

# Metal Mutagenesis in Transgenic Chinese Hamster Cell Lines

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Metals are toxic agents for which genotoxic effects are often difficult to demonstrate. To study metal mutagenesis, we have used two stable *hprt*<sup>-</sup>/*gpt*<sup>+</sup> transgenic cell lines that were derived from Chinese hamster V79 cells. Both the G12 and G10 cell lines are known to be very sensitive to clastogens such as X-rays and bleomycin, with the mutagenic response of the integrated xanthine guanine phosphoribosyl transferase (*gpt*) gene in G10 usually exceeding that of the same gene in the transgenic G12 cells. In studies with carcinogenic insoluble nickel compounds, a high level of mutagenesis was found at the *gpt* locus of G12 cells but not at the endogenous hypoxanthine phosphoribosyl transferase (*hprt*) locus of V79 cells. We have since demonstrated the similar recovery of a high frequency of viable G12 mutants with other insoluble nickel salts including nickel oxides (black and green). The relative mutant yield for the insoluble nickel compounds (G12>G10) is the opposite of that obtained with nonmetal clastogens (G10>G12). In the G12 cells, nickel mutagenesis may be related to the integration of the *gpt* sequence into a heterochromatic region of the genome. For some of the insoluble nickel compounds, significant inhibition of both cytotoxicity and mutant yield resulted when the G12 cells were pretreated with vitamin E. In comparison with the nickel studies, the mutagenic responses to chromium compounds in these cell lines were not as dramatic. Mutagenesis of the *gpt* target could not be demonstrated with other metals such as mercury or vanadium. — Environ Health Perspect 102(Suppl 3):63–67 (1994).

Key words: nickel sulfides, nickel oxides, chromium, mercury, *gpt*, mutagenesis

## Introduction

For many metals, toxicity, genotoxicity, and carcinogenicity have been readily established. To define the mechanisms that produce these deleterious effects, gene-specific mutation analysis can be particularly useful. With metals, however, it has often been difficult to obtain mammalian mutagenesis levels that are high enough to allow definition of the mutagenic spectra. Nickel compounds, for example, are generally weak mutagens in mammalian cells (1–4). In comparison, mutagenesis can usually be demonstrated for chromium compounds in most bacterial and mammalian cell systems; however, chromium does not elicit a high mutagenic response at the *hprt* gene in Chinese hamster V79 cells (5,6).

This paper was presented at the Second International Meeting on Molecular Mechanisms of Metal Toxicity and Carcinogenicity held 10–17 January 1993 in Madonna di Campiglio, Italy.

These studies were supported by NIH grant numbers ES 04895, ES 05512, and ES 04715 from the National Institute of Environmental Health Sciences and grant number R814702 from the US Environmental Protection Agency to M.C.; by NIH grant number CA 51825 from the National Cancer Institute to E.T.S.; and by NIEHS Center grant number 00260.

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One of the rationales for developing transgenic V79 cell lines was to enhance our ability to detect and characterize gene-specific, metal-induced mutations. It was known that integrated plasmid-derived shuttle vector sequences were often more mutable than endogenous mammalian genes such as *hprt* (7,8). Additionally, the introduction of a small bacterial gene target into Chinese hamster V79 cells could provide a readily recoverable mutagenesis target sequence (7). This would facilitate mutation analysis by molecular methods including PCR and DNA sequencing. The enzyme encoded by the *E. coli gpt* gene is functionally equivalent to the enzyme produced by the mammalian *hprt* gene. The bacterial *gpt* gene has previously been introduced into several mammalian host cell lines, generating transgenic cells which have been very useful in mutagenesis studies (9–12). Prior to the transfection of the pSV2*gpt* plasmid into our Chinese hamster V79 cells, these host cells were mutated by UV to generate an irreversible *hprt*<sup>-</sup> phenotype. The successful recovery of numerous stable *gpt*<sup>+</sup> transfectants of V79 cells was subsequently described (13).

