Analysis of Repair and Mutagenesis of Chromium-Induced DNA Damage in Yeast, Mammalian Cells, and Transgenic Mice

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Chromium (Cr) is a widespread environmental contaminant and a known human carcinogen. We have used shuttle vector systems in yeast, mammalian cells, and transgenic mice to characterize the mutational specificity and premutational DNA damage induced by Cr(VI) and its reduction intermediates in order to elucidate the mechanism by which Cr induces mutations. In the yeast system, treatment of vector-containing cells with Cr(VI) results in a dose-dependent increase in mutations in the *SUP4-o* target gene of the vector; mutagenesis is enhanced in an *apn-1* yeast mutant, deficient in the capacity to repair oxidative-type DNA damage. In vector-containing mammalian cells, treatment with Cr(VI) also results in a dose-dependent increase in mutations in the vector target gene *supF*. The Cr-induced mutations in *supF* occurred mostly at G:C base pairs and were widely distributed across the gene, a pattern similar to those observed with ionizing radiation or hydrogen peroxide. These results support the hypothesis that Cr(VI)-induced oxidative-type DNA damage is responsible for Cr mutagenesis in the cell. Recently these studies were extended into the Big Blue transgenic mouse system in which Cr-induced mutagenesis was observed in the lung, the target organ for Cr carcinogenesis in humans. Analysis of the spectrum of these mutations will test whether Cr mutagenesis occurs by similar mechanisms in the intact animal as in cell culture systems and yeast. — *Environ Health Perspect* 106(Suppl 4):1027–1032 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-4/1027-1032cheng/abstract.html*

Key words: chromium, mutagenesis, shuttle vectors, yeast, transgenic mice, mutation spectrum, pZ189, *supF*

Introduction

Chromium (Cr) is an essential nutrient and a human carcinogen (*1,2*). Occupational exposure to hexavalent chromium [Cr(VI)] compounds through inhalation is associated with an increased incidence of bronchogenic carcinoma (*2,3*). In occupationally exposed workers, lung tissue levels of 100 μg/g wet weight have been reported (*4*). Chromium continues to be in widespread use in industry in paints, metal plating, and as a corrosion inhibitor. It has been identified as a primary contaminant at approximately one-third of the Superfund Hazardous Wastesites (*5*). Since Cr is an element, it cannot be removed by biodegradation or incineration methods, and furthermore it may be mobilized into groundwater sources (*6*). The risk to human health from ingestion is unknown, although a drinking water standard of 0.05 mg/l has been established (*7*).

In the environment, Cr exists primarily in the trivalent [Cr(III)] and hexavalent forms; interconversion of the two forms can occur under certain conditions (*8*). Generally, Cr(VI) and not Cr(III) compounds have been found to be mutagenic and carcinogenic in a variety of test systems (*2,9*). This appears to be due to the selective penetration of cells by chromate (CrO_4^{2-}) anions (*9,10*). Once in the cell, Cr(VI) is subject to reduction by a wide variety of cellular reductants (*11–13*). Glutathione (GSH) is thought to be one of the most important intracellular reducing agents for Cr(VI); GSH is present intracellularly at mM levels and alterations in intracellular concentrations has been shown to affect Cr(VI)-induced toxicity (*14,15*). GSH appears to have a dual role: reactive intermediates are generated during reduction of Cr by GSH (*13,16*) and GSH serves as a radical scavenger to protect cells agains Cr-induced oxidative damage (*17*).

Cr induces both oxidative-type DNA damage (e.g., DNA strand breaks and oxidized bases) and cross-links (ternary complexes of GSH or amino acids with Cr and DNA) (*15,18–20*). Cr(VI) has been shown to be mutagenic in a variety of bacterial and mammalian assay systems (*21–23*), but the nature of the premutagenic DNA damage responsible for mutation induction has not been clearly defined. Furthermore, the link between the mutagenic and carcinogenic activities of Cr compounds has not been established.

