Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 4409–4413, July 1984
Cell Biology

Tumor promoters induce a specific morphological signature in the nuclear matrix–intermediate filament scaffold of Madin–Darby canine kidney (MDCK) cell colonies

(cytoskeleton/cytokeratins/phorbol 12-tetradecanoate 13-acetate)

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Communicated by John M. Buchanan, March 23, 1984

ABSTRACT **Tumor promoters such as phorbol 12-tetradecanoate 13-acetate (TPA), mezerein, teleocidin, aplysiatoxin, and benzoyl peroxide, although structurally unrelated, induce similar, profound changes in morphology in differentiated epithelial Madin–Darby canine kidney (MDCK) cell colonies. The alteration is evident in the organization of intermediate filaments in intact cells and in whole mounts of the nuclear matrix–intermediate filament (NM-IF) scaffold of the epithelial sheet. This substructure, obtained by salt extraction of the cytoskeletal framework, represents only 5% of the total cell protein but contains all of the intermediate filaments, nuclear matrix, and desmosomal core proteins arranged essentially as in the intact cell. The NM-IF is profoundly reorganized after exposure to TPA and retains the morphological changes observed in intact cells. These include bundling of the intermediate filaments, disruption of cell–cell borders, and marked deformation of the polygonal geometry of epithelia. Thus, TPA and all other complete or second-stage tumor promoters examined have a characteristic morphological signature that is not induced by mitogens, metabolic inhibitors, or agents known to disrupt microtubules or microfilaments. This signature, characteristic of tumor promoters, occurs in the absence of both protein and RNA synthesis. These results suggest that this response is prior to and independent of other biochemical markers for tumor promoters. Of the major filament systems, the cytokeratin network is implicated as an early or possibly primary site of tumor-promoter action because characteristics of the promoted cytoskeletal signature are observed in epithelial colonies after prior exposure to colchicine or cytochalasin D. Despite the massive reorganization of cytoskeletal morphology induced by TPA, the distribution of prelabeled proteins into structural fractions (i.e., cytoskeletal, chromatin, and the NM-IF) remains essentially unchanged. The sensitivity and specificity of the epithelial cell response suggest its possible use as a screen for promoting compounds.**

The experimental basis for the two-stage model of carcinogenesis, involving separate initiation and promotion steps, has been clearly defined and well reviewed (1, 2). One of the most potent tumor promoters on mouse skin is phorbol 12-tetradecanoate 13-acetate (TPA) (3, 4). Whereas TPA alters a number of biochemical and morphological characteristics of the cells in culture (5, 6), it has been difficult to establish which of these effects is primary and relevant to tumor promotion *in vivo*.

TPA has been shown to significantly alter cell morphology. Fibroblasts display a marked morphological response to TPA (7, 8), exhibiting a rapid loss of actin cables and of multicellular orientation. These morphological changes reflect alterations of the cytoskeleton (8).

Nonfibroblastic cells, particularly those with extensive, well-ordered intermediate filament networks, display a pronounced shape change in response to TPA. Treatment of myotubes with TPA results in a breakdown of myofibrils paralleled by the appearance of dense regions of 10-nm filament bundles (9). Bundles of 10 nm filaments are also the predominant feature in macrophages treated with TPA (10).

Perhaps the greatest sensitivity to TPA is manifested by well-differentiated epithelia. Epidermal keratinocytes display a breakdown of epithelial organization and develop a fibroblastic morphology with many processes (11, 12). Gross morphological changes induced by TPA are particularly apparent in the well-differentiated Madin–Darby canine kidney (MDCK) cell line (13, 14). In these studies, low concentrations of TPA have been shown to induce extensive changes in MDCK morphology concomitant with a breakdown of tight junctional complexes and loss of the differentiated property of vectorial ion transport characteristic of this cell type.

In the present study, we examine alterations of the intermediate filament framework induced by nanomolar levels of the phorbol ester TPA and a number of other, structurally unrelated, tumor promoters. Cell behavior is examined by optical microscopy with time-lapse video and immunofluorescence techniques. MDCK colonies prepared as detergent-extracted whole mounts are also examined by resinless electron microscopy. Promoting agents induce a characteristic morphological response or signature not found with mitogens, metabolic inhibitors, or agents that disrupt cytoskeletal elements. Intermediate filament reorganization is a major component of the morphological response to tumor promoters.

