Chromate-Induced Epimutations in Mammalian Cells

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Epigenetic gene silencing by aberrant DNA methylation of gene promoter regions is a nonmutagenic but heritable epigenetic mechanism that may mistakenly cause the silencing of important cancer-related tumor suppressor genes. Using a transgenic, V79-derived, mammalian cell line (G12) that contains a bacterial *gpt* reporter gene in its DNA, we can study carcinogen-induced gene inactivation by mutagenic as well as epigenetic DNA methylation mechanisms. Whereas numerous carcinogens have previously been shown to be mutagenic in these cells, a few carcinogens, including nickel, diethylstilbestrol, and X-rays, are also capable of silencing the G12 cell *gpt* transgene by aberrant DNA methylation. Here we report for the first time that carcinogenic potassium chromate salts can also induce aberrant DNA methylation in this system. In contrast insoluble barium chromate produced significant level of mutations in these cells but did not cause DNA methylation changes associated with transgene expression. *Key words:* chromate, DNA methylation, *gpt* transgene, V79 Chinese hamster cells. *Environ Health Perspect* 110(suppl 5):739–743 (2002).
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Chromium (Cr) is a well-documented animal and human carcinogen (*1*). The biological and genotoxic effects of Cr exposure vary, depending on intracellular Cr chemistry in the cell type of interest. Due to its rapid uptake by normal cellular anion transport mechanisms, Cr(VI) has been considered the primary carcinogenic form. However, reduced states of Cr are likely to participate in cellular redox interactions contributing to oxidative stress, which can have genotoxic outcomes. Within cells, Cr(VI) is reduced to lower valencies (V to II) by a variety of reductants, including cellular thiols (e.g., glutathione [GSH]), NADPH, and ascorbate, to form reactive oxygen species (ROS), thiyl, and carbon-based radicals [reviewed in Kaltreider et al. (*2*) and Liu et al. (*3*)]. The complex intracellular reduction chemistry of Cr can produce DNA damage, Cr-mediated DNA adducts, DNA–DNA and DNA–protein cross-links, and mutations [reviewed in DeFlora et al. (*4*) and Klein (*5*)]. Cr can also inhibit DNA replication and repair [reviewed in Snow (*6*)], alter gene expression (*7,8*), activate stress-response pathways (*2,9*), trigger apoptosis (*10,11*), and damage chromosomes [reviewed in DeFlora et al. (*4*) and Klein (*5*)].

Using soluble chromate salts and insoluble barium chromate ($BaCrO_4$; Aldrich Chemicals, Milwaukee, WI, USA), we studied the genotoxic effects of Cr in Chinese hamster G12 lung cells. These transgenic cells allow the characterization of a mixed spectrum of genotoxic outcomes that may include genetic mutations, transgene deletions, and epigenetic gene silencing caused by aberrant DNA hypermethylation. In previous studies we have shown that the *gpt* transgene in G12 cells can be mutated or otherwise inactivated by numerous mutagens and carcinogens, including ultraviolet (UV) radiation, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), X-rays, bleomycin, nickel, chromate, 17β-estradiol, diethylstilbestrol (DES), nitric oxide, and oxidant generators such as glucose oxidase. The mutation spectrum induced by each of these agents differs significantly. For example, MNNG and UV induce primarily base-substitution mutations (*12,13*), X-rays and bleomycin induce frequent deletions (*13*), and carcinogenic nickel induces DNA methylation epimutations (variants) almost exclusively (*14*). The nickel effect involves nickel, histone, and chromatin interactions in the DNA that promote condensation of heterochromatin in the vicinity of the transgene (*14,15*).

In support of the concept that chemical and physical carcinogens may exert their effects by interacting with a variety of cellular processes, we found that DES and X-rays can produce a mixed spectrum of DNA mutations (e.g., deletions and point mutations) and epimutations (hypermethylation) in the transgenic *gpt* target of G12 cells (*16*). These observations prompted us to reexamine the mutagenic spectrum induced by Cr in these cells to investigate whether DNA hypermethylation could be detected in any of the nondeletion mutants. Molecular analysis of a panel of chromate-induced mutants shows a mixture of deletions and epimutations (or variants), with the deletion versus methylation frequencies being similar to those induced by X-rays in these cells. In contrast, studies with insoluble $BaCrO_4$ have provided substantial evidence of *gpt* deletions but no evidence of altered DNA methylation.

