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Persistent induction of c-*fos* and c-*jun* expression by asbestos

(mesothelioma/lung cancer/oncogenes/cell proliferation)

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ABSTRACT **To investigate the mechanisms of asbestos-induced carcinogenesis, expression of c-*fos* and c-*jun* protooncogenes was examined in rat pleural mesothelial cells and hamster tracheal epithelial cells after exposure to crocidolite or chrysotile asbestos. In contrast to phorbol 12-myristate 13-acetate, which induces rapid and transient increases in c-*fos* and c-*jun* mRNA, asbestos causes 2- to 5-fold increases in c-*fos* and c-*jun* mRNA that persist for at least 24 hr in mesothelial cells. The induction of c-*fos* and c-*jun* mRNA by asbestos in mesothelial cells is dose-dependent and is most pronounced with crocidolite, the type of asbestos most pathogenic in the causation of pleural mesothelioma. Induction of c-*jun* gene expression by asbestos occurs in tracheal epithelial cells but is not accompanied by a corresponding induction of c-*fos* gene expression. In both cell types, asbestos induces increases in protein factors that bind specifically to the DNA sites that mediate gene expression by the AP-1 family of transcription factors. The persistent induction of AP-1 transcription factors by asbestos suggests a model of asbestos-induced carcinogenesis involving chronic stimulation of cell proliferation through activation of the early response gene pathway that includes c-*jun* and/or c-*fos*.**

Asbestos fibers are occupational carcinogens associated primarily with the development of lung cancers and malignant mesotheliomas (1). Several types of asbestos exist, most notably chrysotile [$Mg_6Si_4O_{10}(OH)_8$], a serpentine fiber that accounts for >90% of the asbestos used industrially, and crocidolite [$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$], a rod-like, more durable amphibole fiber that is associated with a greater risk of pleural mesothelioma (1, 2).

The mechanisms of carcinogenesis by asbestos are unclear. In addition to fiber durability in the lung or pleura over the long latency period of tumor development, several properties of asbestos may contribute to its pathogenicity. Tumorigenic potential, cell transformation, and chromosomal aberrations are more pronounced with long, thin fibers (generally >5 μm in length) in comparison to shorter fibers or particles of similar chemical composition (3, 4). Thus, fiber geometry and size may be critical determinants of fiber carcinogenicity. Other factors, such as the generation of active oxygen species from redox reactions catalyzed on the fiber surface, or the release of active oxygen species or growth factors from cells of the immune system, also may mediate the toxic or carcinogenic effects of asbestos (5).

Asbestos displays a variety of biological effects in different cell types. Human mesothelial cells are exquisitely sensitive to the cytotoxic effects of asbestos (6), and asbestos causes both chromosomal aberrations and morphological transformation in human (6) and rodent (7) mesothelial cells. In fibroblasts (8) and epithelial cells (9), but not mesothelial cells (10), asbestos also enhances cell transformation by other agents such as polycyclic aromatic hydrocarbons. These results, and the lack of correlation between smoking history and the development of mesothelioma in humans (1), suggest that asbestos fibers may be complete carcinogens in the development of mesothelioma.

Asbestos, in comparison to cigarette smoke, is weakly carcinogenic in epithelial cells of the respiratory tract. In experimental models using rodent tracheal grafts, asbestos appears to act primarily as a cocarcinogen (11) or tumor promoter (12), perhaps by facilitating the uptake, metabolism, and/or DNA binding of chemical carcinogens (13). Alternatively, asbestos causes chronic inflammation and may foster the development of tracheobronchial neoplasms by acting as a mitogen in a manner similar to the tumor promoter phorbol 12-myristate 13-acetate ("12-*O*-tetradecanoylphorbol 13-acetate," TPA) (1). At sublethal concentrations, chrysotile and crocidolite fibers cause proliferation of epithelial (14) and mesothelial (15) cells. Crocidolite asbestos causes increased accumulation of diacylglycerol, hydrolysis of phosphatidylinositol, and stimulation of protein kinase C (PKC) activity in hamster tracheal epithelial (HTE) cells (16, 17), suggesting that asbestos fibers initiate proliferation through second messenger pathways similar to those activated by TPA.

