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Advances in Brief

Transcriptional Activation of the Proto-oncogene c-*jun* by Asbestos and H_2O_2 Is Directly Related to Increased Proliferation and Transformation of Tracheal Epithelial Cells[1]

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Abstract

Asbestos causes persistent increases in c-*jun* mRNA and AP-1 DNA binding activity in hamster tracheal epithelial (HTE) cells, the progenitor cell type of asbestos-induced bronchogenic carcinoma. Studies here were designed to determine mechanisms of c-*jun* induction by asbestos and the phenotypic consequences of Jun expression in HTE cells. To examine whether asbestos or H_2O_2 induced transcription of c-*jun*, we transiently transfected HTE cells with a plasmid containing a fragment of the c-*jun* promoter coupled to a luciferase reporter gene. In addition, c-*jun* was overexpressed in cells using a full-length human c-*jun* construct, and effects on proliferation and transformation were examined. HTE cells transfected with the *jun*-luciferase construct showed increased luciferase activity when exposed to crocidolite asbestos or H_2O_2. These results demonstrate that asbestos and H_2O_2 activate AP-1-dependent gene transcription. Overexpression of c-*jun* led to increased proliferation and enhanced ability of HTE cells to grow in soft agar, an indication of cellular transformation. Data suggest that overexpression of c-*jun* may contribute to asbestos and oxidant-induced proliferation and carcinogenesis.

Introduction

Asbestos, a family of hydrated silicates, gives rise to asbestosis and malignancies (mesothelioma and bronchogenic carcinoma) in humans following occupational exposures (1, 2). Common features of asbestos-related diseases are chronic inflammation and unregulated proliferation of target cells of the lung and pleura, *i.e.*, epithelial and mesothelial cells (1–5). The mechanisms of asbestos-induced carcinogenesis are not clearly understood. Many of the asbestos-induced effects appear to be mediated by the production of AOS[3] from redox reactions catalyzed on the fiber surface and/or release of AOS from inflammatory cells (6). Studies *in vitro* have demonstrated that asbestos-induced cytotoxicity can be ameliorated by concomitant exposure to antioxidants (7). Moreover, administration of polyethylene glycol-conjugated catalase, a scavenger of AOS, during inhalation of asbestos ameliorates pulmonary toxicity, inflammation, and the development of asbestosis (3). These studies indicate the importance of AOS in the pathogenesis of asbestos-induced diseases. Yet how asbestos and AOS interact with cells at the molecular level to cause alterations in cell growth and their relationship to tumor development are unknown.

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[3] The abbreviations used are: AOS, active oxygen species; HTE, hamster tracheal epithelial; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BrdUrd, 5-bromo-2′-deoxyuridine; RSV, Rous sarcoma virus; β-gal, β-galactosidase.

Previous work in our laboratory has shown that asbestos causes persistent increases in mRNA levels of the proto-oncogene c-*jun* and increased DNA binding activity of the AP-1 transcription factor in HTE cells and rat pleural mesothelial cells, target cells of asbestos-induced disease (8). c-*jun* is a member of a multigene family that is transiently expressed in response to a variety of stimuli and encodes subunits (Jun homodimers or Jun/Fos heterodimers) of the transcription factor, AP-1. c-*jun* and members of the c-*fos* gene family are immediate early response genes involved in the transition of the G_1 phase and entry into the S phase of the cell cycle (9). More importantly, deregulated expression of c-*jun* can lead to aberrant proliferation and transformation of a number of different cell types (9).

In experiments here, we transiently transfected HTE cells with a *jun*-luciferase reporter construct that contains a single AP-1 binding sequence to examine whether asbestos caused transcriptional induction of c-*jun*. In comparative experiments, we examined whether H_2O_2 also transactivated AP-1-dependent gene expression, indicating a common molecular mechanism of oxidants and asbestos in initiation of cell replication. We used the tumor promoter, TPA, as a positive control because it has been used to study regulation of c-*jun* and c-*fos* gene families and AP-1 DNA binding in a number of cell types (9). Moreover, the effects of TPA also mimic many asbestos-induced responses in organ and cell cultures of tracheal epithelium (2). To determine the phenotypic ramifications of c-*jun* induction by asbestos and oxidants (10), we next examined whether overexpression of c-*jun* resulted in altered growth characteristics of HTE cells. Our results indicate that asbestos and H_2O_2 directly activate transcription of AP-1-dependent genes and that overexpression of c-*jun* leads to increases in cell proliferation and transformation in tracheobronchial epithelial cells, the progenitor cells of bronchogenic carcinomas (lung cancers).

