanced primer extension by polymerase I–KF. For this experiment, M13mp2 single-stranded DNA was incubated with $CrCl_3$ for 10 min at 37 °C, and excess Cr(III) was removed by passing over Sephadex G-50. Following hybridization with ^{32}P-5′ end-labeled 15-mer primer, 0.1 μg of DNA template was replicated for 10 min at 37 °C in the presence of 50 μM each of dATP, dCTP, dGTP, and dTTP, 10 mM $MgCl_2$, and 0.02 unit of pol I–KF. The polymerase reaction was then stopped by the addition of 2 μL of 0.5 M EDTA. The samples were run on a 12% sequencing gel adjacent to dideoxy sequencing reactions prepared using an end-labeled primer and untreated M13mp2 template and autoradiographed.

calculated that the rate of nucleotide incorporation is greatest on a DNA template with approximately 25 molecules of Cr(III) bound per template (or 1 Cr ion per 300 nucleotides). Even concentrations of $CrCl_3$ as high as 50 μM, at which over 1000 molecules of Cr(III) are associated with each template molecule (or an average of 1 chromium per 6 nucleotides), produce over a 4-fold increase in nucleotide incorporation relative to the untreated control. Control experiments showed that under these conditions the chromium does not cause precipitation of the nucleotide precursors and that the background due to nonspecific cross-linking of the unincorporated ^{32}P is less than 1% of the observed incorporation.

This contrasts with the results obtained during replication in the presence of free chromium(III). When $CrCl_3$ is added shortly before the replication of M13mp2 single-stranded DNA (primed with 17-mer), a dose-dependent decrease in the rate of nucleotide incorporation is seen (not shown). Pretreatment of pol I–KF alone for 15 min at room temperature with similar concentrations of Cr(III) did not significantly inhibit or enhance replication relative to controls incubated without chromium (data not shown).

$CrCl_3$ and Processivity of pol I–KF. DNA polymerase I and pol I–KF are processive at low ionic strength (Bambara et al., 1978; Uyemura et al., 1975), and it has been shown that metal ions such as $MnCl_2$ can both increase processivity (Hohn & Grosse, 1987) and decrease the fidelity of DNA replication by this enzyme (Beckman et al., 1985; Hillebrand & Beattie, 1984). We have examined the effects of $CrCl_3$ on DNA polymerase I–KF processivity by primer-extension analysis. This method also allows us to investigate sequence-specific pausing and polymerase termination.

Under our conditions, at low ionic strength (40 mM Tris-HCl and 12.5 mM NaCl), the processivity of pol I–KF is approximately 30–60 nucleotides per binding event (see below). This agrees well with the values of 20–50 nucleotides reported by Bambara et al. (1978). Figure 3 presents an autoradiograph of a polyacrylamide sequencing gel showing the effect of chromium(III) on the processivity of DNA polymerase I–KF on a primer-template treated with 0, 1, or 5 μM $CrCl_3$. Under conditions where polymerase is limiting, the length of

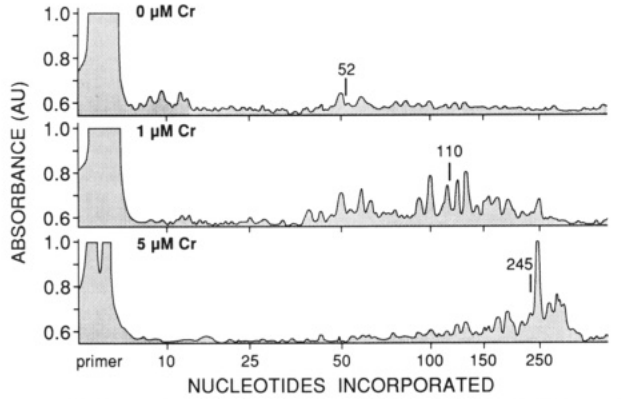

FIGURE 4: Laser densitometer scans of the gel shown in Figure 3. The length of the extended primers was determined from the dideoxy sequencing reactions run on the same gel. The median length of the extended primers for each reaction is also shown.

