

Induction of Internucleosomal DNA Fragmentation by Carcinogenic Chromate: Relationship to DNA Damage, Genotoxicity, and Inhibition of Macromolecular Synthesis

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Hexavalent chromium (Cr) compounds are respiratory carcinogens in humans and animals. Treatment of Chinese hamster ovary cells with 150 and 300 μM sodium chromate (Na_2CrO_4) for 2 hr decreased colony-forming efficiency by 46 and 92%, respectively. These treatments induced dose-dependent internucleosomal fragmentation of cellular DNA beyond 24 hr after chromate treatment. This fragmentation pattern is characteristic of apoptosis as a mechanism of cell death. These treatments also induced an immediate inhibition of macromolecular synthesis and delayed progression of cells through S-phase of the cell cycle. Cell growth (as evidenced by DNA synthesis) was inhibited for at least 4 days and transcription remained suppressed for at least 32 hr. Many of the cells that did progress to metaphase exhibited chromosome damage. Chromate caused the dose-dependent formation of DNA single-strand breaks and DNA-protein cross-links, but these were repaired 8 and 24 hr after removal of the treatment, respectively. In contrast, Cr-DNA adducts (up to 1/100 base-pairs) were extremely resistant to repair and were still detectable even 5 days after treatment. Compared with other regions of the genome, DNA-protein cross-links and Cr adducts were preferentially associated with the nuclear matrix DNA of treated cells, which was 4.5-fold enriched in actively transcribed genes. Chromium adducts, formed on DNA *in vitro* at a similar level to that detected in nuclear matrix DNA, arrested the progression of a DNA polymerase in a sequence-specific manner, possibly through the formation of DNA-DNA cross-links. Total RNA and mRNA synthesis and induction of expression of the inducible GRP78 gene were suppressed in a concentration- and time-dependent manner by chromate. The effects of chromate on GRP78 induction correlated most closely with the presence of DNA-protein cross-links but suppression of total RNA and mRNA synthesis correlated with the presence of DNA-Cr adducts in cells. These results suggest that the persistent Cr-DNA adducts may be responsible for the protracted cell cycle delay and transcriptional inhibition caused by chromate. Escape from apoptosis may be one of the steps involved in chromate-induced carcinogenesis. — Environ Health Perspect 102(Suppl 3):159-167 (1994).

Key words: apoptosis, DNA damage, chromate, transcription, replication, carcinogenesis, gene expression

Introduction

Hexavalent (VI) chromium compounds are carcinogenic to humans, potent inducers of tumors in experimental animals (1,2), and can neoplastically transform cells in culture (3-5). They are also genotoxic (4,6,7) and can induce a spectrum of DNA damage (8-13), gene mutations (4,5,7), sister chromatid exchanges (4,14), and chromo-

somal aberrations (4,15). The mechanisms by which hexavalent chromium-induced toxicity and carcinogenicity are mediated is, however, currently poorly understood.

Potentially, carcinogen toxicity may occur by one of two mechanisms: necrosis or apoptosis (16). These two types of cell death differ morphologically and biochemically. Necrotic cells exhibit an early characteristic cell swelling with the loss of the ionic gradient across the cell membrane and subsequent DNA degradation by released lysosomal enzymes (17). Necrotic cell death does not appear to be cell cycle related. In contrast, cells dying by apoptosis undergo shrinkage while the ionic gradient across the cell membrane is maintained until later stages of the apoptotic process (18). A characteristic feature of apoptosis is that DNA is fragmented internucleosomally before the loss of cell membrane integrity occurs. This is believed to result from the activation of a nuclear, $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent or pH-sensitive endonuclease, possibly DNase II (16,19). In many cases the

process of apoptosis appears related to arrest of cells in a distinct phase of the cell cycle (18,20,21). Carcinogen-treated cells that do overcome the cell cycle block may begin the process of tumor formation, suggesting that apoptosis could serve as a method of escape from the process of multistage carcinogenesis initiated by genotoxic agents.

Recently, several carcinogenic and chemotherapeutic agents, including methylprednisolone, 4-hydroxytamoxifen, methotrexate, 5-fluorouracil, 5-fluorodeoxyuridine, etoposide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, cisplatin, γ -irradiation, and nitrogen mustard, have been shown to induce apoptosis (18,20-24). Recent studies in this laboratory have sought to understand the role of apoptosis in hexavalent chromium-induced toxicity and carcinogenicity. Treatment of Chinese hamster ovary (CHO) cells with toxic doses of sodium chromate was found to result in an immediate inhibition of cell growth, and in macromolecular synthesis; however, cell

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death was delayed for at least 24 hr. Between 24 and 72 hr post-treatment, considerable cell death occurred following an accumulation of cells in the S-phase of the cell cycle. Many of the cells that did progress to metaphase exhibited chromosome damage. Internucleosomal fragmentation of cellular DNA was detected beyond 24 hr after chromate treatment. This fragmentation pattern is characteristic of apoptosis as a mechanism of cell death. Although treatment with 150 μ M chromate induced several types of DNA damage, only chromium adducts remained unrepaired throughout the period when cell death occurred. This lesion was found to be preferentially associated with the nuclear matrix, the putative site of replication and transcription in the cell (25). Chromium-DNA adducts formed on a synthetic DNA template *in vitro* inhibited progression of a DNA polymerase in a dose-dependent manner. These results suggest that the persistent chromium-DNA adducts may be responsible for the protracted cell cycle delay and transcriptional inhibition caused by chromate. They also suggest that potentially, escape from chromate-induced apoptosis may be one of the steps involved in chromate-induced carcinogenesis.

Materials and Methods

Cell Culture, Chromate Treatment, and Cytotoxicity Assays

CHO AA8 cells were cultured as monolayers as described previously (13). Cells were treated with 150 or 300 μ M Na_2CrO_4 for 2 hr in complete medium.