Materials and Methods

Cell Culture, Plasmids, and Transfection. HTE cells isolated from the tracheal epithelium of a neonatal hamster and characterized previously by our laboratory (11) were grown in Ham's F-12 (GIBCO-BRL, Gaithersburg, MD) medium supplemented with 10% newborn bovine serum (GIBCO-BRL). Plasmids containing c-*jun* cDNA (RSV-cJun; Ref. 12) or promoter fragments (*jun*-luciferase) and the empty expression vector (RSV-0) were obtained from Dr. Michael Karin (University of California, San Diego, CA). The reporter plasmid, *jun*-luciferase, which contains 249 bp of the c-*jun* promoter (from position −70 to +170 bp encompassing a single AP-1 binding sequence) coupled to the luciferase reporter gene, was used to determine transcriptional activation of AP-1-dependent genes by various agents. RSV-cJun, which contains the full-length human c-*jun* constitutively expressed from the RSV long terminal repeat, was used in experiments to evaluate overexpression of c-*jun*. The empty expression vector, RSV-0, was used as a control in all experiments. Cells were transfected for 5–6 h with the calcium phosphate-DNA coprecipitation technique (13). In all transfection experiments, 2 μg pSVβ-gal (Promega, Madison, WI) were used to control for transfection efficiency. Following transfection, cells were washed with HBSS

(GIBCO-BRL), fed with fresh growth medium, and allowed to recover overnight.

Exposure to Agents. A reference sample of National Institute of Environmental Health Sciences processed crocidolite [$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$] asbestos fibers was obtained from the Thermal Insulation Manufacturers Association Fiber Repository (Littleton, CO). Asbestos fibers were sterilized at 225°F for 12–15 h, suspended in HBSS at 1 mg/ml, and triturated eight times through a 22-gauge needle before addition to cultures at a 1-, 2.5-, or 5-$\mu g/cm^2$ area of culture dish. These concentrations of asbestos have been shown previously to induce c-*jun* mRNA in a dose-dependent fashion (8). TPA (Consolidated Midland, Brewster, NY) was added to medium at a final concentration of 100 ng/ml. H_2O_2 (Sigma Chemical Co., St. Louis, MO) was diluted in PBS (pH 7.4) and added directly to medium at 10 and 100 μM. The concentrations of TPA and H_2O_2 used in these experiments have been shown previously to induce c-*jun* mRNA in HTE cells (8, 10).

Assay for Transcriptional Activation of c-*jun*-dependent Gene Expression. Following an overnight recovery, transfected cells were changed to media containing 2% serum for 8 h before exposure to various test agents as described above. Total cell extracts were prepared 16 or 40 h after the addition of various agents and assayed for luciferase activity (Luciferase Assay System; Promega, Madison, WI), β-gal activity (14), and total cellular protein (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA). The amount of luciferase activity was expressed as total luciferase/β-gal/μg protein. Five individual transfection experiments were performed. Luciferase activity in RSV-cJun transfected cells was significantly higher at 16 and 40 h posttransfection compared to RSV-0 controls (data not shown). This observation demonstrates successful overexpression of c-*jun* in HTE cells.

Proliferation Assays. Incorporation of BrdUrd labeling reagent [BrdUrd and 5-fluoro-2′-deoxyuridine (10:1); Zymed, San Francisco, CA], a specific marker of DNA synthesis (15), was used to examine the effect of c-*jun* overexpression on HTE cell proliferation. Sixteen h posttransfection, cells were trypsinized and plated for BrdUrd studies or growth curves. For BrdUrd incorporation, cells were plated in 10% serum-containing medium at high density (4.3×10^4 cells/ml) in two-chamber Lab-Tek slides (Nunc, Inc., Naperville, IL), allowed to attach overnight, and switched to 2% serum-containing medium with BrdUrd (1:1000 dilution) for 8 h. After exposure to crocidolite asbestos or H_2O_2 for an additional 16 h, cells were fixed in 70% methanol and stained for BrdUrd using an anti-BrdUrd antibody (Zymed BrdUrd Staining Kit; Zymed, San Francisco, CA). To determine growth rates, transfected cells were plated at 50,000 cells/60-mm dish in 10% serum-containing medium and allowed to attach overnight; then designated dishes were exposed to crocidolite at 0.25 $\mu g/cm^2$ area of culture dish. Cells were trypsinized, and total cell counts were determined at 24, 48, and 72 h. A total of four dishes were counted for each data point. Experiments were repeated in triplicate.

