DNA-Protein Complexes Induced by Chromate and Other Carcinogens

by Max Costa*

DNA-protein complexes induced in intact Chinese hamster ovary cells by chromate have been isolated, analyzed, and compared with those induced by cis-platinum, ultraviolet light, and formaldehyde. Actin has been identified as one of the major proteins complexed to DNA by chromate based upon its molecular weight, isoelectric point, positive reaction with an actin polyclonal antibody, and proteolytic mapping. Chromate and cis-platinum both complex proteins of similar molecular weight and isoelectric points, and these complexes can be disrupted by chelating agents and sulfhydryl reducing agents, suggesting that the metal itself is participating in binding rather than having a catalytic or indirect role (i.e., oxygen radicals). In contrast, formaldehyde complexed histones to the DNA, and these complexes were not disrupted by chelating or reducing agents. An antiserum raised to chromate-induced DNA-protein complexes reacted primarily with 97,000 kDa protein that did not silver stain. Slot blots, as well as Western blots, were used to detect formation of p97 DNA crosslinks. This protein was complexed to the DNA by all four agents studied.

Introduction

Chromate compounds are well established as human carcinogens and are potently genotoxic at a number of in vitro and in vivo end points (1-4). The most toxicologically active form of chromium is the hexavalent oxidation state, which at physiological pH exists as an oxyanion (3,4). Oxyanions, such as chromate, are actively transported into cells by the sulfate transport system, resulting in a high intracellular accumulation of chromium. The hexavalent chromium reacts with a number of reducing agents in cells, including glutathione, hydrogen peroxide, microsomal enzymes, and ribonucleotides. Chromium(VI) is eventually reduced to the kinetically inert and stable trivalent form (5,6). During its reduction, intermediate oxidation states of chromium are formed, and the reduction process itself, as well as the formation of these intermediate states of chromium, is thought to be important in chromium genotoxicity.

The hexavalent form of chromium has been shown to produce a variety of lesions in the DNA of mammalian cells, including single-strand breaks, alkali-labile sites, DNA-DNA, and DNA-protein crosslinks (7-9). Chromate is a very broad-acting genotoxic agent, as evidenced by its ability to directly induce lesions as well as to indirectly generate oxygen radicals and reactive intermediates (6). In fact, chromate is positive in almost every genotoxicity assay in which it has been tested.

DNA-protein complexes produced by chromium compounds have not been well studied in the past. These lesions, unlike the strand breaks and other DNA lesions that are readily repaired, are relatively persistent in the cell (8,9). Due to their lack of repair, DNA-protein complexes are likely to be present during DNA replication and may constitute a block to the replication machinery. Deletions which may result could be important in chromium carcinogenesis if the deleted DNA sequences code for a tumor-suppressor gene or are involved in the regulation of these important genes. The persistence of the chromate-induced DNA-protein complex makes it an interesting lesion to study as a marker for assessing chromate exposures. Due to the diversity of specific chemical agents that produce these lesions, it is likely that many different proteins can be complexed with DNA. In this regard, the specific proteins complexed with DNA and the nature of their bonding may produce a “fingerprint” from which the chemical nature of exposure might be deduced.

Neither the nature of DNA-protein complexes nor the number of agents that can cause the formation of this type of lesion has been well studied, primarily because the methodology to examine DNA-protein complexes is limited. The primary method currently in use is alkaline elution (10), which detects these lesions indirectly and cannot characterize their composition.

In the present study, we have the examined DNA-
protein complexes formed by chromate, formaldehyde, ultraviolet (UV) light, and cis-platinum, agents which have previously been shown to produce these lesions (11). We describe methods for isolating and characterizing these complexes with respect to the stability of the protein crosslinked to the DNA. We have also studied the specific proteins that are crosslinked to the DNA and have used immunological methods to detect the formation of DNA-protein complexes.

**Methods**

Chinese hamster ovary cells were treated in culture with chromate compounds as well as UV, formaldehyde, and cis-platinum by methods previously described (12). Following treatment, DNA-protein complexes were isolated from intact cells, as well as from isolated nuclei, by sodium dodecyl sulfate (SDS) lysis followed by

**Figure 1.** Analysis of crosslink stability with chemicals and enzymes. DNA and protein components of purified crosslinks were recovered from untreated cells and from cells exposed to 100 μM cis-platinum, 6 mM formaldehyde, or 200 μM potassium chromate. Samples were treated with the various enzymatic or chemical agents listed in the figure prior to centrifugation to determine whether protein or DNA recovery was sensitive to these disruptive agents. Reproduced with permission from *Molecular Toxicology* (12).