Table II: Median Length of Extended Primers on Cr(III)-Treated Templates[a]

$[Cr^{3+}]$ (μM)	lane[b]	median no. of added nucleotides	% control	% unused primer[c]
		Cr-Treated Template, Only[d]		
0	A	52		79
1	B	110	212	76
5	C	245	471	76
		Cr-Treated Template plus Primer[e]		
0	D	51		72
1	E	64	126	77
5	F	107	210	85

[a] M13mp2 single-stranded DNA, treated with $CrCl_3$ either before (lanes A–C) or after (lanes D–F) hybridization of a ^{32}P-end-labeled 15-mer primer, was used as a template for DNA synthesis by pol I–KF, and the primer extension products were analyzed on a 12% sequencing gel as shown in Figure 3. The autoradiograph of the gel was then scanned on an LKB laser densitometer as shown in Figure 4. The areas under the curves corresponding to extended primers of various lengths were determined by summing up the areas under the peaks, and the percent of extended primers corresponding to the various length intervals was determined. [b] Lane number on the scanned gel. Lanes A, B, and C are shown in Figures 3 and 4. [c] This value is an underestimate due to overexposure of the autoradiogram. On the basis of available data, the actual percent of unused primers is estimated to be at least 10 or 25% greater than the calculated value for experiments 1 and 2, respectively. [d] Template alone was treated with $CrCl_3$; then the labeled primer was hybridized to the template. [e] Template and labeled primer were hybridized and treated together with $CrCl_3$ as described under Experimental Procedures.

the extended primers is greatly increased by prior treatment of the template with chromium(III) chloride. A scanning densitometer tracing of this gel is presented in Figure 4. The mean number of nucleotides added to the extended primers after treatment of the template with 0, 1, and 5 μM $CrCl_3$ was 52, 110, and 245 nucleotides, respectively (Table II). In this experiment, a maximum of 24% of the total available primers were used in the reaction, suggesting that these extended primers truely represent the product of the initial processive phase of replication and not multiple rounds of replication on each primer. Table II also shows the results obtained in this same experiment using a set of primer-templates which were treated with chromium after hybridization of the primer. The amount of nucleotide incorporation and degree of chromium-enhanced processivity are slightly less (but still greater than twice the control value) under these conditions, possibly due to inhibition of the initial priming step by chromium bound to the primer itself. These results using chromium–treated templates after removal of the unbound chromium ions further suggest that it is the Cr(III) ions which are bound to the DNA