We have undertaken a systematic analysis of Cr mutagenesis to clarify the role of GSH in Cr activation and to better define the Cr-induced DNA damage responsible for the mutagenic activity of Cr compounds. We have used shuttle vectors in yeast (*24*) and mammalian systems (*25,26*) and the Big Blue transgenic mouse system (*27*) to investigate the mechanism of Cr mutagenesis and to characterize Cr-induced mutations. The ultimate goal of this research is to elucidate the mechanism of Cr carcinogenesis, thereby clarifying the risk to human health from Cr exposure.

Materials and Methods

Chemicals

Potassium dichromate ($K_2Cr_2O_7$, 99%), glutathione (98–100%), L-buthionine sulfoximide (BSO), L-glutamate, L-cysteine, and glycine were all purchased from Sigma Chemical Company (St. Louis, MO). 3,5-Diaminbenzoic acid (98%) was purchased from Aldrich Chemical Company (Milwaukee, WI).

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Abbreviations used: BSO, buthionine sulfoximide; GSH, glutathione; IPTG, isopropyl-β-D-thiogalactoside; NER, nucleotide excision repair; 5-bromo-4-chloro-3-indoyl-β-D-galactoside.

Yeast *SUP4-o* System

The yeast *SUP4-o* mutagenesis assay system was developed by Pierce and colleagues (*28*). The plasmid YCpMP2 (*28*), which carries the *SUP4-o* mutagenesis target, was introduced into *Saccharomyces cerevisiae* strains MKP-o [*MATa,can1-100,ade2-1,lys2-1,ura3-52,leu2-3,112,his3-D200,trp1-D901* (*28*)], KAM1 [*rad1*; defective in nucleotide excision repair (*29*)], and DRY373 [*apn1*; defective in oxidative DNA damage repair (*30*)]. These strains were then cultured in Uracil-deficient medium and treated during exponential growth with Cr. Mutations in the *SUP4-o* gene were detected on the basis loss of suppression of three *ochre* markers; mutant colonies were pink or red on cavanine-containing plates and failed to grow in the absence of lysine. Mutant plasmids were transferred to *Escherichia coli* strain MBM7070 (*31*) for preparation of DNA for sequencing.

Mammalian *SupF* System

The pZ189 shuttle vector (*31*) carries the bacterial *supF* gene as the mutational target, pBR322 sequences for replication and selection in bacteria, and SV40 virus sequences for replication in primate cells. The *E. coli* tester strain MBM7070 (*31*) carries the *lacZ*(Am)CA7020, which can be suppressed by the *supF* tRNA suppressor. When MBM7070 carries pZ189, it forms blue colonies in the presence of isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal); bacteria with *supF* mutant plasmids form white or light blue colonies. Almost any mutational change in the *supF* gene causes loss of suppressor function.

For mutagenesis testing, the vector was transfected into African green monkey kidney CV-1 cells grown in monolayer (*24,25*). For *in vitro* treatment of the vector, DNA was first washed in Tris–EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.6) and then treated with potassium dichromate in the presence of a 5-fold excess of GSH in 0.1 M phosphate buffer, pH 7.0, for 90 min at 37°C; DNA was washed with 100 mM EDTA before transfection. The EDTA treatment removes bound Cr from the DNA. Vector DNA was isolated 48 hr after transfection. For treatment of the transfected cells, potassium dichromate was added to the growth medium for 12 hr, starting 6 hr after transfection; vector DNA was harvested from the cells 72 hr after transfection. In all cases, vector DNA harvested from transfected cells was treated with *DpnI* (to degrade input DNA) and transformed into *E. coli* MBM7070 by electroporation.

Big Blue Transgenic Mouse System

The C57BL/6 Big Blue transgenic mouse carries the bacterial *lacI* gene in a bacteriophage λ-based vector integrated into the mouse chromosome (*32*). For chromium treatment, mice (4- to 6-week- old females) were anesthetized, and a small surgical cut was made in the ventral neck to expose the trachea; a chromium solution was injected with a 30.5-gauge needle through the wall of the trachea into the lungs. High molecular weight lung DNA was isolated as described by Stratagene (*33*) and the λ-based vector DNA was recovered by λ packaging using the Stratagene Transpack (Stratagene, La Jolla, CA). Vector *lacI* mutants were identified as blue plaques on agar plates with SCS-8 *E. coli* in the presence of X-gal; wild-type vector produced colorless plaques. Mutant sequence analysis was carried out as described by Erfle et al. (*34*).