Cytotoxicity Assays. Cytotoxicity was assayed by measuring reduction in plating efficiency relative to controls. Two hundred cells were seeded in 5 ml of basal media Eagle's onto 60 mm dishes and allowed to grow for 17 hr. Cultures were treated with Na_2CrO_4 for 2 hr. After treatment the medium was removed, cells were washed with PBS and then refed with 5 ml of fresh medium. Seven to 10 days later colonies were stained with crystal violet and counted. Experiments were performed in triplicate. There were three replicates per treatment group in each experiment.

DNA Fragmentation Analysis. Cellular DNA was analyzed by the method of Martikainen et al. (26). Cell pellets were suspended in 5 ml of 10 mM Tris-Cl pH 8.0, 0.5% (v/v) Triton X-100, 5 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and digested with 300 μ g/ml proteinase K for 18 hr at 37°C. After digestion, the DNA was sequentially extracted with phe-

nol, phenol/chloroform, chloroform, and ethanol precipitated. Purified DNA (10 μ g) was electrophoresed on a 1.6% agarose gel. The gel was stained with 0.5 μ g/ml ethidium bromide in 10 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.5 mg/ml of RNase A for 4 to 16 hr and photographed.

Karyotype Analysis. Chromosome preparations and scoring of individual types of damage were as described by Wise et al. (15).

Measurement of Macromolecular Synthesis. To measure DNA and protein synthesis, cells were pulse labeled with 1 μ Ci/ml [methyl- ^3H]thymidine (60–90 Ci/mMole, ICN Biomedicals, Inc., Costa Mesa, CA) or 1 μ Ci/ml [^{35}S]methionine (1000 Ci/mMole, ICN Biomedicals) for 1 hr. Cells were harvested and counted using a model Zf Coulter counter. Incorporation of radioactivity into cellular macromolecules (cpm/cell) was determined by scintillation counting of acid-insoluble material by the method of Lehmann and Stevens (27). RNA synthesis was assessed by incorporation of [^3H]uridine into total cytoplasmic RNA or mRNA as described previously (13). GRP78 transcript levels were determined by Northern blot hybridization analysis as described by Manning et al. (13).

Preparation of Nuclei and Nuclear Matrix Preparation

CHO cells were suspended in RSB buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl_2 , 1 mM phenyl methyl sulfonyl fluoride [PMSF]) and incubated on ice for 20 min. Nonidet P-40 (Sigma Chemical Co., St Louis, MO) was added to a final concentration of 0.05% and the cells were homogenized with 10 to 12 strokes of a glass-teflon homogenizer. The nuclei were collected by centrifugation (750 $\times g$, 4°C for 5 min), and washed twice with RSB containing 0.25 M sucrose. Finally, nuclei were separated from whole cells by centrifugation at 75,000 $\times g$, 4°C for 1 hr through a cushion of 1.8 M sucrose in RSB. Nuclear pellets were stored at -80°C until required. Chromatin fractionations were performed essentially as described by Obi et al. (28) with minor modifications. PMSF (1 mM) was added to each buffer immediately before use. Purified CHO nuclei (20 OD₂₆₀ units in 0.1 M NaOH) were suspended in 1 ml of micrococcal nuclease digestion buffer (10 mM Tris-Cl, pH 7.4, 25 mM KCl, 5 mM MgCl_2 , 0.5 mM CaCl_2 , and 0.35 M sucrose) and digested with 1 to 2 μ g/ml of micrococcal nuclease (Sigma) at 37°C for 5 min.

Digestions were terminated by the addition of EGTA to a final concentration of 3 mM. The nuclei were pelleted (14,000 $\times g$, 2 min, 4°C) and the supernatant decanted to a fresh tube. The pellets were sequentially extracted with 1 ml of 10 mM Tris-Cl pH 7.4, 0.2 mM MgCl_2 and 1 ml of 10 mM Tris-Cl, pH 7.4, 2 M NaCl, 0.2 mM MgCl_2 for 15 min on ice. In each case the supernatant, containing solubilized chromatin, was decanted to a fresh tube. The final pellet obtained, containing the residual nuclear matrix fraction, was washed with 1% Triton X-100, 10 mM Tris-Cl pH 7.4, 0.2 mM MgCl_2 to remove nuclear membranes.

Analysis of DNA Damage

Analysis of DNA damage by alkaline elution was performed by the method of Kohn et al. (29,30) as previously described by Patierno et al. (10,13,31).

DNA-protein cross-links were isolated from whole nuclei and nuclear matrix fractions were prepared from cells which had been prelabeled with 0.5 μ Ci/ml of trans-[^{35}S]metabolic labeling reagent (a mixture of L-[^{35}S]methionine and L-[^{35}S]cysteine; 1066 Ci/mM, ICN Biomedicals) for 24 to 48 hr prior to treatment with Na_2CrO_4 . Samples were mixed with 15 vol of urea extraction buffer (8 M urea, 1% Triton X-100, and 10 mM Tris, pH 7.4) and rocked for 4 hr at room temperature. These mixtures were then applied to a Qiagen tip-500 column (Qiagen, Chatsworth, CA) which had been equilibrated with 10 ml of buffer A (0.75 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol, 0.15% Triton X-100, and 0.1 mM PMSF). The column was then washed with 30 ml of buffer B (1 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol, and 0.1 mM PMSF). DNA and urea-resistant DNA-associated proteins were eluted with 15 ml of buffer C (1.25 M NaCl, 50 mM Tris, pH 8.2, 15% ethanol, and 0.1 mM PMSF). The eluted fractions were further extracted with 2% SDS overnight rocking at room temperature. DNA and DNA-protein cross-links were recovered from the retentate after filtration through Centricon-100 filters (Amicon, Beverly, MA) at 1000 $\times g$ for 30 min. DNA was quantified spectroscopically from its absorbance at 260 nm. The specific activity ($^{35}\text{S}/\mu\text{g}$ DNA) of the Centricon filtration retentate was determined by scintillation counting. The data were expressed as a ratio of the specific activity (cpm/ μg DNA) of DNA-protein cross-links recovered from treated cells over that obtained with control cells in the same experiment.