**Figure 2.** Nonequilibrium two-dimensional electrophoresis of DNA-protein complexes isolated from nuclei of chromate-treated cells. DNA-protein complexes were isolated from nuclei of control or potassium chromate-treated cells. (A) Two-dimensional gel of the control DNA-protein complexes. (B,C) Proteins dissociated from DNA-protein complexes generated by treating cells with 33 and 100 μM potassium chromate, respectively, prior to isolation. For each gel, 200μg DNA was loaded. Reproduced with permission from *Molecular Carcinogenesis* (18).
ultracentrifugation collection of the DNA complexed with protein, as previously described (12–14). The DNA-containing complexed proteins were washed extensively with SDS, urea, and high salt to dissociate noncovalently bound proteins from the DNA (13). The proteins covalently complexed to the DNA could be released by degrading the DNA with DNase or, in the case of metal-induced complexes, by treatment with specific agents that are known to dissociate proteins from the DNA. The mercaptoethanol present during protein separation by electrophoresis was sufficient in the case of chromium and cis-platinum-induced DNA-protein complexes to cause this dissociation. In contrast, complexes formed by formaldehyde or UV light were not affected by the presence of mercaptoethanol and required treatment with DNase in order to resolve the complexed proteins in polyacrylamide gels (12,14).

An antiserum to DNA-protein complexes was raised in rabbits, as previously described (13). Western blotting and slot blotting were also performed, as previously described (13,14).

**Results**

Figure 1 illustrates the stability of protein and DNA components of DNA-protein complexes formed by chromate, cis-platinum, and formaldehyde in intact Chinese hamster ovary cells. Figure 1 also characterizes the background of DNA-protein complexes that were present in untreated cells. Treatment of the DNA-protein complexes with DNase caused decreased recovery of the DNA component in parallel with the loss of protein. This important control, which is routinely performed in these experiments, illustrates that protein recovery depends upon the integrity of the DNA. This indicates that the centrifugation recovery of protein is DNA-complex dependent and is not an artifact of altered protein solubility due to metal binding. Figure 1 also shows the effect of RNase treatment, which did not significantly reduce recovery of either component of the complex. Protease K, however, eliminated the protein recovery.

As expected, EDTA treatment did not affect recovery of DNA; however, with metal-induced complexes, the recovery of the protein component was substantially reduced by EDTA (Fig. 1). In contrast, recovery of this protein component from protein-DNA complex formed by formaldehyde or those present in the background of untreated cells were not affected by EDTA. Similarly to EDTA, thiourea also dissociated the protein-DNA complexes induced by metals, but not those present in untreated cells or those formed by formaldehyde. These results illustrate that a substantial portion of the DNA-protein complexes formed by cis-platinum or chromium involved direct participation of the metals in the complex formation. Additionally, the effect of EDTA on chromate complexes suggests that the trivalent form of chromium is involved in the DNA-protein complex, since this is the only form of chromium that can be chelated by EDTA.

Figure 2 examines the protein components of the DNA-protein complex induced by chromate. Figure 2A shows the absence of proteins associated with 200 μg of DNA from untreated cells. Following treatment with chromate at 33 μM, there were three major proteins
Figure 4. Two-dimensional gel electrophoresis of nuclear protein lysate and formaldehyde-induced crosslinks. (A) Silver-stained, nonequilibrium two-dimensional gel of 40 μg of a nuclear protein lysate run for approximately 1200 V/hr. The H denotes the position of the core histones. (B) Silver-stained gel following electrophoresis of two A38 units of crosslink material from untreated cells; no proteins were detectable under these conditions or when control material was digested with DNase I before electrophoresis (not shown). (C, D) DNA-protein crosslinks isolated from cells treated with 13 mM HCHO for 1.5 hr. The gel in (C) shows no protein has entered the gel, while histones are visible in (D) (labeled H). The crosslinks in (D) were digested with DNase I prior to electrophoresis. Contaminating DNase I proteins are indicated by d. Reproduced with permission from Molecular Toxicology (12).
complexed with DNA. Enhanced levels of these same proteins, plus a number of additional proteins, were found following 100 μM chromium treatment (Fig. 2C).

Figure 3 illustrates that the proteins complexed to DNA by chromate were released during electrophoresis without prior treatment with DNase or RNase. Degradation of DNA did not enhance the number of proteins that were resolved in the gels, suggesting that the conditions of electrophoresis, as well as the presence of mercaptoethanol during electrophoresis, were sufficient to release the complexed protein from the DNA. cis-Platinum complexed proteins of similar molecular weight and isoelectric point to those found with chromate.