Among other events, activators of PKC, including TPA, induce the expression of AP-1, a family of accessory transcription factors that interact with regulatory DNA sequences known as TPA response elements (TREs) or AP-1 sites (18, 19). The family of AP-1 transcription factors that act through TREs includes homo- and heterodimeric protein complexes encoded by the c-*fos* and c-*jun* families of protooncogenes (19). In cell culture systems, expression of both c-*fos* and c-*jun* is required for transition of the G_1 phase and entry into the S phase of the cell cycle (18). By analogy to viral systems, Fos and Jun proteins are considered immediate early (or early response) gene products that regulate the expression of other genes required for progression through the cell cycle (18, 19).

Here we report the effects of crocidolite and chrysotile asbestos on the expression of c-*fos* and c-*jun* mRNA in rat pleural mesothelial (RPM) cells and a diploid HTE cell line, a progenitor cell type of bronchogenic carcinoma (20). Our results show that in RPM cells crocidolite asbestos is a potent and persistent inducer of c-*fos* and c-*jun* mRNA and AP-1 DNA-binding activity. Although in HTE cells induction of c-*jun* mRNA by asbestos is not accompanied by coincidental increases in c-*fos* gene expression, increases in AP-1 DNA-binding activity are observed. These studies suggest that asbestos may act as a mitogen in carcinogenesis by persistently activating the early response gene pathway.

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Abbreviations: FSE, fat-specific element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HTE, hamster tracheal epithelial; PKC, protein kinase C; RPM, rat pleural mesothelial; TPA, "12-*O*-tetradecanoylphorbol 13-acetate" (phorbol 12-myristate 13-acetate); TRE, TPA response element.

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MATERIALS AND METHODS

Asbestos Fibers. Reference samples of National Institute of Environmental Health Sciences (NIEHS) processed crocidolite and chrysotile (Jeffrey Mines, Quebec) asbestos fibers were obtained from the Thermal Insulation Manufacturers Association Fiber Repository (Littleton, CO). Fiber numbers per unit weight of NIEHS chrysotile (courtesy of A. Wylie, University of Maryland) were ≈10-fold greater than those for NIEHS processed crocidolite asbestos (courtesy of G. Hart, Schuller Mountain Technical Center, Littleton, CO) as calculated by using data from scanning electron microscopy. Preparations of riebeckite, a nonfibrous particle similar chemically to crocidolite asbestos (21), and polystyrene beads (average diameter, 1.05 μm; Polyscience) were used to assess the influence of fiber geometry on gene expression.

Cells and Exposure to Test Agents. RPM cells were isolated from the parietal pleura of Fischer 344 rats by methods reported previously (22). Cells were propagated for four passages in F12 medium (GIBCO) containing 10% fetal bovine serum and hydrocortisone (100 ng/ml), insulin (2.5 μg/ml), transferrin (2.5 μg/ml), and selenium (2.5 ng/ml) (Sigma). HTE cells, isolated from the tracheal epithelium of a neonatal hamster (20), were propagated in F12 medium with 10% fetal bovine serum. Twenty-four hours prior to exposure of confluent cultures to test agents, the growth medium was replaced with medium containing 2% serum. Asbestos fibers and other particulates were suspended in Hank's balanced salt solution (GIBCO) at 1 mg/ml and added directly to medium at final concentrations of 1.25–5.0 μg/cm^2 area of dish. These concentrations of asbestos fibers were nontoxic to both cell types for at least 24 hr as determined by total cellular protein (data not presented). TPA (Consolidated Midland, Brewster, NY) was added to medium at 100 ng/ml from a stock solution of 1 mg/ml in acetone. Untreated cultures were removed from the incubator and subjected to mock manipulations without changing the maintenance medium.