The MDCK cell line was chosen for these studies because it is highly differentiated, closely resembling "normal" kidney distal tubule cells (15). It has a remarkably stable karyotype and forms highly differentiated epithelial colonies that are nontumorigenic when injected into nude mice (16). This line has proven remarkably sensitive to promoting compounds with a marked and reproducible morphological response to subnanomolar levels of tumor promoters.

MATERIALS AND METHODS

Cell Culture. MDCK cells were grown as described (17) and used at a subconfluent density of 6–8 $\times$ 10^6 cells per 100-mm diameter plastic tissue culture plate. Dimethyl sulfoxide or acetone was added to control plates at a concentration of 0.1%. Tumor promoters and other compounds were added at concentrations indicated in the figure legends. Mezerein, aplysiatoxin and teleocidin were generously provided by I. B. Weinstein. Epidermal growth factor and benzoyl per-

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Abbreviations: TPA, phorbol 12-tetradecanoate 13-acetate; MDCK cells, Madin–Darby canine kidney; NM-IF, nuclear matrix–intermediate filament.

oxide were obtained from Collaborative Research (Waltham, MA) and Aldrich, respectively.

Radiolabeling. Cell protein was prelabeled for 24 hr with 15 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (10 mCi/ml; 1107 Ci/mmol; New England Nuclear) in Dulbecco's minimal essential medium with 10% fetal bovine serum. Cells were washed three times in unlabeled complete medium prior to the addition of unlabeled medium and further incubation in the presence of TPA at 5 ng/ml.

Cell Fractionation. MDCK colonies, grown on plastic plates or glass coverslips were rinsed twice with phosphate-buffered saline, extracted with cytoskeleton buffer (CSK buffer) (100 mM NaCl/300 mM sucrose/10 mM Pipes, pH 6.8/3 mM $MgCl_2$/0.5% Triton X-100/1.2 mM phenylmethylsulfonyl fluoride), and the supernatant was removed as the "soluble" fraction. The "cytoskeleton" fraction was obtained after incubation in an extraction buffer (identical to CSK buffer except for 250 mM $(NH_4)_2SO_4$ in place of NaCl) for 10 min at 0°C. The "chromatin" fraction was obtained after a 20-min digestion at 20°C in CSK buffer with 50 mM NaCl containing both 400 μg of bovine pancreatic DNase I (EC 3.1.4.5, Worthington) and 400 μg of pancreatic RNase A (EC 3.1.4.22, Sigma) per ml, followed by a 5-min incubation in 250 mM $(NH_4)_2SO_4$ (final concentration). The remaining insoluble structure is the "nuclear matrix–intermediate filament" (NM-IF) fraction.

Light Microscopy. Time-lapse video micrographs of MDCK colonies were obtained by phase-contrast optics on a Zeiss Universal microscope coupled to an RCA Nuvicon TC 1005 video camera.

Immunofluorescence Microscopy. Rabbit anti-keratin antibody was generously provided by J. Rheinwald. Immunofluorescence microscopy of NM-IF fractions was performed by use of a double-antibody technique as described (17).

Electron Microscopy. Whole-mount transmission electron microscopy was done on MDCK colonies grown on gold grids previously coated with Formvar and carbon. NM-IF fractions were prepared as described above, fixed in 2.5% gluteraldehyde, postfixed in 1% OsO_4, and dehydrated through an ethanol series. The grids were dried through the CO_2 critical point and coated with gold-palladium prior to examination in a JOEL 100B transmission electron microscope.

Electrophoresis. Polyacrylamide gels (10%) were run by the protocol of Laemmli (18). Equivalent cell numbers were loaded onto each gel track. Gels were dried and exposed to preflashed Kodak X-Omat film.

RESULTS

The gross morphological alterations produced by TPA at 5 ng/ml are shown in Fig. 1. Fig. 1 *a–d* shows a time course of morphological changes observed on living MDCK colonies with phase micrographs taken from time-lapse video recordings. The control MDCK colony (Fig. 1*a*) represents a typical 30-cell epithelial colony that displays a tight cuboidal morphology. After 30 min in TPA (Fig. 1*b*), the colony enlarged, and the cells became flattened and elongated. After a 1-hr incubation in TPA (Fig. 1*c*), the cells elongated and developed crescent-shaped processes, which became more pronounced as the cells became motile. At 2 hr (Fig. 1*d*), long cellular processes became a predominant feature of the cell morphology, and the MDCK cells displayed a morphological "signature" that is a characteristic response unique to tumor-promoting agents.

