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Chrysotile Fiber Is a Strong Mutagen in Mammalian Cells[1]

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ABSTRACT

Although chrysotile asbestos is a proven human carcinogen, several studies have concluded that these fibers are not mutagenic to cultured mammalian cells. We show here, on the other hand, that when tested using the A_L cell system that detects both intragenic and multilocus mutations, chrysotile is indeed mutagenic and comparable in strength to that of γ-rays. Southern analysis of the induced mutants shows that the majority contains large deletions ranging in size from a few thousand to several million base pairs. Results of our study demonstrate that, while chrysotile may be less durable *in vivo* than the amphibole fibers such as crocidolites and amosites, it can effectively create genetic damage involved in the cancer process.

INTRODUCTION

The carcinogenicity of asbestos fibers has been well established in both humans and experimental animals (1–3). The mechanisms by which asbestos produces malignancy, however, are not clear. Various *in vitro* and *in vivo* studies suggest that fiber dimensions, surface properties, and physical durability are important criteria for the carcinogenicity of asbestos (2, 4). Studies using oncogenic transformation as an end point have shown that asbestos fibers can induce malignantly transformed foci in certain rodent cells (5) and that asbestos fibers, in combination with either benzo(*a*)pyrene (6) or ionizing radiations (7, 8), can synergistically enhance the oncogenic transforming incidence by these agents. Recent studies, however, suggest that the interaction between asbestos and benzo(*a*)pyrene may be cell line dependent (9). In addition, there is evidence to suggest that oxygen radicals may be important in the toxicity and oncogenic transforming effects of asbestos fibers (10–12).

Data available for genotoxicities of asbestos fibers are variable and the results appear to depend on the end point examined. Several types of asbestos fibers have been shown to induce chromosomal aberrations (13–15) and sister chromatid exchanges in cultured rodent and human cells (16, 17). Reports on the mutagenicity of several types of asbestos have largely been negative in both mammalian cells (18–20) and bacteria (21). Huang *et al.* (22) using the HGPRT[4] locus in Chinese hamster lung cells have, thus far, reported the only positive, although marginal, mutagenic effect of crocidolite fibers (22).

Recent studies based on epidemiological data of asbestos miners and the analysis of specific fiber types recovered from the lungs of mesothelioma patients have suggested that amphibole asbestos such as crocidolites and amosites may be more potent than chrysotile fibers in the induction of mesothelioma and other lung diseases (23). The possibility that chrysotile fiber is less durable than the amphible fibers due to leaching may be a factor of concern in human risk analysis. In the present study, we quantified mutations induced by graded doses of chrysotile fibers using the A_L human- hamster hybrid cell in an antibody complement mediated cytotoxicity assay to determine the genotoxic potential of the fibers. The A_L cell contains a single copy of chromosome 11 as its only human chromosome that encodes a series of human cell surface antigens. The genes for these antigenic markers have been regionally mapped on the human chromosome (24, 25). This assay can detect sensitively both intragenic and multilocus mutations since virtually the entire human chromosome serves as a target for mutation. Since only a small portion of the human chromosome 11 is essential for viability of the hybrid cell, even large chromosomal deletions involving millions of base pairs are not lethal.

We have examined the mutational events at both the *HGPRT* and the human chromosome marker genes within the same chrysotile-treated A_L cell population in order to quantitate a spectrum of genetic events, ranging from point mutations to small and large deletions. We show here that chrysotile is, in fact, a strong mutagen at the S_1 locus of the human chromosome and that the principal class of mutations it induces are large, multilocus types.

MATERIALS AND METHODS

Cell Cultures

The human-hamster hybrid cell line (A_L), developed by Puck *et al.* (25), was used in these studies. The hybrids were formed by fusion of human fibroblasts and the gly⁻A mutant of the Chinese hamster ovary cells. In addition to the standard set of hamster chromosomes, these hybrid cells contain a single copy of human chromosome 11. Cell surface antigenic markers such as S_1, S_2, S_3, lactic dehydrogenase, and β-globulin have been identified on these cells and have been regionally mapped on chromosome 11 (24). Normal rabbit serum was used as a source of complement and specific monoclonal antibody against the S_1 antigenic marker was produced as described (26). These antibodies have been shown to be highly specific for their respective human antigens and display no cross-reactivity with any hamster antigens under the conditions used here. All complement preparations were screened and those displaying nonspecific toxicity were rejected (27). Cells were maintained in Ham's F-12 medium supplemented with 8% heat inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 2 × normal glycine (2×10^{-4} M), and 25 μg/ml gentamycin.