RNA Isolation and Northern Blot Analysis. Total RNA was prepared and Northern blot hybridization was performed as described by Shull *et al.* (23). cDNA probes for human c-*fos* and c-*jun* were from Richard Gaynor (University of California, Los Angeles) and were labeled with [α-^{32}P]dATP by random hexamer priming. Hybridization signals were quantified by measuring the radioactivity on blots either directly with a model 603 Betascope (Betagen, Waltham, MA) or indirectly by densitometric analysis of autoradiographs with a Microscan densitometer (Technology Resources, Nashville). To ascertain the specificity of the c-*fos* and c-*jun* response, Northern blots were rehybridized with a ^{32}P-labeled cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (a gift from Ph. Jeanteur, Laboratoire de Biochimie, Centre Paul Lamarque, France).

Gel Mobility-Shift Assays for AP-1 DNA-Binding Activity. One nanogram of a ^{32}P-end-labeled double-stranded oligodeoxynucleotide representing the fat-specific element (FSE) that contains a TRE consensus sequence (i.e., TGACTCA) (24) was incubated with 2 μg of whole-cell extract (0.5–2.0 mg of protein per ml) in 40 mM Hepes buffer, pH 7.8/80 mM KCl containing poly[d(I-C)] at 200 μg/ml in a total volume of 20 μl for 20 min at room temperature. Whole cell extracts were prepared as described (25) except that the final extract was not dialyzed. Protein–DNA complexes were resolved by electrophoresis in nondenaturing 4% polyacrylamide gels and visualized by autoradiography.

RESULTS

To explore the effects of asbestos on the induction of early response genes, confluent cultures of RPM cells were first exposed to crocidolite asbestos at a concentration that causes cell proliferation *in vitro* (14). At intervals from 30 min to 24 hr, total RNA was isolated for Northern blot analyses to ascertain the levels of c-*fos* and c-*jun* mRNA. In several experiments we compared c-*fos* and c-*jun* mRNA levels in cells treated with asbestos to the levels in untreated RPM cells and in cells exposed to TPA. In all groups, including untreated cultures not exposed to test agents, increased levels of c-*fos* and c-*jun* mRNA were observed at 30 min (Fig. 1). Because the increases that occurred by 30 min were similar in all groups, it is likely that these initial responses are nonspecific, as has been observed by others (18, 19). In comparison to controls, RPM cells treated with TPA yielded increased amounts of c-*fos* mRNA that reached peak levels after 1 hr and rapidly diminished to control levels thereafter (Fig. 1 *A* and *B*). TPA induced a similar accumulation of c-*jun* mRNA in RPM cells; peak levels of c-*jun* mRNA were observed by 1 hr after exposure and diminished thereafter (Fig. 1 *A* and *C*). The induction of c-*fos* and c-*jun* by TPA in RPM cells was potentiated by cycloheximide (data not shown), suggesting that TPA induces a rapid and transient increase in the expression of c-*fos* and c-*jun* mRNA in RPM cells; such an increase is the hallmark of the early response gene pathway (18, 19).

In contrast, the kinetics of induction of c-*fos* and c-*jun* mRNA in RPM cells by asbestos differed from that observed with TPA. After 2 hr of exposure to crocidolite fibers, the levels of c-*fos* and c-*jun* mRNA increased steadily for at least 24 hr (Fig. 1). Quantification of the Northern blot hybridization signals showed that at 24 hr the levels of c-*fos* and c-*jun* mRNA in RPM cells exposed to crocidolite asbestos were 2- to 5-fold higher than those in control cultures or cultures treated with TPA (Fig. 1 *B* and *C*). To determine whether the cellular responses leading to the accumulation of c-*fos* and c-*jun* mRNA were specific, we reprobed the Northern blots presented in Fig. 1 with a cDNA probe for the "housekeeping" gene encoding GAPDH. In RPM cells, GAPDH mRNA levels varied <15% between control and experimental groups.