Figure 4 examines the proteins that were complexed to the DNA by formaldehyde. In Figure 4A, a nuclear protein lysate analyzed by two-dimensional gel electrophoresis shows the location of histone proteins. Figure 4B illustrates a silver-stained gel used to analyze crosslinked material from untreated cells. No proteins were be detected in untreated cells with the DNA load that was utilized in this gel. Figures 4C and 4D shows the DNA-protein crosslinks at a similar load of DNA from cells treated with formaldehyde. In Figure 4C, DNA-protein complexes were not treated with DNase prior to analysis on the gel, whereas in Figure 4D the DNA was previously degraded. Figure 4D demonstrates that histones were the predominant proteins that were complexed to the DNA by formaldehyde. The spots identified in the figure by the letter d are those proteins that were introduced by the addition of DNase enzyme.

One of the major proteins complexed to the DNA by cis-platinum and chromate was a 45,000-Da protein with the same molecular weight and isoelectric point as actin. In fact, a purified actin standard migrates to the same location in the gels as this p45 protein. We have recently confirmed the identity of this protein as actin by its positive reaction with an actin polyclonal antibody and by V8 protease mapping (data not shown).

Another approach taken to study DNA-protein complexes uses an antiserum raised by rabbits against the chromate-induced DNA-protein complexes formed in Chinese hamster ovary cells (13). Figure 5 illustrates the reactivity of this antiserum toward a Chinese hamster nuclear protein lysate. Figure 5A shows the silver staining of the nuclear protein lysate, while Figure 5B is a Western blot reacted with the DNA-protein complexed antiserum and 125I protein A. As depicted in Figure 5B, the antibody recognized a 95,000-Da protein, which is not silver stained, in the left-hand side of Figure 5A. Recently, more accurate molecular weight determinations of this protein confirm a 97,000 Da molecular weight. The lack of silver staining of this protein, however, precluded its detection when the proteins of chromate-induced DNA-protein complexes were initially studied (Figs. 2, 3). Figure 6 illustrates a one-dimensional gel of DNA-protein complexes induced by chromate in Chinese hamster ovary cells whose proteins were radiolabeled with 35S-methionine prior to chromate treatment. In this Figure 6, a 35S-methionine radiolabeled protein is seen complexed to the DNA in the molecular weight range of p97.
Figure 9 illustrates the use of the same DNA-protein complex antibody to detect complexes formed by various agents in a slot-blot protocol. In Figure 9, the antiserum reacted with DNA-protein complexes from cells treated with cis-platinum, chromate, and formaldehyde. Figure 9 illustrates antibody detection of those complexes formed by all three chemical agents, including untreated cellular DNA at high DNA loading, but not at the lower level of 5 μg DNA. As previously noted in Figure 8, the antiserum reacted primarily with the protein component of the complex. This is supported by Figure 9, which shows protease K eliminated the antibody reaction. In contrast, treatment with DNase did not substantially affect the reactivity of this antibody, although there was a slight reduction in the background reactivity of untreated cells.

Discussion

DNA-protein complexes formed by metals have previously been shown to involve the direct participation of the metal in the complex (12). In the case of chromate, the trivalent form of chromium was implicated in the formation of these complexes (12). One possible mechanism for complex formation involves the trivalent chromium-mediated reaction of the N7 position of guanine with a sulfur of the amino acid cysteine. This model is derived from data regarding the sensitivity of the complex to sulfhydryl reducing agents, the effects of EDTA on the complex, and the binding constants of chromium for various ligands.

Chromate and cis-platinum DNA-protein complexes were very similar in their protein composition, since two-dimensional gels separating these proteins are virtually indistinguishable. One of these proteins has been positively identified as actin, by virtue of its identical molecular weight and isoelectric point as actin, the reactivity of the p45 protein from chromate-induced complexes with an actin polyclonal antibody, and the proteolytic map of this protein, which was similar to that of bovine muscle and human platelet actin (data not shown).