Two *hprt*<sup>-</sup>/*gpt*<sup>+</sup> transgenic V79 cell lines, G12 and G10, have since been characterized in extensive mutagenesis studies (13,14). Each of these cell lines maintains a single integration site for the plasmid sequence in different locations of the

Chinese hamster genome. Both the G12 and G10 cell lines have been shown to be very sensitive to clastogens such as X-rays and bleomycin, and the mutagenic response of the integrated *gpt* gene in G10 is greater than that of the same gene in the transgenic G12 cells (14). For both cell lines, X-ray mutagenesis of the integrated *gpt* locus exceeds that which can be recovered at the endogenous *hprt* locus of the parental Chinese hamster V79 cells. These cell lines are not generally hypermutable, however, as demonstrated by spontaneous and alkylation mutagenesis of the endogenous Na<sup>+</sup>K<sup>+</sup>/ATPase locus (14). Having established the utility of the transgenic cell lines for mutagenesis studies, the G12 and G10 cells were employed to investigate whether significant levels of *gpt* mutagenesis could be induced by carcinogenic and toxic metals. This manuscript summarizes data that was obtained from our studies of several metals, including soluble and insoluble nickel compounds, chromate, vanadium, and mercury.

## Materials and Methods

### Cell Culture

The *hprt*<sup>-</sup>/*gpt*<sup>+</sup> transgenic cell lines G12 and G10, and the parental Chinese hamster V79 cells, are cultured in F12 medium (GIBCO, Grand Island, NY) with 5% fetal bovine serum (GIBCO) and 1% peni-

cillin/streptomycin (GIBCO) at 37°C and 5% CO<sub>2</sub> (13,14). To ensure low spontaneous mutagenesis levels, the G12 and G10 cultures are initiated every 6 weeks from frozen stocks and are supplemented with HAT (100 μM hypoxanthine, 1 μM aminopterin, 100 μM thymidine). V79 cells are also frequently defrosted, but are not supplemented with HAT.

### Mutagenesis

The mutagenesis protocol for detecting 6-thioguanine (6-TG) resistance as an indicator of genetic alteration or nonexpression of the *gpt* gene in the transgenic cell lines has been previously reported (13,14). The protocol is a standard reseeding assay which is essentially similar to that used to detect *hprt*<sup>-</sup> mutants among V79 cells. Optimum mutant yields can be obtained when the treated cells are reseeded into selection media (F12 with 10 μg/ml 6-TG) 6 days after treatment, and the mutants are permitted to grow for an additional 13 days. The reseeding plating efficiency is also determined at this time, by seeding a small number of cells into F12 without 6-TG. At the end of the selection period, the mutants are simultaneously fixed and stained with Giemsa (50% Giemsa solution, 50% methanol). Mutant frequencies are calculated by totaling the number of mutants obtained on 10 mutagenesis plates per metal dose, and dividing that number by the reseeding plating efficiency of each treated and recovered population of cells. The cytotoxicity of each treatment is measured by a colony-forming assay in which 300 or 500 cells are seeded in triplicate in 60 mm dishes. Following cell attachment, the cells are treated, rinsed, supplemented with fresh F12, and allowed to grow undisturbed for 7 days before staining and counting.

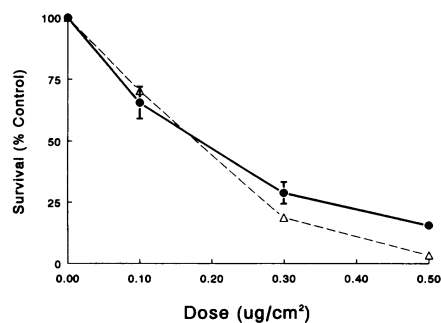
Nickel mutagenesis studies were performed as previously described (15). Briefly, V79 cells and the transgenic cells were exposed to sterilized, ground (<5 μm) particles of insoluble nickel compounds (nickel sulfide, nickel subsulfide, nickel oxides; INCO, Ltd., Toronto, Ontario) for 24 hr in complete media. The nickel doses are expressed as μg nickel/cm<sup>2</sup> surface area of the tissue culture dishes, since these insoluble particles tend to settle out during the 24-hr treatment (16,17). The nickel doses chosen for the mutagenesis studies were determined by preliminary cytotoxicity studies, such that all the different nickel compounds were investigated at generally equitoxic dose ranges. As a positive mutagenesis control, *N*-methyl-*N*'-nitro-*N*'-