To determine whether differences existed between responses of RPM cells to either crocidolite or chrysotile asbestos, cells were exposed to different doses of each fiber type for 8 hr, and the levels of mRNA encoding c-*jun* and c-*fos* were measured by quantitative Northern blot analysis. Both crocidolite and chrysotile asbestos caused dose-dependent, persistent increases in c-*fos* and c-*jun* mRNA (Fig. 2). When the response is evaluated as a function of fiber mass (microgram of particulate per unit area) these two fiber types appear similar in their ability to induce c-*fos* and c-*jun* gene expression. However, if the dose is evaluated as a function of the number of fibers per unit area (as there are more chrysotile than crocidolite fibers per unit mass), crocidolite asbestos is a more potent inducer of c-*fos* and c-*jun* mRNA expression in RPM cells than is chrysotile asbestos. Increases in c-*fos* and c-*jun* mRNA were not observed with polystyrene beads (Fig. 2) or with riebeckite (data not shown) at a range of particle concentrations.

We next asked whether asbestos induces c-*fos* and c-*jun* mRNA in HTE cells. Like RPM cells, untreated HTE cultures and cultures exposed to asbestos displayed rapid and nonspecific increases in both c-*fos* and c-*jun* mRNA at 30 min (Fig. 3). After a 30-min exposure to TPA, HTE cells exhibited c-*fos* mRNA levels 4- to 5-fold greater than those observed in control cells (Fig. 3*B*). In contrast to RPM cells, elevated levels of c-*fos* mRNA were not observed in HTE cells at any later time after treatment with TPA, chrysotile, or crocidolite asbestos (Fig. 3*B*). The c-*jun* response in HTE cells also differed from that observed in RPM cells. In HTE cells, the accumulation of c-*jun* mRNA in response to TPA occurred rapidly but persisted for 4–8 hr before falling to control levels at 24 hr. With crocidolite asbestos, elevated levels of c-*jun* mRNA were first observed 4 hr after addition of fibers and increased steadily for at least 24 hr. These results clearly