Assay for Anchorage-independent Growth. Transfected cells also were examined for their ability to grow in soft agar, an indication of cell transformation. Briefly, 24 h following transient transfection with the full-length c-*jun* construct, cells were trypsinized, and 20,000 cells/well were plated into 12-well culture plates in normal growth medium with 0.33% Noble agar (DIFCO Laboratories, Detroit, MI) onto a 2-ml support layer containing 0.5% Noble agar (16). Plates were examined for colony formation 21 days later, and four wells for each group were photographed for colony counting. Colony formation was scored by two individuals using a blind coding system by determining the number of colonies greater than 100 μm in five random fields/well. The data were expressed as mean ± SEM for each group.

Statistical Analysis. Data were examined by ANOVA to determine differences between groups using Duncan's procedure to adjust for multiple comparisons.

Results and Discussion

Previously, our laboratory has demonstrated that asbestos causes dose-related and persistent increases in mRNA levels of c-*jun* and DNA binding activity of AP-1 consensus sequences in HTE cells (8, 10). To demonstrate directly that asbestos activates AP-1-dependent gene transcription, we used a luciferase reporter gene driven by a 249-bp fragment of the c-*jun* promoter containing an AP-1 binding site. Fig. 1 shows luciferase activity in HTE cells after 16 h (Fig. 1*A*) or 40 h (Fig. 1*B*) of exposure to asbestos, H_2O_2, or the tumor promoter, TPA. Significant ($P < 0.05$) and dose-dependent increases in luciferase activity were observed following exposure of cells to increasing amounts of asbestos (Fig. 1).

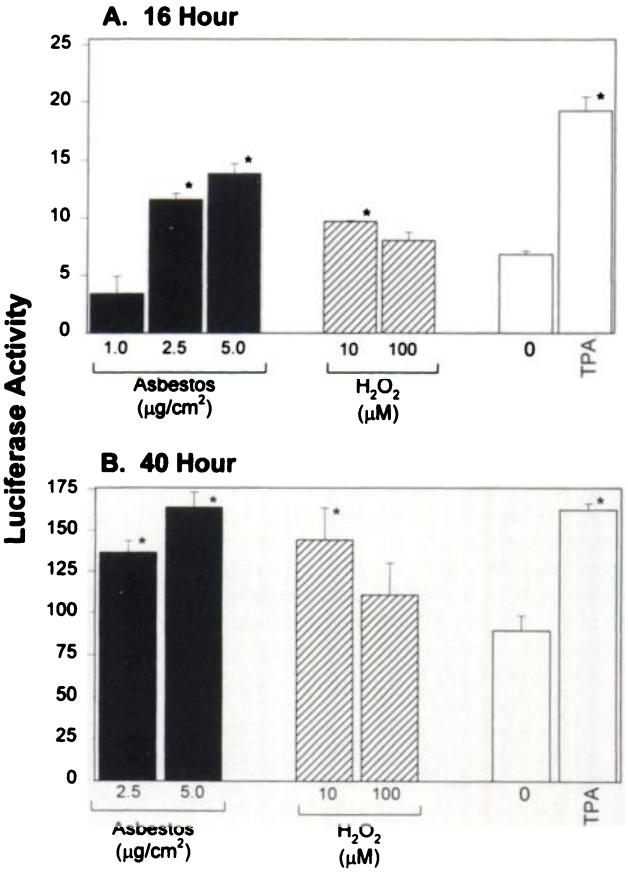

Fig. 1. Transcriptional activation of c-*jun* by asbestos and H_2O_2 in HTE cells. HTE cells were transfected with calcium phosphate-DNA coprecipitates containing 4 μg *jun*-luciferase, 4 μg RSV-0, and 2 μg pSVβ-gal, harvested at 16 h (*A*) or 40 h (*B*) after treatment with the indicated agent and assayed for luciferase and β-gal activity. Luciferase activity is normalized to β-gal activity and protein levels and expressed as relative units (mean ± SEM). The graphs shown are representative of five independent transfection experiments. Each experimental point was performed in duplicate. *, $P < 0.05$ as compared to untreated controls.