To measure the formation of chromium-DNA adducts, CHO cells were treated with [^{51}Cr]-labeled sodium chromate. Chromium bound to DNA purified from whole cells; nuclei and nuclear matrix fractions were quantified by scintillation counting as described previously (13).

In Vitro Binding of Chromium (III) to DNA

Chromium (III) chloride (Sigma Chemical Co.) was dissolved in 10 mM Tris-Cl, pH 7.6, immediately before use. pSV2neoTS DNA (7 μg) at a concentration of 840 μM DNA-phosphate was treated with CrCl_3 in a total volume of 25 μl for 16 hr at room temperature. For polymerase arrest assays, chromium-treated template DNA was denatured with 0.2 M NaOH at 37°C for 30 min. Unbound chromium was removed by passing samples over Chromaspin-30 columns in nuclease-free water (Clontech Laboratories Inc., Palo Alto, CA). To measure chromium binding, DNA was treated with $^{51}\text{CrCl}_3$ (specific activity 200–900 Ci/g Cr; ICN Biomedicals). Following treatment, samples were diluted 50-fold in 10 mM Tris, pH 7.6, and aliquots were precipitated with an equal volume of 20% trichloroacetic acid (TCA) on ice for 5 min. The precipitated sample was applied to a GF/C glass microfiber filter (Whatman International Ltd., Maidstone, England) under vacuum. Filters were sequentially washed with 10% TCA (15 ml), 100% ethanol (5 ml), and air dried. The filters were scintillation counted using the tritium channel of a Beckman LS 7000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA). To measure counting efficiency, aliquots of a dilution series of $^{51}\text{CrCl}_3$ were also applied to filters, air dried, and scintillation counted. Data were expressed as mmole of ^{51}Cr molecules bound per mole of DNA-phosphate.

Polymerase Arrest Assay

A synthetic 88-bp template was synthesized using a Cyclone Plus DNA Synthesizer (Millipore, Bedford, MA) (Figure 7) and cloned into the plasmid pSV2neo using standard techniques (32) at the site created by digestion of the vector with *Eco* R1 and *Bam* H1 restriction endonucleases to generate pSV2neoTS. A 25-base oligonucleotide primer [5'-CGTATCACGAGGC-CCTTTCGTCTTC-3'] complementary to the region two bases upstream of the mutational target sequence was synthesized. Ten pmole of primer were end-labeled with 1 μl of [γ - ^{32}P]-ATP (20 pmole, 170 μCi) (ICN Biochemicals) in a total volume of 20 μl

containing 2 μl of 10 \times T4 polynucleotide kinase buffer (400 mM Tris-Cl, pH 7.5, 100 mM MgCl_2 , 50 mM DTT), and 1 μl (8 units) of T4 polynucleotide kinase (Promega, Madison, WI) at 37°C for 10 min. The reaction was stopped by incubating tubes at 90°C for 2 min and the labeled primer was stored at -20°C. An aliquot of chromium-treated, denatured pSV2neoTS (2 μg in 7 μl) was combined with 2 μl of stop-assay reaction buffer (200 mM Tris-Cl, pH 7.5, 50 mM MgCl_2 , 250 mM NaCl) and 1 μl of end-labeled primer (0.5 pmole). This mixture was heated to 65°C for 2 min and cooled slowly to below 30°C. After annealing, each reaction was mixed with 1 μl of 0.1 M DTT, 2.5 μl of water and 2 μl of Sequenase Version 2.0 T7 polymerase (United States Biochemical, Cleveland, OH) diluted 1:8 in the manufacturer's enzyme dilution buffer (10 mM Tris-Cl, pH 7.5, 5 mM DTT, 0.5 mg/ml bovine serum albumin). The tubes were mixed well, briefly centrifuged, and 3.5 μl of each mixture was transferred to fresh tubes containing 2.5 μl of 160 μM each of dATP, dCTP, dTTP, and dGTP. These elongation reactions were incubated at 45°C for 5 min. Stop solution (4 μl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was then added and the tubes heated to 78°C for 2 min and placed on ice. Aliquots of each reaction (2 μl) were analyzed on a 6% denaturing polyacrylamide sequencing gel. To determine the position of polymerase arrests, sequencing reactions of the DNA template were also analyzed on the same gel. Gels were run for approximately 2 hr at 2000 V in 1 \times TBE buffer (0.045 M Tris-borate, 1 mM EDTA). Following electrophoresis, gels were wrapped in cellophane and exposed to X-ray films between two intensifying screens for 5 to 17 hr at -70°C.

Results

Toxicity and Effects on Cell Growth

Treatment of CHO cells with 150 or 300 μM Na_2CrO_4 for 2 hr reduced colony-forming efficiency by 46 and 92%, respectively compared with untreated controls (not shown). Colonies in chromate-treated cultures were notably smaller than the corresponding untreated control colonies indicating that cell growth had been inhibited (not shown). Consistent with this observation, Figure 1 shows that immediately after treatment with sodium chromate the growth of CHO cells was suppressed. There was, however, no decrease in the

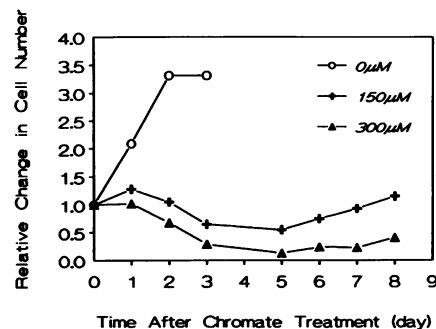


Figure 1. The change in cell number in cultures of CHO cells after treatment with chromate. Cells (1×10^6) were seeded into 10 cm dishes. After 48 hr in culture, the cells were treated with 0 μM , 150 μM , or 300 μM sodium chromate for 2 hr. Cells were harvested at various times after this treatment and duplicate samples were counted with a model Zf Coulter counter. These data are representative of three independent experiments.

number of adherent cells for at least 24 hr. Between days 1 and 3 after treatment the number of adherent cells in the 150- and 300- μM treatment groups decreased by 35 and 85%, respectively, indicating that considerable cell death occurred during this period. In both treatment groups greater than 95% of adherent and nonadherent cells excluded trypan blue up to at least 48 hr suggesting that extensive cell lysis had not occurred during this period. By 72 hr, none of the nonadherent cells excluded trypan blue, indicating that they were no longer viable. The number of adherent cells did not begin to increase until at least 5 days after treatment.