Materials and Methods

Cell Culture and Generation of *gpt*⁻ Mutants or Methylation Variants

The G12 *gpt*⁺ cells were grown in F12 medium (Invitrogen Corp., Life Technologies, Inc./Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Omega Scientific, Tarzana, CA, USA) and 1% penicillin/streptomycin (Invitrogen Corp., Life Technologies, Inc., Grand Island, NY, USA) at 37°C in a humid atmosphere (5% CO_2). Standard mutagenesis protocols for the selection of the 6-thioguanine resistance ($6TG^R$) *gpt*⁻ phenotype in G12 cells were followed as previously described (*12,13,17*). In brief, cells were treated with soluble potassium chromate (K_2CrO_4; Fisher, Pittsburgh, PA, USA) (5–50 μM) or insoluble $BaCrO_4$ (0.05–0.25 μg/cm^2), then allowed a 7-day expression period in nonselective F12 medium prior to being plated for mutant selection in 10 μg/mL 6TGF12 medium [reviewed in Klein et al. (*18*)]. K_2CrO_4 treatments were for 2 hr (*12,19,20*) at 37°C in magnesium-supplemented salts glucose medium (SGM; 50 mM HEPES buffer, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgSO_4$, pH 7.2). Insoluble $BaCrO_4$ treatments were for 24 hr at 37°C in complete F12 medium rather than in SGM because the G12 cells do not tolerate salt buffers for lengthy exposures, even when supplemented with glucose, as we have previously reported (*19*). In contrast to the K_2CrO_4 exposures (2 hr), the $BaCrO_4$ exposures were for 24 hr to allow either dissolution of Cr from the particles, if it occurs, or to allow time for phagocytic uptake of the particles by the G12 cells similar to that which occurs for insoluble nickel compounds (*20, 21*). Also similar to the way in which we have previously studied insoluble nickel compounds (*20,21*), the insoluble

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$BaCrO_4$ doses are expressed as micrograms $BaCrO_4$ per square centimeter surface area of the tissue culture dish, as these insoluble particles (sized at 0.4–0.6 μM) will tend to settle out during the 24-hr exposure time. The dose range of K_2CrO_4 or $BaCrO_4$ chosen for mutagenesis studies corresponds to those doses that fell within the comprehensive 20–100% survival curve generated for each Cr compound.

For the collection of independent chromate-induced $6TG^R$ clones, individually treated minicultures of 10^4 G12 cells were treated with various doses of K_2CrO_4 in 35-mm wells of 6-well dishes, and the cells were handled as independent cultures throughout the entire 6TG selection process. Only one mutant per treated population was isolated and frozen for further analysis. The doses of each compound chosen for subsequent mutant isolation were either the maximal mutagenic dose ($BaCrO_4$) or a variety of doses (K_2CrO_4). All reagents and biochemicals were acquired from Sigma Biochemical Company (St. Louis, MO, USA), unless otherwise specified.

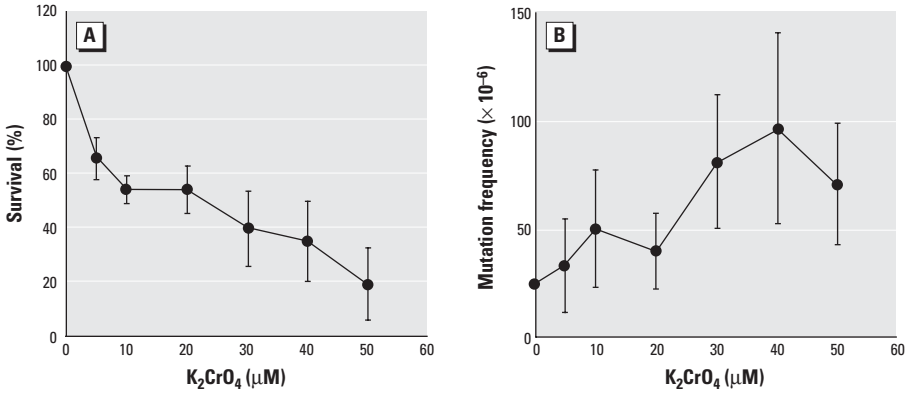

Figure 1. Survival and mutagenesis of G12 cells treated with K_2CrO_4. K_2CrO_4 exposures were for 2 hr at 37°C in magnesium-supplemented SGM. Clonal cell survival (% control) is depicted in (*A*), and the corresponding mutagenesis is shown in (*B*). The data points represent the mean of 3–5 independent experiments, with error bars denoting standard error of the means.