Results

To investigate Cr mutagenesis, we used three mutagenesis assay systems. Each system allowed the examination of a particular aspect of Cr mutagenesis. In the yeast *SUP4-o* system (*28*), we took advantage of the availability of particular DNA repair-deficient strains to aid in the identification of the types of premutagenic DNA damage responsible for Cr mutagenesis. In the mammalian cell pZ189 system (*31*), we compared the mutation spectrum that resulted from treatment of the plasmid *in vitro* under controlled conditions with that observed when the DNA was damaged intracellularly. In the Big Blue transgenic mouse system (*32*), we examined the mutagenic potential of Cr in the lung, the target organ for Cr carcinogenesis in humans. By comparing results from all three systems, we have determined mutational mechanisms that appear to be common to all systems and that likely reflect the mechanisms that contribute to human disease.

Yeast *SUP4-o* System

In the yeast system (*28*), the mutational target is the yeast *SUP4-o* suppressor tRNA gene (*25*) carried on the single-copy YCpMP2 plasmid. To characterize premutagenic DNA damage in this system, we introduced this plasmid into two repair-deficient yeast strains. These were the DRY373 strain, which carries the *apn1* mutation and lacks apurinic endonuclease/3′-diesterase activity (*30*), and the KAM-1 strain, which carries the *rad1* mutation and is deficient in the incision step of nucleotide excision repair (NER) (*29*). Thus, the DRY373 strain is sensitive to agents that cause oxidative DNA damage (e.g., hydrogen peroxide) and the KAM-1 strain is sensitive to agents that cause bulky DNA adducts (e.g., UV radiation). We used these strains to determine the sensitivity to Cr toxicity and Cr mutagenicity.

For toxicity measurements, these strains were treated with Cr compounds during exponential growth, and survival was measured by a colony assay. For the mutagenesis studies, the plasmid-containing yeasts were treated with Cr and then screened for mutants. *SUP4-o* mutant plasmids were further analyzed by DNA sequencing. To investigate the role of GSH in Cr toxicity and mutagenesis, we used BSO to lower the intracellular level of GSH (*35*) and the precursor amino acids (L-glutamate, L-cysteine, and glycine) to raise the intracellular level of GSH (*36*).

We found that Cr toxicity was the greatest in the *apn1* DRY strain and under conditions of lowered GSH (Table 1). Cr mutagenesis was the highest in the DRY strain (Figure 1) and was increased in all strains when GSH levels were raised by treatment of cells with precursor amino acids (*26*). These results suggest that Cr causes premutagenic DNA damage that is normally repaired by the product of the *APN1* gene, but not by NER. Furthermore, GSH appears to play a role in protecting cells against Cr toxicity; but at the same time, GSH appears to enhance Cr mutagenesis.

In the DRY strain, Cr induced base substitution mutations, deletions, and insertions (Table 2). Base substitution mutations likely arise from DNA base damage. About

Table 1. Survival[a] of yeast strains after a 3-hr exposure to 0.5 mM Cr(VI).

Yeast strains	No pretreatment	+aa[b]	+BSO[c]
MKP-o	0.9	0.9	0.2
DRY (*apn1*)	0.7	0.7	0.01
KAM (*rad1*)	0.9	0.9	0.2

[a]Survival is the yeast cell density (measured by colony formation) in log phase cultures treated with Cr for 3 hr compared to comparable cultures without Cr treatment. [b]Cells pretreated with 20 mM L-glutamate, L-cysteine, and glycine for 1 hr; GSH rose to 580% of control; control value was 1.2 + 0.1 μmol GSH/mg cell DNA. [c]Cells pretreated with 1 mM BSO for 8 hr; GSH reduced to 28% of control.