Since AOS are implicated as important mediators of asbestos-induced cell proliferation (17), we were also interested in determining whether the oxidant, H_2O_2, could induce AP-1-dependent gene expression in HTE cells. As shown in Fig. 1, exposure of cells to low concentrations of H_2O_2 (10 μM) caused an increase in luciferase activity at a level comparable to that of asbestos. Slightly reduced activity in cells treated with higher concentrations (100 μM) of H_2O_2 is most likely due to cytotoxicity, as indicated by cell sloughing over a subsequent 40-h time interval. These results indicate that oxidant-mediated events can lead to activation of AP-1-dependent genes in HTE cells.

In cells exposed to TPA, significant ($P < 0.05$) increases in luciferase activity were observed in comparison to controls, indicating activation of signaling pathways that lead to AP-1-mediated gene transcription (Fig. 1). This result extends our previous observation that TPA induces the early response gene pathway in HTE cells as shown by increases in c-*jun* mRNA and AP-1 DNA-binding activity (8).

Aberrant proliferation of target cells of the lung and pleura is a prominent feature of asbestos-induced diseases (1, 3). Since AP-1 plays a central role in mediating cellular proliferation in response to

extracellular signals (9) and asbestos directly activates AP-1-dependent gene transcription, we next determined whether overexpression of c-*jun* in HTE cells could lead to increased cell proliferation. Moreover, we investigated whether the biological effects of asbestos were modified in c-*jun*-overexpressing cells.

To assess the effect of c-*jun* overexpression in contact-inhibited cells, confluent cultures of RSV-cJun or RSV-0 (control vector) transfected cells were incubated for 16 h in 2% serum-containing medium in the presence of BrdUrd. Additional groups of RSV-cJun or RSV-0 transfected cells were used to determine if RSV-cJun transfected cells were altered in their responses to asbestos when compared to RSV-0 controls. As shown in Fig. 2*A*, transient overexpression of c-*jun* resulted in an increase in BrdUrd-positive cells compared to RSV-0 control cells, indicating that overexpression of c-*jun* leads to increased proliferation of HTE cells. The addition of asbestos to transfected cells resulted in decreases in the percentage of BrdUrd-positive cells relative to untreated RSV-0 controls (Fig. 2, *A* and *B*). In contrast to RSV-0 cells exposed to asbestos, the percentage of BrdUrd-positive cells in RSV-cJun transfectants exposed to asbestos was significantly increased ($P < 0.05$; Fig. 2*B*). These data indicate that overexpression of c-*jun* leads to increased proliferation in cells exposed to asbestos. In all groups examined, including untreated, asbestos-exposed, and H_2O_2-exposed HTE cells, the percentage of BrdUrd-positive cells was increased in RSV-cJun transfected cells compared to RSV-0 cells ($P < 0.05$; Fig. 2).

We next examined whether overexpression of c-*jun* altered rates of cell growth in control (Fig. 3*A*) and asbestos-exposed (Fig. 3*B*) cells. RSV-0 and RSV-cJun-transfected cells were plated at low density, and cell numbers were determined after trypsinization at 24-, 48-, and 72-h intervals. At 48 h, but not at other times, there was a significant ($P < 0.05$) increase in the numbers of RSV-cJun cells compared to RSV-0 controls (Fig. 3*A*). These results indicate that cells overexpressing c-*jun* proliferate at an increased rate during logarithmic growth. To examine the combined effects of c-*jun* overexpression and exposure to asbestos, we exposed transfected cells to a concentration of asbestos previously shown to increase colony-forming efficiency