Analysis of Genomic DNA

Genomic DNA prepared from pooled unattached and adherent cells at various times after treatment with Na_2CrO_4 was analyzed by agarose gel electrophoresis (Figures 2, 3). DNA isolated from untreated cells was of high molecular weight (greater than 24 kb) and unfragmented at all times examined (Figures 2, 3). Up to 24 hr after chromate treatment, little DNA fragmentation was apparent (Figure 2) even on highly overloaded gels (not shown). In contrast, dose-dependent internucleosomal DNA fragmentation, indicative of apoptosis, was detectable 48 hr after treatment of cells with either 150 or 300 μM chromate (Figure 2). By 72 hr posttreatment a considerable amount of DNA fragmentation was apparent in both treatment groups (Figure 3). Comparison of the size of fragmented genomic DNA with molecular weight marker DNA indicated that the average size difference between the DNA fragments was approximately 180 bp. This fragmentation pattern is consistent with

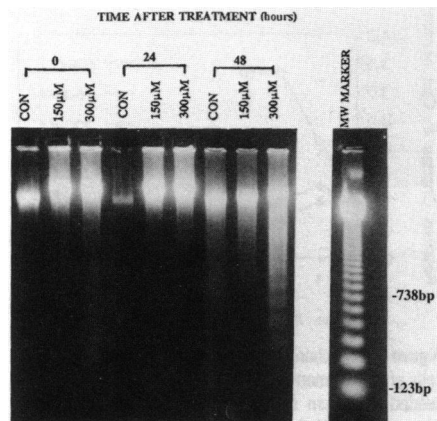


Figure 2. Analysis of genomic DNA isolated from CHO cells up to 48 hr after treatment with sodium chromate. Cells were treated with 0, 150, or 300 μM chromate for 2 hr. Either immediately after this treatment (0 hr), 24 or 48 hr later, DNA was isolated from pooled adherent and nonadherent cells and analyzed on a 1.6% agarose gel. Molecular weight markers were a 123 bp ladder (Gibco, BRL, Gaithersburg, MD). These data are representative of five independent experiments.

that expected to result from the internucleosomal DNA cleavage that occurs during apoptotic cell death (16). Seven days (168 hr) after chromate treatment, no DNA fragmentation was apparent, suggesting that the process of apoptosis was completed in these cell populations by this time (Figure 3).

Effects on Macromolecular Synthesis

Figure 4 shows the residual DNA synthesis occurring in Na_2CrO_4 -treated CHO cells expressed as a percentage of that occurring in logarithmic phase control cells. Treatment with 150 and 300 μM chromate for 2 hr immediately suppressed DNA synthesis to 30 and 10% of control values, respectively. This inhibition continued for at least 4 days in both treatment groups. By day 5, there was some recovery of DNA synthesis, to 70 and 50% of control values in the 150 and 300 μM treated cells, respectively.

Figure 5 shows the effect of Na_2CrO_4 on the synthesis of cytoplasmic RNA and mRNA in CHO cells expressed as a percentage of that occurring in control cells. The extent of [^3H]uridine incorporation into total cytoplasmic RNA and mRNA in a 1 hr pulse was measured up to 32 hr after treatment with Na_2CrO_4 . Treatment with 150 and 300 μM chromate suppressed total RNA synthesis by approximately 50 to 60% and 75 to 90%, respectively compared with controls (Figure 5). These concentrations also suppressed mRNA synthesis to approximately 30 and 20% of control levels, respectively (Figure 5). Suppression of transcription of both total and mRNA

synthesis occurred immediately after treatment (13) and this suppression persisted for at least 32 hr (Figure 5).

Chromate was found to inhibit the induction of expression of the inducible GRP78 gene (33) by tunicamycin in a time- and concentration-dependent manner. CHO cells were exposed to 150 or 300 μM chromate for 2 hr. At various times after this treatment, cultures were incubated with tunicamycin for 8 hr to induce GRP78 gene expression. Increases in GRP78 mRNA levels were measured by Northern blot hybridization analysis. Immediately after treatment of cells with 150 and 300 μM chromate, induction of GRP78 expression was suppressed to approximately 10 and 2% of that occurring in nonchromate treated cells, respectively (Figure 5, 8-hr column). In contrast, 24 hr after treatment with 150 μM chromate, there was a partial recovery of GRP78 inducibility to approximately 56% of that occurring in untreated cells (Figure 5, 32-hr column). There was, however, no induction of GRP78 expression in cultures treated with 300 μM chromate at this time.

Chromate-Induced DNA Damage

DNA damage induced in CHO cells by 2 hr of treatment with 150 or 300 μM Na_2CrO_4 was measured by alkaline elution. DNA single-strand breaks (13) and DNA-protein cross-links (Figure 6A) were detected immediately after treatment of cells with chromate. The single-strand breaks were essentially repaired within 8 hr (13). DNA-protein cross-links were more persistent but were repaired in cells treated with 150 μM chromate by 24 hr after treatment (Figure 6A). Cells treated with the 300 μM dose contained a much reduced level of cross-links at 24 hr compared with 0 hr, indicating that DNA repair had occurred.

Figure 6B shows that chromium-DNA adducts were formed immediately after treatment of cells with [^{51}Cr]-labeled Na_2CrO_4 . After a slight reduction in adduct levels between 0 and 8 hr post-treatment, little further decrease occurred up to at least 32 hr (Figure 6B).