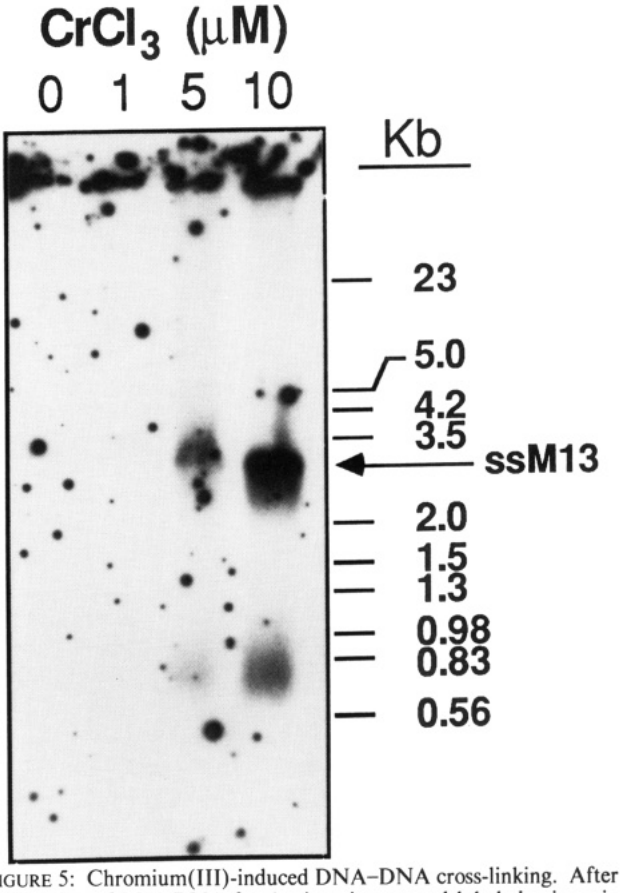

FIGURE 5: Chromium(III)-induced DNA–DNA cross-linking. After replication of M13 DNA for 4 min using an end-labeled primer in the presence of activated calf thymus DNA, as described above, the denatured reaction products were analyzed on an 0.8% agarose gel. The gel was then dried overnight and autoradiographed in the presence of an enhancer screen for 5 days at −70 °C.

template, and not free chromium, which produce increased pol I–KF processivity.

Analysis of Primer-Extension Products on an Agarose Gel. In some experiments, a dose-dependent increase in label remained in the loading wells (not shown). This nonmigrating material may represent either a subset of greatly extended primers (greater than 300 nucleotides) or cross-linked material. To determine whether the counts remaining in the well at the top of the gel were due to DNA–DNA cross-linking or to long extended primers, the polymerase reactions were carried out on chromium-treated templates for either 30 s or 4 min, and the reaction products were analyzed on 0.8% agarose gels. Both denatured and undenatured samples were analyzed to determine the percentage of cross-linked and long primers relative to the total amount of primers in the reaction. Figure 5 shows an autoradiograph of an agarose gel on which the heat-denatured products of replication were run. When the products of replication are not denatured prior to their being run on an agarose gel, the labeled DNA primer migrates with the M13mp2 single-stranded DNA template and there is no significant difference between control and $CrCl_3$-treated DNA samples (not shown). Denaturation displaces the primers, and after DNA replication in the presence of 0 or 1 μM chromium, none of the primers, which average less than 200 nucleotides in length (see Table II), can be visualized on the gel. In contrast, primer extension in the presence of greater amounts of $CrCl_3$ results in the formation of two bands of radioactivity on the agarose gel. A major band is at the position of M13mp2 single-stranded DNA (as determined by ethidium bromide staining), indicating that the chromium formed heat-stable

Table III: Extended Primers Are Visible on Agarose Gels after Denaturation

$[Cr^{3+}]$ (μM)	lane[a]	undenatured primers[b,c]	long primers ±800 nucleotides[b,d]	"cross-linked" primers[d,e]
		30-s Reaction		
0	1	135	0.16 (0.1)[d]	3.04 (2.2)[d]
1	2	162	nd[g]	nd
5	3	165	0.51 (0.3)	2.39 (1.5)
10	4	[164][f]	1.58 (1.0)	5.28 (3.2)
		4-min Reaction		
0	5	135	nd	nd
1	6	170	0.25 (0.2)	0.40 (0.3)
5	7	[176][f]	1.16 (0.7)	2.30 (1.3)
10	8	182	2.59 (1.4)	8.11 (4.5)

[a] Lane number on the gel. [b] Area under the peak on day 5 of autoradiographic exposure. [c] The total amount of undenatured primer migrating with the template on day 5 was determined using a 1-day exposure and multiplying by a factor of 9.0 (±26%), as determined by quantitating the relative areas under the peaks of the denatured samples on day 1 versus day 5. [d] Values in parentheses are the percentage of primers migrating in this position after denaturation relative to the total amount of undenatured primers migrating with the M13 template (i.e., the total amount of available primers). [e] The primers remaining associated with the M13 template after denaturation. [f] Estimated based on the average of the two closest lanes. Not directly measurable due to technical difficulties. [g] nd, not determined due to experimental artifacts (blobs) on the autoradiogram.

DNA–DNA cross-links between some of the primers and the template DNA. The remainder of the labeled primers are located in a broad band with an average length of about 800 bases. The relative amount of radioactivity in each region of the gel was determined by scanning the gel and determining the areas under the peaks (Table III). Both overnight and 5-day exposures of the gel were measured to ensure that the exposures were within the linear range of the film. Both the amount of extended primers and the amount of cross-linked primers (those migrating with the template after heat denaturation) increased in a dose-dependent fashion. The total amount of highly extended and cross-linked primers remaining on the gel after denaturation relative to the amount associated with the template prior to denaturation averaged 1.9% for the 5 μM Cr-treated samples and 4.8% for the 10 μM Cr-treated samples (Table III).

