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Analysis of DNA–Protein Complexes Induced by Chemical Carcinogens

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DNA–protein complexes induced in intact cells by chromate have been isolated and compared with those formed by other agents such as cis-platinum. Actin has been identified as one of the major proteins that is complexed to the DNA by chromate based upon a number of criteria including, a molecular weight and isoelectric point identical to actin, positive reaction with actin polyclonal antibody, and proteolytic mapping. Chromate and cis-platinum both complex proteins of very similar molecular weight and isoelectric points and these complexes can be disrupted by exposure to chelating or reducing agents. These results suggest that the metal itself is participating in rather than catalyzing the formation of a DNA–protein complex. An antiserum which was raised to chromate-induced DNA–protein complexes reacted primarily with a 97,000 protein that could not be detected by silver staining. Western blots and slot blots were utilized to detect p97 DNA–protein complexes formed by cis-platinum, UV, formaldehyde, and chromate.

Other work in this area, involving studying whether DNA–protein complexes are formed in actively transcribed DNA compared with genetically inactive DNA, is discussed. Methods to detect DNA–protein complexes, the stability and repair of these lesions, and characterization of DNA–protein complexes are reviewed. Nuclear matrix proteins have been identified as a major substrate for the formation of DNA–protein complexes and these findings are also reviewed.

Key words: **cis-platinum, chromate, formaldehyde, antibody detection**

In a normal cell, there are very few proteins which are covalently bound or tightly complexed with DNA [1]. An example of such a protein is topoisomerase [1]. Following exposure to certain chemical agents such as chromate, cis-platinum, UV-light, and formaldehyde, other proteins become covalently bound to DNA. The identity of these proteins has not been well-studied. DNA–protein complexes may be important lesions in the genotoxicity of metals and certain chemicals, because DNA–protein complexes are, in general, persistent and not as readily repaired as other lesions [2,3]. This is likely to be important in carcinogenesis, since deletions of DNA may result from attempts to replicate DNA buried in such protein–DNA complexes. These deletions may result in the loss or inactivation of tumor suppressor genes.

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The lack of knowledge about DNA–protein complexes is probably the result of inadequate methodology for detection and characterization of these lesions. In the past, alkaline elution has been primarily utilized [4], but more recently, a few other methods have been applied [1,5,6]. However, in general, adequate methods for the analysis and detection of these lesions do not exist.

In the present study, we have examined the DNA–protein complexes induced by chromate and compared them to those formed by UV-light, cis-platinum, and formaldehyde. The protein content of these complexes has been separated by two-dimensional gel electrophoresis and analyzed by silver staining or immunological reaction. The stability of the complexes to various reagents has been examined in order to identify the potential structure of the DNA–protein complex [6]. Finally, actin has been identified as one of the major proteins complexed to the DNA by cis-platinum and chromate [7].

Characterization of DNA–Protein Complexes

DNA–protein complexes induced by metals such as cis-platinum and chromate were disrupted by exposure to EDTA or sulfhydryl reducing reagents such as thiourea, whereas the background DNA–protein complexes induced by UV-light and formaldehyde were not similarly disrupted [6,8]. DNA–protein complexes were isolated by lysing cells or nuclei in SDS and the DNA was subsequently collected by ultracentrifugation [9]. The DNA pellet was washed extensively with SDS and urea to dissociate proteins that were not covalently bound. As a control, the DNA was also degraded by treatment with DNase to assure that, during the analysis, the recovery of the protein from DNA–protein complexes induced by the various chemical agents was eliminated [9]. This is an important control because proteins may appear associated with DNA by means other than actual complexation [9].