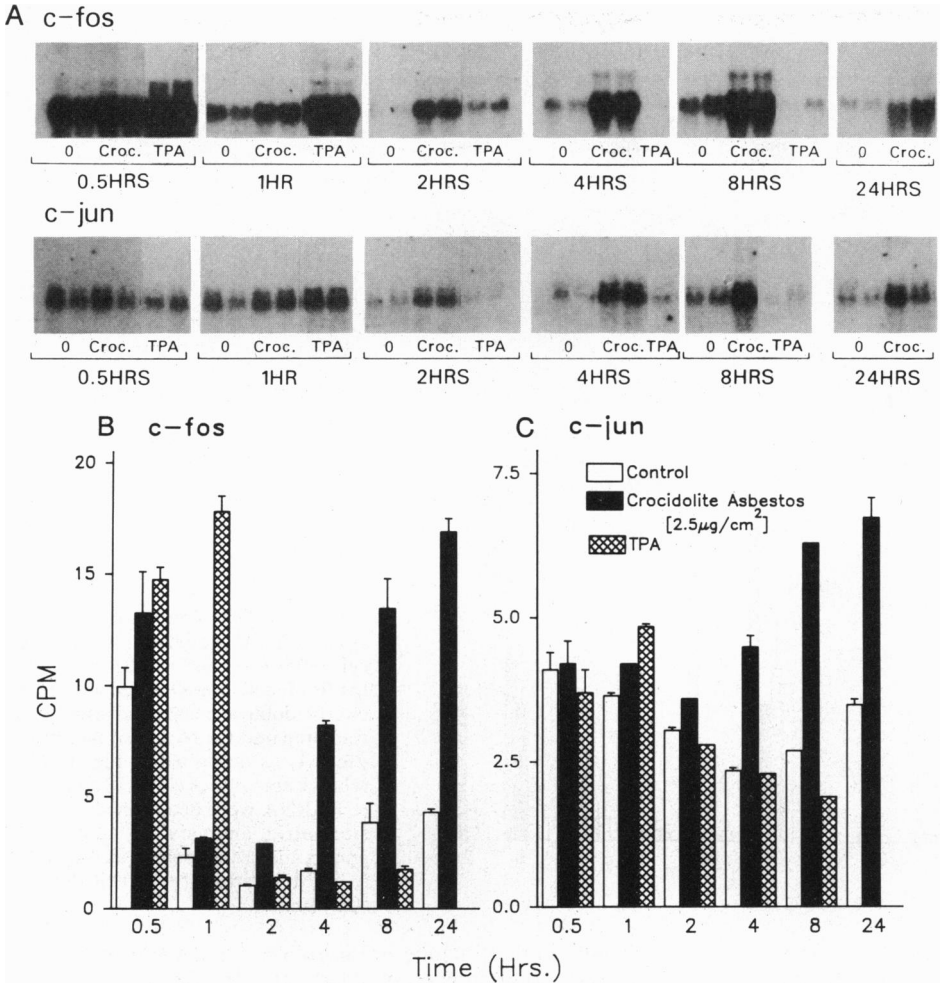

FIG. 1. Persistent induction of c-*fos* and c-*jun* mRNA accumulation in confluent RPM cells after exposure to crocidolite (Croc.) asbestos. Total RNA was extracted from RPM cells at various times after treatment with the indicated agent and 12.5-μg samples were analyzed by Northern blot hybridization to measure the relative steady-state levels of c-*fos* and c-*jun* mRNA (*A*). The Northern blot hybridization signals for c-*fos* (*B*) and c-*jun* (*C*) were quantified with a model 603 Betascope apparatus and are expressed in counts per minute. Each experimental point was performed in duplicate and the relative amounts of mRNA are expressed as mean ± SEM.

show that crocidolite asbestos induces elevated levels of mRNA for early response gene products that persist for at least 24 hr. Moreover, they indicate that the regulation of c-*fos* mRNA levels in response to asbestos differs in HTE and RPM cells. In HTE cells, the levels of GAPDH mRNA varied <5% between control and test groups (data not shown).

We used gel mobility-shift assays to ascertain whether the induction of c-*fos* and/or c-*jun* mRNA was accompanied by increased levels of AP-1 transcription factors. Whole cell extracts from treated and untreated cells were incubated with a radiolabeled probe (FSE) containing the consensus sequence for AP-1 DNA binding, and the resulting protein–