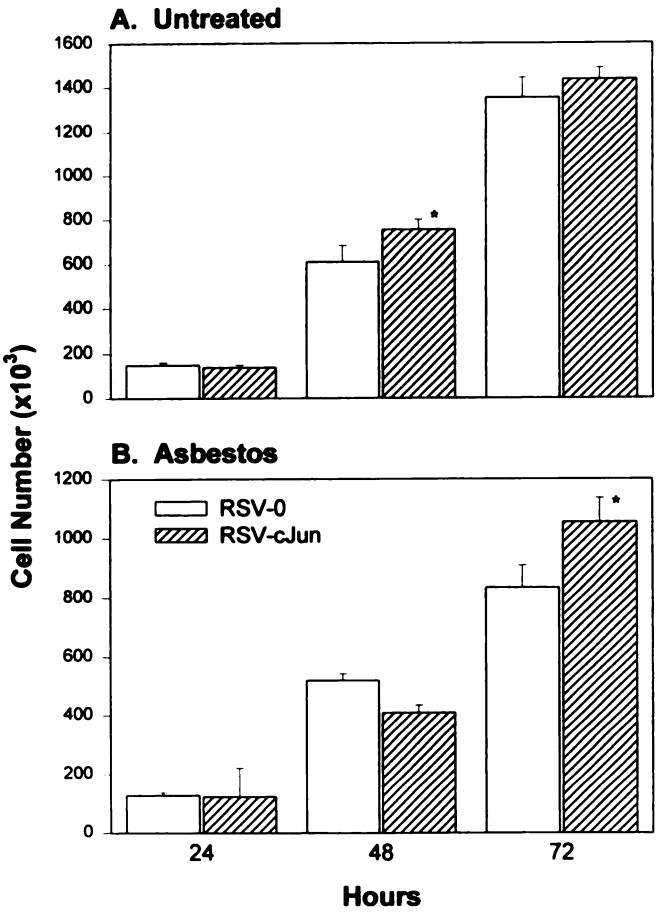

Fig. 3. Transient overexpression of c-*jun* increases the growth rate of HTE cells. HTE cells transfected with calcium phosphate-DNA coprecipitates containing 4 μg RSV-cJun and 2 μg pSVβ-gal or 4 μg RSV-0 and 2 μg pSVβ-gal were plated at 50,000 cells/60-mm culture dish and exposed to 0.25 μg/cm² crocidolite asbestos (*B*) or left untreated (*A*). Cells were harvested by trypsinization at 24, 48, and 72 h, and total cell number was determined by counting. The data represent the average of three independent experiments and are expressed as mean ± SEM. *, $P < 0.05$ as compared to RSV-0 cells.

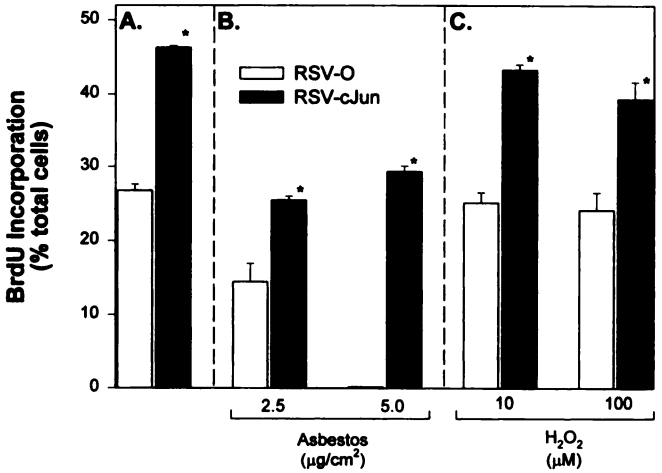

Fig. 2. Transient overexpression of c-*jun* induces proliferation in HTE cells. HTE cells transfected with calcium phosphate-DNA coprecipitates containing 4 μg RSV-cJun and 2 μg pSVβ-gal or 4 μg RSV-0 and 2 μg pSVβ-gal (control) were plated at high density, allowed to attach overnight, switched to 2% serum-containing medium plus BrdUrd, and incubated 8 h, followed by exposure to the indicated agents (*A*, untreated; *B*, asbestos; *C*, H_2O_2) for an additional 16 h. Cells were then fixed in 70% methanol and stained for BrdUrd using an anti-BrdUrd antibody. The percentage of BrdUrd incorporation was determined by counting the number of BrdUrd-positive and total cells in 10 fields of two independent treatments and is expressed as mean ± SEM. *, $P < 0.05$ as compared to RSV-0 cells.