Figure 1 illustrates the proteins associated with the DNA from untreated cultured Chinese hamster ovary cells and from those cells treated for 24 h with 100 μM cis-platinum or 50 μM chromate. The molecular weight and isoelectric points of the proteins complexed to the DNA by these two metals were very similar. Similar proteins were found after exposure to lower concentrations of these metals, however, proteins were more consistently complexed at the higher concentrations. The 45,000 Da protein with a slightly acidic p*I* had the same molecular weight and isoelectric point as actin. Further experiments were conducted to confirm whether this protein was actin [7]. These experiments included isolating the p45 protein, radioiodinating it with ^{125}I, and comparing proteolytic maps of this protein with those of iodinated bovine muscle or human platelet actin [7]. The proteolytic maps of these two proteins were almost identical, whereas the map of ovalbumin, a protein of similar molecular weight to actin, was very different [7]. Using a highly specific commercially available polyclonal antibody to actin, the p45 protein reacted with this antibody [7]. Thus, based upon isoelectric point, molecular weight, proteolytic maps, and positive reaction with actin polyclonal antibody, the p45 protein cross-linked to the DNA by chromate and cis-platinum has been identified as actin [7].

Figure 2 illustrates a two-dimensional gel of DNA–protein cross-links induced by formaldehyde. In order to release the proteins from DNA and resolve them on gels, complexes induced by formaldehyde and UV required treatment with DNase. However, resolution of proteins from complexes induced by chromate and cis-platinum did not require DNase treatment, since the proteins could be disrupted from the DNA

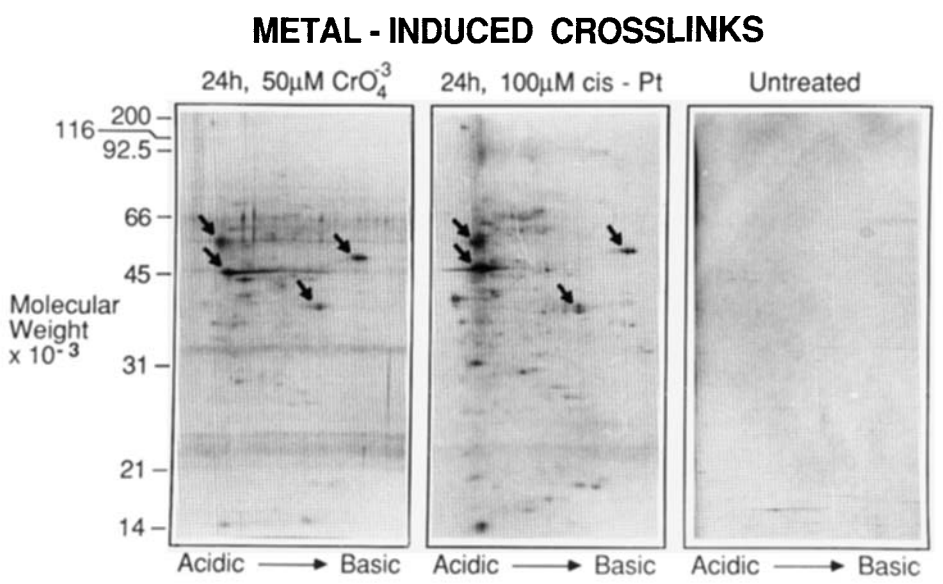


Fig. 1. Analysis by two-dimensional gel electrophoresis of the proteins complexed with DNA from untreated or cells treated for 24 h with 100 μM cis-platinum or 50 μM chromate. DNA–protein complexes were isolated, as previously described [9]. Complexed proteins were separated by two-dimensional gel electrophoresis, as previously described, and visualized by silver staining. Shown are the proteins associated with similar DNA loads from Chinese hamster cells (100 μg DNA was loaded in each lane).

following adequate exposure to mercaptoethanol prior to electrophoresis. In Figure 2, the right-hand panel shows resolution of proteins from formaldehyde cross-links without DNase digestion on the left, proteins are resolved following DNase digestion. Histones, indicated by the "h" on the left panel of Figure 2, were the major proteins complexed to DNA by formaldehyde. The other spots on this gel were attributed to the addition of the DNase I protein. In the absence of DNase treatment, no proteins were resolved in the formaldehyde cross-links. These results illustrate that formaldehyde primarily complexes histones to DNA whereas cis-platinum and chromate did not. Actin has been identified as one of the major proteins complexed to DNA by chromate and cis-platinum.