Chromium-DNA adducts and DNA-protein cross-links isolated from the nuclei and nuclear matrix of CHO cells treated with 150 μM chromate were quantified. Immediately after treatment with chromate, chromium-DNA adducts were markedly enriched in the nuclear matrix (approximately 4-fold compared with total nuclear DNA) (Table 1). Twenty-four hours after treatment, chromium-DNA

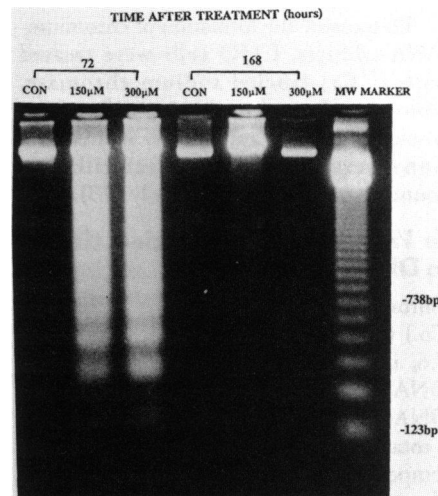


Figure 3. Analysis of genomic DNA isolated from CHO cells 3 days (72 hr) and 7 days (168 hr) after treatment with 0, 150, or 300 μM chromate for 2 hr. DNA was isolated from pooled adherent and nonadherent cells and analyzed on a 1.6% agarose gel. Molecular weight markers were a 123 bp ladder (Gibco, BRL, Gaithersburg, MD). These data are representative of three independent experiments.

adducts were still enriched in nuclear matrix DNA but at a slightly decreased level compared with 0 hr. Consistent with the data presented in Figure 6B; however, there was little decrease in adduct levels in total nuclear DNA (Table 1). Chromium-induced DNA-protein cross-links, which were stable to 8 M urea and 2% SDS, were also enriched in the nuclear matrix (Table 1). Consistent with the alkaline elution data presented in Figure 6A, the matrix-associated cross-links reached a

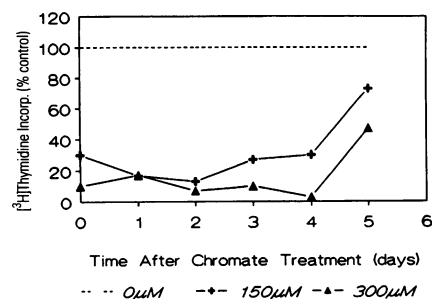


Figure 4. The effect of sodium chromate on DNA synthesis in CHO cells. Cells were treated with the indicated concentrations of sodium chromate for 2 hr and then transferred to fresh medium. For 1 hr before the indicated times, cells were incubated with [^3H]thymidine to assess DNA synthesis. [^3H]thymidine incorporation (cpm/cell) occurring in chromate-treated cells is presented as a percent of that occurring in logarithmic phase control cells (broken line). These data represent three independent experiments.

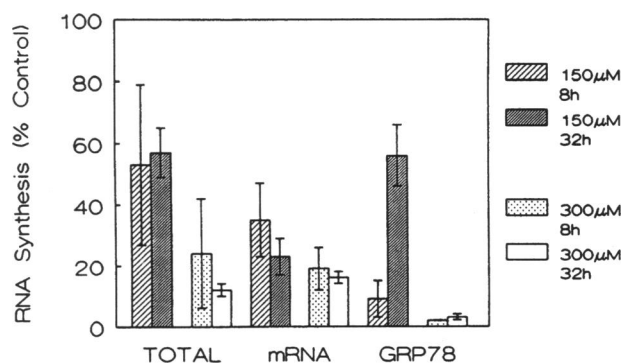


Figure 5. Cells were treated with 150 or 300 μM Na_2CrO_4 for 2 hr and then transferred to fresh medium (0 hr). For 1 hr prior to the indicated times, cells were incubated in the presence of [^3H]uridine to assess total cytoplasmic RNA synthesis and messenger RNA synthesis. Transcription occurring in chromate-treated cells was expressed as a percentage of that occurring in control cultures at the same time \pm SEM of two to four independent experiments. Either immediately after treatment with chromate (0 hr) or 24 hr later, cells were incubated with 10 $\mu\text{g}/\text{ml}$ tunicamycin for 8 hr to induce GRP78 expression. At the end of these incubation periods (8 and 32 hr, respectively), RNA was isolated and GRP78 transcript levels were assayed by Northern blot hybridization analysis. Results are expressed as a percentage of the increase in GRP78 mRNA levels occurring in nonchromate-treated control cells exposed to tunicamycin \pm SEM of three determinations. Data reproduced with permission from Manining et al. (13).

Table 1. Chromium-DNA adduct and DNA-protein cross-link levels in CHO cells treated with 150 μM chromate for 2 hr.^a

Time after treatment, hr	Chromium adducts/DNA phosphate (mmole/mole)	
	Total nuclear DNA	Nuclear matrix DNA
0	1.05 \pm 0.19	3.86 \pm 0.93
24	0.84 \pm 0.25	2.57 \pm 0.22
Relative DNA-protein cross-link levels ^b		
Untreated control ^c	1	6.56 \pm 2.60
0	2.15 \pm 0.38	12.84 \pm 0.78
24	1.92 \pm 0.44	8.87 \pm 0.65

^aThe presented data are the mean \pm SE of two to four determinations in each case. ^bDNA-protein cross-links stable to extraction with urea and SDS were quantified as described in Materials and Methods. ^cResults were standardized to the value obtained with nonchromate-treated, unfractionated nuclei in each experiment. The level of protein bound to the nuclear matrix of untreated cells is also shown.

Table 2. Clastogenicity of sodium chromate.^a

Concentration of sodium chromate (μM)	0	150
Percent metaphases with damage	2 \pm 1	33 \pm 0
Aberrations per 100 metaphases	2 \pm 1	47 \pm 5
Chromatid lesions ^b	2 \pm 1	38 \pm 3
Isochromatid lesions ^c	0	4 \pm 0
Chromatid exchange	0	3 \pm 2
Dicentric	0	1 \pm 1
Triradial figures	0	2 \pm 0
Quadriradial figures	0	1 \pm 1

^aResults are the average of two experiments \pm range (100 metaphases analyzed per experiment). ^bAchromatic lesions

maximum level at the end of the 2 hr treatment but were mostly repaired 24 hr later.