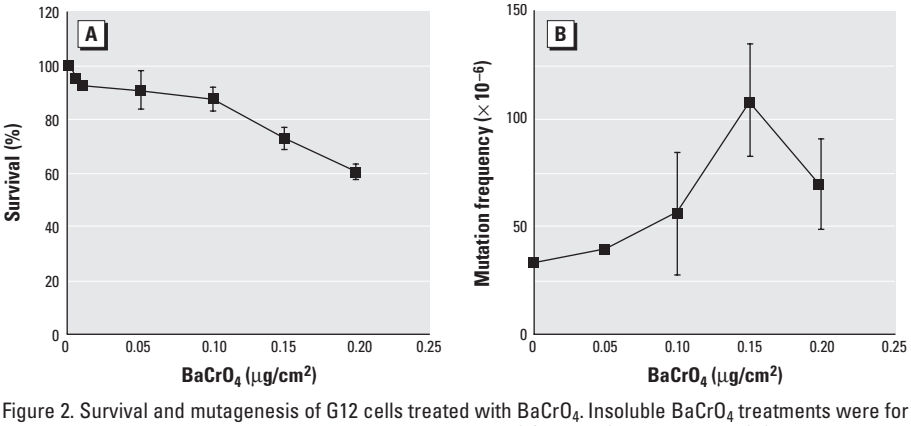

Figure 2. Survival and mutagenesis of G12 cells treated with $BaCrO_4$. Insoluble $BaCrO_4$ treatments were for 24 hr at 37°C in complete F12 medium. Clonal cell survival (% control) is depicted in (*A*), and the corresponding mutagenesis is shown in (*B*). The data points represent the means of 2–3 independent experiments, with standard error of the means shown by error bars for doses where at least three data points were available.

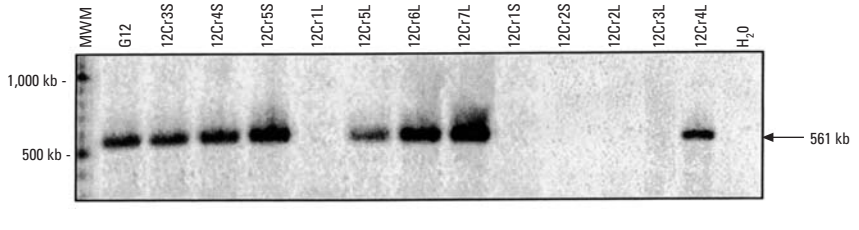

Figure 3. PCR amplification of the *gpt* sequence in G12 cells and K_2CrO_4-induced G12 mutants. MWM, molecular weight marker. High molecular weight DNA was extracted from mutant G12 cells and was screened by PCR to detect total or partial deletions of the *gpt* target genes as shown on agarose gels. The single PCR primer set used generates a single 561-bp PCR amplification product from G12 cells and any nondeleted mutants. K_2CrO_4 exposure doses (2 hr in SGM) were as follows: 12Cr1L and 1S = 5 μM; 12Cr2L and 2S = 10 μM; 12Cr3L and 3S = 20 μM; 12Cr4L and 4S = 30 μM; 12Cr5L and 5S = 40 μM; and 12Cr6L and 6S = 50 μM.

Analysis of Deletion Mutations and DNA Methylation in G12 Cells

A panel of independent $6TG^R$ mutants/epimutants derived from the various chromate treatments was evaluated for deletion or methylation of the transgene. High molecular weight DNA was extracted from 0.5 to 1 × 10^7 mutant G12 cells following standard nonphenol extraction protocols and was screened by polymerase chain reaction (PCR) to detect total or partial deletions of the *gpt* target gene as per Klein et al. (*13*). The single PCR primer set used generates a single 561-bp PCR amplification product from G12 cells and their nondeleted mutants.

