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Genetics

Asbestos fibers mediate transformation of monkey cells by exogenous plasmid DNA

(carcinogenesis/chrysotile/cell transfection/DNA replication/oncogene p53)

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ABSTRACT **We have tested the ability of chrysotile asbestos fibers to introduce plasmid DNA into monkey COS-7 cells and the ability of this DNA to function in both replication and gene expression. Chrysotile fibers are at least as effective as calcium phosphate in standard transfection assays at optimal ratios of asbestos to DNA. After transfection with chrysotile, a minor percentage of introduced plasmid DNA bearing a simian virus 40 origin of replication replicates after 24 hr. Fragmentation of entering DNA is more prominent with asbestos than with calcium phosphate, and after 72 hr most DNA introduced by asbestos is associated with chromosomal DNA. Cells transfected with plasmid p11-4, bearing the p53 protooncogene, express this gene. Cells transfected with pSV2-neo express a gene conferring resistance of antibiotic G418, allowing isolation of colonies of transformed cells after 18 days. The introduction of exogenous DNA into eukaryotic cells could cause mutations in several ways and thus contribute to asbestos-induced oncogenesis.**

A large body of epidemiological and experimental evidence clearly indicates that asbestos fibers are carcinogenic (1–6). Nonetheless, there is currently no cogent mechanism that relates asbestos-induced oncogenesis to changes in DNA at the molecular level (see ref. 5). In this study we test the hypothesis that asbestos fibers, specifically chrysotile fibers, can act as transfection agents that mediate the uptake of exogenous DNA into cells in such a way that genes on this DNA are subsequently expressed. This hypothesis was prompted by several known properties of asbestos fibers (5–12), particularly reports that similar silicate minerals can enhance the uptake of viral RNA into cells (11) and the observation that asbestos fibers readily enter phagocytic and parenchymal cells (12). Measurable concentrations of DNA are present in normal extracellular fluids (13), and these concentrations are undoubtedly much higher at sites of tissue necrosis and in inflammatory exudates (14). The precedent of another mineral agent, calcium phosphate, being able to transfect DNA into cells is well known and widely exploited (15–18).

More than 90% of asbestos in commercial use is chrysotile. Chrysotile consists of adjoining sheets of silicate and magnesium oxide, which are tightly rolled in a scroll-like fashion to form a hollow tube or filament that is the basic crystalline unit (5, 6). These unit filaments have diameters that may vary between 10 and 60 nm and lengths that may approach 2 mm. Thicker chrysotile fibers consist of unit filaments packed in parallel arrays (or polyfilamentous bundles). The magnesium oxide layer always forms the outermost sheet of chrysotile filaments and bears a net positive charge (5, 6).

We demonstrate, using electron microscopy, the interaction of plasmid DNA molecules with chrysotile fibers. We then examine the ability of these fibers to introduce plasmid DNA molecules into cells, and we examine the fate of entering DNA with respect to replication and association with chromosomal DNA. Using selected plasmid vectors, we examine the ability of DNA transfected by chrysotile to express a known oncogene and to transform cells to antibiotic resistance.

MATERIALS AND METHODS

Preparation of Mineral Particulates. All experiments with chrysotile asbestos used Union Internationale Contre le Cancer (UICC) Canadian chrysotile sample B (19). Chrysotile fibers were sterilized dry by heating at 250°C for 6 hr prior to suspension in sterile 50 mM Hepes, pH 7.0/0.14 M NaCl (HBS) for transfection as described. Glass fibers were prepared by extensive pulverization of Pyrex wool filtering fiber (catalog no. 3950, Corning). The average diameter of fibers was approximately 7 μm, and their length ranged from 130 μm down to 3 μm as determined by electron microscopy. The fibers were autoclaved in HBS prior to transfection. Calcium phosphate precipitates were formed in the presence of transfecting DNA essentially as described by Graham and Van der Eb (16). DNA was dissolved in HBS containing 0.7 mM sodium phosphate (pH 7.0). To this was added 2.0 M $CaCl_2$ to give a final concentration of 125 mM, and the precipitate was allowed to form for 30 min at 20°C before addition to cells.