Chromate-Induced Chromosome Damage

Table 2 shows that chromosomes prepared from metaphase CHO cells 24 hr after 2 hr of treatment with 150 μM Na_2CrO_4 contained high levels of damage (33% of metaphases with damage) compared with chromosomes obtained from untreated

controls (2% metaphases with damage). A high percentage of cells that manifested damage also contained multiple chromosomal aberrations. Metaphase chromosomes could not be obtained 24 hr after treatment of cells with 300 μM chromate.

Effects on *in Vitro* Replication

To investigate the effect of chromium-DNA adducts on *in vitro* replication, a synthetic 88 bp DNA template was synthesized

and cloned into the *Eco* R1-*Bam* H1 site of the plasmid pSV2neo to form pSV2neoTS. The sequence of this template is shown in Figure 7 and includes a 24 bp sequence 67% enriched in AT residues, a 17 bp sequence 100% enriched in GC residues, and a 17 bp self-complementary inverted repeat. These unique regions were included in the target sequence because each had been demonstrated to be particularly susceptible to chemical mutation (34).

The plasmid pSV2neoTS was linearized by digestion with Not I to generate a discrete 105 bp full length elongation product in the polymerase arrest assay. Linearized plasmid was treated at a concentration of 840 μM with 0, 35, 105, 210, 420, or 840 μM CrCl_3 for 16 hr at room temperature. These doses are lower than the intracellular concentration of chromium occurring in CHO cells treated with 150 μM Na_2CrO_4 for 2 hr which was determined to be 5.2 mM (Xu J, Manning FCR, Patierno SR, unpublished). They are also comparable to the intracellular concentration of chromium occurring in cells treated with lead chromate for 24 hr (35). The selected treatments correspond to DNA-phosphate: Cr^{3+} ratios of 1:0, 1:0.04, 1:0.13, 1:0.25, 1:0.5, and 1:1, respectively. After denaturation with NaOH and removal of unbound chromium by passage over Chromaspin-30 chromatography columns, the treated DNA was analyzed by polymerase arrest assay using Sequenase Version 2.0 T7 DNA polymerase. Few arrests occurred when untreated DNA was used as a template in this assay. The majority of synthesized DNA was of a size that corresponded to the expected 105 bp full-length fragment (Figure 7, control lane). In contrast, treatment of pSV2neoTS with CrCl_3 resulted in the concentration-dependent synthesis of truncated DNA molecules and a reduction in the amount of full-length product (Figure 7, 35 to 840 μM lanes). Comparison of the size of these truncated molecules with the corresponding sequencing reactions (Figure 7; G, A, T, and C lanes) indicated that the polymerase arrests occurred in a sequence-specific manner, in the GC-rich (100% GC) and palindromic (65% GC) sequences and to a lesser extent in the AT-rich region (27% GC). This effect was most pronounced after treatment with the lower concentrations of CrCl_3 (Figure 7, 35 and 105 μM lanes). The template sequence prior to the AT-rich region, in which a number of arrests occurred, is also enriched in GC (61%). At the highest concentrations of CrCl_3 employed, almost every base was affected to some extent

Table 3. Binding of chromium (III) to template DNA *in vitro*.

CrCl ₃ , μM ^a	35	105	210	420	840
Cr binding; mmole/mole DNA-phosphate ^b	5 ± 3	23 ± 5	57 ± 3	96 ± 9	169 ± 9

^aDNA at a concentration of 840 μM DNA-phosphate was treated with the indicated concentrations of CrCl₃ for 16 hr at room temperature. ^bThe data are the average ± the range of two independent experiments.

(Figure 7, 420 and 840 μM lanes). Identical polymerase arrests were detected when heat denaturation was substituted for alkaline denaturation in the arrest assay (36). Treatment of pSV2neoTS with concentrations of [³¹Cr]-labeled CrCl₃ that produced polymerase arrests indicated that chromium-DNA adducts were formed on template DNA in a dose-dependent manner (Table 3).

Discussion

The mechanisms of toxicity and carcinogenicity of hexavalent chromium compounds are currently poorly understood. Recently, however, several carcinogenic agents have been reported to induce apoptotic cell death in mammalian cells (18,20-24). Studies in this laboratory have therefore investigated whether apoptosis could be a mechanism by which chromium (VI) toxicity is mediated.

Treatment of CHO cells with cytotoxic concentrations of Na₂CrO₄ for 2 hr immediately inhibited cell growth (Figure 1) and DNA synthesis (Figure 4), however no immediate cell death, as judged by trypan blue exclusion, occurred. Rapid inhibition of DNA replication by chromate has previously been reported by Levis et al. (37). Bakke et al. (38) have previously reported that cells treated with chronic, high concentrations of chromate arrest in S-phase.

In agreement with these observations, 24 hr after treatment with 150 μM chromate, cells were found to accumulate in the S-phase of the cell cycle (not shown). An S-phase delay has also been reported after treatment of cells with the toxic metal salts, CdCl₂ and NiCl₂ (39). In addition, nitrogen mustard blocks progression of cells through this phase of the cell cycle prior to the onset of apoptosis (21).

Many of the cells that did progress to metaphase exhibited a high level of chromosome damage 24 hr after treatment with 150 μM chromate (Table 2). The majority of the damage observed were chromatid lesions. These results are consistent with our previous finding that the relatively insoluble hexavalent chromium salt, lead

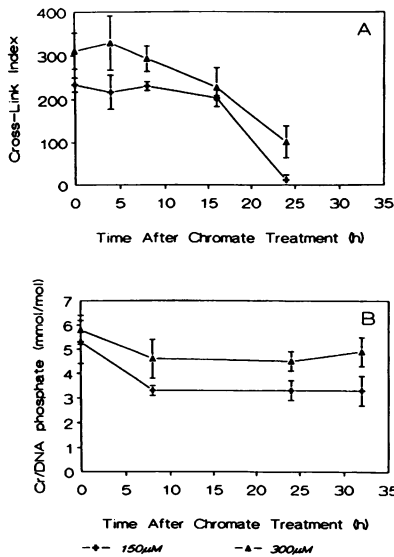


Figure 6. Formation and repair of DNA damage in Na₂CrO₄-treated CHO cells. Cells were treated with 150 or 300 μM Na₂CrO₄ for 2 hr and then were refed with fresh medium (0 hr). DNA-protein cross-links (A) were measured by alkaline elution at the indicated times after this treatment. To assess chromium adduct formation, cells were incubated with [⁵¹Cr]-labeled Na₂CrO₄ and the amount of radioactivity associated with total cellular DNA determined (B). Error bars represent the SEM of three independent experiments. Data reproduced with permission from Manning et al. (13).

