Use of Denaturing-gradient Gel Electrophoresis to Study Chromium-induced Point Mutations in Human Cells

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A large number of hprt-mutants were obtained by treating human lymphoblast cells (TK6) with 5 μM K₂Cr₂O₇ for 5 hr and selecting by growth in 6-thioguanine. A combination of high fidelity polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) allowed us to measure mutant frequencies as a function of DNA sequence. Chromium(VI) induced four hotspots in a 104 bp domain of hprt exon 3. Substitutions at G:C base pairs were the predominant mutations. One of the chromium-induced hotspots was located at the same position as previously determined hydrogen peroxide and benzo(a)pyrene diol epoxide hotspots. — Environ Health Perspect 102(Suppl 3):227–229 (1994).

Key words: DGGE, mutational spectrum, chromium (VI)

Introduction

Chromium(VI) is a human and animal carcinogen (1). Exposure to chromium(VI) compounds is common in industries like steel manufacturing and leather tanning, and has been linked to excess risk for lung cancer among the workers exposed to various forms of chromium(VI) (2,3). It is believed that chromium(VI) exerts its mutagenic effect by being rapidly transported into cells and reduced intracellularly. However, it is not clear whether the ultimate mutagen is a chromium intermediate or an oxygen radical generated during chromium(VI) reduction.

Mutational spectra, the unique patterns of DNA alterations associated with a mutagen, provide useful information for the study of mechanisms of mutations (4–7). Such cell-culture spectra are usually obtained by treating cells with a mutagen and analyzing a large set of mutant colonies to avoid the bias of sib selection. This “clone by clone” approach demands an inordinate amount of labor to generate a statistically valid spectrum. We have previously described an approach to obtain a mutational spectrum of great statistical precision by treating several large cell cultures with a mutagen to induce large number of independent mutations, amplifying the target DNA sequence, and then separating the separated DNA sequences from nonmutant sequences by denaturing gradient gel electrophoresis (DGGE) (8). The positions, types, frequencies and sequence specificity are studied by sequencing the mutant DNA. Such an approach reduces the time and labor necessary to obtain the mutational spectrum.

Obtaining Chromium-induced Mutants

To achieve a precision of ±20% for the induced frequency of a particular mutation, one must analyze at least 100 independent mutants for that particular mutant type. If we select the hprt-mutants by 6-thioguanine (6TG), and consider 1% of 6TG-resistant (6TG') mutations as a hotspot, then we need to induce 10,000 independent 6TG' mutants to confirm the existence of such a hotspot. In addition, assuming 50% of the 6TG' mutants are due to large DNA alteration (e.g., large deletion), one needs to analyze 20,000 independent mutants to obtain a point mutation spectrum. In this study, 6 liters of human lymphoblast cells (TK6) at a concentration of 1 x 10⁶ cells/ml were treated with 5 μM K₂Cr₂O₇ for 5 hr. Survival and mutant fraction were determined (9) to be 33% and 1.2 x 10⁻⁵, respectively. The treatment resulted in:

(treated cell no.) x (survival fraction) x (mutant fraction)

= (6 liters) x (1 x 10⁹ cells/liter) x (0.33) x (1.2 x 10⁻⁵)

= 23760 6TG' mutants.

The experiment was performed twice. The number of chromium induced mutants met our requirement to obtain a mutational spectrum with good statistical precision.

Application of DGGE to Examine hprt-Mutations

DGGE is a technique developed by Fischer and Lerman (10) to separate DNA sequences that differ by a single base pair. The technique is based on the fact that the electrophoretic mobility of DNA in polyacrylamide gel is sensitive to the secondary structure of the molecule. When a DNA sequence containing both a high-melting and a low-melting domain is run on a polyacrylamide gel with increasing concentrations of denaturant, the low-melting domain will make the transition from helix to random coil (melted), resulting in greatly reduced mobility of the molecule. The melting property of a domain is extremely sequence-dependent. We extended this approach by boiling and renaturing mutant sequences together with wild-type sequences to create mutant/wild-type heteroduplexes. By this means, all point mutations in the low-

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The wild-type domain of hprt exon 3 in K₂Cr₂O₇ treated cultures.

**Figure 1.** DGGE analysis of K₂Cr₂O₇-induced mutations in the low-melting domain of hprt exon 3. The sequence was amplified with primers P1 and P2. Lane 1: control; lane 2: untreated and 6TG-selected culture; lanes 3 and 4: K₂Cr₂O₇-treated and 6TG-selected cultures. Additional bands in lanes 3 and 4 represent chromium-induced mutations.