nitrosoguanidine (MNNG, Sigma Chemical Co., St. Louis, MO) was routinely used. Potassium chromate (K<sub>2</sub>CrO<sub>4</sub>, Fisher Scientific, Chemical Division, Fair Lawn, NJ) treatments were for 2 to 5 hr (18), at 37°C in either Earle's Balanced Salt Solution (EBSS; GIBCO, Grand Island, NY) or magnesium-supplemented salts glucose media (50 mM HEPES buffer, 100 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, pH 7.2). With chromium oxide (Fisher), the cells were exposed 1–6 μM CrO<sub>3</sub> for 24 hr in complete media. Vanadium exposures were with ammonium metavanadate, NH<sub>4</sub>VO<sub>3</sub> (J.T. Baker Chemicals, Phillipsburg, NJ), for 2 or 24 hr in serum-free F12, SGM, or EBSS as previously reported (5). For the mercury studies, V79, G12, and G10 cells were treated with 3 to 50 μg/mL mercuric chloride (Sigma Chemical Co.) or methyl mercury(II) chloride (Johnson Matthey Electronics, Ward Hill, MA) for 2 hr in EBSS.

### Results and Discussion

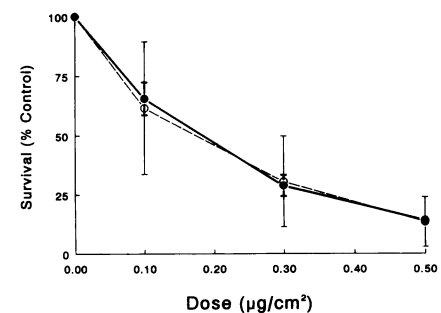
In preliminary studies with nickel compounds, a high level of 6-TG was found to be induced in G12 cells with insoluble nickel sulfide (NiS) but not with soluble nickel sulfate (NiSO<sub>4</sub>) (16). These compounds were not mutagenic at the *hprt* locus of the parental V79 cells. In another study, a high level of *gpt* mutagenesis was also found with insoluble Ni<sub>3</sub>S<sub>2</sub> in G12 cells (17). We have since confirmed these findings, and have recently shown similar levels of induction of 6-TG resistance following the exposure of G12 cells to insoluble nickel oxides (15). We have also investigated the nickel-induced response of the integrated *gpt* gene in the G10 cell line. Figure 1 shows that the cytotoxicity of NiS is similar for the two transgenic cell lines, G12 and G10. These data agree with those from our studies with X-rays, bleomycin, UV, and MNNG (14) in which large cell-line differences in cytotoxicity are not generally observed, whereas the site of *gpt* integration and its mutability differ.

Figure 2 compares the relative toxicity of insoluble NiS and Ni<sub>3</sub>S<sub>2</sub> in the G12 cells. We have studied all the nickel compounds over essentially equitoxic dose ranges (10–100% survival), and find that much higher particle concentrations are required for the nickel oxides than for the nickel sulfides (Figures 3,4). Green nickel oxide was significantly less toxic than the black oxide particles. The high levels of induction of 6-TG resistance by nickel sulfide and subsulfide in G12 cells are shown

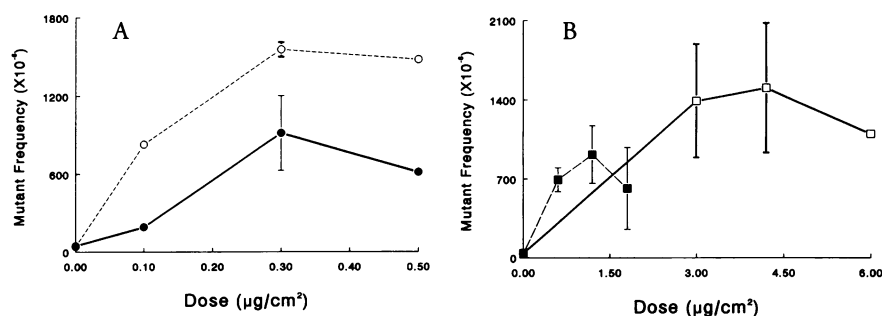


**Figure 1.** NiS toxicity in G12 and G10 cells. Cytotoxicity for insoluble NiS is shown for G12 (●) and G10 (Δ) cells, as derived from one to three experiments per cell line according to the protocol described in "Materials and Methods." Standard errors of the mean are depicted by error bars in all figures where sufficient data exist.