Electron Microscopy of DNA Bound to Asbestos. Chrysotile fibers were suspended in 50 mM Tris·HCl, pH 7.0/0.14 M NaCl at a concentration of 2.0 μg/ml. Plasmid DNA was added to a concentration of 1.0 μg/ml. After incubation for 20 min at 20°C, 1/10th volume of 1.0 M Tris·HCl/0.1 M EDTA, pH 8.5, and then an equal volume of formamide were added. The DNA-chrysotile was coated with cytochrome *c*, spread, fixed, stained with uranyl acetate, and shadowed with Pt–Pd as described (20).

Transfection of Monkey COS-7 Cells. COS-7 cells, 10^6 per 100-mm Petri dish, were transfected with 5.0 μg of pSV2-neo plasmid together with 50 μg of chrysotile or glass fibers or the standard calcium phosphate coprecipitate formed with the DNA as described. After a 30-min incubation, the transfection mixture was added to dishes containing cells in 5.0 ml of fresh Dulbecco's modified Eagle's medium with 10% fetal calf serum and 0.1 mM chloroquine diphosphate (21). After 4 hr, dishes were rinsed twice with sterile saline, and fresh medium was applied without chloroquine. Assays for activities of transfected plasmids were conducted as described in the figure legends.

Assay for Replication of Transfected Plasmid DNA. COS-7 cells contain a partial, integrated simian virus 40 (SV40) genome and express the gene for tumor antigen, thus allowing

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Abbreviation: SV40, simian virus 40.

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replication of plasmids bearing an SV40 origin of replication (22). After transfection, replication is assayed by resistance to restriction endonuclease *Dpn* I. This enzyme cleaves at GATC sites only if adenosine bases on both strands are methylated, as they are when plasmids are propagated in a dam^+ strain of *Escherichia coli* (23). We transfect with plasmid pSV2-neo, which bears an SV40 origin (24) and was isolated from dam^+ *E. coli* strain HB101. At the times indicated in the figure legends, low molecular weight DNA was isolated as described (20), and plasmid DNA was linearized with restriction endonuclease *Bam*HI and further treated with *Dpn* I prior to agarose gel electrophoresis and hybridization to plasmid pBR322, which is complementary to much of pSV2-neo but not to COS-7 cellular DNA.

Assay for Transient Expression of Protooncogene p53. Cells were transfected as described with plasmid p11-4, which carries the p53 protooncogene linked to an SV40 promoter-enhancer (25). At various times after transfection, cells were fixed with freshly prepared 3.75% formaldehyde in PBS for 15 min at 20°C and then permeabilized with 0.1% Triton X-100 in PBS for 3 min. Cells were then incubated with anti-p53 monoclonal antibody pAB122 (26) 1:500 in PBS containing 2% (vol/vol) fetal calf serum at 37°C for 1 hr. The cells were then washed three times with PBS and incubated with the second antibody, either fluorescein- or horseradish peroxidase-conjugated goat anti-mouse IgG/IgM (Boehringer Mannheim) diluted 1:100 in PBS containing 1% (vol/vol) bovine serum albumin. Samples were washed as before. Peroxidase samples were developed with 0.3 mg of diaminobenzidine per ml of PBS and 0.05% H_2O_2.

RESULTS

DNA and RNA (9) and chromatin (10) have been reported to bind to chrysotile fibers. The amphibole fibers crocidolite and amosite also bind DNA, although the affinity and capacity are considerably lower than that described for chrysotile. Binding experiments, not presented in detail in this paper, indicated that the DNA-binding capacity of 100 μg of UICC sample B Canadian chrysotile was saturated by 10 μg of DNA, and that binding was linear with increasing DNA to that point.

To further characterize the binding of DNA to chrysotile, we visualized DNA molecules on the surface of chrysotile fibers. Fig. 1 shows plasmid DNA molecules bound to chrysotile. In Fig. 1*A*, a single chrysotile filament, ≈650 nm long and 45 nm in diameter, is seen with DNA strands splaying off either end. DNA also appears to be wrapped diagonally around the surface of the filament in at least two places (indicated by the triple arrows and double arrows). The three strands near the left end of the filament are ≈35 nm apart, and the long axes of the two left-most strands make a 60° angle with the long axis of the chrysotile filament. The