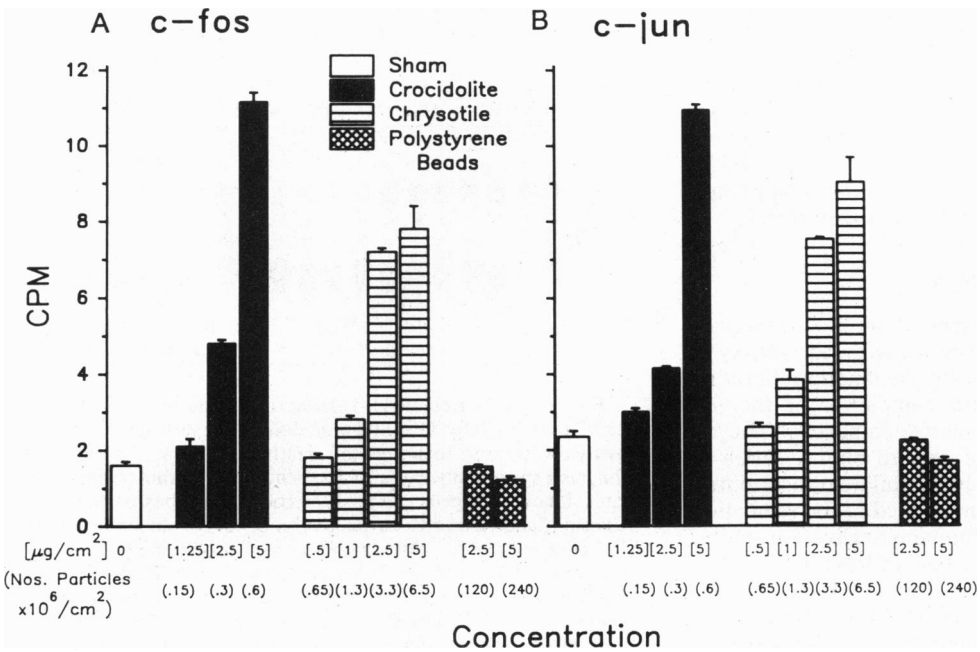

FIG. 2. Dose–response studies measuring steady-state levels of c-*fos* mRNA (*A*) and c-*jun* mRNA (*B*) in RPM cells. Cells at confluence were exposed to NIEHS processed crocidolite or NIEHS chrysotile asbestos or polystyrene microspheres for 8 hr and RNA was isolated for Northern blot analyses. The hybridization signals for c-*fos* and c-*jun* mRNA were quantitated directly with a model 603 Betascope. Data are expressed as mean cpm ± SEM (n = 2 lanes per group). Concentrations of particulates on the abscissa are expressed as micrograms and corresponding numbers of fibers or polystyrene particles (per unit weight) per cm² of surface area of dish.

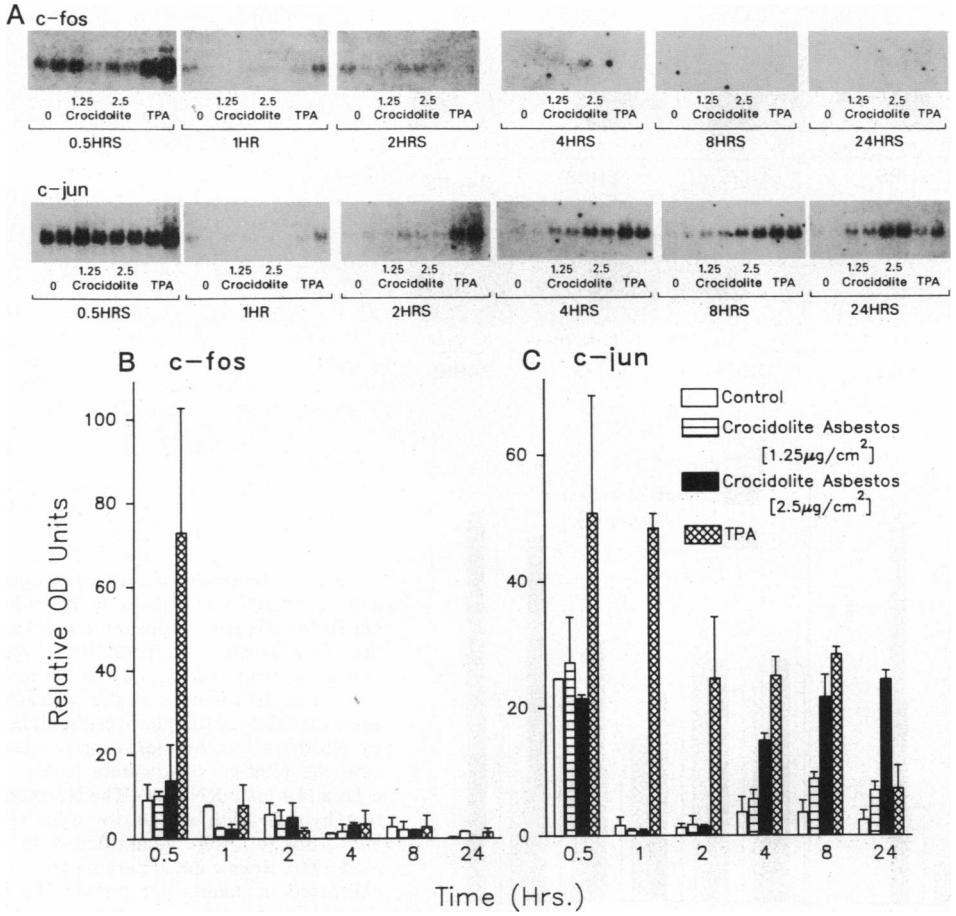

FIG. 3. Time course of c-*fos* and c-*jun* mRNA accumulation in HTE cells after exposure to TPA or asbestos. HTE cells were treated with TPA or crocidolite asbestos and total RNA was prepared for Northern blot analyses (*A*) as described in Fig. 1. The relative amounts of c-*fos* (*B*) and c-*jun* (*C*) mRNA were determined by densitometric analysis of autoradiographs and are expressed as the mean ± SEM in arbitrary optical density (OD) units.