Detection of DNA–Protein Cross-Links

An antibody to chromate-induced DNA–protein complexes from Chinese hamster ovary cells was raised in rabbits. The reaction of this antibody toward a two-dimensional gel of nuclear protein lysate is shown in Figure 3. The left-hand portion of the gel shows the silver staining of the proteins in this nuclear extract, whereas the right-hand portion of the gel shows the reaction of the antibody with these proteins. As shown in the figure, the antibody reacts primarily with a 95,000 Da protein (more accurate molecular weight determinations indicate a p 97,000 Da). This protein is interesting because, as shown in the figure, it is negatively stained with silver. As a result, this protein was not detected in the earlier experiments when proteins complexed to the DNA by chromate and cis-platinum were analyzed.

Figure 4 shows the reaction of this antibody to complexes induced by chromate. Lanes 1 and 2 are from untreated cells, while lanes 3 and 4 are from cells treated with

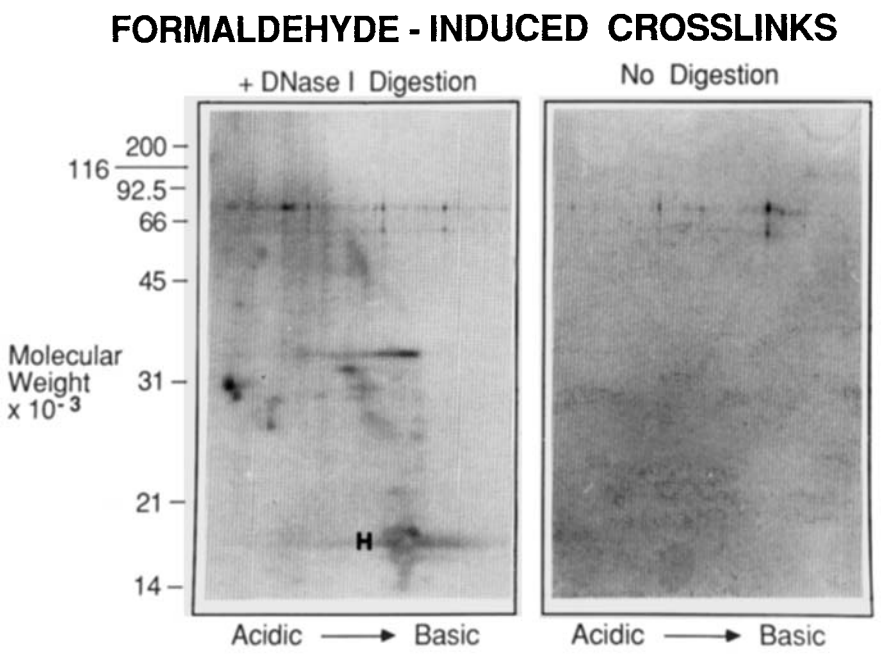


Fig. 2. Proteins cross-linked to the DNA by formaldehyde. DNA complexes from intact cells treated with formaldehyde (13 mM for 1.5 h) were isolated, as previously described [9]. Proteins were separated on two-dimensional gels, also as previously described [9]. In the left portion of the gel, the DNA–protein complexes were treated with DNase I prior to analysis. The complexes analyzed on the right-hand gel were not treated with DNase. Two A_{260} units were loaded for each lane of DNA–protein complexes analyzed in the figure shown above.

chromate. The proteins were separated on a one-dimensional gel, western blotted, and reacted with the complex specific antibody as shown in the figure. The antibody recognized primarily a p95 (p97 protein) complexed to the DNA by chromate.