Asbestos Fibers and Cell Treatment

International Union Against Cancer standard reference chrysotile fibers were used in these studies. The compositional analysis, size distribution, and preparation of the fibers have been described previously (28).

Exponentially growing A_L cells were trypsinized and replated into 25-cm^2 area tissue culture flasks at 1×10^5 cells/flask. Forty-eight h after plating, the cultures were treated with graded doses of chrysotile fibers in 5 ml of culture medium/flask for 24 h. Following treatment, the culture were washed twice with buffered salt solution, trypsinized, counted, and replated into 75-cm^2 area flasks at a density such that approximately 1×10^5 cells were included. The actual number of cells

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[4] The abbreviations used are: HGPRT, hypoxanthine-guanine phosphoribosyltransferase; D_0, the concentration that reduces the cell survival to 1/*e* in the log-linear portion of the survival curve.

plated per flask depended on the survival level and ranged from 1.2×10^5 cells for no killing to 8×10^5 cells for 10% survival. Corresponding dishes were plated at a lower cell number to determine survival.

Mutagenesis Assay

The asbestos treated cultures were incubated for 1 week before mutagenesis testing began as described (27, 29). This expression period permitted the surviving cells to multiply to the point at which the progeny of the mutated cells no longer contain lethal amounts of the surface antigens. The cultures to be tested were then trypsinized and counted. Aliquots containing 5×10^4 cells were plated into each of six 60-mm-diameter dishes in 2.0 ml of growth medium. The dishes were incubated for 2 h to allow cell attachment. Subsequently 0.2% antibody together with 1.5% freshly thawed complement were added to each dish. After overnight incubation, the medium was changed in all dishes. Culture were incubated for 8–10 days at which time they were fixed, stained, and scored for surviving colonies. The control included identical sets of dishes, with appropriate number of cells, containing antiserum alone, complement alone, or neither agent. The cultures were tested each week for 2 consecutive weeks to ensure full expression of the mutants. During this period, the cultures were subcultured twice per week. New dishes were seeded with approximately 2×10^5 cells, a number large enough to minimize possible distorting effects that could result from random fluctuations in the number of mutants present in small inocula. As such, the induced mutant fractions remain fairly constant for several weeks (26, 27). Mutation frequencies were determined as the number of surviving colonies divided by the total number of cells plated after correction for any nonspecific killing due to complement.

To assay for the induction of HGPRT$^-$ mutants, exponentially growing A_L cultures that had been subcultured after the fiber treatment described above were trypsinized and replated into 100-mm diameter dishes at a density of 1×10^5 cell/dish (30). Each dish contained 12 ml of completed F-12 medium, together with 40 μM 6-thioguanine. Corresponding dishes were plated at lower cell densities in normal medium to determine plating efficiencies. A total of 30 dishes for mutant selection and 12 dishes/dose point for plating efficiency were plated. After incubation for 9 to 10 days, all dishes were fixed and stained as described above. Mutation frequencies were expressed as the number of mutant cells/10^5 survivors. The treated cultures were tested for mutagenesis at the HGPRT locus for 2 consecutive weeks after the initial 1-week expression period.

Molecular Characterization of the S_1 and HGPRT$^-$ Mutants

Preparation of High-Molecular-Weight DNA. Isolation of the S_1^- and HGPRT$^-$ mutants was achieved by cloning. High-molecular-weight DNA from various asbestos-treated and spontaneous mutants were prepared according to the method modified from Maniatis *et al.* (31). Briefly, mutant cells were lyzed by treatment with sodium dodecyl sulfate (0.5% by volume) and proteinase K (200 μg/ml) in Tris buffered saline at 37°C. The cell lysates were then extracted once with an equal volume of buffered phenol, once with buffered phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The final DNA precipitate was then washed twice with 95% ethanol, air dried, redissolved in sterile 10 mM Tris-EDTA buffer at pH 7.5, and stored at 4°C until use.