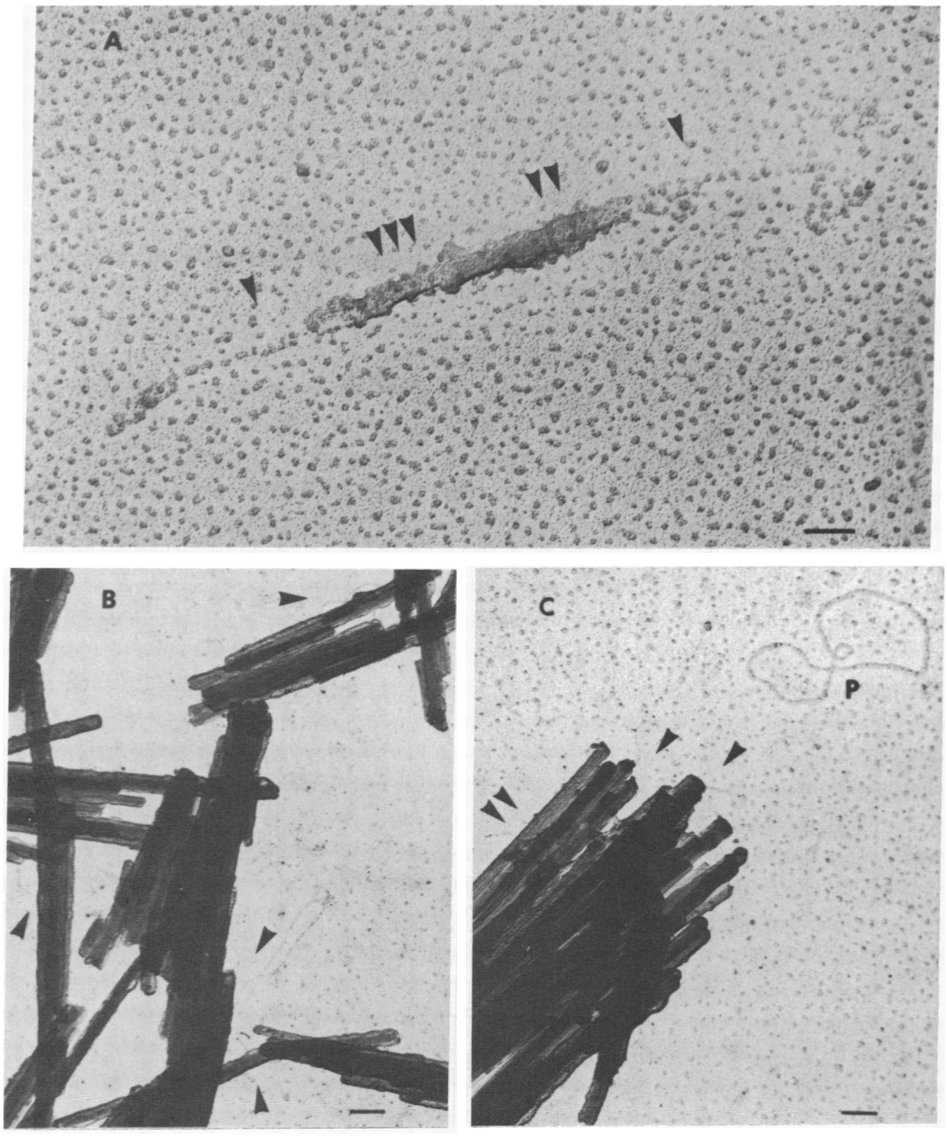

FIG. 1. Electron microscopic visualization of plasmid DNA bound to chrysotile asbestos fibers. DNA was bound to chrysotile fibers at approximately physiological NaCl concentration and prepared for electron microscopy as described. (*A*) Single chrysotile filament with pSV2-neo DNA bound to the surface. Arrows point to DNA extending from the ends of the fiber and also indicate the regular spacing of DNA strands coiled on the surface. (*B* and *C*) Array of chrysotile fibers with bound pSV2-neo DNA. In several places DNA can be detected on the surface of ordinarily smooth fibers. The arrows point to places at which this DNA extends from the fibers. (Bars = 100 nm.)

regular pattern of wrapping of DNA around the chrysotile filament may reflect in part the ordered spacing of magnesium ions in the surface brucite layer. In control experiments, chrysotile filaments to which no DNA was added appeared uniformly smooth.