The methylation status of the *gpt* transgene and its proximal 5′ promoter region was examined on Southern blots for all nondeletion mutants as previously described (*14*). In brief, Hpa II (CCGG) or Hae II(GCGC) digested Eco RV fragments of G12 DNA containing the *gpt* transgene and proximal promoter regions were separated in agarose, then transferred to Nytran Supercharge (Schleicher and Schuell, Keene, NH, USA) membranes by capillary transfer or vacuum blotting and UV linked (Stratalinker, Stratagene, La Jolla, CA, USA) to the membrane. Blots were prehybridized for 6 hr at 68°C (in 1% nonfat dry milk, 0.1 mM EDTA, 6% NaH_2PO_4, 7% sodium dodecyl sulfate, pH 7.0), followed by overnight probe hybridization in a fresh solution of the same buffer with 10% dextran sulfate. Probes derived from the pSV2gpt plasmid that was originally transfected into V79 cells to create the G12 cells were labeled to a high specific activity (>10^9 counts per min/μg DNA) by random priming or PCR incorporation of ^{32}P. The hybridized membranes were imaged and analyzed using a Storm 860 PhosphoImager (NYU/NIEHS Center, Molecular Biology Facility Core).

Reversion Studies in G12 Cells

To confirm the methylation silencing of the *gpt* transgene induced by chromate, 5-azacytidine–induced reversion to HAT (100 μM hypoxanthine, 1 μM aminopterin, 100 μM thymidine) resistance was examined. In brief,

Table 1. Chromium-induced deletion frequencies.

Cells	Gene	Mutagen	Deletion (% total)
G12	*gpt*	K_2CrO_4	8/15 (53)
G12	*gpt*	$BaCrO_4$	8/23 (33)
V79	*hprt*	K_2CrO_4	14/27 (52)[a]

[a]Only exons 3 and 6 of the *hprt* gene were screened in these mutants.

a representative sampling of G12 epimutants that exhibited evidence of DNA methylation on Southern blots were treated with 5-azacytidine (5 μM) for 48 hr followed by a 24-hr recovery period prior to selection of revertants in F12HAT medium (*14,21*). HAT-resistant clones were stained and counted after 2–3 weeks in selection.

Results

Figure 1 shows that K_2CrO_4 (5–50 μM) was mutagenic in G12 cells over a completely toxic dose range, with maximal mutation induction (3× background levels) peaking at 40 μM (about 40% survival), as we have previously reported (*19,20*). Similarly, insoluble $BaCrO_4$ was mutagenic (Figure 2) in G12 cells, with a maximal mutation peak (3.5× background) observed at 0.15 μg/cm^2 (75% survival). Notable in both of these figures (Figures 1B, 2B) is the peak in mutagenesis levels followed by a decline, and the broadness of the error bars. These features are characteristic of the mutagenesis profiles generated by us and others in various mammalian cells by Cr (*22,23*), by other metals such as nickel oxides (*12,20*), and by oxidants including nitric oxide, hydrogen peroxide, and glucose oxidase (*24*). Interestingly, chromate-induced mutagenesis at the Na^+K^+-ATPase (ouabain resistance) gene was not recoverable in either G12 or their parent V79 cells (data not shown). These results support our findings, described below, showing that chromate primarily induced a mixture of deletion mutants and methylation variants that would not be recoverable at the Na^+K^+-ATPase gene due to the tight limitations of the ouabain resistance selection mechanisms (*12*).

K_2CrO_4 generated *gpt* transgene deletions in 5 of the 12 6TG^R G12 mutants depicted in the PCR panel in Figure 3. These mutants were induced by an increasing range of K_2CrO_4 doses as follows: 12Cr1L and 1S by 5 μM; 12Cr2L and 2S by 10 μM; 12Cr3L and 3S by 20 μM; 12Cr4L and 4S by 30 μM; 12Cr5L and 5S by 40 μM; and 12Cr6L and 6S by 50 μM. Among 15 K_2CrO_4-generated G12 mutants we have examined to date, 53% exhibited complete deletion of the *gpt* transgene. This frequency of chromate-induced deletions was in the same range of transgene deletion frequencies previously described for approximately equitoxic doses of X-rays (48%) and bleomycin (57%) in these same cells (*13*). In comparison, about 20% of spontaneous G12 mutants exhibit transgene deletions (*13*). Additionally, the chromate-induced deletion frequency in G12 cells was identical to that observed for deletions of exons 3 and 6 of the *hprt* gene (14/27 = 52%) in the parental V79 cells (Table 1). Figure 4 shows PCR-deletion screening of a sampling of $BaCrO_4$–induced mutations in G12 cells. In total, we have identified 33% (8/24) deletion mutants from G12 cells exposed to insoluble $BaCrO_4$. This deletion frequency is slightly less than that noted above for K_2CrO_4 mutants. Until now, very little was known about the type of mutations that would be induced by insoluble $BaCrO_4$ (*4*).