Results and Discussion

**Mutational Spectrum of K₂Cr₂O₇**

Results of DGGE analysis of mutations in the 104-bp low-melting domain of hprt exon 3 are shown in Figure 1. Results of sequence analysis of the mutant bands are displayed in Table 1. Four hotspot mutations induced by K₂Cr₂O₇ treatment were found. The frequencies of these mutations ranged from 2 to 4.5% of 6TG² mutants and contained 475 to 1069 independent mutants in each hotspot. Three of the 4 mutations were G:C base substitutions. Mutations occurred at base-position 243, and 289 mutations shared a common local sequence, 5'-TGTA-3', where G was mutated.

**DGGE as a Tool to Study Chromium Mutagenesis**

This study demonstrated a clear reproducible spectrum for chromium(VI). The number of mutants studied was large enough to permit statistical analysis of the mutant frequency as a function of DNA sequence.

The approach of high-fidelity PCR and DGGE can now be further applied to study the mechanism of chromium mutagenesis. The mutational spectra of chromium(VI) and the suspected ultimate mutagen could be compared. The comparison could reveal either no common hotspot, suggesting that the suspected ultimate mutagen does not contribute significantly to chromium mutagenesis; or some common hotspots, suggesting a shared pathway related to the suspected ultimate mutagen. It is interesting to see that chromium(VI), as well as hydrogen peroxide (13) and benzo(a)pyrene diol epoxide (8), induced mutations at position 243. Chromium(VI) and benzo(a)pyrene diol epoxide hotspots are both G:C → A:T; but the hydrogen-peroxide hotspot is G:C → C:G. These results suggest either that all three mutagens may share some but not all mutagenic pathways or that position 243 is particularly prone to DNA damage leading to hprt-mutations.

| base substitution | Number of mutants | % of 6TG² cells | Local sequences | Positions | Amino acid change |
|-------------------|------------------|----------------|----------------|----------|------------------|
| CG → AT           | 1069             | 4.5            | TTATAC          | 243      | Tyr → stop       |
| AT → TA           | 475              | 2.0            | ATCAAG          | 247      | Lys → stop       |
| GC → AT           | 594              | 2.5            | ACTGTA          | 288      | Val → Ile        |
| CG → TA           | 950              | 4.0            | GAGCTAT         | 312      | Ser → Ser        |

*The sequences indicated are the non-transcribed strand (5' → 3') of hprt gene. Bases in bold face represent the mutated positions.

**REFERENCES**

1. IARC. IARC Monographs on the evaluation of carcinogenic risks to humans. Vol. 49. Chromium, Nickel and Welding. Lyon: International Agency for Research on Cancer, 1990.
2. Norseth T. The carcinogenicity and chromium. Environ Health Perspect 40:213–214 (1981).
3. Sundermann, F.W. Carcinogenicity and mutagenicity of some metals and their compounds. IARC Scientific Publication No. 71, Lyon: International Agency for Research on Cancer, 1986:17–44.
4. Coulondre C, Miller JH. Genetic studies of the lac repressor: IV. Mutagenic specificity in the lac gene of E. coli. J. Molec Biol 117:577–606 (1977).
5. Coulondre C, Miller JH, Farabaugh PJ, Gilbert BW. Molecular basis of base substitution hotspots in Escherichia coli. Nature 247:775–780 (1978).
6. Fuchs RPP, Schwartz N, Daune MP. Hotspots of frameshift mutations induced by the ultimate mutagen N-acetoxy-N-2-acetylaminofluorene. Nature 294:657–659 (1981).
7. Glickman BW, Ripley LS. Structural intermediates of deletions mutagenesis: a role for palindromic DNA. Proc Natl Acad Sci USA 81:512–516 (1984).
8. Keohavong P, Thilly WG. Mutagenic spectrometry: a general approach for hotspot point mutations in selectable genes. Proc Natl Acad Sci USA 89:4625–4627 (1992).
9. Furth EE, Thilly WG, Penman B, Liber WHL, Rand WM. Quantitative assay for mutation in diploid human lymphoblasts using microtitre plates. Anal Biochem 110:1–8 (1981).
10. Fischer S, Lerman LS. DNA fragments differing by single base-pair substitutions separated in denaturing gradient gels: correspondence with melting theory. Proc Natl Acad Sci USA 80:1579–1583 (1983).
11. Thilly WG. Potential use of gradient denaturing gel electrophoresis in obtaining mutational spectra from human cells. In: Carcinogenesis: The Role of Chemicals and Radiation in the etiology
11. Huberman E, Barr SH, eds. New York: Raven Press, 1976; 511-528.
12. Ling LL, Keesavong P, Dias C, Thilly WG. Optimization of the polymerase chain reaction with regard to fidelity: modified T7, Taq, and Vent DNA polymerases. PCR Meth and Appl 1: 63-69 (1991).
13. Oller AR, Thilly WG. Mutational spectra in human cells: spontaneous, oxygen and hydrogen peroxide-induced mutations at the hprt gene. J Mol Biol 228:813-826 (1992).