After transfection into COS-7 cells, pSV2-neo, if present as a full-length circle, will replicate several times within 48 hr. Our assay for transient plasmid replication used the restriction endonuclease *Dpn* I, which selectively cleaves unreplicated plasmid DNAs at multiple sites (20, 23). Fig. 2 shows transient replication of pSV2-neo molecules in asbestos-transfected COS-7 cells within 24 hr. In Fig. 2 *Left*, Hirt-supernatant DNA hybridizing to the pSV2-neo probe is seen in lanes C and D. Lane C shows hybridization to uncleaved DNA. The broad hybridizing band near the top of lane C

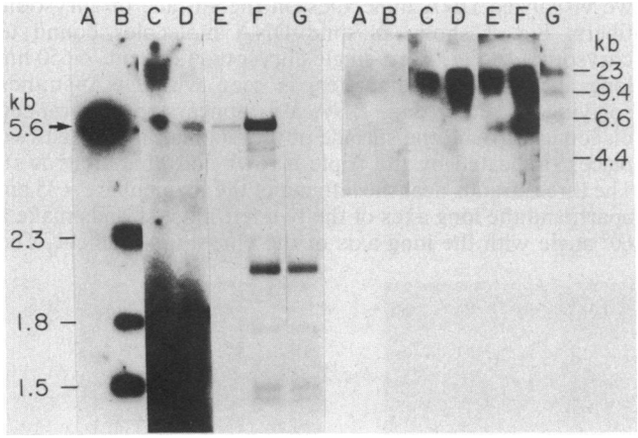

FIG. 2. Transient replication of pSV2-neo DNA in COS-7 cells. COS-7 cells were transfected with 5.0 μg of pSV2-neo DNA together with 20 μg of herring sperm carrier DNA and either chrysotile or calcium phosphate precipitate as the transfecting agent as described. Pulsing with chloroquine diphosphate and isolation of low molecular weight Hirt-supernatant DNA (47) also has been described (20). Total cellular DNA was isolated by lysing cells in 50 mM Tris·HCl (pH 8.0) containing 10 mM EDTA and 0.5% sodium dodecyl sulfate and extracting with phenol and chloroform. (*Left*) *Dpn* I-resistant plasmid DNA in Hirt supernatants. DNA in each Hirt supernatant was purified by successive extractions with phenol, chloroform, and ether, and one-fourth of each sample was subjected to electrophoresis on a 1.2% agarose gel either directly or after restriction enzyme cleavage. DNA in agarose was blotted onto a GeneScreen*Plus* filter (New England Nuclear). Filter-bound DNA was hybridized with pBR322 DNA labeled with [α-^{32}P]dCTP to approximately 2×10^8 cpm/μg of DNA. Hybridization conditions and washing of the resulting filter were as described (20). Filters were blot-dried and exposed to Kodak x-ray film at -70°C with one intensifying screen for 24 hr. Lanes: A, pSV2-neo DNA linearized with *Eco*RI; B, pSV2-neo DNA digested with *Pst* I; C, 24 hr after transfection with asbestos, Hirt-supernatant DNA untreated with restriction enzymes; D, 24 hr after transfection with asbestos, Hirt-supernatant DNA digested with *Bam*HI (40 units for 2 hr at 37°C) and *Dpn* I (150 units for 4 hr at 37°C); E, pSV2-neo DNA (5.0 ng) linearized with *Bam*HI; F, 48 hr after transfection with calcium phosphate, Hirt-supernatant DNA digested with *Bam*HI and *Dpn* I as above; G, 0 hr after transfection with calcium phosphate, Hirt-supernatant DNA digested with *Bam*HI and *Dpn* I as above. (*Right*) Distribution of pSV2-neo DNA sequences in chromosomal and plasmid DNA forms. Total COS-7 cell DNA was isolated by lysis of the transfected cells and extraction as described above. Digestions were performed by using *Dpn* I (15 units/μg of DNA) as described for *Left*. Hybridizations were also performed as described for *Left*, and autoradiographs were exposed 24 hr. Lanes: A and B, 48 and 72 hr, respectively, after pSV2-neo transfection with no mineral agent; C and D, 48 and 72 hr, respectively, after pSV2-neo transfection with chrysotile as transfecting agent; E and F, 48 and 72 hr, respectively, after transfection with calcium phosphate as transfecting agent; G, labeled λ phage DNA digested with *Hin*dIII. kb, Kilobases.

