

# Investigating the Nature of the Chromium-DNA Complex

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*Past experiments have shown that some chromium compounds are potent mutagens and carcinogens. Although these Cr(III)-DNA complexes have been studied intensely for the past fifteen years, there are virtually no data on their molecular level behavior. The purpose of this scientific study was to explore the experimental conditions in which to study the nature of interaction between Cr(III) and DNA at a molecular level, especially at the guanine base, that results when DNA is exposed to Cr(III) directly. Such knowledge is essential to understanding and determining the potential of these complexes to lead to deleterious effects. Results suggest that the chosen experimental conditions allow interaction between chromium and DNA to occur and be detected; however, more research is necessary to characterize these interactions.*

## Introduction

Workers in a number of occupations experience exposure to chromium (Cr) compounds. The highest exposure to the toxic Cr(VI) form is usually detected in the chrome plating industry, among chromate production workers and stainless steel welders. The most common form of cancer seen in these people is squamous cell carcinoma of the lung. [1] Chromium(VI) complexes, most notably including chromate,  $\text{CrO}_4^{2-}$ , dichromate,  $\text{Cr}_2\text{O}_7^{2-}$ , are well known carcinogens and mutagens when inhaled. The potential for harmful effects from orally consumed Cr(VI) species is an actively debated area of research. The mechanism by which Cr(VI) causes chromosomal damage leading to mutations and cancer is also a matter of debate, despite decades of research. Part of the difficulty lies in the number of potential mechanisms for damaging DNA. In addition to redox damage brought on by Cr(VI), the generation of Cr(III)

can also be deleterious. Cr(III) forms almost exclusively octahedral complexes, generally with hard oxygen- and nitrogen- based ligands. If this occurs in the proximity of DNA, the chromic center could coordinate to phosphate groups and bases of DNA. Binding of a Cr atom with two ligands leads to inter- or intra-strand crosslinks or, given the presence of other biomolecules, crosslinks between DNA and other molecules. Cr(III) species are generally regarded as safe and are present in foods at very low concentration. “Free” Cr(III) does not readily cross the membrane barrier into cells, as opposed to chromate, which is readily transported across the membrane into a cell.

Understanding the relative significance of oxidative damage versus the formation of chromium-DNA adducts is important to determining the major pathway(s) leading to mutations and, ultimately, cancer from Cr(VI) exposure. So, although Cr(III) remains outside the cell, Cr(VI) is taken up and is eventually reduced to Cr(III) inside the cell where it can interact with cellular DNA. [2, 3] Oxidation appears to occur primarily at the guanine bases, the most readily oxidized of the bases. The site is susceptible to further oxidation, resulting in the formation of spiroiminodihydantoin and guanidinohydantoin. [4] Incorrect DNA repair of these lesions can lead to mutations. Mutations in certain cancer-associated genes of the cell are believed to be the basis for initiating cancer.

Research by one of the leading contributors to DNA and chromium research, Anatoly Zhitkovich, involves the characterization of molecular mechanisms responsible for cell death and mutagenicity of DNA-reactive carcinogenic chemicals and anticancer drugs. In his published works, Zhitkovich often includes information on chromium in the environment. In “Importance of Chromium-DNA Adducts in Mutagenicity and Toxicity of Chromium(VI),” Zhitkovich examines the potential role of chromium-DNA adducts. In this article, he states that several million industrial workers worldwide are potentially exposed to Chromium (Cr) and Cr-containing compounds. Environmental contamination with Cr has also become a significant concern due to continuous industrial emissions and the presence of heavily contaminated sites in the vicinity of residential areas.

It has long been known that chromium-exposed cells contain abundant Cr(III)-DNA adducts, although their chemical nature and mutagenic potential remain unknown. Zhitkovich et al. have recently found that the treatment of cells with chromate leads to the formation of stable ternary Cr-DNA adducts involving amino acid ligands. [5] Ternary cysteine-Cr(III)-DNA adducts were identified as the principal mutagenic lesions. Cr(VI) itself is unreactive toward DNA at physiological pH and requires reductive activation to produce DNA-damaging species. The most important

intracellular reducers of Cr(VI) are ascorbate and nonprotein thiols such as glutathione and cysteine (Cys). [6] Cysteine is considered the primary thiol because it has the highest rate of Cr(VI) reduction. [7] However, this proposed ternary structure for the Cr-DNA adduct is based on essentially no spectroscopic or other evidence at a molecular level.