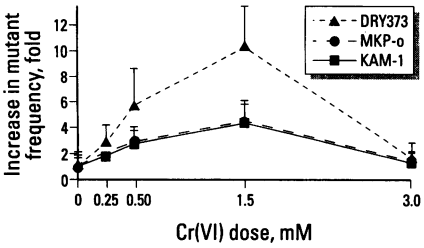

Figure 1. Mutation induction by Cr in yeast. Three yeast strains carrying the *SUP4-o* plasmid were treated with Cr(VI) for 3 hr, then the yeast were plated on indicator plates to identify *SUP4-o* mutants. MKP-o is the parental strain, DRY373 is mutant in the *apn1* gene, and KAM-1 is mutant in the *rad1* gene; the *SUP4-o* mutant frequencies in untreated cells were 3×10^{-6}, 1×10^{-5}, and 1×10^{-5}, respectively. Cell survival at 1.5 and 3 mM Cr(VI) was 20 to 30% and 15 to 20%, respectively, for all three yeast strains.

Table 2. Types of mutations induced in *Sup4-o* by Cr(VI) treatment of DRY373p.

Types of mutation	Number of mutations detected (%)
Transitions	
GC → AT	1 (2.9)
AT → GC	4[a] (11.4)
Transversions	
AT → TA	0
AT → CG	5 (14.3)
GC → TA	3 (8.6)
GC → CG	5 (14.3)
Single base insertion	0
Deletions	5 (14.3)
TY element insertion	12 (34.3)
Total	34 (100)

[a]Double mutant.

one-third of the mutations analyzed were insertions of the yeast Ty transposable element into one of two sites within the *SUP4-o* gene. In previous studies, mobilization of the Ty element was found to be induced primarily by agents that cause DNA strand breaks (*37*). These results suggest that DNA strand breaks are another important type of Cr-induced premutagenic DNA damage.

Mammalian pZ189 System

In the mammalian system, the mutational target was the bacterial *supF* gene carried by an SV40-based shuttle vector plasmid, pZ189 (*31*) (Figure 2). In this system, the plasmid DNA was treated with Cr and GSH under controlled *in vitro* conditions and then introduced into the cells for replication and mutation fixation (*24*); or the untreated plasmid was introduced into the cells, and the cells were treated with Cr (*25*). Under the latter conditions, the Cr will be activated by normal cellular constituents and