Mutagenesis Using Chromium-Treated Templates. It has previously been shown that chromium salts decrease the fidelity of DNA polymerases during replication of poly(dA-dT) (Sirover & Loeb, 1976; Tkeshelashvili et al., 1980) and increase base substitution mutagenesis during replication of *ϕX174* (Schaaper et al., 1987) in vitro and M13mp2 DNA in vivo (Snow & Xu, 1989; Snow, 1991). In order to determine whether the fidelity of replication of chromium-treated M13 DNA is decreased as a function of increased polymerase processivity, we examined whether in vitro replication of of Cr(III)-bound M13 DNA results in increased mutagenesis when the copied templates are transfected into mismatch repair defective SOS-induced *E. coli* and the progeny phage are scored for survival and mutagenesis. Under the conditions used for this experiment (replication for 30 min with 0.2 unit of pol I–KF per reaction), most of the templates were fully copied, as evidenced by the fact that the DNA migrated as form III (relaxed double-stranded circles) on an agarose gel. Replication of Cr-treated templates in vitro and subsequent transfection of the replicated templates into mismatch repair deficient host cells give a 2-fold increase in mutagenesis relative to control templates replicated in the absence of chromium (Table IV). This increase is similar to the increased mutagenesis observed when chromium-treated templates are transfected into *E. coli* and copied in vivo by *E. coli* polymerase III [Table IV; also see Snow and Xu (1989)]. The mutagenesis following in vitro replication on a chromium-treated template persists, even if the replicated DNA is stored before transfection. In contrast, either storage or EDTA treatment of the chromium-treated, but uncopied, DNA promotes dissociation of the chromium from the template and results in restoration of both phage survival and mutagenesis to control levels (Table IV).

Table IV: Chromium-Treated DNA Templates Are Mutagenic When Replicated in Vitro or in Vivo in the Presence of Bound Cr(III)[a]

$CrCl_3$ treatment	DNA replication in vitro	total plaques[b]	mutant plaques	% survival vs control[c]	mutant frequency ($\times 10^4$)
0	no	30134	19	100 ± 30	6.4 ± 0.2
5 μM (day 1)[d]	no	16054	37	40 ± 17	16.6 ± 0.3
5 μM (stored)[e]	no	50232	35	116	7.0
5 μM + EDTA[f]	no	47071	30	120	6.3
0	yes	26215	17	123	6.5
5 μM (day 1)	yes	11067	14	51.8	12.6
5 μM (day 10)[g]	yes	10486	19	49.1	18.2

[a] Single-stranded M13mp2 DNA was treated ±5 μM $CrCl_3$ and hybridized to a 15-nucleotide primer complementary to the template sequence corresponding to amino acids 46–50 of the *lacZ* gene. The primed DNA template was then copied by DNA polymerase I–KF for 30 min as described under Experimental Procedures. Copied and uncopied, control, DNA was transfected into mismatch repair defective *E. coli* (strain NR9064; Schaaper et al., 1989) and scored for survival and mutagenesis. [b] The sum of one or more experiments. The total amount of DNA transfected may vary in different experiments. [c] The plating efficiency of untreated and uncopied, control, DNA was 436 ● 130 plaques per nanogram of DNA transfected. [d] Chromium-treated DNA was desalted by Sephadex G50 chromatography and transfected into $CaCl_2$-treated *E. coli* within 30 min of treatment. [e] Chromium-treated DNA was desalted and then stored at −20 °C for 1–8 days prior to transfection. [f] Chromium-treated DNA was prepared as above and then incubated with 20 mM EDTA overnight to chelate and remove the bound chromium prior to transfection. [g] Chromium-treated DNA was copied in vitro and stored at −20 °C prior to transfection.