Southern Blot Hybridization. Fifteen μg of high-molecular-weight DNA were digested with restriction endonuclease in the buffer specified by the supplier (Biolabs, Beverly, MA) for 5 h at 37°C. *Pst*I and *Eco*RI were used for the HGPRT$^-$ and S_1^- mutants, respectively. The fragments were then fractionated on a 0.8% agarose gel. The slab gel was photographed, hydrolyzed in 0.25 M HCl, denatured in 1 M NaCl-0.5 M NaOH for 30 min, and neutralized in 0.5 M Tris-HCl buffer (pH 7.4) for 60 min. The blotting was done overnight in 20 × standard saline citrate buffer onto a nitrocellulose sheet. After baking for 2 h at 80°C, the blot was prehybridized and then hybridized with ^{32}P-labeled complementary DNA probe (pHPT12 for the HGPRT locus and a mixture of three probes, D_{16}, FTH, and APO-A1, for the S_1 locus) using a Robbin Scientific incubator (Sunnyvale, CA). The pHPT12 was kindly provided to us by Dr. Richard Okinaka, Los Alamos National Laboratory. whereas the probes for the human chromosome were obtained from the American Type Culture Collection (Rockville, MD). The filter was then washed and exposed to X-ray film at −80°C for 2 days in the presence of intensifying screens.

RESULTS

Chrysotile fibers induced a concentration dependent toxicity in A_L cells as shown in Fig. 1, where the surviving fractions, after a 24-h treatment period, are plotted against fiber concentration. The highest concentration examined, 40 μg/ml, was equivalent to ~8 μg/cm^2 of area of the culture flask. A fiber concentration of 13 μg/ml killed 50% of the cells. The mean lethal dose, D_0, is ~20 μg/ml.

Wild-type A_L cells express a set of human surface antigen markers including S_1 and S_2. A gene locus at 11p13 (*MIC1*) encodes the S_1 antigen, formerly called a_1 (32, 33). In the presence of complement and specific monoclonal antibody against the S_1 antigen, wild-type A_L cells are quantitively lysed, while mutated cells that have lost the S_1 antigen survive to form colonies (26, 27, 34). The few that survived come from preexisting mutants in the populations and represent the background mutant fractions. The average number of preexisting S_1^- mutants/10^5 survivors ranged from 57 to 150. Fluctuation analysis shows that the spontaneous rate of loss of the S_1 marker is $\sim 1.5 \times 10^{-6}$/cell/generation (34).

The mutant fractions induced by graded doses of chrysotile fibers at the S_1 and HGPRT loci of the A_L cells are shown in Fig. 2. Although the number of induced HGPRT$^-$ mutants (observed minus background) in the high dose groups was, in some experiments, greater than zero, the difference in the mutant fraction between the highest and lowest doses was not statistically significant so that pooled data from four experiments produced no consistent dose response for the yield of mutants at that locus. The background HGPRT$^-$ mutant fraction in our A_L cells ranged from 0.16 to 5.5 mutants/10^5 survivors, a value within the range of that reported in other cell lines (18, 19). When 6-thioguanine resistant mutants were cloned and retested in hypoxanthine-aminopterin-thymidine medium, none survived, indicating that they were true HGPRT$^-$ mutants.

In contrast to the inconsistent and weak response at the HGPRT locus, chrysotile fibers induced a dose dependent mutagenesis at the S_1 locus. At the lowest fiber concentration

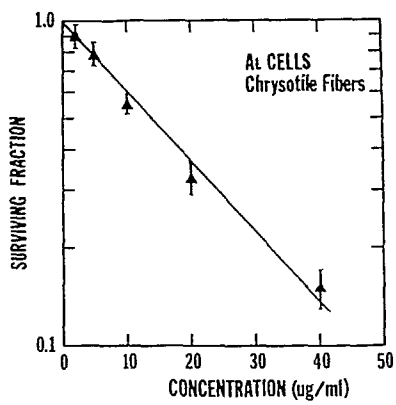


Fig. 1. Effects of graded doses of chrysotile fibers on the surviving fractions of A_L cells. Exponentially growing A_L cells were treated with chrysotile fibers for 24 h. Cultures were washed, trypsinized, and replated for colony formation. Each data point represents an average of 4 to 8 dishes from 4 experiments. *Bars*, SEM.