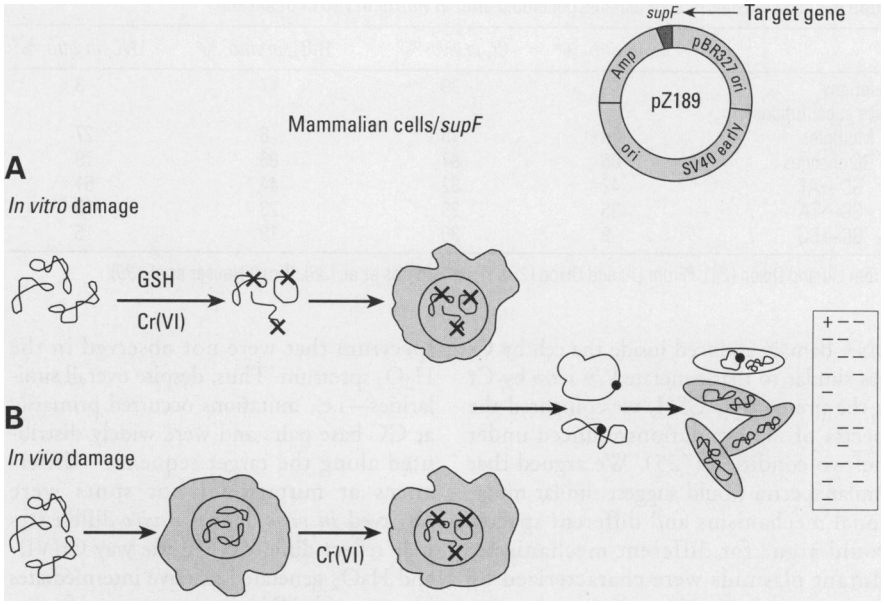

Figure 2. The mammalian/*supF* system. The pZ189 vector carries the bacterial *supF* gene as the mutational target. (*A*) The plasmid DNA is treated with Cr *in vitro* and then transfected into the mammalian cells where it replicates in the nucleus. Replicated plasmid is recovered and mutants are identified by the failure to suppress an *amber* mutation in the bacterial *lacZ* gene. (*B*) Cells are transfected with undamaged plasmid and then treated with Cr. Replicated plasmid is recovered and tested for mutations as in section *A*.

interact with the chromatized replicating plasmid in the cell nucleus. If Cr-induced mutations that were generated after *in vitro* treatment of the plasmid are similar to those generated when plasmid-containing cells are treated with Cr, this would suggest that similar mechanisms are involved.

When the pZ189 plasmid was treated *in vitro* with Cr(VI) in the presence of GSH (1:5 molar ratio) there was a Cr dose-dependent increase in single-strand and double-strand breaks (assayed on agarose gels) and a concomitant loss in biologic activity (assayed as transformation efficiency in *E. coli*) (*24*). In contrast, there was no measurable effect when the DNA was treated with Cr(III) in the presence of GSH, or with either Cr(VI) or Cr(III) in the absence of GSH. Replication of this vector DNA in CV-1 monkey cells led to a Cr dose-dependent increase in *supF* mutants with Cr(VI) plus GSH-treated DNA, but not Cr(III)-treated DNA (*24*) (Table 3). These results demonstrate that reactive intermediates which cause premutational DNA damage are generated in the reduction of Cr(VI) by GSH.

We next asked whether Cr(VI) was mutagenic in the *supF* system when vector-containing cells were treated with Cr(VI). Here again, mutagenesis increased in a dose-dependent manner with Cr(VI) [(*25*); Table 4). To determine whether premutational

Table 3. Frequency of *supF* mutants generated in CV-1 cells transfected with Cr/GSH-treated pZ189.

Plasmid	Mutants/total	Mutation frequency
Untreated	16/63198	0.03
Cr(VI), μM		
1	51/49581	0.10
5	76/21864	0.35
10	97/9274	1.05
20	47/12046	1.22
Cr(III), μM		
1	9/11000	0.08
20	14/13940	0.10

[a]Treatments were conducted in the presence of a 5-fold excess of GSH in phophate buffer, pH 7.0, at 37°C for 90 min; then DNA was washed with 100 mM EDTA and then with 10 mM Tris/1 mM EDTA before transfection.

Table 4. Frequency of *supF* mutants generated in vector-containing CV-1 cells after treatment with Cr.

Cell treatment[a]	Mutant frequency
Untreated	0.05 ± 0.02[b]
Cr(VI), μM	
10	0.15 ± 0.03
20	0.25 ± 0.08
40	1.6 ± 0.03

[a]Treatments were for 12 hr after transfection; plasmid was harvested after an additional 72 hr. [b]Values are the mean of six separate transfections ± standard error.

Table 5. Characteristics of *supF* mutants generated after *in vitro* or *in vivo* Cr treatment.

	Cr, *in vitro* ,%[a]	Cr, *in vivo*, %[b]	H_2O_2, *in vitro*, %[c]	UVC, *in vitro*, %[d]
Deletions	43	39	47	3
Base substitutions				
Multiples	9	18	6	27
GC changes	86	87	86	79
GC→AT	42	32	44	61
GC→TA	35	25	23	13
GC→CG	9	30	19	5

[a]From Liu and Dixon (*24*). [b]From Liu and Dixon (*25*). [c]From Moraes et al. (*38*). [d]From Hauser et al. (*39*).