Figure 5 shows the reactivity of the antibody toward DNA–protein complexes formed by cis-platinum, UV-light, and formaldehyde. All of these agents cross-link p97 to the DNA. However, only with cis-platinum, proteins can be detected on gels without DNase treatment. For UV-light and formaldehyde, DNase treatment was a prerequisite to release the p97 protein from DNA and its resolution on the gels.

Figure 6 illustrates the use of this antiserum to detect DNA–protein complexes by slot blotting. The p97 protein was cross-linked to the DNA by cis-platinum, chromate, and formaldehyde. Antibody selectivity for this protein is illustrated in this figure, since degradation of the sample with DNase did not affect antibody reaction while protease K eliminated the immunological reaction. DNase treatment, however, did reduce some of the background antibody reaction.

These results illustrate that different chemical agents complex specific proteins to DNA. For example, actin is complexed to DNA by chromate and cis-platinum but not by formaldehyde, which complexes histones. The use of a p97 antibody to detect DNA–protein complexes by slot blotting potentially represents a very sensitive and reproducible method for the detection of DNA–protein complexes since p97 is

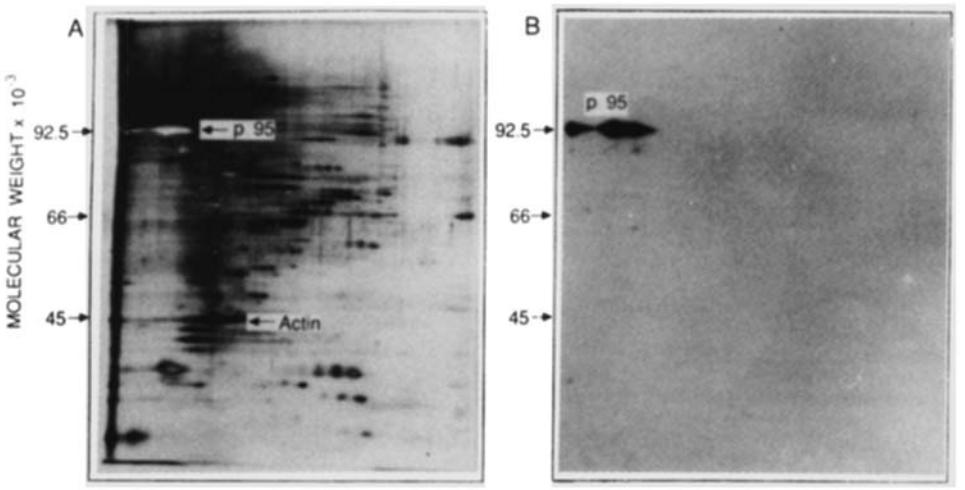
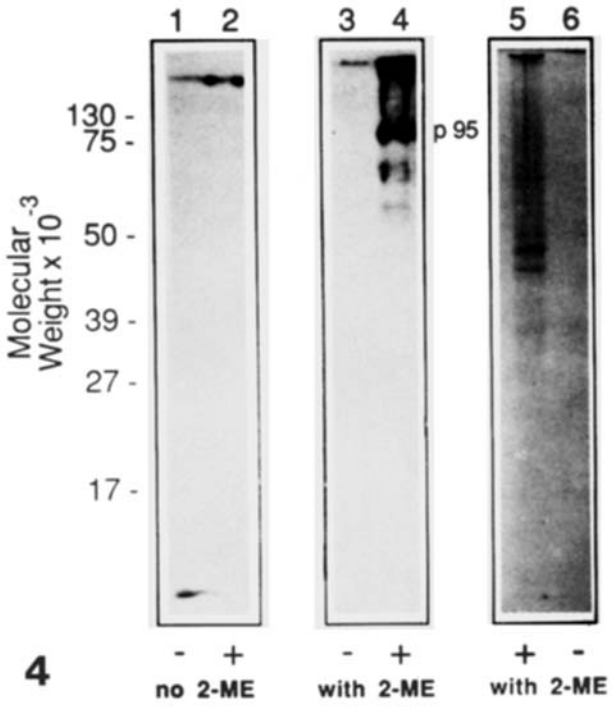