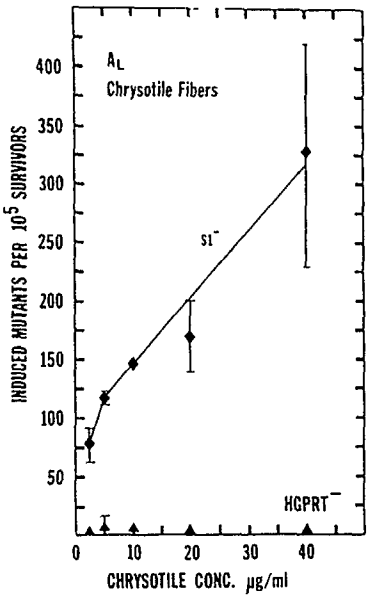


Fig. 2. Mutation induction by chrysotile fibers, expressed as number of mutants/10^5 survivors, at the S_1 (♦) and HGPRT (▲) loci measured in the same population of A_L cells. Each point represents data pooled from 3 to 4 experiments. Induced mutant fractions = total mutant yield minus background. Mutation was determined at 7–14 days after exposure to chrysotile fibers. These induced mutant fractions were reasonably constant over the assay period (26, 27). *Bars*, SEM.

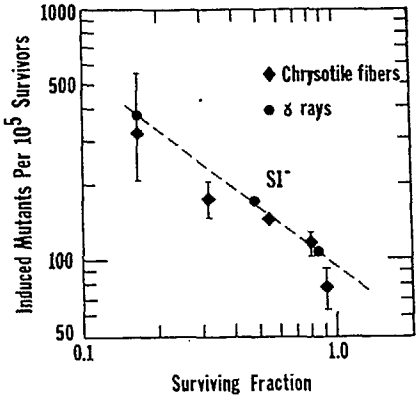


Fig. 3. Induced S_1^- mutant fractions as a function of cell survival for chrysotile fibers and γ-rays. Data for radiation are replotted from Ref. 27. The mutagenic potency of chrysotile fibers is comparable to that of γ-irradiation.

examined (2.5 μg/ml), the mutant fraction at the S_1 locus was at least 100-fold higher ($78 \times 10^5 \pm 10$) than the corresponding HGPRT$^-$ mutant yield. Since the rate of spontaneous loss of the S_1 marker is low compared to the frequency of induced mutations, the background mutant fraction was subtracted at each asbestos dose to give the induced frequencies.

The mutagenic potency of asbestos fibers is comparable to that of γ–irradiation as shown in Fig. 3 where the mutant frequencies for the 2 agents are plotted against the corresponding surviving fractions. An additional perspective is provided when the mutagenicity of chrysotile fibers is compared in terms of mutants per mean lethal dose, D_0, with such recognized mutagens such as γ-rays, UV irradiation, or the chemical ethyl methanesulfonate. The rationale for this comparison has been discussed (34). The values of D_0 for these four agents are 20 μg/ml, 145 cGy, 11 J/m^2, and 100 μg/ml, respectively, so that the mutant yields/D_0 are ~165, 130, 45, and 120 mutants/10^5 survivors. As such, chrysotile can readily be classified as a strong mutagen in mammalian cells.

We used Southern analysis to investigate the kinds of lesions that underlie the HGPRT$^-$ and S_1^- phenotypes. Fig. 4 shows representative Southern blots obtained by probing *Pst*I restriction digests of DNA extracted from individual HGPRT$^-$ mutant clones with the ^{32}P-labeled pHPT12 probe which contains the full length HGPRT complementary DNA of Chinese hamster cells (35). Greater than 60% of the spontaneous mutants from untreated populations had no detectable change in their *HGPRT* gene, presumably because the inactivating mutation was too small to be detected. The remaining clones had a partial deletion of the gene. Although the pooled data for induction of HGPRT$^-$ mutants did not produce a reliable dose response curve, the number of mutants in population exposed to a dose of 20 μg/ml of fibers was, in individual experiments, often higher than background. To study these "induced mutants," we picked clones from dishes where the increase over background was such that 2 out of 3 clones isolated would be expected to be induced based on statistical grounds. Southern analysis of the limited number of clones available by this criterion revealed that 80% (12 of 15) had deletions involving large portions of their *HGPRT* gene, while the remaining three clones only lost 1 out of the 6 functional sequences. This result agrees with our earlier finding that crocidolite fiber efficiently induces large deletions (36).