The binding of Cr(III) to DNA has been known for over fifteen years to result in increases of the number of nucleotide misincorporations by polymerases in vitro [8] and recently was shown to also result in polymerase arrest. [2, 3] The amount of interstrand DNA crosslinks appears to correlate with the degree of polymerase inhibition [3, 9], while polymerase inhibition does not correlate with the degree of oxidative damage. [6] Hence, interstrand crosslinks probably promote a terminal cell fate such as apoptosis and terminal growth arrest. [10] This area has been thoroughly reviewed by Zhitkovich. [1]

Much more work is needed to understand the base-specificity of these lesions and how the phosphate backbone is incorporated into the Cr-DNA adducts. The sugar-phosphate backbone of DNA is one of the most common sites of damage by oxidizing species. [11] The lack of evidence has been addressed by past studies, but they have all lacked the binding restrictions of double-stranded DNA and thus have failed to provide much of value in understanding how Cr(III) might coordinate within a cell. The understanding of the nature of these crosslinks is simply not likely to advance significantly without the aid of spectroscopic and related studies that can explore the chemistry of the complex formation.

The aim of this project was to explore the nature of the interaction in double-stranded DNA between Cr(III) and the components of the DNA at a molecular level. Chromium(III) ions are expected to bind to guanine within a double-stranded DNA complex. However, in order to find any evidence that chromium is binding to DNA, the experimental conditions in which this experiment will take place are of primary concern. This report will include the steps taken to determine the proper conditions that will be used for future Cr(III) binding studies.

## Materials and Methods

### 2.1 Design and Synthesis of Custom DNA Sequences

Many factors were taken into consideration when designing a sequence of DNA to work with. First, a 14 base-pair sequence was designed because of its estimated  $T_m$  of 36°C and due to its self complementary ordering. There is one guanine site on each strand, but because they are

self complementary, there will appear to be only one guanine site in spectroscopic studies.

Strand A 5'-TAT TGT TAA CAA TA-3'  
Strand B 3'-ATA ACA ATT GTT AT-5'

Although this duplex was initially thought to be workable, it did not produce the needed results during testing and was not considered for further use.

We decided to change the sequence based on the notion that Cr(III) binds to DNA at specific sites (guanines, specifically, NGG sequences, see reference 1). A sequence was prepared that possesses one putative binding site. The limitation of the number of unique binding sites should simplify characterization of the DNA-Cr complexes. The DNA sequence was carefully designed to produce a duplex that is stable at room temperature and slightly elevated temperatures. The following 15-mer duplex was chosen because it has only one putative GG chromium binding site and was predicted to have a  $T_m$  of 39 °C (at 4  $\mu$ M oligo and 100 mM NaCl concentrations, pH = 6).

Strand A 5'-AAT TAA TGG TTA ATA-3'  
Strand B 3'-TTA ATT ACC AAT TAT-5'

These two non-self-complementary sequences were prepared separately. Syntheses of the oligonucleotides were performed using solid phase methods on an ABI 391 DNA Synthesizer. The resulting oligonucleotides were purified via the cartridge purification method. Following lyophilization, aqueous stock solutions of each oligonucleotide were made, and the concentrations of each were determined by measuring the absorbance of diluted samples at 260 nm.

Absorbance was measured for both strands at wavelength 260;

$$A_{\text{strand A}} = 54.659$$

$$A_{\text{strand B}} = 50.356$$

Then concentration calculations were done using Beer's Law and estimated extinction coefficients;

$$c_{\text{A strand}} = 2.97 \times 10^{-4} \text{ M}$$

$$c_{\text{B strand}} = 2.98 \times 10^{-4} \text{ M}$$

Finally, using the  $M_1V_1=M_2V_2$  rule, the volume of DNA to be used in a 200  $\mu\text{L}$  solution of 4  $\mu\text{M}$  DNA concentration was determined to be 2.7  $\mu\text{L}$  of each strand.