DNA damage induced inside the cell by Cr was similar to that generated *in vitro* by Cr in the presence of GSH, we compared the spectra of *supF* mutations induced under the two conditions (*25*). We argued that similar spectra would suggest similar mutational mechanisms and different spectra would argue for different mechanisms. Mutant plasmids were characterized by agarose gel electrophoresis to screen for large insertions and deletions and by DNA sequencing to identify smaller changes. A total of 141 mutants were analyzed from the *in vitro* Cr treatment (*24*) and 89 mutants were analyzed from the treatment of vector-containing cells (Table 4) (*25*).

Comparison of the mutants induced following *in vitro* treatment of the vector with Cr plus GSH and the mutants induced when vector-containing cells were treated with Cr *in vivo* revealed many similaritites (Table 5) (*24,25*). A large percentage of the mutations were deletions, similar to that observed with H_2O_2-treated plasmid (*38*) and unlike UVC-treated plasmid (*39*). Most of the mutations occurred at GC base pairs with a large fraction of GC→TA as well as GC→AT base substitutions; this distribution is also similar to H_2O_2-treated plasmid and not UVC-treated plasmid. The fraction of multiple mutations appeared to be somewhat higher when the cells were treated with Cr versus treatment of plasmid.

To compare the mutation spectra with respect to the distribution of mutations within the *supF* gene, we carried out an analysis (*25*) using the method described by Adams and Skopek (*40*). A comparason of the spectra of mutations within the tRNA coding sequence generated by Cr(VI) *in vivo* and *in vitro* revealed no significant difference ($p > 0.05$). A similar comparison of the two H_2O_2 spectra revealed no significant difference. In contrast, comparison of the Cr(VI) and H_2O_2 *in vivo* spectra revealed significant differences ($p < 0.001$); in particular, several hot spots were observed in the Cr(VI) spectrum that were not observed in the H_2O_2 spectrum. Thus, despite overall similarities—i.e., mutations occurred primarily at GC base pairs and were widely distributed along the target sequence—differences at mutational hot spots were observed *in vivo*. These *in vivo* differences may reflect differences in the way Cr(VI)- and H_2O_2-generated reactive intermediates interact with DNA in the context of other cellular proteins.

Big Blue Transgenic Mouse System

To relate mutagenesis data from cell culture to Cr mutagenesis in the target organ relevant to carcinogenesis, we next used the Big Blue transgenic mouse system (Figure 3). In this system, the *lacI* mutagenesis target is carried in a bacteriophage λ-based vector tandemly integrated into the mouse chromosome. These mice were treated with Cr(VI) by intratracheal instillation into the lung, and 1 to 4 weeks later the mice were sacrificed, the lungs were removed, and high molecular weight DNA was isolated. The vector DNA was recovered by λ packaging and *lacI* mutants were identified by plaque color on indicator plates.

We observed a time-dependent (1–4 weeks; data not shown) and Cr(VI) dose-dependent (1.7–6.8 mg/kg) increase in the frequency of *lacI* mutants in vector DNA from the lungs of treated mice (Table 6). This is the first demonstration of Cr(VI)-induced mutations in an experimental animal. Further study will be required to relate this mutagenesis to the carcinogenic activity of Cr compounds. Nevertheless, these results suggest that Cr has the mutagenic potential to be a genotoxic carcinogen.

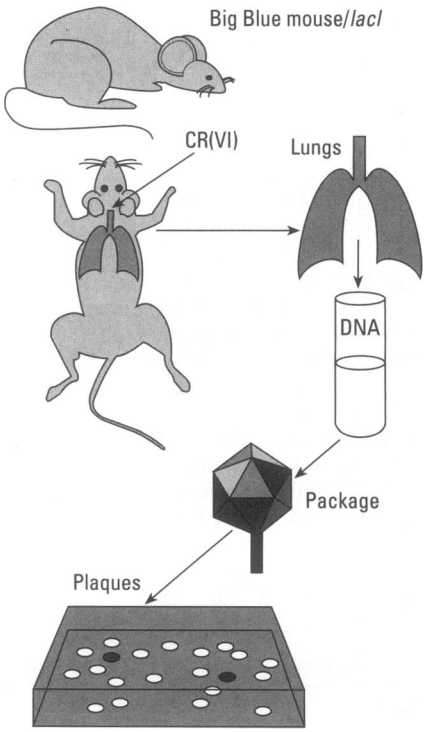

Figure 3. The Big Blue mouse system. The transgenic mice carrying the *lacI* target gene in an integrated λ-based vector, are treated with Cr by instillation into the lung. Later, animals are sacrificed, the lungs are removed, and lung DNA is isolated. Target gene sequences are recovered by λ packaging and *lacI* mutants are identified by plaque color on indicator plates.