DNA methylation analysis of the *gpt* transgene and 5′ promoter region in several K_2CrO_4-induced mutants is shown in Figure 5. Compared with the completely unmethylated status of control untreated G12 DNA (lanes 2 and 3), there is evidence of partial methylation of the *gpt* transgene in Cr variants Cr3S, Cr4S, Cr5S, Cr5L, Cr6L, and Cr7L, as detected by the presence of a 0.98-kb band in the HpaII lanes (Figure 5A) and the 1.7-kb band in the HaeII lanes (Figure 5B). In these studies, almost all nondeleted chromate-induced variants exhibit partial methylation of the transgene. In comparison, only 10% of spontaneous G12 mutants have their transgene silenced by DNA hypermethylation. The Cr mutant Cr1L was previously shown to be deleted by PCR (Figure 3). In contrast to this K_2CrO_4 data, insoluble $BaCrO_4$ did not induce any DNA methylation changes in G12 cells, as shown in the representative panel of mutants examined (Figure 6).

Similar to the partial DNA methylation pattern shown above (Figure 5) for the Cr methylation variants and for some X-ray G12 variants (Figure 7, lanes 3 and 4), nickel-induced variants also exhibited some degree of incomplete or partial methylation of the G12 transgene by nickel (*14*), as evidenced by acquisition of 1.7-kb HaeII bands accompanied by incomplete loss of the unmethylated 1.2-kb bands. This could represent either incomplete methylation or reversion loss of methylation in some cells. In the Cr studies it is possible that the partial methylation we observe is due to the short exposure to chromate (2 hr), and we are presently examining the DNA methylation status of G12 cells exposed to chromate for longer exposures (24–48 hr) as well as chronic exposures (2–6 weeks) to low chromate doses. However, others have reported that 1- to 2-hr exposures of mammalian cells to soluble chromate is sufficient to induce a variety of transcription regulators, including AP1, nuclear factor kappa B, and Sp1 (*2,8*).

A measurable degree of spontaneous reversion, accompanied by demonstrable loss of DNA methylation, occurs in nickel-induced methylation variant cell populations, allowing the *gpt* transgene to regain functional expression in a substantial proportion of the cells (*14*). However, the Cr-induced variant cells discussed herein were continuously grown in 6TG^R selection until the time of DNA extraction, thus eliminating the possibility of any spontaneous reversion at this point. Furthermore, epigenetic silencing of *gpt* in G12 cells can be completely reversed by treatment of silenced cells with the methylation inhibitor 5-azacytidine (*14*) or with a combination of 5-azacytidine and the histone deacetylase inhibitor trichostatin A (*15*), as demonstrated for nickel-silenced cells. We show here (Table 2) that

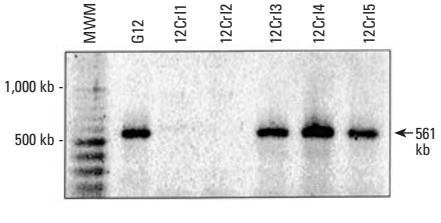

Figure 4. PCR amplification of the *gpt* sequence in G12 cells and $BaCrO_4$-induced G12 mutants. High molecular weight DNA was screened by PCR to detect total or partial deletions of the *gpt* target gene, as shown on agarose gels. All $BaCrO_4$ exposures were 0.15 μg/cm^2 (24 hr) in complete F12.