in Figure 3A; while similar results with the nickel oxides are shown in Figure 3B. With soluble NiCl<sub>2</sub>, mutagenesis of the *gpt* locus did not exceed 2 to 4 times the spontaneous levels in either G12 or G10 cells (15). This is in agreement with the data obtained previously for soluble NiSO<sub>4</sub> in G12 cells (16,17), and correlates well with carcinogenicity studies in which the insoluble nickel compounds are the most active due to their phagocytic uptake (19). In our studies with the G12 cells, we could microscopically visualize phagocytized intracellular particles of NiS and Ni<sub>3</sub>S<sub>2</sub> at the end of the 24-hr treatment, but could not see phagocytized particles of NiO (15). Although nickel oxide particles may not be phagocytized as readily as the nickel sulfides (20), there are indications that these particles may remain associated with the cell membranes for long periods (21). Since the treated cells are not disturbed in our assay for up to 6 days after the end of



**Figure 2.** NiS and Ni<sub>3</sub>S<sub>2</sub> toxicity in G12 cells. Cytotoxicity curves for two insoluble nickel compounds, NiS (●) and Ni<sub>3</sub>S<sub>2</sub> (○), are depicted. The data were derived from two or three experiments per compound. For the mutagenesis studies, all nickel compounds were studied over equitoxic dose ranges yielding approximately 10 to 100% survival as shown here.



**Figure 3.** Mutagenesis of insoluble nickel compounds in G12 cells. Comparative mutagenesis of NiS (●) and Ni<sub>3</sub>S<sub>2</sub> (○) at the *gpt* locus of G12 cells, is shown in Panel 3A. Mutagenesis is also shown for NiO (black ■) and NiO (green □) at the *gpt* locus of G12 cells in Panel 3B. The data were derived from two to three experiments per compound. The difference in dose ranges yielding 10 to 100% cell survival is quite evident for the sulfides versus the two oxides.

**Table 1.** Mutagenesis of *gpt* in transgenic cell lines.

Mutagen	Cell line	6-TG <sup>†</sup> Mutagenesis*(times background)
X-rays, 250–750 cGy	G12	18 X
	G10	32 X
Bleomycin, 10–125 µg/ml, 1 hr	G12	8 X
	G10	17 X
mAMSA, 10–100 ng/ml, 3 hr	G12	10 X
	G10	15 X
UV, 2–10 J/M <sup>2</sup>	G12	10 X
	G10	10 X

\*Highest observed mutagenesis expressed as a function of the background mutagenesis for each cell line (induced/spontaneous).

the treatment, the opportunity exists for prolonged cellular association of the nickel oxides.

What is the nature of these nickel-induced mutants, and why do they arise at such a high frequency? Preliminary studies of the mutagenic alterations within the *gpt* sequence of several 6-TG-resistant G12 colonies have thus far failed to show base changes or deletions involving the coding region of the *gpt* gene (17). In studies with another set of mutants, preliminary data strongly suggest that most of the nickel-induced 6-TG-resistant colonies were variants, whose phenotype could be reverted to 6-TG sensitivity by subsequent treatment with nickel or 5-azacytidine (22). Nickel appears to be unique in this regard, since DNA sequence changes, as well as total or partial deletions of the *gpt* gene sequence, among spontaneous, X-ray, bleomycin, and even chromate-induced 6-TG-resistant mutants of both G12 and G10 cells have been observed (14,23). In fact, the *gpt* locus in the G12 cells may be an important nickel-sensitive locus, and studies of the modulation of *gpt* gene expression may be

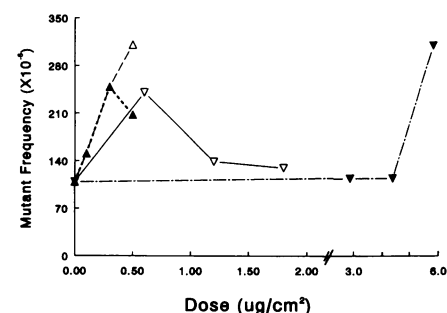
useful for defining the mechanisms of nickel carcinogenesis.