Discussion

The carcinogenic potential of certain chromium compounds has been recognized for several decades. This has led to the implementation of restrictions on human exposures and concern about the high levels of chromium contamination at many waste sites. Despite these concerns, chromium compounds are still used in large quantities in industry and the potential for human exposure remains. Although chromium compounds have been shown to be mutagenic in a variety of test systems, the mechanism of mutagenesis has not been well understood. Furthermore, it is not clear what level of danger is posed by environmental sources of chromium. The work described here focuses on an analysis of

Table 6. *LacI* mutant frequency in mouse lung DNA 4 weeks after Cr(VI) instillation.

Treatment	Mutants/PFU	Mean MF ± SD[a]
Saline	13/492,080	$2.6 \pm 0.5 \times 10^{-5}$
1.7 mg/kg Cr(VI)	21/511,310	$4.1 \pm 0.8 \times 10^{-5}$
3.4 mg/kg Cr(VI)	31/400,690	$7.7 \pm 2.2 \times 10^{-5}$
6.8 mg/kg Cr(VI)	45/361,380	$12.5 \pm 1.7 \times 10^{-5}$

Abbreviations: PFU, plaque-forming unit; MF mutant frequency. [a]Four mice were analyzed per group.

mechanisms by which chromium induces DNA damage and causes mutations. The long-range goal of this work is to enhance our understanding of the links between chromium exposure, DNA damage, mutagenesis, and carcinogenesis to provide a rationale for assessing the risks associated with human environmental exposures.

By using both yeast and mammalian cells we have demonstrated that Cr(VI)-induced DNA damage leads to deletions, DNA rearrangements, and base substitution mutations. The pattern of base substitution mutations resembles that induced by agents, such as hydrogen peroxide and ionizing radiation, that cause oxidative DNA damage. In particular, mutations were observed primarily at GC base pairs and were distributed widely within the target gene. This is the first comparison of the mutational specificity of chromium to other oxidative agents in a system that allows the detection of a broad spectrum of mutational events. We did observe differences among these spectra at a few mutational hot spot sites (*25*). The mutational specificity of chromium in human cells was also examined using the denaturing gradient gel electrophoresis method developed by Chen and colleagues (*23*). Of the four sites at which chromium-induced base substitutions were detected in that system, three were GC base pairs; these hot spots differed from those detected in that system after H_2O_2 treatment (*23*). This difference in mutational hot spots is consistent with our observations in the shuttle vector systems. Yang et al. (*22*) also examined the mutational specificity of chromium in the *hprt* gene of Chinese hamster ovary-K1 cells. They reported dominant base changes at AT sites with mainly T→A and T→G transversions. These data may indicate that the base substitution specificity of chromium mutagensis is species specific.

Our studies with transgenic mice demonstrate that Cr(VI) is mutagenic in the target organ (lung) relevant to human chromium carcinogenesis. Despite the relative insensitivity of this system to mutagenesis, we detected a large increase in mutagenesis after exposure to chromium. While the levels of chromium used in this study are well above those associated with human exposure, these studies confirm that chromium has the potential to act as a genotoxic carcinogen in the intact organism. Further analysis of the spectrum of mutations in this system should allow a better understanding of the mutational mechanisms operative in the intact organism. In addition, this system can be utilized to determine the potential for chromium genotoxicity in other organs and by other routes of exposure. In particular, it will be of interest to determine whether exposures that are more relevant to environmental contamination (e.g., drinking water) also cause mutagensis in this system.

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