## 2.2 Buffer Selection

Many factors were considered in choosing the appropriate buffer solution. To avoid competition of the buffer salt with metal binding sites on DNA, strongly coordinating buffers such as phosphate (PES) or TRIS were to be avoided. In addition, the rate of ligand exchange on chromium centers can be increased by the use of acidic conditions. Thus, 50 mM MES (morpholineethanesulfonic acid) at pH 6 was chosen; a 10X stock solution was prepared by adding aqueous sodium hydroxide to a 50 mM solution of MES in double-deionized water ( $\text{ddH}_2\text{O}$ ) until the solution was pH 6. In order to improve the stability of the DNA duplexes, a magnesium salt (0.1 mM  $\text{MgCl}_2$ ) was added to the MES solution in some experiments. The divalent cation magnesium was believed to have been competing with chromium by binding to the DNA and not being easily removed.

Therefore, we switched to sodium salt because it has a lesser charge and can be removed from binding to DNA more easily than magnesium. A 200mM concentration of NaCl was added to the 50 mM MES buffer. The resulting  $T_m$  was 41.7  $^\circ\text{C}$  which was stable enough to continue the study. The next step was to add chromium to the buffer salt in varying concentrations. We chose to start with 0.4  $\mu\text{M}$ , 4.4  $\mu\text{M}$ , and 24.4  $\mu\text{M}$  Cr(III) concentrations. We used thermal denaturation studies to observe whether Cr(III) was interacting with DNA in the presence of  $\text{Na}^+$ . Any change in the melting temperature suggests the existence of an interaction that affects the structure and stability of the DNA complex.

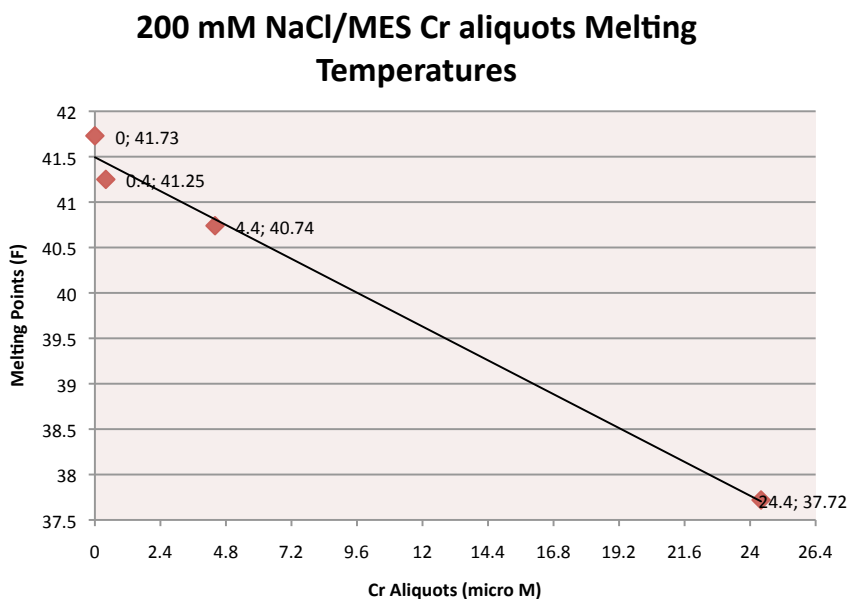
## 2.3 Thermal Denaturation

The stabilities of the DNA duplexes alone and in the presence of added metal ions [Cr(III) or Co(III)] were determined using UV thermal denaturation experiments. For each run, a 200.0  $\mu\text{L}$  solution was prepared by combination of each DNA solution (2.7  $\mu\text{L}$  of each strand) with 20.0  $\mu\text{L}$  of 10X MES/ $\text{Na}^+$  buffer solution, and 174.6  $\mu\text{L}$  of  $\text{ddH}_2\text{O}$ . The solutions were placed in a quartz cuvette and overlaid with mineral oil. The absorbance of the solutions was monitored at 260 nm while the temperature was increased from 25 $\rightarrow$ 75  $^\circ\text{C}$ . The absorbance data were fit using Meltwin software; the  $T_m$  of each curve represents the temperature at which half of the duplex DNA molecules had “melted” into single strands. After  $T_m$  was

determined for DNA in buffer salt solution with no chromium ( 41.7 °C) (see Appendix, Fig. A), we added a simple chromic salt,  $\text{CrCl}_3$  [a source of  $\text{Cr(III)}$ ], to the 200.0  $\mu\text{L}$  DNA/ MES/ $\text{Na}^+$  solution. Increasing aliquots of  $\text{CrCl}_3$  were added to the DNA and buffer salt solution. This was followed by thermal denaturation measurements for each solution ranging from 0.4  $\mu\text{M}$ , 4.4  $\mu\text{M}$ , and 24.4  $\mu\text{M}$ .