Discussion

Many different experimental approaches have been utilized to determine the mutagenicity and mutagenic mechanism of Cr(III) compounds. However, because of the poor ability of Cr(III) ions to cross the cell membrane in vivo, these studies have often proven negative. In contrast, DNA replication in vitro using synthetic polynucleotide and/or *ϕX174* DNA templates in the presence of $CrCl_3$ or $CrCl_2$ [which rapidly becomes Cr(III) in aqueous solution] shows a chromium-dependent increase in the frequency of nucleotide misincorporation (Schaaper et al., 1987; Sirover & Loeb, 1977; Tkeshelashvili et al., 1980). We show here (Figure 2) that as little as 10–25 mol of Cr(III) bound per mole of single-stranded M13mp2 DNA template enhances nucleotide incorporation by the Klenow fragment of *E. coli* polymerase I. This increase is not seen immediately after the addition of acqueous $CrCl_3$ (not shown) because the chromium chloride (a kinetically inactive compound) has not yet had time to bind to the DNA (e.g., Figure 1).

The enhanced nucleotide incorporation does not appear to be due to the substitution of chromium ions for $MgCl_2$, the normal divalent metal cofactor for DNA polymerases. Although the chromium does increase DNA replication substantially in the absence of added magnesium (Snow & Xu, 1989), the maximum rate of incorporation under these con-

ditions is considerably below the rate achieved in the presence of $MgCl_2$. It cannot be ruled out that low concentrations of unbound Cr(III) in equilibrium with the template-bound Cr(III) or small amounts of $CrCl_3$ that pass through the gel filtration column could also act to enhance the polymerase activity. However, as shown in Table IV, Cr(III)-induced mutagenesis requires that the chromium remain bound to the DNA. Allowing the chromium to dissociate from the template by long-term storage in the absence of free chromium restores both survival and mutagenicity to control levels, unless the mutations are fixed by replication of the chromium-treated template in vitro, prior to transfection. Under these conditions, the chromium is bound to the template by ionic interaction but is not readily available for chelation by EDTA (Table I).

We have previously shown that low concentrations of $CrCl_3$ (1–5 μM) can increase synthesis by pol I–KF into and through the hairpin sequence of M13mp7 (Snow & Xu, 1989) and can also increase the rate of primer extension of eukaryotic polymerase α (Snow & Xu, 1989) and polymerase β (Snow, 1991). In the present study using single-stranded M13mp2 as the template, we show that low (1–5 μM) concentrations of chromium also increase pol I–KF processivity up to 4.7-fold. Chromium(III) can also increase the amount of nonspecific transcription by RNA polymerase in vitro (Okada et al., 1981, 1983; Ohba et al., 1986). This suggests that $CrCl_3$ has the ability to increase the strength of polymerase–template DNA binding, in general, so that either the rate or the extent of nucleotide incorporation is increased during each initiation and termination cycle.

As with increased nucleotide incorporation, increased polymerase processivity is caused by Cr(III) bound to the DNA template, while somewhat higher concentrations of aqueous Cr(III) ions inhibit DNA polymerase I–KF (not shown). This result is very similar to the preferential binding of micromolar concentrations of $MnCl_2$ to DNA and to the effects of manganese on polymerase processivity and DNA replication fidelity (Beckman et al., 1985). Increased binding by DNA polymerase due to Cr(III) may also result in a decrease in polymerase proofreading ability and may increase bypass across DNA lesions (Snow, 1991). An increase in DNA replication infidelity during DNA replication across oxidative damage produced during the intracellular reduction of chromium, for example, could account in part for the high mutagenic and carcinogenic potential of chromate in vivo. Alternatively, at high concentrations, chromium(III) is known to bind preferentially to guanine-containing DNA (Tsapakos & Wetterhahn, 1983; Wolf et al., 1989) and may either cause mispairing directly or promote oxidation (Aiyar et al., 1989) or depurination (Schaaper et al., 1987) of the guanine, causing subsequent mispairing of the modified (or missing) base. However, the concentrations of chromium necessary to cause depurination are, in our hands, inhibitory to replication.