The transgenic G10 cells did not respond to the insoluble nickel compounds with the same intensity as seen above for G12 cells (Figure 4), in contrast to our data with X-rays and bleomycin (14). Nickel mutagenesis could only be induced to about three times that of the spontaneous frequency for the G10 cell line. The cell line differences in *gpt* mutagenesis are clearly evident as summarized on the tables for both nonmetal mutagens (Table 1) and metals (Table 2). In the G12 cells, the nickel effects may be related to the integration of the *gpt* sequence into a heterochromatic region of the genome. The specificity for nickel interactions with heterochromatin are well documented (19,24). Using fluorescent *in situ* hybridization of a biotin-labeled *gpt* probe to the metaphase chromosomes of G12 cells, we have localized the integrated *gpt* sequence to a subtelomeric region of chromosome 1 (25). Based on the C-banding pattern of Chinese hamster V79 chromosomes (25), the *gpt* sequence of the V79-derived G12 cells

appears to be localized within a heterochromatic region of chromosome 1. In contrast, localization studies of the *gpt* sequence in G10 cells suggest a chromosomal position which is neither chromosome 1 or X (Klein, unpublished) and this location may not be sensitive to transcription modulation of *gpt* gene expression in the same manner as in G12 cells.

For some of the insoluble nickel compounds, significant inhibition of both cytotoxicity and mutant yield resulted when the G12 cells were pretreated for 24 hr with a nontoxic (20 µM) dose of vitamin E (15). Vitamin E is an antioxidant, which is capable of protecting cells against some of the genotoxic effects of ionizing radiation (26) and chemicals, including chromate (6,27,28). Vitamin E was previously shown to inhibit some nickel-induced chromosomal aberrations in Chinese hamster ovary cells (29) and we have extended those finding to demonstrate inhibition of cytotoxicity and *gpt* mutagenesis in G12 cells (15). Enhanced cell survival was most notable for NiS and NiO (black); and *gpt* mutagenesis by NiS, Ni<sub>3</sub>S<sub>2</sub>, and NiO (black) could be reduced by up to 50% at some doses (data not shown). With soluble NiCl<sub>2</sub>, significant vitamin E protection was not detected. These results suggests that the process of phagocytosis, and the ongoing generation of reactive oxygen radicals, may play important roles in nickel genotoxicity.

In comparison with the nickel studies, the mutagenic responses to chromium compounds in the transgenic cell lines were not as dramatic. Relative to spontaneous mutagenesis levels, the maximal chromate-induced mutant yield is only three times the background yield for both G12 and



**Figure 4.** Mutagenesis of insoluble nickel compounds in G10 cells. Mutagenesis of insoluble NiS (▲), Ni<sub>3</sub>S<sub>2</sub> (Δ), NiO (black ■), and NiO (green □) particles is compared at the *gpt* locus of G10 cells. The data for NiS and NiO (black) are the means of two independent experiments, while the data for Ni<sub>3</sub>S<sub>2</sub> and NiO (green) are derived from single experiments. The different biologically effective dose ranges for these compounds are clearly evident.

**Table 2.** Comparative metal mutagenesis in transgenic cell lines.

Mutagen	Cells	6-TG <sup>R</sup> mutagenesis (times background) <sup>a</sup>
Nickel sulfide, 0.1–0.5 µg/cm <sup>2</sup> , 24 hr	G12	30 x
	G10	2 x
Nickel subsulfide, 0.1–0.5 µg/cm <sup>2</sup> , 24 hr	G12	40 x
	G10	3 x
Nickel oxide (black), 0.6–1.8 µg/cm <sup>2</sup> , 24 hr	G12	30 x
	G10	2 x
Nickel oxide (green), 3.0–6.0 µg/cm <sup>2</sup> , 24 hr	G12	40 x
	G10	3 x
Nickel chloride, 200–600 µM, 24 hr	G12	4 x
	G10	4 x
Chromate, 5–30 µM, 2 hr	G12	3 x
	G10	3 x
Vanadate, 5–40 µM, 24 hr	G12	2 x
	G10	ND
Methyl mercury, 3–50 ng/ml, 1 hr	G12	<2 x
	G10	<1 x

<sup>a</sup>Highest observed mutagenesis expressed as induced/spontaneous.