Histones were complexed to the DNA by formaldehyde, but were not complexed by the other agents examined in this study. This is probably due to the high reactivity of formaldehyde at the ε-amino group of lysine. The proteins complexed to the DNA by chromate and cis-platinum are similar in molecular weight and isoelectric points to those proteins which are present at very high concentration in the nuclear matrix. Association of DNA with the nuclear matrix is known to be important in DNA repair and transcription. Actin, which is known to be an important component of transcriptional complexes and is involved in DNA repair, is also an abundant protein in the nuclear matrix (15,16). The metal-induced complexing of actin to the DNA is of interest in light of recent studies demonstrating that chromate can selectively inhibit inducible gene expression (17).
The rabbit antiserum raised to chromate-induced DNA-protein complexes reacted primarily with a protein that was not initially detected on the silver-stained gels in which the DNA-complexed proteins were analyzed. This antiserum reacted with a 97,000 Da protein that was apparently complexed to the DNA by formaldehyde, cis-platinum, UV light, and chromate. This protein is very interesting for future studies due to its ability to be complexed with DNA by a variety of chemical agents and due to its lack of silver staining. The antiserum to p97 has also been used to detect the formation of DNA-protein complexes by various chemical agents on slot blots (14). This is a simple method which appears to be useful in monitoring the formation of these complexes. Currently, these methods are being applied to the detection of chromate-induced DNA-protein crosslinks formed in vivo, which may be sensitive biomarkers of chromate exposure.

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REFERENCES

1. Sunderman, F. W. Carcinogenicity and mutagenicity of some metals and their compounds. In: IARC Monographs: Environmental Carcinogens—Selected Methods of Analysis (I. K. O’Neill, P. Schuller, and L. Fishbein, Eds.), Oxford University Press, Oxford, England, 1986, pp. 17–43.

2. Hertel, R. F. Sources of exposure and biological effects of chromium. In: IARC Monographs: Environmental Carcinogens—Selected Methods of Analysis (I. K. O’Neill, P. Schuller, and L. Fishbein, Eds.), Oxford University Press, Oxford, England, 1986, pp. 63–77.

3. Levis, A. G., and Bianchi, V. Mutagenic and cytogenetic effects of chromium compounds. In: Biological and Environmental Aspects of Chromium (S. Langard, Ed.), Elsevier Biomedical Press, Amsterdam, 1982, pp. 171–208.
Heck, J. D., and Costa, M. In vitro assessment of the toxicity of metal compounds. I. Mammalian cell transformation. Biol. Trace Elem. Res. 4: 71–82 (1982).

Aslan, P., Beltrame, M., and Tomasi, A. Intracellular chromium reduction. Biochim. Biophys. Acta 963: 10–15 (1987).

Connett, P. H., and Wetterhahn, K. Metabolism of the carcinogen chromate by cellular constituents. Structure Bond. 54: 93–124 (1989).

Tsapakos, M. J., Hampton, T. H., and Wetterhahn, K. E. Chromium(VI)-induced DNA lesions and chromium distribution in rat kidney, liver, and lung. Cancer Res. 43: 5662–5667 (1983).

Sugiyama, M., Wang, X. W., and Costa, M. Comparison of DNA lesions and cytotoxicity induced by calcium chromate in human, mouse, and hamster cell lines. Cancer Res. 46: 4547–4551 (1986).

Oleinick, N. L., Chiu, S., Ramakrishnan, N., and Xue, L. The formation, identification, and significance of DNA-protein cross-links in mammalian cells. Br. J. Cancer 55: 135–140 (1987).

Miller, C. A., III, and Costa, M. Analysis of proteins crosslinked to DNA after treatment of cells with formaldehyde, chromate, and cis-diamminedichloroplatinum(II). Mol. Toxicol. 2: 11–26 (1989).

Miller, C. A., III, and Costa, M. Immunological detection of DNA-protein complexes induced by chromate. Carcinogenesis 10(4): 667–672 (1989).

Miller, C. A., III, and Costa, M. Immunodetection of DNA-protein crosslinks by slot blotting. Mutat. Res. 234: 97–106 (1990).

Pienta, K. J., Partin, A. W., and Coffey, D. S. Cancer as a disease of DNA organization and dynamic cell structure. Cancer Res. 49: 2525–2532 (1989).

Eastman, A. Characterization of the adducts produced in DNA by cis-diamminedichloroplatinum(II) and cis-dichloro(ethylenediamine) platinum(II). Biochemistry 22: 3927–3933 (1983).

Hamilton, J. W., and Wetterhahn, K. E. Differential effects of chromium(VI) on constitutive and inducible gene expression in chick embryo liver in vivo and correlation with chromium(VI)-induced DNA damage. Mol. Carcinog. 2: 267–268 (1989).

Miller, C. A., III, and Costa, M. Characterization of DNA protein complexes induced in intact cells by the carcinogen chromate. Mol. Carcinog. 1: 125–133 (1988).