includes form II (nicked circular) plasmid DNA and potential replication forms. A well-defined *Dpn* I-resistant band at 5.6 kb can be seen in lane D after treatment with that enzyme and *Bam*HI, which linearizes any replication forms. Note that in the lower half of lanes C and D, a broad dark smear of hybridizing, fragmented pSV2-neo DNA is present. This is seen even in samples not treated with restriction enzyme (lane C), indicating that much of the plasmid DNA is broken down upon or before entry into the cells. Neither this smear nor the *Dpn* I-resistant band is seen when asbestos was omitted from the transfection. We did not detect significant cytotoxicity at this dose of chrysotile in 24 hr, as determined by monitoring cell death, suggesting that breakdown is not due to release of nucleases from dying cells. With increasing times after chrysotile-mediated transfection, continued breakdown of plasmid DNA was observed together with evidence of decreasing transient replication. DNA recovered from cells transfected by the standard calcium phosphate technique after 48 hr (the optimal time for observing replication after transfection with this agent) did not show the extensive degree of fragmentation that was apparent in DNA transfected by chrysotile (lane F). Control lane G affirms that *Dpn* I as used does cleave unreplicated pSV2-neo DNA isolated from Hirt supernatants. Additional controls, not shown, indicated that chrysotile does not inhibit *Dpn* I cleavage of the plasmid DNA.

In Fig. 2 *Right*, total COS-7 cell DNA recovered 48 and 72 hr after chrysotile-mediated transfection is compared with control DNA after calcium phosphate-mediated transfection with respect to hybridization to the labeled pSV2-neo DNA probe. Prior to electrophoresis, the DNA was treated with *Dpn* I, which cleaves unreplicated plasmid DNA but does not cleave chromosomal DNA. Lanes A and B show that no appreciable DNA was detected in cells transfected without a mediating agent and attest to the specificity of our hybridization probe, which did not significantly hybridize to cellular DNA sequences. After transfection mediated with either asbestos or calcium phosphate, the pSV2-neo DNA was detected prominently in the extracted DNA (lanes C–F). In all four C–F lanes, most of the pSV2-neo DNA is associated with bands larger than plasmid size; a faint heterogeneous smear is also seen extending below this size. The higher molecular weight fragments represent plasmid molecules, either partial or complete, integrated with cellular DNA, as also confirmed by treatment with other restriction enzymes (not shown). These bands were heterogeneous although not highly diffuse because of their large size. *Dpn* I-resistant DNA bands of plasmid size, indicating transient replication, were evident only in the calcium phosphate lanes. At 72 hr, the intensity of hybridization of pSV2-neo to high molecular weight chromosomal DNA after asbestos-mediated transfection was slightly higher than that after calcium phosphate-mediated transfection, whereas the *Dpn* I-resistant replicated plasmid band, representing both plasmid forms I and II, was very prominent after calcium phosphate transfection. Taken together, Fig. 2 *Left* and *Right* indicates that asbestos introduces highly fragmented plasmid DNA into COS-7 cells. While some of this DNA is capable of replication, a high proportion is chromosomally integrated. The DNA damage and fragmentation associated with asbestos-mediated transfection may contribute to its lack of persistent replication and to its enhanced chromosomal integration (27). This fragmentation may represent a marked augmentation of the DNA damage that presumably causes the increased frequency of mutations on plasmid DNAs after their transfection into mammalian cells (28, 29). Moreover, asbestos fibers are known to enhance the formation of reactive species of oxygen (30, 31), and these oxygen radicals can damage DNA (32). However, simply incubating plasmid DNA with chrysotile fibers in buffered saline for several hr at 20°C did not lead

to any appreciable fragmentation of the plasmid DNA. Consequently, we conclude that additional factors present in the cell culture media or in the cells themselves are required for the observed DNA fragmentation.