S_1^- mutants from control and exposed populations were also selected for molecular analysis. An advantage of the A_L hybrid is the availability of a large number of mapped, chromosome 11 DNA probes (32, 33, 37), the use of which allows one to define the sizes of mutations. Molecular analysis of the asbestos induced S_1^- mutants using probes for marker genes that mapped on both the long or short arms of chromosome 11 indicates that

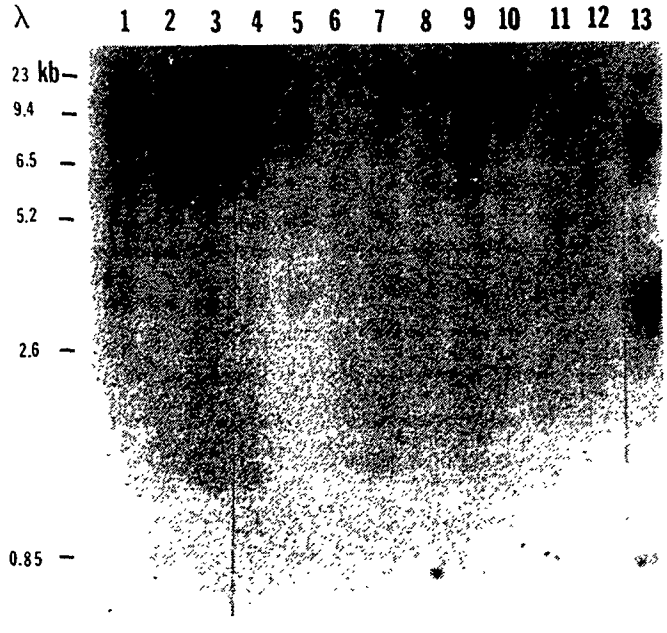


Fig. 4. Southern analysis of DNA from 9 fiber induced HGPRT$^-$ mutants selected from populations treated with a dose of 20 μg/ml (*Lanes 4–12*), 3 spontaneous mutants (*Lanes 1–3*), and from control A_L cells (*Lane 13*). *Hind*III digested DNA was used as molecular size markers. The full-length Chinese hamster HGPRT complementary DNA probe (pHPT12) hybridized to DNA from normal A_L cells as 6 fragments of 8.8, 8.2, 7.6, 5.2, 3.2, and 0.85 kilobases. In some samples, a weakly hybridizing fragment of 2.6 kilobases could also be identified. This and the fragment at 5.2 kilobases represent pseudogenes (35).

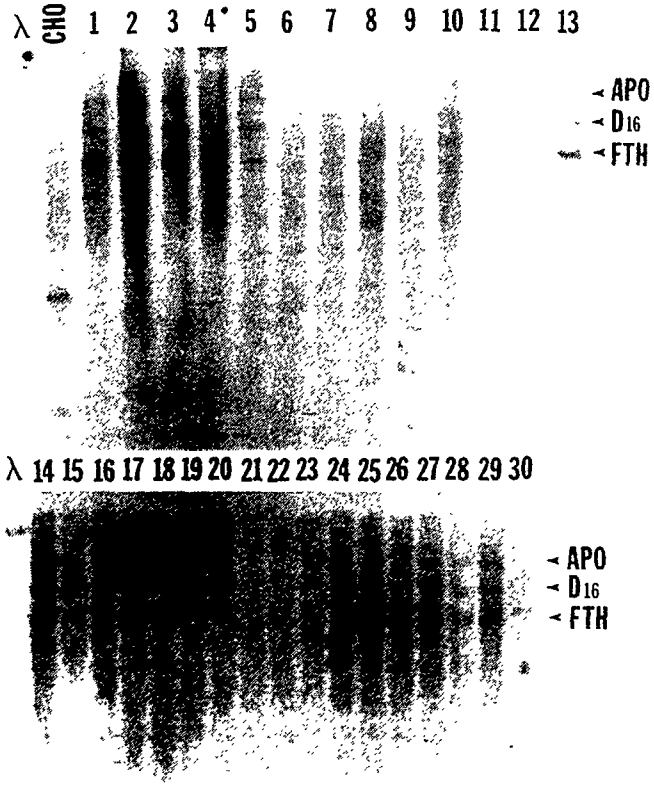
the majority of these mutations had suffered massive chromosomal damage involving loss of millions of base pairs (Fig. 5). Three probes, D_{16}, FTH, and APO-A1, which were mapped, respectively, to p13, q13, and q23, were used. They were chosen because of their map positions relative to S_1. Under the hybridization conditions used, they do not cross-hybridize with hamster genes so that mutations can be detected by the loss of a band or bands rather than by shifts in the positions of the bands. Of the 101 spontaneous S_1^- clones, 33% retained all three probes, D_{16} was absent but FTH and APO-A1 were present in 27%; combinations of probes were missing or rearranged in 14%; and all 3 were missing in 26%. By comparison, 14 of 24 (58%) of the S_1^- mutants from chrysotile treated populations had lost the 3 marker probes. This difference in the percentage of total marker loss between mutants from fiber treated and unexposed pupulations is significant at the 99.5% confidence level when analyzed using the χ^2 test of homogeneity.