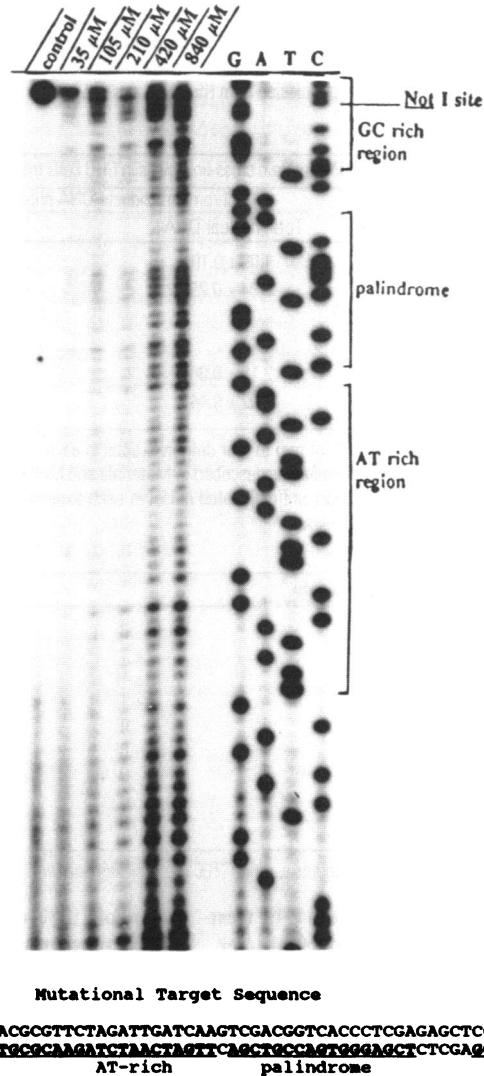


Figure 7. Analysis of chromium (III)-treated DNA by polymerase arrest assay. The plasmid pSV2neoTS was treated with the indicated concentrations CrCl₃ and replicated *in vitro* with Sequenase Version 2.0 T7 DNA polymerase. The products of these reactions were analyzed on a sequencing gel. Also shown are the corresponding sequencing reactions (G, A, T, and C lanes). The lower panel shows the sequence of the synthetic template used in these reactions.

chromate, is clastogenic to CHO cells (15). Metaphase chromosomes could not be obtained 24 hr after treatment with 300 μM chromate. This may be due to the extreme suppression of DNA synthesis produced by this dose (Figure 4). In agreement with this possibility, although cells treated with 150 μM chromate grew slightly in the 24 hr following treatment, no growth was detected after treatment with the 300 μM concentration (Figure 1) (13).

A characteristic feature of apoptosis is that genomic DNA is fragmented internucleosomally before the loss of cell membrane integrity occurs (18). The size of DNA isolated from chromate-treated CHO cells was therefore analyzed on agarose gels. Dose-dependent internucleosomal DNA fragmentation was detected 48 hr after treatment of cells with Na_2CrO_4 (Figure 2). Beyond 24 hr, a large number of cells became detached from the culture dishes (Figure 1). However, the membranes of these cells excluded trypan blue until between 48 and 72 hr after treatment. This is consistent with the known parameters of apoptotic cell death (16). By 72 hr after treatment with either 15 or 300 μM chromate, considerable internucleosomal DNA fragmentation was apparent (Figure 3), suggesting that a large number of cells were undergoing apoptosis at this time.

Following a protracted inhibition of growth, chromate-treated cells began to recover, as evidenced by increased DNA synthesis on day 5 after treatment (Figure 4). This was followed by an increase in cell number between days 6 and 8 (Figure 1). By day 7 after treatment, no DNA fragmentation was detected (Figure 3) suggesting that cells were no longer undergoing apoptosis at this time.

Treatment of CHO cells with 150 and 300 μM Na_2CrO_4 produced an immediate inhibition of protein synthesis (40). Although the role of protein synthesis in apoptosis is unclear, the protein synthesis inhibitor cycloheximide has been reported to induce apoptosis in some instances (41). It is therefore possible that the inhibition of protein synthesis produced by chromate could be involved in the initiation of apoptosis.

The formation and repair of DNA damage of chromate-treated CHO cells was examined. DNA single-strand breaks, DNA-protein cross-links, and chromium DNA adducts were formed in a concentration-dependent manner; subsequently each type of damage was repaired at a different rate. Single-strand breaks were rapidly repaired, essentially within 8 hr (13). The DNA-protein cross-links were more persis-

tent than the strand breaks but were also repaired. By 24 hr no cross-links remained in cells treated with 150 μM chromate, while cells exposed to the 300 μM dose contained considerably lower levels of this type of damage at this time (Figure 6A).

The most persistent form of damage was chromium-DNA adducts that remained unrepaired for at least 32 hr (Figure 6B). This was the only type of damage to persist after the onset of cell death induced by both 150 and 300 μM chromate. In fact, adducts were still detectable in cells 5 days after treatment with the 150 μM dose (13). Potentially therefore, chromium-DNA adducts could be involved in the mechanism of chromate-induced toxicity or carcinogenicity. Alternatively, DNA single-strand breaks or DNA-protein cross-links, although repaired prior to the onset of cell death, may cause the induction of a signal that induces cells to undergo apoptosis. A further possibility is that free radicals, produced as a result of the intracellular reduction of hexavalent chromium (8,42-44), may trigger apoptosis in affected cells.