We transfected COS-7 cells with the plasmid p11-4, which contains the murine p53 protooncogene linked to the SV40 early-region promoter-enhancer (25) to see if a gene introduced by asbestos could be expressed. At various time points (0–48 hr), cells were fixed as described and incubated with the anti-p53 monoclonal antibody pAB122 (26). Cells that produced p53 protein were visualized either by fluorescence or peroxidase staining via a second antibody reaction. Many positive cells stained by each method were pierced by asbestos fibers; representative examples are shown in Fig. 3. Positive cells stained most intensely in the nucleus, but reacting p53 protein was visible out to the edge of the cells. COS cells constitutively express the p53 gene, which is present at low copy number. Relative to the transfected gene, which can replicate to high copy number, the endogenous gene contributed only minor background. Under various growth conditions, we observed various levels of this background staining in nuclei of untransfected cells, but in COS-7 cells transfected with p11-4, the enhanced fluorescent staining was localized in the nucleus 48 hr after transfection. At 24 hr, as seen in Fig. 3*A*, some cells showed extranuclear staining, possibly because p53 protein was still in transit or because asbestos fibers could disturb the normal transport and localization of the protein. Nuclear localization of p53 protein has been reported in transformed cells (25). Comparative transfection efficiencies are listed in Table 1. Asbestos plates contained ≈1.4 times as many positive cells as the calcium phosphate plates. Use of glass fibers as a mediating agent did not result in significant levels of transfection. Plates incubated with asbestos fibers without p11-4 DNA did not show significant numbers of peroxidase-staining cells, indicating that asbestos is not merely facilitating the entry of antibody into cells or activating the endogenous gene. We conclude that the p53 gene is expressed on plasmid DNA introduced by asbestos.

Since expression of the "*neo*" gene present on the pSV2-neo plasmid will confer resistance to the neomycin analog G418 (24), stable genotypic transformation can be quantitated after transfection of COS-7 cells with pSV2-neo by selecting and counting colonies resistant to G418. After transfection as described in Table 1, G418 was added. Antibiotic-induced cell death was maximal at 3–7 days. After 10 days, resistant colonies could begin to be resolved; by 18 days, stable colonies of 50–200 cells were clearly defined against an acellular background. (Results presented are not corrected for asbestos-induced cytotoxicity. At higher concentrations of asbestos, 250 μg per 100-mm plate, the rate at which G418 killed sensitive cells was enhanced approximately 2-fold over the rate seen with no transfecting agent or with calcium phosphate.) Control plates incubated with DNA and glass fibers, with asbestos but no DNA, or with DNA but no mineral transfection agent were completely free of colonies at 18 days. The numbers of stable cell colonies at 18 days are listed in Table 1. Asbestos-treated plates contained ≈3 times as many colonies as the calcium phosphate-treated plates.

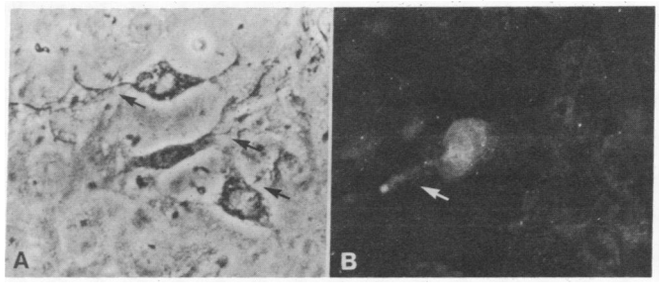

FIG. 3. Expression of protein p53 in monkey COS-7 cells after transfection with plasmid p11-4 bearing the p53 oncogene. COS-7 cells (10^5 per 35-mm Petri dish) were transfected with 2.5 μg of plasmid p11-4 DNA after 20 min of incubation with 25 μg of either sterile chrysotile or glass fibers or standard calcium phosphate precipitates formed with the DNA as described for Fig. 2. At times from 4 to 36 hr, cells were fixed and treated with anti-p53 monoclonal antibody pAB122 and a second antibody—either horseradish peroxidase-conjugated (*A*) or fluorescein-conjugated (*B*) goat anti-mouse antibody as described. A second series was run in parallel without the pAB122 incubation to monitor background staining levels. Arrows point to chrysotile fibers impaling positively staining cells.