## Results and Discussion

The effect of the addition of  $\text{CrCl}_3$  to DNA in MES/ $\text{NaCl}$  buffer was assayed by UV/vis thermal denaturation. The results are summarized in Fig 1.



*Fig. 1 Cr additions and resulting Melting Point Temperature*

The addition of 0.4 and 4.4  $\mu\text{M}$   $\text{Cr(III)}$  resulted in a small decrease in the stability of the DNA duplex (see Appendix, Fig. B and Fig. C). However, at the highest chromium concentration that was studied (24.4  $\mu\text{M}$ ) (see Appendix, Fig. D), a significant decrease in the stability of the DNA double helix was observed. Because  $\text{Cr}^{3+}$  ions have a greater charge than  $\text{Na}^{1+}$  ions, the duplex should be more stabilized if the Cr was interacting solely with the negatively charged phosphates. Thus, the large decrease in stability suggests that a significant structural change is occurring upon

Cr binding. The nature of this change cannot be determined from these experiments; thus, additional studies will be necessary to understand how the Cr is interacting with the DNA.

### Additional Studies

Further research will include pursuing more chromium with NaCl concentration studies in order to produce a broader range of melting temperature data. Afterwards, there will be structural studies involving NMR and EPR spectroscopy (with the addition of labeled and modified bases) and equilibrium dialysis to provide further binding data. DNA targets with unique chromium binding sites can be probed using labels introduced near or at the site of coordination. Using label  $^{15}\text{N-G}$ , an NMR-active isotope (unlike the  $^{14}\text{N}$  isotope usually found in nature), will facilitate spectroscopic characterization of chromium binding to DNA with the help of NMR and EPR spectroscopic experiments. With the ability to synthesize oligonucleotides containing a single unique Cr binding site (NGG), it becomes possible to extend this work toward a survey of structure-activity relationships. In particular, the introduction of modified guanine residues into synthetic oligonucleotide duplexes can provide direct evidence for the participation of those residues in Cr binding. Since most metals interact with the N-7 on the guanine, replacement of this nitrogen atom with a C-H group will eliminate the possibility of coordination by this residue. Thus, the role of each guanine within a chromium-binding duplex can be assayed by examining the chromium binding of a modified DNA. The 7-deaza-2'-deoxyguanosine (7-deazaG) residue can be incorporated using commercially available phosphoramidite reagent (Glen Research). The binding constant for chromium binding to DNA will be determined by equilibrium dialysis. Aliquots of a mixture of non-radioactive "cold" Cr and radioactive  $^{51}\text{Cr}$  will be added to a solution of DNA in a MES buffer which poorly coordinates metal ions in an Amicon ultrafiltration unit (with a suitable molecular weight cutoff membrane) at  $4^\circ\text{C}$ . After addition of each aliquot, the solution will be slowly stirred until equilibrium is reached. The ultrafiltration unit is pressurized and effluent is collected. The content of free chromium, not bound to DNA, in the effluent will be determined by gamma counting. Gamma counting measures radioactivity in free chromium solution (effluent). Using these measurements, the amount of Cr(III) bound to DNA can be calculated. These data will verify that Cr(III) is, in fact, binding to DNA. The binding constant for Cr binding to the DNA will be analyzed using the appropriate equilibrium and mass balances equations and also by the Hill method. [12] These studies will be performed in three different manners in order to determine the extent to which Cr(III) is presented to the DNA.

## Acknowledgments

Dale Becker greatly appreciates the support of The Ronald E. McNair Post-Baccalaureate Achievement Program and Woski labs.