Fig. 3. Reactivity of chromate-induced DNA–protein complex antiserum with Chinese hamsters nuclear proteins. Shown in (**A**) is a silver-stained two-dimensional gel of a control nuclear protein fraction (25 μg protein). The 95-kDa (p97) protein is indicated as the unstained area. Actin is also indicated for purposes of comparison. (**B**) A radiograph of a duplicate blot of the gel in (**A**) reacted with the DNA–protein complex antiserum and ^{125}I-labeled protein A. (Reproduced with permission from Miller CA III, Costa M (1989). Carcinogenesis 104:667–672, Oxford University Press, Oxford, United Kingdom.)

complexed by all these cross-linking chemicals. In the future, even greater sensitivity will be achieved by the use of monoclonal antibodies that react more specifically with the cross-linked proteins.

Other studies have examined DNA–protein cross-link induced in mammalian cells by X-rays, UV-light, and cis-platinum. The chemistry of the linkages between DNA and protein has not been well characterized. Thymine–cysteine adducts, for example, have been isolated from UV-radiated bacteria [10] and phosphotyrosine is known to be involved in the covalent linkage of topisomerase to DNA [11]. Cis-platinum and trivalent chromium are known to react with the N7 position of guanine and are likely to bind to cysteine residues on proteins [12]. Therefore, the DNA–protein complex is likely to involve a bridge between trivalent chromium, cysteine, and the N7 position of guanine, but this has not been proven [12].

Using a nitrocellulose binding assay, the background levels of cellular DNA–protein complexes have been studied [1]. This background was comprised of about 1–3% of the cellular DNA, and represents about 6,000 DNA–protein complexes per cell [1]. Following treatment with radiation, these complexes increased to a maximum of 60,000–70,000 per cell [1]. However, the isolation of these complexes included all protein–DNA complexes that resists dissociation by 1 M sodium perchlorate, 1% sarcosyl, and 10 mM EDTA at 65°C for 15–20 min [13,14]. These conditions will not effect noncovalent protein–DNA complexes intact, since these agents are not as stringent in dissociating proteins from DNA as SDS which has been used in our studies [5,6], as well as in others [15]. Therefore, the background levels of truly covalent DNA–protein complexes are probably less than the estimated value by these authors.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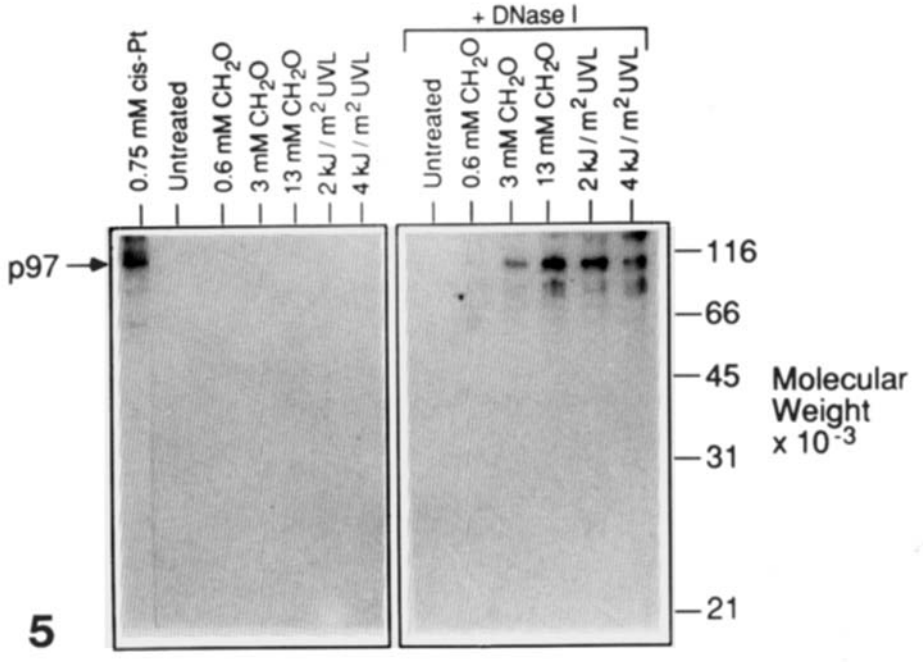
+ DNase I
0.75 mM cis-Pt
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13 mM CH2O
2 kJ / m2 UVL
4 kJ / m2 UVL
Untreated
0.6 mM CH2O
3 mM CH2O
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2 kJ / m2 UVL
4 kJ / m2 UVL
p97
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66
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5