DNA complexes were resolved in neutral polyacrylamide gels (24). Induction of c-*fos* and c-*jun* mRNA in RPM cells by asbestos was accompanied in time by increased levels of protein factors that bound specifically to the AP-1 sequence (Fig. 4). Note that the protein–AP-1 DNA complexes induced by TPA and asbestos comigrated and that after 24 hr, the level of AP-1 DNA-binding activity induced by asbestos was about twice that observed with TPA (Fig. 4*A*). Using a variety of specific and nonspecific competitors, as well as purified c-Fos and c-Jun proteins, we have demonstrated that the protein–AP-1 DNA complex induced by asbestos comigrates with that produced by c-Fos/c-Jun heterodimers (data not shown). Although we have not observed induction of c-*fos* mRNA in HTE cells by asbestos, exposure of HTE cells to asbestos fibers is accompanied by increases in protein factors that bind specifically to the AP-1 DNA consensus sequence (Fig. 4*B*). The induction of AP-1 binding activity in HTE cells may result from the induction of Fra1 or another Fos-related gene product that is able to dimerize with c-Jun (26).

DISCUSSION

The c-*fos* and c-*jun* genes are members of multigene families that are transiently expressed in response to a wide variety of environmental cues in both proliferating and nonproliferating cells (18, 19). Both homodimeric complexes of the *jun* gene-family products and heterodimeric complexes of the *jun* and *fos* gene-family products are able to bind a series of related DNA sequences and thereby modulate transcription. Although *fos* and *jun* have been implicated in the regulation of cell growth *in vitro* and *in vivo*, immunolocalization studies show that Fos is present at high levels in terminally differentiated cells (27). Thus, the members of the *fos* and *jun* gene families may couple cell signaling events at the cell surface to changes in gene expression that modulate cell-type-specific responses that include proliferation, changes in phenotype, or even programmed cell death (18, 19, 27).

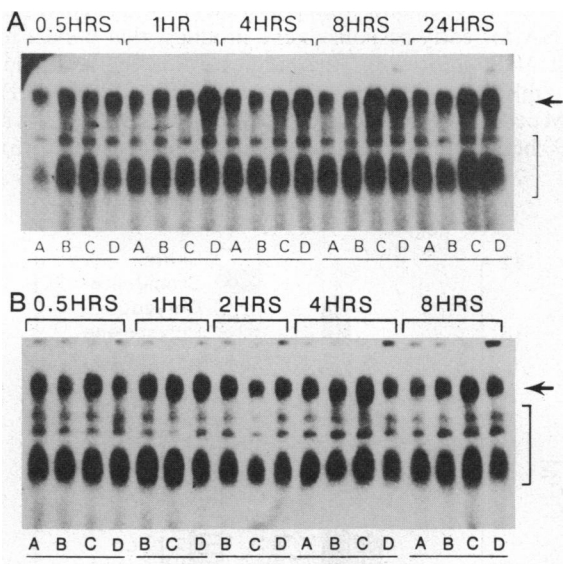

FIG. 4. Induction of AP-1 transcription factors in RPM cells (*A*) or HTE cells (*B*) by TPA and crocidolite asbestos. Gel mobility-shift assays were used to assess the relative amounts of protein factors that bind specifically to the TREs. Whole cell extracts from untreated cells (lanes A) or cells exposed to crocidolite asbestos at 1.25 or 2.5 μg/cm² (lanes B and C, respectively) or to TPA at 100 ng/ml (lanes D) were incubated with an end-labeled probe (FSE) containing a consensus TRE. Protein–DNA complexes were resolved by nondenaturing gel electrophoresis and visualized by autoradiography. The specific AP-1–FSE DNA complex is indicated by an arrow; nonspecific protein–DNA complexes are indicated by a bracket.