DISCUSSION

Apart from the induction of lung fibrosis, asbestos fibers have been shown to cause lung cancers and pleural and peritoneal meosthelioma in occupational settings (23). Furthermore, cigarette smoke can enhance the lung cancer incidence among asbestos workers in a synergistic fashion (38). Although the incidence is relatively low, the danger of developing asbestos related diseases has been documented in family members of asbestos workers (39) and in individuals living in the neighorhood of industrial source of asbestos (40).

While the exact mechanism(s) of fiber carcinogenesis is not clear, several studies have shown that asbestos can induce aneuploidy in both human and rodent mesothelial cells (13, 14). The physical interaction of fibers with chromosomes on structural proteins of the spindle apparatus and chromosomal disjunctional processes have been proposed to account for these observations. In addition, asbestos fibers at certain intermediate doses can alter the growth properties of some human and rodent cells in a manner similar to that of the tumor promoter, 12-*O*-tetradecanoyl-phorbol-13-acetate (23, 41). However, these fiber associated growth changes fail to explain the many aspects of fiber carcinogenesis. The identification in human meosthelioma of loss of specific chromosomal regions suggests that loss of tumor suppressor genes may play a role in these cancers (42). The fact that these genes are often inactivated by large deletions or chromosomal loss further reinforces the idea that asbestos may cause cancer by creating chromosomal mutations.

Results of the present studies indicate that chrysotile fibers are highly mutagenic to cultured mammalian cells. We show that, while few or no mutants are induced at the HGPRT locus, there is, at equivalent fiber concentrations, a substantial dose dependent increase in mutant induction at the S_1 locus. These data are consistent with our previous findings on the mutagenicity of crocidolite fibers (International Union against Cancer standard reference sample) using the A_L cells (36). While the use of gene markers such as the HGPRT located on essential, monosomic chromosomes may provide an accurate and convenient assay for agents that produce mainly small gene mutations, chromosomal mutations such as large deletions and nondisjunctional process may be underestimated as has been shown for ionizing radiations and certain chemicals (27, 34, 37, 43). The fact that earlier studies failed to detect mutation by asbestos at the HGPRT or ouabain loci (18–20) may be a



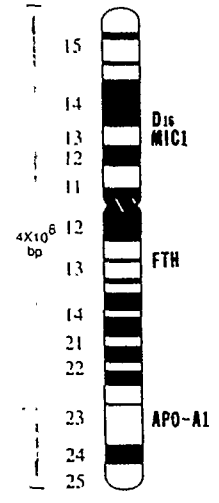


Fig. 5. Southern analysis of DNA from S_1^- mutants and wild-type A_L cells probed with complementary DNA for APO-A1, D_{16}, and FTH. Map locations of these probes and surface antigen loci are shown on the adjacent human chromosome 11 map. The S_1 antigen is encoded by the *MIC1* gene. D_{16} is an anonynous segment; APO-A1 is from apolipoprotein A1; and FTH is a ferritin gene probe. *Lanes 1–24*, patterns for fiber treated A_L cultures; *Lanes 25–30*, spontaneous mutants.

consequence of its induction of multilocus deletions that are not compatible with the survival of the mutants in the cells used. These larger kinds of damages cannot be ignored since they are implicated in a number of human mutational diseases including cancers (44).

Although the mechanism of asbestos induced genetic damage is not known, various *in vitro* and *in vivo* studies have implicated reactive oxygen species (23, 45). The fact that oxygen radicals are mutagenic in bacteria (46, 47), while mineral fibers are not (21), suggests the importance of fiber-cell interaction. Furthermore, superoxide dismutase has been shown to protect mammalian cells against the mutagenic effects of chemically produced superoxide anions (48). The utilization of these enzymes will undoubtedly help to clarify the involvement of oxygen radicals in mutagenesis of mineral fibers.

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