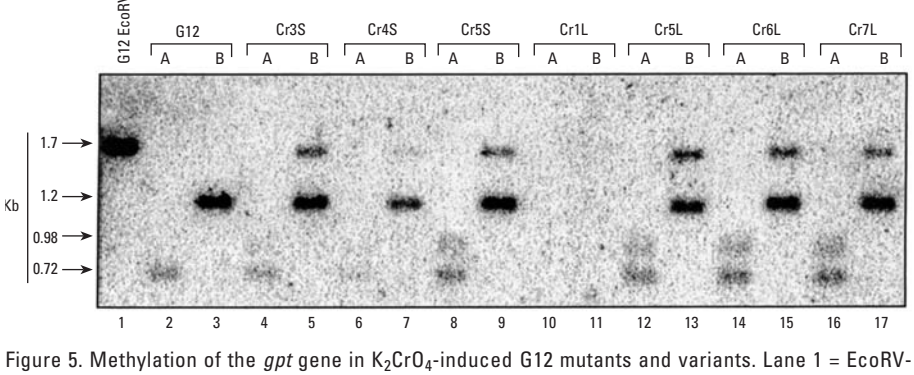

Figure 5. Methylation of the *gpt* gene in K_2CrO_4-induced G12 mutants and variants. Lane 1 = EcoRV-digested control G12 DNA. Lanes 2 and 3 are unmethylated control G12 DNA. All genomic DNA was first digested with EcoRV, then DNA in lanes labeled A were subsequently digested with Hpa II, and those labeled B were digested with Hae II. Cr3S to Cr7L are K_2CrO_4 -induced 6TG-resistant G12 variants. All variants except the Cr1L deletion mutant show methylation of the *gpt* gene, as observed by the 1.7-kb band in HaeII digests (B) and 0.98-kb band in HpaII digests (A). In Cr1L, the *gpt* gene was deleted, as previously shown by PCR. The mutants denoted by shorthand in this figure as Cr3S, Cr1L, etc., are identical to those labeled as 12Cr3S, 12Cr1L, etc. described elsewhere.

chromate-induced methylation variants are also subject to 5-azacytidine–mediated reversion of transgene silencing, as are methylation variants induced by X-rays and DES (data not shown). The reversion frequencies shown in Table 2 are similar to those previously reported for nickel-induced methylation variants (*14*). Whereas spontaneous reversion of nickel-induced variants occurs at a frequency of about 10^{-4}, we have not yet investigated the spontaneous reversion of the chromate variants, but we anticipate it will be similar.

Discussion

Based on our knowledge of the myriad Cr-induced mutagenic, clastogenic, and carcinogenic effects (*5*), including evidence characterizing numerous Cr-induced base substitution mutations (*22,25*), it was not surprising that such a high frequency of *gpt* deletions was recovered in the G12 mutation experiments. Although it was possible that Cr-induced mutagenic base substitutions could have occurred in the PCR primer sites to eliminate *gpt* sequence amplification, the transgene deletions observed by PCR screening were confirmed on Southern blots of genomic DNA. These deletions are not inconsistent with the activity of reactive oxygen or other reactive radicals that may be generated during GSH or other reductant-mediated intracellular reduction of Cr(VI) to Cr(III). In support of this, Cr induced DNA strand breaks when reduced by ascorbate or glutathione [reviewed in Klein (*5*)]. Furthermore, Cr(VI)/GSH- or Cr(VI)/peroxide-induced oxidation-mediated deletions of the pZ189 vector *supF* gene in CV-1 cells have been reported for 43% of the mutants recovered and analyzed in the experiments of Dixon and collaborators (*3,26*).

Similar to K_2CrO_4, insoluble $BaCrO_4$ also yielded a generous proportion of G12 deletion mutants. Indeed, little is known about the mutagenic spectrum that can be induced in mammalian cells by $BaCrO_4$ (*4*). Compared with sodium, calcium, and lead chromates, which generate reactive oxygen radicals in serum-supplemented culture medium, $BaCrO_4$ was reported to be inactive in ROS generation under similar circumstances (*27*). Also in contrast to soluble K_2CrO_4, $BaCrO_4$ was much less active in inducing pulmonary inflammation in rat inhalation studies (*28*). It will be interesting to identify the DNA sequence mutations within the nondeleted transgene in the as yet uncharacterized $BaCrO_4$ mutants.