Here we have examined the effects of asbestos on the expression of c-*fos* and c-*jun* mRNA in RPM and HTE cells. The asbestos was present at sublethal amounts that induce the proliferation of both cell types (14, 15). In response to TPA, both RPM and HTE cells displayed the transient increase in c-*fos* and c-*jun* mRNA that is the hallmark of the early response gene pathway. In contrast, the patterns of expression of these protooncogenes were dissimilar in the two cell types after exposure to asbestos. While crocidolite and chrysotile asbestos induced a dramatic increase in c-*fos* mRNA in RPM cells, c-*fos* mRNA was not induced by these agents in HTE cells, showing that the regulation of c-*fos* gene expression in response to asbestos varies in different cell types. In both RPM and HTE cells, asbestos induced increases in c-*jun* mRNA that persisted at high levels (nearly 5-fold over untreated controls) for at least 24 hr. The persistent induction of c-*jun* and/or c-*fos* mRNA is significant, as induction of transcription by AP-1 binding proteins requires sustained expression of these factors (28).

The induction of gene expression in RPM cells by asbestos is dose-dependent and not observed with polystyrene beads (Fig. 2) or riebeckite (data not shown), nonfibrous and noncarcinogenic particles which do not induce proliferation in cell or organ cultures (14, 21). These data suggest that fiber geometry is a critical determinant in the sustained induction of c-*fos* and c-*jun* transcription and/or mRNA stabilization.

Our results suggest a model for the induction of neoplastic disease by asbestos (Fig. 5). By persistently activating the early response gene pathway, asbestos may induce chronic cell proliferation that subsequently contributes to carcinogenesis in lung and pleura (29, 30). The model is consistent with data that show increases in diacylglycerol and stimulation of PKC precede asbestos-induced cell replication (16, 17). Here we have shown that these cell signaling responses are accompanied by the accumulation of c-*jun* and/or c-*fos* mRNA and the concomitant formation of AP-1 transcription factors that bind specific regulatory DNA sequences. Based on studies in other systems (18, 19), we suggest that these early gene responses are representative of the events that initiate progression through the cell cycle. Durable asbestos fibers may therefore provide a persistent growth stimulus during long latency periods of tumors and thereby contribute to the eventual fixation of genetic changes caused by asbestos itself or other agents. Alternatively, induction of c-*fos* and c-*jun* may affect changes in cell phenotype that contribute to neoplastic transformation.

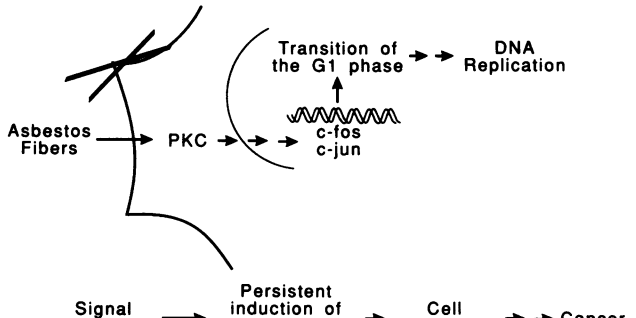

FIG. 5. Model for asbestos-induced cell proliferation and carcinogenesis. Asbestos fibers are proposed to stimulate cell signaling pathways that result in activation of the early response gene pathway, thereby triggering the regulatory lattice of gene expression required for transition through the cell cycle. Chronic stimulation of cell proliferation leads to the fixation of genetic damage caused by asbestos itself or other agents, eventually culminating in the selection of neoplastic cells.

It is noteworthy that the accumulation of c-*jun* and/or c-*fos* mRNA occurs in RPM and HTE cells in the absence of intermediary cells of the immune system. This suggests that growth factors or other substances released from immune cells are not required to elicit the early gene response by asbestos fibers. Rather, our data suggest that the induction of chronic cell proliferation may be an inherent property of asbestos fibers. Thus, both an enhanced capacity to activate the early response gene pathway and pronounced biopersistence may be critical factors in the increased pathogenicity of crocidolite as compared to chrysotile asbestos (1, 2).

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