G10 cells following a 2-hr exposure to potassium dichromate (5–30 µM). This mutagenesis yield could be increased to five or seven times the background levels, if the chromate treatment times were increased. With chromium oxide, *gpt* mutagenesis could be induced to about four times the spontaneous levels in G12 cells, and was slightly higher in some studies with G10 cells (S. Cosentino, unpublished). Chromium mutagenesis studies, however, are complicated for several reasons. We have observed that the prolonged exposure of attached cells to chromium compounds often results in cellular detachment from the treatment dishes (18). As a result, pre-mutant cells are likely to be lost from the

chromium-treated populations. It is also difficult to define an effective chromium treatment dosing regime, since chromium compounds are often mutagenic over a very narrow dose range (5,6,18,28). This may be partly related to residual toxicity that could result from trivalent Cr that is trapped within the cells (30). Residual chromate toxicity, as indicated by reduced plating efficiency, has been observed up to one week after the treatment of V79, G12 and G10 cells (18).

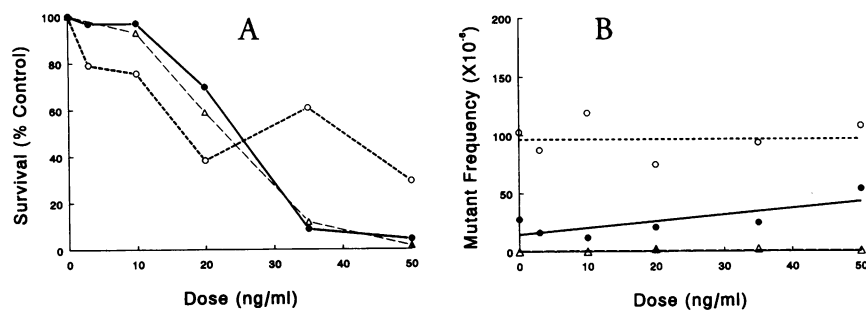
Mutagenesis of the *gpt* targets in G12 and G10 cells could not be demonstrated with other metals such as mercury or vanadium. Figure 5 shows the toxicity and mutagenesis data obtained in experiments

with methyl mercury chloride. As noted in Figure 5A, the G10 cells seem to exhibit a slight resistance to the toxicity of this agent. This effect has not been further studied. Mutagenesis could not be induced at the *gpt* locus of either G12 or G10 cells, nor could it be demonstrated at the *hprt* gene in the parental V79 cells. These findings suggest that mercury compounds are generally too toxic to permit cell survival as required for mutagenesis, and are consistent with the literature on mercury genotoxicity (31,32). In studies with vanadate (Table 2), weak mutagenesis was demonstrated at the *hprt* gene of V79 cells, and at the *gpt* locus of G12 cells, but was not investigated with G10 cells (5).

In conclusion, metal mutagenesis is difficult to quantitate in mammalian cell systems. For most of the metal studies summarized here, the level of induced *gpt* mutagenesis was less than five times the spontaneous frequency. The transgenic G12 cell line, however, is sensitive to insoluble nickel compounds, and will be used in future studies of nickel modulation of *gpt* gene expression. These studies may ultimately be useful in determining the mechanisms involved in nickel carcinogenesis.

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**Figure 5.** Methyl mercury toxicity and mutagenesis in G12, G10 and V79 cells. The cytotoxicity (Figure 5a) of MeHg is depicted for G12 (●), G10 (○), and V79 (△) cells. Mutagenesis could not be induced at either the *gpt* locus of G12 (●) or G10 (○) cells, or at the *hprt* locus of the parental V79 cells (△) as shown in Figure 5b. The data were derived from two independent experiments for each transgenic cell line, and a single experiment with V79 cells.

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