$CrCl_3$ decreases DNA polymerase fidelity 2-fold during DNA replication in vitro (Table IV). This result, obtained using an M13mp2 template under conditions that enhance polymerase processivity, is similar to the results obtained using the *ϕX174* mutagenesis assay which measures polymerase incorporation fidelity (base substitution mutagenesis) at a single site in the *ϕX174* genome (Schaaper et al., 1987). Phage survival and fidelity of DNA replication in vivo on a chromium(III)-treated template are similarly decreased (Table IV; Snow & Xu, 1989; Snow, 1991). The fact that both of these forms of genotoxicity are fixed during replication and that the genotoxicity is eliminated by removal of the chromium prior to replication implies that the increased mutagenesis occurs as the result of replication on the Cr(III)-bound DNA and that replication under conditions that promote increased processivity is less faithful. It is also apparent that Cr(III) does not produce lasting DNA damage and that the increased mutagenesis and toxicity are not due to the production of DNA lesions, such as apurinic sites or oxidative damage. Thus, our data, including the relatively low (2-fold) increase in mutagenesis due to chromium(III), are consistent with an effect on polymerase processivity due to increased (nonspecific) binding of the polymerase to the template DNA. The stronger polymerase-template binding, in turn, contributes to decreased fidelity in subtle ways, including, as we have shown in more recent data not presented here, an increased K_m for the dNTP substrate. (Again, this effect is similar to that seen with manganese.)

Consistent with reports from other laboratories (Bianchi et al., 1983), it was observed that $CrCl_3$ can also induce dose-dependent DNA–DNA cross-linking. Such cross-links are expected to stop DNA replication (Bernges & Holler, 1988; Villani et al., 1988) and may correlate with the toxicity of $CrCl_3$ in *E. coli* mutagenesis assays (Snow & Xu, 1989; Snow, 1991). DNA–protein cross-linking is also mediated by Cr(III) in vitro (Cohen et al., 1990) and might be involved in the inhibition of DNA replication at higher concentrations of chromium.

The role of chromium(III) species in chromium genotoxicity in vivo relative to the role of chromium-mediated cross-links or oxidative damage is far from clear; however, the effects of chromium(III) are shown here to occur at extremely low concentrations and could be significant at the low doses of chromium(III) that may result from environmental exposure to chromate.

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Sulfhydryl-Selective Fluorescence Labeling of Lipoprotein(a) Reveals Evidence for One Single Disulfide Linkage between Apoproteins(a) and B-100†

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ABSTRACT: Human lipoprotein(a) and low-density lipoprotein were labeled with two different sulfhydryl-selective fluorescence markers. The hydrophilic fluorophore lucifer yellow iodoacetamide and the apolar compound 6-acryloyl-2-(dimethylamino)naphthalene were used to derivatize free -SH groups in the lipoproteins. Three sulfhydryls could be detected in low-density lipoprotein, whereas only two cysteines were available in lipoprotein(a). One of the three -SH groups in low-density lipoprotein was shown to be located in close proximity to the particle surface. We suggest that this surface-exposed cysteine of apoprotein B-100 serves as a component for the disulfide linkage to apoprotein(a) in lipoprotein(a).

Lipoprotein(a) [Lp(a)][1] represents a class of human plasma lipoprotein particles associated with premature coronary heart disease and stroke. When the plasma level of Lp(a) is above 30 mg·dL^{-1}, the relative risk of coronary atherosclerosis rises about 2-fold (Kostner et al., 1981; Armstrong et al., 1986; Dahlen et al., 1986). The molecular basis of these findings is still under investigation.

Lipoprotein(a) consists of two polypeptide chains. One of these is identical with apolipoprotein B-100 (apo B), the

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[1] Abbreviations: Lp(a), lipoprotein(a); LDL, low-density lipoprotein; apo(a), apolipoprotein(a); apo B, apolipoprotein B-100; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; LY, lucifer yellow iodoacetamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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