Although Cr is mutagenic in a variety of bacterial and mammalian systems [reviewed in DeFlora et al. (*4*) and Klein (*5*)], the mutagenic potential of Cr(VI) exposures is ambiguous in the literature. Yang et al. (*22*) analyzed the mutagenic specificity of base substitution mutations caused by Cr(VI) in the *hprt* gene of Chinese hamster ovary cells, finding predominantly AT→TA or AT→CG mutations. These results correlated with previous studies of chromate mutagenesis in *Salmonella* strain TA102, which is sensitive to mutations in A/T-rich sequences (*29*). In contrast, Chen and Thilly (*25*) described four hotspots for Cr mutagenesis in the endogenous human *hprt* gene, specifically observing mutations of CG→AT or TA, CG→AT, and AT→TA. Similarly, Liu et al. (*3*) thoroughly characterized the mutations induced by potassium dichromate in the *supF* gene of pZ189 shuttle vectors in cultured mammalian (CV-1 monkey) cells, finding a predominance of mutations at GC base pairs (GC→AT, GC→CG, GC→TA). These results are in general agreement with Chen and Thilly (*25*). In closely examining the sequence context of the reported mutations in the Liu study (*3*), it becomes evident that many but not all of the chromate mutations are located at guanines and cytosines in CpG DNA sites. Our ongoing examination of the entire *gpt* sequence in nondeleted methylation variants will reveal whether intragenic CpG sites within the transgene are similarly mutated by Cr at guanine or cytosine bases. This would not be unexpected, as there is already sufficient precedent for some other carcinogens to react at CpG sites within relevant cancer genes. For example, studies of benzo[*a*]pyrene mutations in *p53* have defined predominant mutation hotspots at guanines in methylated CpG sites within the coding exons of *p53* (*30–32*). Although the mutation of a CpG site can easily be envisioned to result in the aberrant loss of normal DNA methylation, it is also possible that mutation of CpG sites may lead to aberrant DNA hypermethylation, perhaps through as yet undefined DNA repair-mediated processes or by perturbations of those normal processes.

Our observations of DNA methylation silencing and 5-azacytidine–mediated reversion of transgene expression following chromate exposure were unexpected. Previously we reported that carcinogenic nickel (*14*) and the synthetic estrogen DES (*16*) could silence expression of the *gpt* transgene in G12 cells.

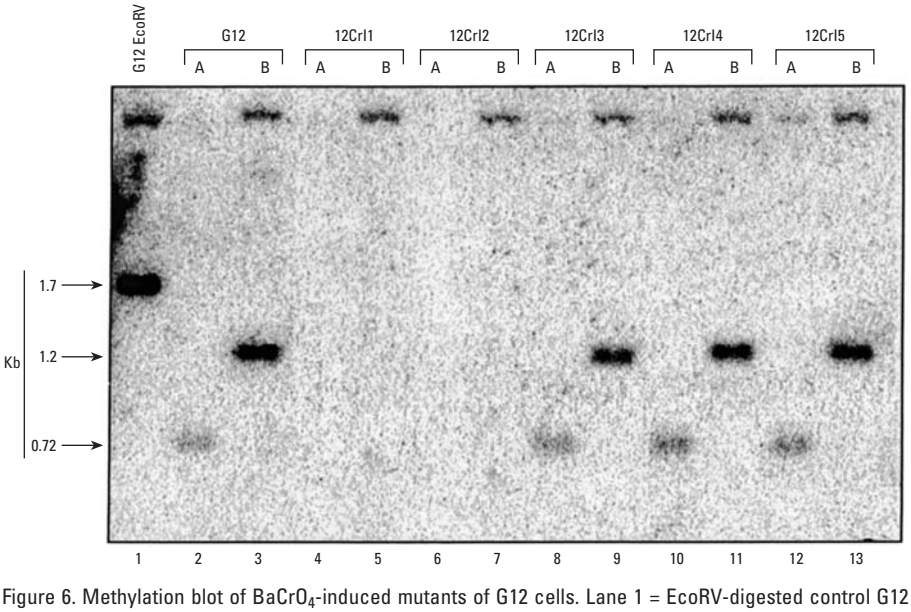

Figure 6. Methylation blot of $BaCrO_4$-induced mutants of G12 cells. Lane 1 = EcoRV-digested control G12 DNA. Lanes 2 and 3 are unmethylated control G12 DNA. All lanes labeled A are digested with Hpa II, and those labeled B are digested with Hae II, after intial digestion with EcoRV. 12Crl1 to 12Crl5 are $BaCrO_4$-induced, 6TG-resistant G12 variants. The *gpt* gene was deleted in 12Crl1 and 12Crl2, as shown above by PCR. There is a complete lack of induced DNA methylation in mutants 12Crl3, 12Crl4, and 12Crl5.