(18). As shown in Fig. 3*B*, significant increases ($P < 0.05$) in cell number were observed in RSV-cJun transfected cells at 72 h. This 24-h delay in the time frame of increased cell proliferation when compared to untreated controls (Fig. 3*A*) may be due to a difference in normal proliferation kinetics in cells exposed to asbestos.

Since asbestos exposure leads to transcriptional activation of c-*jun*, we examined the possibility that overexpression of c-*jun* could lead to transformation of epithelial cells. RSV-0 and RSV-cJun transfected cells were plated in soft agar, and the number of colonies greater than 100 μM in size were scored after 21 days. As shown in Fig. 4, a significant ($P < 0.05$) increase is observed in the number of colonies formed by RSV-cJun cells in comparison to RSV-0 control cells, indicating that overexpression of c-*jun* is directly involved in transformation of tracheal epithelial cells.

Our results demonstrate that overexpression of c-*jun* in HTE cells leads to increased proliferation and changes in cell phenotype that are consistent with cellular transformation as reported by others in embryonic fibroblasts (19). The ability of c-*jun* alone to alter the phenotype of tracheal epithelial cells towards a more malignant state is significant since asbestos persistently increases c-*jun* mRNA and AP-1 DNA-binding activities in this cell type. Our observation that H_2O_2 also activates AP-1-dependent gene expression suggests that asbestos-mediated transcriptional activation of AP-1 may be associated with the production of AOS from asbestos fibers directly or during phagocytosis of fibers by cells (17). Mechanisti-

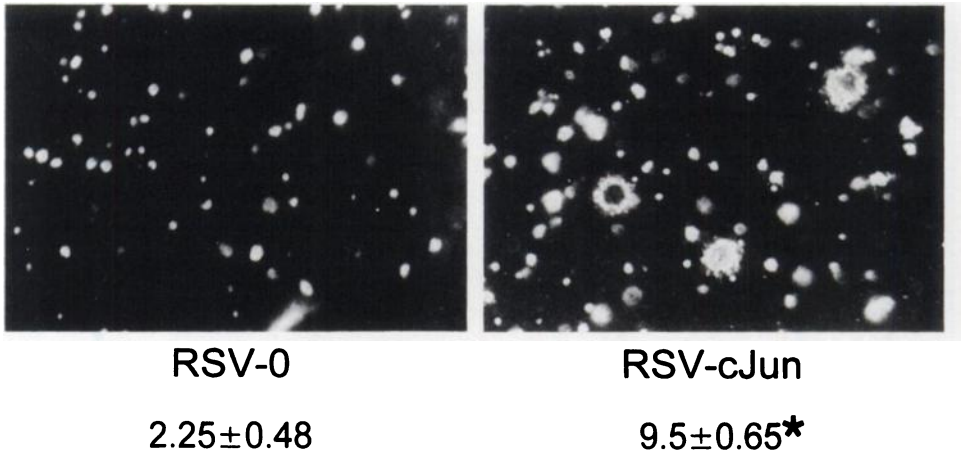

Fig. 4. Overexpression of c-*jun* enhances anchorage-independent growth in HTE cells. HTE cells transfected with calcium phosphate-DNA coprecipitates containing 4 μg RSV-cJun and 2 μg pSVβ-gal or 4 μg RSV-0 and 2 μg pSVβ-gal (control cells) were plated in soft agar as described in "Materials and Methods." Plates were examined for colony formation 21 days later, and four wells for each group were photographed for colony counting. The data are expressed as the mean ± SEM and represent the number of colonies >100 μm in five random fields for each of $n = 4$ wells/group. *, $P < 0.05$ as compared to RSV-0 cells.

cally, little is known about the regulation of AP-1 by oxidants. However, induction of the early response gene, c-*fos*, by AOS has been shown to be through a mechanism involving poly-ADP-ribosylation and is distinct from that of phorbol esters or serum (20).

Our working model of asbestos-induced disease suggests that persistent induction of the early response pathway (*i.e.*, c-*jun*) may lead to chronic cell proliferation and changes in cell phenotype indicative of neoplastic transformation. Here we have shown that asbestos can directly activate transcription of the c-*jun* gene and that overexpression of c-*jun* alone is sufficient to phenotypically change tracheal epithelial cells. Our results suggest that persistent induction of c-*jun* may contribute to asbestos-induced proliferation, a feature of associated lung cancers and mesotheliomas.

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