DISCUSSION

These results indicate that chrysotile fibers can bind plasmid DNA molecules and promote their entry into primate cells in culture. Although this process is associated with fragmentation of the entering plasmid DNA, some of the plasmids are capable of transient replication and gene expression. Transformation frequencies observed are consistent with the possibility that transfection is an aspect of asbestos carcinogenesis. Table 1 indicates that DNA enters many more cells than are actually transformed to antibiotic resistance (≈4 cells in 10^5). Transformation by asbestos may be qualitatively different from that mediated by calcium phosphate in several respects: (*i*) the damage sustained by the DNA during or after entry, (*ii*) the release mechanism of the DNA from the mineral carrier, (*iii*) the durability and persistence of the mineral carrier within the cell, and (*iv*) the route taken by the released DNA through intracellular compartments. Other mineral agents that can bind DNA and are small enough to enter cells might also promote DNA uptake; however, their ability to cause mutations and cancer may be influenced by

Table 1. Comparison of asbestos- and calcium phosphate-mediated transfection efficiency: Transient gene expression and genotypic transformation

	Peroxidase-stained cells 24 hr after transfection with plasmid p11-4 bearing the p53 protooncogene*				Cells transformed to G418-resistance 18 days after transfection with plasmid pSV2-neo†			
Transfecting agent	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 1	Exp 2.	Exp. 3	Exp. 4
Asbestos	125	130	140	130	45	36	37	40
Calcium phosphate	95	85	102	88	12	13	10	14
Glass fibers	0	10	6	0	0	0	0	0
DNA, no mineral	ND	ND	ND	ND	0	0	0	0
Asbestos without added DNA	5	3	0	0	0	0	ND	ND

*Positively stained cells per 2.5 μg of DNA per 25 μg of mineral agent per 10^5 transfected cells. ND, not done.
†On day 18, colonies of 100 cells or more were scored: G418-resistant colonies per 5 μg of DNA per 50 μg of mineral agent per 10^6 transfected cells.

the factors listed above and by their ability to enter and persist inside the body.

Most studies using short-term *in vitro* assays to identify some mutagenic or DNA-damaging activity for asbestos fibers have yielded negative or equivocal results (33–36). However, several studies suggest that asbestos fibers can affect DNA in chromosomes (37–39). In none of these studies was DNA intentionally added to asbestos. Nonetheless, variable concentrations of DNA, derived from cell lysis, may have been present. Our data reveal a new property of asbestos fibers that clearly has the potential for causing mutations and inducing the progression towards the neoplastic state. Asbestos-mediated DNA entry with subsequent random insertion of the entering DNA into the genome of the host cell could result in mutations and oncogenesis by a variety of mechanisms. First, genes essential for the maintenance of normal growth control (40, 41) might be inactivated by insertional mutagenesis (42). Second, protooncogenes present in the entering DNA may be mutated to active oncogenes during the asbestos-mediated transfection process and then inserted into the host-cell genome. Third, a protooncogene in the host cell might be activated by a promoter insertion mechanism (see ref. 43). Fourth, it is possible that the introduction into a cell of DNA that is extensively damaged and fragmented might trigger the induction of DNA repair enzymes, some of which might be error-prone (see ref. 44).

Normal human plasma contains DNA (45), and bronchoalveolar lavage fluid recovered from the lungs of healthy volunteers contains substantial concentrations of free DNA (13), comparable to those used here for *in vitro* transfection. Moreover, asbestos fibers themselves may further increase local concentrations of free DNA by promoting inflammation and cell lysis (5, 6). Therefore, all components are available for asbestos, when inhaled in minute quantities, to induce transformation of cells in the lung. Among asbestos minerals, chrysotile, with its positively charged surface, binds naked DNA with the highest affinity (9). However, DNA in extracellular fluid is likely to be complexed with histone and nonhistone proteins, which may further enhance its binding to amphibole fibers. Our data show that chrysotile fibers introduce exogenous DNA into cells and that much of the entering DNA becomes fragmented and inserted into host-cell chromosomes. Although of obvious oncogenic potential, this transformation mechanism does not fully account for certain aspects of asbestos oncogenesis (e.g., the long latency of onset and the synergism with other carcinogens) (5, 6). Carcinogenesis is now viewed as a multistep process and specific carcinogens might only be able to accelerate a subset of the requisite steps. Our findings point to a potential new direction for efforts to illuminate the precise molecular events underlying asbestos oncogenesis.

After this manuscript was submitted, a paper appeared (47) reporting that chrysotile and amphibole asbestos fibers can mediate the transfection of deproteinized viral RNA into cultured monkey cells with an efficiency at least that of calcium phosphate.

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