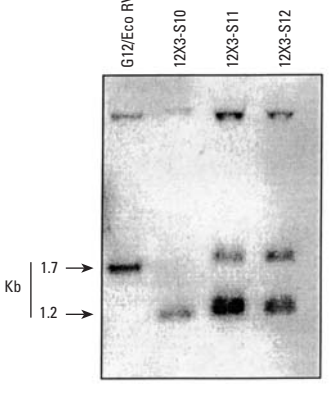

Figure 7. DNA Methylation of *gpt* in X-ray–induced G12 epimutants-Hae II digests. Lane 1 = EcoRV-digested control G12 DNA. Lane 2 is a nonmethylated, nonrevertible X-ray–induced mutant (12X3-S10). Lanes 3 and 4 are partially methylated, 5-azacytidine–revertible X-ray-induced G12 variants (12X3-S11 and 12X3-S12).

Subsequently, we added X-rays and now chromate to the growing list of carcinogens that have the capacity to induce DNA methylation silencing of susceptible target genes. These carcinogens, with the exception of nickel, all induce a mixed spectrum comprising varying proportions of classic gene mutations, transgene deletions, and altered transgene promoter methylation that result in the inactivation, loss, or silencing of the G12 reporter transgene. All epigenetically silenced variants examined to date can be reactivated by 5-azacytidine to reexpress the transgene, regardless of the carcinogen (nickel, chromate, DES, X-rays) that caused the aberrant promoter or transgene methylation. Some degree of spontaneous reversion also occurs for most variants. In contrast to nickel-induced methylation, which causes formation of tightly condensed heterochromatin, our preliminary data suggest that the methylation induced by chromate, DES, and X-rays may be transient, and perhaps weaker, as suggested by DNase resistance studies. Temporal studies are underway to further characterize the persistence of the altered DNA methylation state, as the methylation blots shown herein clearly demonstrate partial transgene methylation, as discussed above.

The relevance of these findings can be applied to the human cancer scenario, for which the loss of expression of a multitude of tumor suppressor and DNA repair has now been shown to result from aberrant DNA methylation (*33*). In human tumors, aberrant DNA hypermethylation of the *CDKN2/p16/MTS1* gene, for example, has been described for about 33% of breast cancers, 60% of prostate cancers, 23% of renal cancers, and 92% of colon cancers cell lines, as well as in 31% of primary breast cancer tumors and 40% of primary colon cancers (*34*). DNA repair genes including O^6-methylguanine-DNA methyltransferase and the mismatch repair genes *hMLH1*and *hMSH6* (*35,36*) are also subject to aberrant transcription silencing in tumors. Even the human telomerase gene is subject to epigenetic DNA methylation silencing, which may have significant ramifications in the carcinogenic process (*37*). Recent studies of DNA hypermethylation in certain cancers suggest that methylation changes in gene expression may in fact be early events in the carcinogenic process (*35,38*).

Table 2. 5-Azacytidine–induced reversion of chromate-induced methylation variants.

Cells	Mutagen	Reversion frequency
12Cr3S	20 μM K_2CrO_4	8.16×10^{-2}
12Cr5S	40 μM K_2CrO_4	8.75×10^{-2}
12Cr5L	40 μM K_2CrO_4	1.05×10^{-2}
12Cr6L	50 μM K_2CrO_4	6.33×10^{-2}
12Cr7L	50 μM K_2CrO_4	3.16×10^{-2}

In conclusion, accumulating evidence from retrospective studies of archived and fresh breast, ovarian, colon, and many other human tumors has provided substantial evidence that a variety of tumor suppressor and other crucial DNA repair and damage control genes can be inactivated by DNA methylation silencing. However, it is unknown from these tumor studies whether these DNA methylation changes occur early in tumorigenesis, possibly resulting from environmental exposures, or if they are late effects perhaps related to establishment, selection, and perpetuation of the tumorigenic phenotype (*39*). Our data support the emerging concept that gene-specific environmental exposure–related changes in DNA methylation may be early events in the tumorigenic process.

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