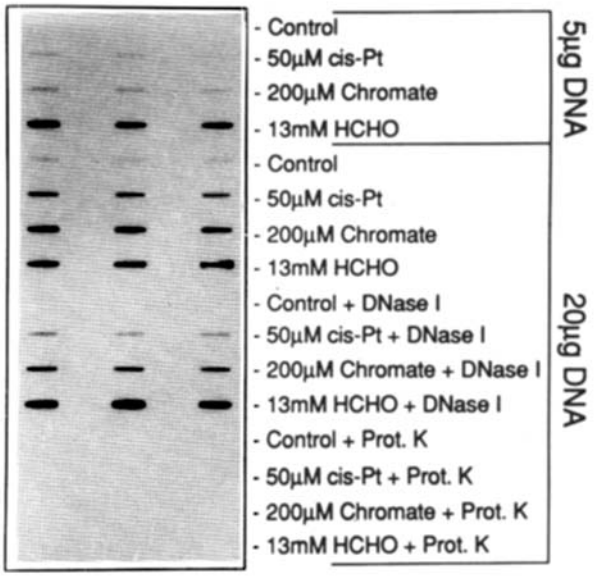

Fig. 6. Immunological detection of p97 complexes formed in cultured cells. CHO cells were treated with metals or formaldehyde and DNA–protein complexes were isolated, as previously described [5–9]. The complexes were bound to the nitrocellulose sheets (in triplicate) at the DNA concentrations shown in the figure and the antigen was detected with antiserum and ^{125}I-labeled protein A. In some instances the complexes were digested with DNase I or proteinase K prior to their application to the filter to assess the nature of the antigenic reactivity. (Reproduced with permission from *Mutation Research* [Miller CA III, Costa M: Immunodetection of DNA-protein crosslinks by slot blotting. Mutation Res 234:97–106, 1990].)

This same group of investigators [1] has also characterized the proteins complexed with the DNA using a more extensive purification procedure which involves centrifugation in the presence of sarcosyl and banding on a cesium chloride density gradient. These studies have identified nuclear matrix proteins as the major component that complexes to the DNA in both untreated and X-irradiated exposed cells. These studies utilized one-dimensional gel electrophoresis to separate proteins. Since there was no substantial increase in the proteins associated with DNA in cells treated

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Fig. 4. Sensitivity of p97 DNA complexes to mercaptoethanol. Undigested DNA–protein complexes (250 μg DNA/lane) were separated by one-dimensional gel electrophoresis and blotted to nitrocellulose in the absence (lanes 1, 2) or presence of 2-mercaptoethanol (lanes 3–6). The autoradiograph of the blot probed with anti-DPC serum and ^{125}I-labeled protein A is shown in lanes 1–4. Lanes 5 and 6 were stained with amido black. Lanes marked (−) are from untreated cells and lanes marked (+) are from chromate treated cells (200 μM, 24 h). (Reproduced with permission from Miller CA III, Costa M (1989). Carcinogenesis 104:667–672, Oxford University Press, Oxford, United Kingdom.)