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### Appendix

#### Melt Curve Results from Meltwin

Fig. A Melting Curve of DNA with no Chromium

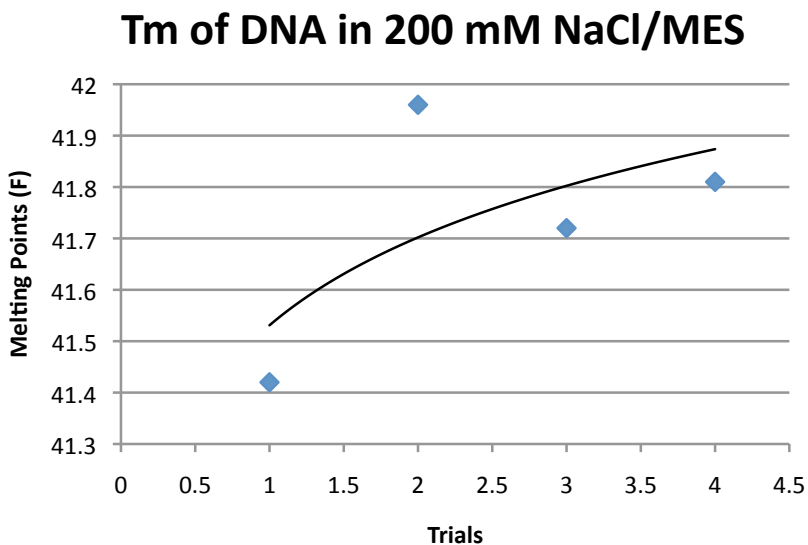


Fig. B Melting Curve of DNA with 0.4 micM Cr

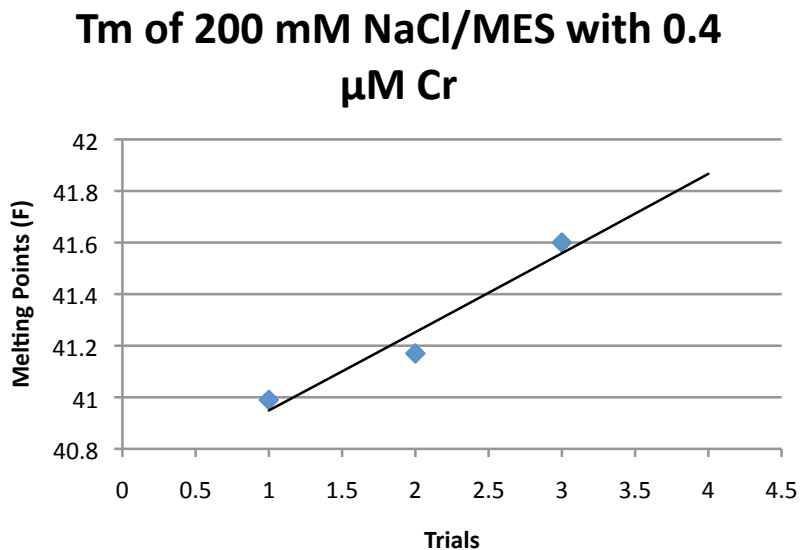
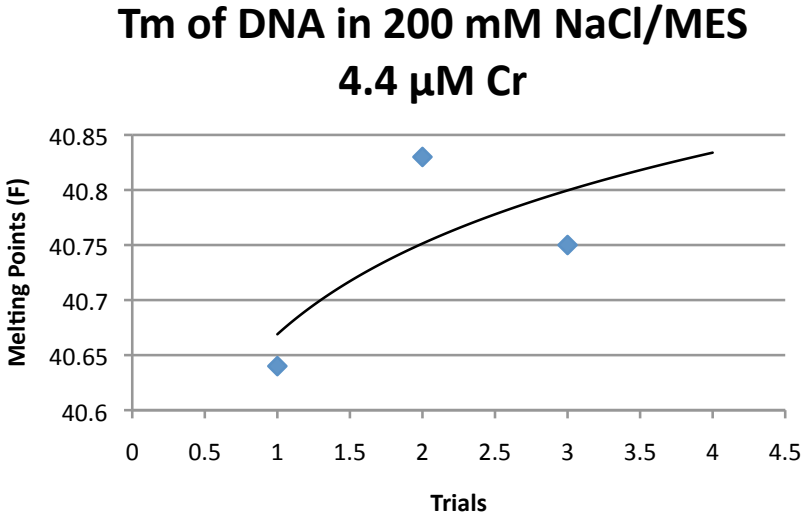


Fig. C Melting Curve of DNA with 4.4  $\mu\text{M}$  CrFig. D Melting Curve of DNA with 24.4  $\mu\text{M}$  Cr