Compared with total nuclear DNA, chromium-DNA adducts were found to be enriched in the nuclear matrix of CHO cells both immediately after treatment with 150 μM Na_2CrO_4 and 24 hr later (Table 1). Both these data and those presented in Figure 6B suggest that chromium-DNA adducts are very poorly repaired in CHO cells.

The nuclear matrix is believed to be the site of replication in the cell (25). Potentially, chromium-DNA adducts associated with this fraction could interfere with replication and hence cause the inhibition of DNA synthesis and cellular growth observed after treatment of cells with Na_2CrO_4 (Figures 1, 4). One mechanism by which this may occur would be if chromium-DNA adducts inhibited the action of DNA polymerases. To test this possibility, the effect of chromium-DNA adducts on the *in vitro* replication of DNA by Sequenase Version 2.0 T7 DNA polymerase was examined. For these experiments a synthetic template containing GC- and AT-rich regions and a palindrome sequence (pSV2neoTS) was employed. A reduced trivalent form of chromium is believed to be the species which binds to cellular DNA (45). Treatment of pSV2neoTS with CrCl_3 resulted in the dose-dependent formation of truncated molecules (Figure 7). After treatment with relatively low concentrations of CrCl_3 (35, 105, and 210 μM), these polymerase arrests occurred preferentially in the GC-rich regions of the template

molecule. This is consistent with the known affinity of chromium binding for guanine residues (46). Notably, DNA treated *in vitro* with 35 μM CrCl_3 contained a similar level of adducts (Table 3) to that detected in the nuclear matrix of cells treated with 150 μM chromate (Table 1).

Recent data obtained in this laboratory suggest that the CrCl_3 -induced polymerase arrests may result from the formation of DNA-DNA cross-links (36). A similar inhibition of polymerase action via the formation of chromium (III)-induced DNA-DNA cross-links has previously been reported by Snow and Xu (45). DNA-DNA cross-links formed in the nuclear matrix may therefore be a potential mechanism by which the chromate-induced inhibition of DNA synthesis is mediated.

As an alternative to the alkaline elution technique (29,30) we have developed a procedure to isolate and quantify DNA-protein cross-links that are stable to sequential extraction with urea and SDS (Xu J, Manning FCR, Patierno SR, unpublished). Using this procedure, chromium-induced DNA-protein cross-links were detected predominantly in the nuclear matrix fraction immediately after treatment of cells with 150 μM Na_2CrO_4 (Table 1). Preferential cross-linking of nuclear matrix proteins to DNA has also been reported by other workers (47,48). Consistent with the alkaline elution data presented in Figure 6A, the cross-links in the nuclear matrix reached a maximum level immediately after treatment with 150 μM Na_2CrO_4 but were essentially repaired 24 hr later (Table 1).

Treatment of CHO cells with 150 or 300 μM Na_2CrO_4 suppressed both total RNA and mRNA synthesis for at least 32 hr (Figure 5). In cells treated with the lower chromate concentration, DNA-chromium adducts were the only damage which persisted over this entire period (Figure 6B). *In vitro*, binding of chromium (III) has been found to interfere with the function of DNA templates, causing an increased number of initiation sites and the formation of truncated transcription products (49). The chromium adducts formed in CHO cells may therefore exert an inhibitory effect on transcription, possibly by interfering with the action of RNA polymerases.

Immediately after treatment with 150 μM chromate, induction of expression of the inducible gene GRP78 by tunicamycin was almost completely inhibited (Figure 5). In contrast, by 24 hr after treatment a recovery in GRP78 induction occurred (Figure 5). This recovery correlated with the repair of DNA-protein cross-

links at this time (Figure 6A; Table 1) but not with a general recovery of RNA synthesis (Figure 5). Repair of single-strand breaks did not result in a recovery of GRP78 induction (13). These results are similar to those of Hamilton and Wetterhahn (47) who reported that dichromate inhibited induction of the inducible genes 5-aminolevinate synthase and cytochrome PB₁ P450 in chick embryo liver *in vivo* and that this inhibition correlated with the presence of DNA-protein cross-links.

It has been suggested that transcription of inducible genes may be particularly sensitive to DNA-protein cross-link formation because these genes typically have complex regulatory regions and often require numerous DNA-protein interactions for proper gene expression (50). Potentially therefore, DNA-protein cross-links formed within the GRP78 promoter may inhibit the action of transacting factors required

for gene induction. In fact, a number of transcription-factor binding sites, which are required for inducible gene expression, have been identified within the promoter region of the GRP78 gene (51–54). DNA-protein cross-links occurred predominantly in the nuclear matrix fraction after treatment with 150 μ M chromate (Table 1); and repair of these cross-links 24 hr after treatment correlated with recovery of GRP78 induction (Figure 5; Table 1, respectively). It is therefore interesting that the nuclear matrix fraction was found to be enriched in GRP78 gene sequences compared with total nuclear DNA (Xu J, Manning FCR, Patierno SR, unpublished observations).

Only a partial recovery of GRP78 induction (56%) occurred when DNA-protein cross-links were repaired 24 hr after treatment with 150 μ M chromate (Figure 5). This suggests that chromium-DNA adducts, which were the only DNA

damage still detectable at this time (Figure 6B; Table 1), may contribute to the observed inhibition of GRP78 induction. Alternatively, this effect may be due to the large number of cells undergoing apoptosis beyond 24 hr (Figure 1). In cells treated with 300 μ M chromate, no recovery of GRP78 induction occurred even 24 hr after treatment (Figure 5), probably because of the extreme toxicity of this dose.

The results presented in this study indicate that the toxicity of hexavalent chromium compounds can occur via apoptosis. Cells which do not undergo apoptosis may contain chromate-induced chromosome damage in genes associated with tumor formation such as oncogenes or tumor suppressor genes. Escape from chromate-induced apoptosis could therefore represent an early step in chromate-induced carcinogenesis.

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