Fig. 5. p97 DNA–protein complexes induced by various agents. Prior to electrophoresis and blotting, the samples on the right portion of the gel were digested with DNase I as indicated. p97 was detected with antiserum and ^{125}I-labeled protein A followed by autoradiography. (Reproduced with permission from Miller CA III, Costa M (1990). Mutation Res 234:97–106, Elsevier Science Publishers, Amsterdam, The Netherlands.)

with 100 Gy of radiation, compared with untreated cells, the protein analysis was probably qualitative [1].

Other investigators have similarly observed nuclear matrix proteins complexed to the DNA, including our studies with complexes induced by chromate and cis-platinum [7]. Still other investigators have studied proteins complexed by metals to the DNA of intact mammalian cells, including copper sulfate, lead nitrate, mercury chloride, and aluminum chloride [15]. However the concentrations of these metals that complex proteins to DNA range between 0.5 and 5 mM, and are quite high and extremely toxic to cells [15]. These studies have contributed more toward our understanding of chromatin structure, rather than the physiological or toxicologically relevant effects of these metals in cells. This is especially true in the case when cells were exposed to the metal ions in a salt glucose medium, which yields a more effective dose of metals to cells, than if they had been exposed in the presence of fetal bovine serum or other metal chelators [15] such as the amino acids present in serum. If DNA–protein cross-links were formed by a free-radical mechanism, it is likely that a large number of proteins can potentially be cross-linked to the DNA, including those that associate with the DNA, such as histones, and those that do not, such as albumin [1]. Studies in our lab have shown the cross-linking of actin to the DNA of intact cells by chromate and cis-platinum [7]. Although actin is not known to be a DNA-binding protein, it is associated with the nuclear matrix and with actively transcribed DNA. It is, therefore, interesting to note that chromate has been shown to selectively suppress the expression of inducible genes in mammalian cells [16]. These results correlate well with our studies showing chromate-induced cross-linking of actin to DNA [16].

There is not much known about the DNA sequences involved in DNA–protein complexes [1]. It is unlikely that there will be sequence specificity involved in the cross-linking of proteins to DNA, however, a series of studies has demonstrated that proteins are selectively complexed with transcriptionally active DNA by X-ray, while UV-light does not complex proteins to actively transcribed DNA [1]. The cross-linking of actively transcribed DNA sequences by radiation may be related to the chromatin structure of actively transcribed genes which may be more relaxed and accessible to oxygen radicals and subsequent DNA–protein cross-linking [1]. In contrast, UV-radiation is absorbed by the DNA bases, forming reactive intermediates in the DNA which will react with appropriately adjacent proteins and, therefore, is not dependent upon the structure of chromatin [1]. The fact that UV-induced DNA–protein cross-links are not present in actively transcribed regions suggests that the characteristics of the radiation-induced DNA–protein cross-links were not attributable to the isolation methodology, but were a property of the ionizing radiation [1]. This is further supported by the fact that radiation selectively induces single strand breaks in actively transcribed DNA, rather than in inactive or bulk DNA [1].

With regard to the repair of DNA–protein cross-links, there are a number of studies demonstrating preferential repair of DNA–protein cross-links formed in actively transcribed regions versus those that are not transcribed [1], but this repair occurs at a much slower rate than the repair of single strand breaks. The persistence of DNA–protein cross-links suggests that they may be important lesions in carcinogenesis, and also suggests their utility as biomarkers of exposure (i.e., in lymphocytes). In this regard, it is important to reduce the background levels of DNA–protein complexes present in untreated cells, so that the chemically induced covalent DNA–protein

complexes can be detected. Attempts to reduce this background may disrupt noncovalent DNA–protein complexes induced by chemicals. However, background reduction may not be necessary if a specific protein that is not normally part of the background becomes complexed with DNA, or if there is some DNA sequence specificity based upon chromatin structure that results in the complexing of a new DNA sequence. These factors must be considered in studying the DNA–protein complex and utilizing it as a biomarker to assess the exposure of crosslinking agents.

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