The images shown in Fig. 1 *e–h* were derived from MDCK colonies from a time-course identical with that shown in Fig. 1 *a–d*. In this case, the cells were extracted at each time point, and NM-IF scaffold was prepared *in situ*. These structures retain only 5% of the original cell proteins and are devoid of nucleic acids and phospholipid, yet retain the original morphology of the cytokeratins. The NM-IF preparations were stained by use of a double-antibody immunofluorescence technique in which the first antibody is to keratin proteins. These images represent, therefore, the distribution of cytokeratin filaments after exposure to TPA. In the control colony (Fig. 1*e*), the cuboidal morphology is apparent in the keratin filaments that are concentrated at the regions of intercellular junctions (17). As the morphological signature developed in response to TPA, the distribution of the cytokeratin filaments was altered in a manner that closely parallels the formation of cellular processes observed in the intact cells. Thus, the pattern of cytokeratin distribution in the NM-IF preparation may be used to monitor the morphological changes induced by TPA.

More detailed examination of the alterations in NM-IF organization in response to TPA or other promoting agents is facilitated by the use of whole-mount transmission electron microscopy. Fig. 2 shows the detailed organization of the control NM-IF scaffold as previously reported (17). The nuclear matrix was observed in association with cytokeratin filaments, which terminate at residual desmosomal structures. After a 2-hr exposure to TPA (5 ng/ml), the ordered arrangement of filaments in the control NM-IF scaffold was drastically altered (Fig. 2*b*). The filaments, which are normally distributed in a well-spread, radial pattern, formed dense bundles. The distorted nuclear matrices became elongated and enlarged and were observed in close association with the newly formed filament bundles (Fig. 2*b*).

That the morphological alterations induced by TPA may represent a primary response to the tumor promoter is suggested by the experiment described in Fig. 3. MDCK colonies were treated with either cycloheximide (Fig. 3*a*) at 50 μg/ml or actinomycin D (Fig. 3*c*) at 5 μg/ml for 30 min. The inhibition of protein and RNA synthesis did not alter the morphology of the MDCK colonies. These colonies were in-

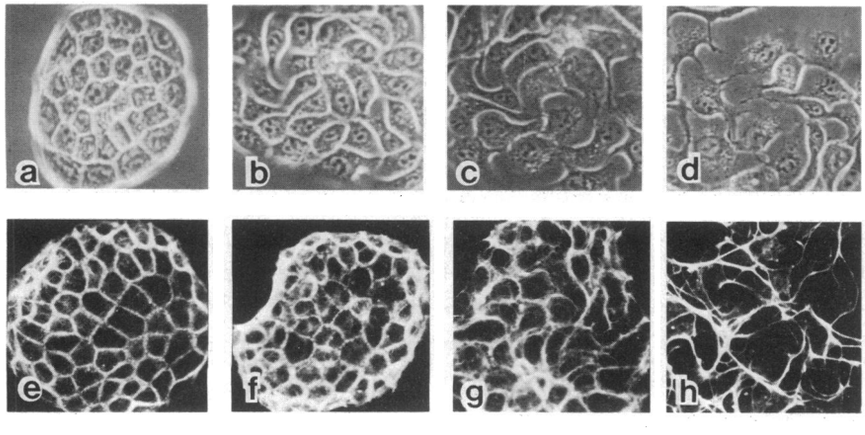

FIG. 1. Video time-lapse micrographs (*a–d*) showing the progression of morphological alterations induced in a living MDCK cell colony after exposure to TPA at 5 ng/ml. These phenotypic changes are faithfully retained in purified NM-IF scaffolds (*e–h*) stained for keratins and visualized by immunofluorescence microscopy. The control colonies (*a* and *e*) show typical epitheloid morphology, which is seen to deform at 30 min (*b* and *f*). At 1 hr (*c* and *g*), cytoplasmid processed and cytokeratin bundles develop. These become the predominant features after 2 hr (*d* and *h*). (×200.)

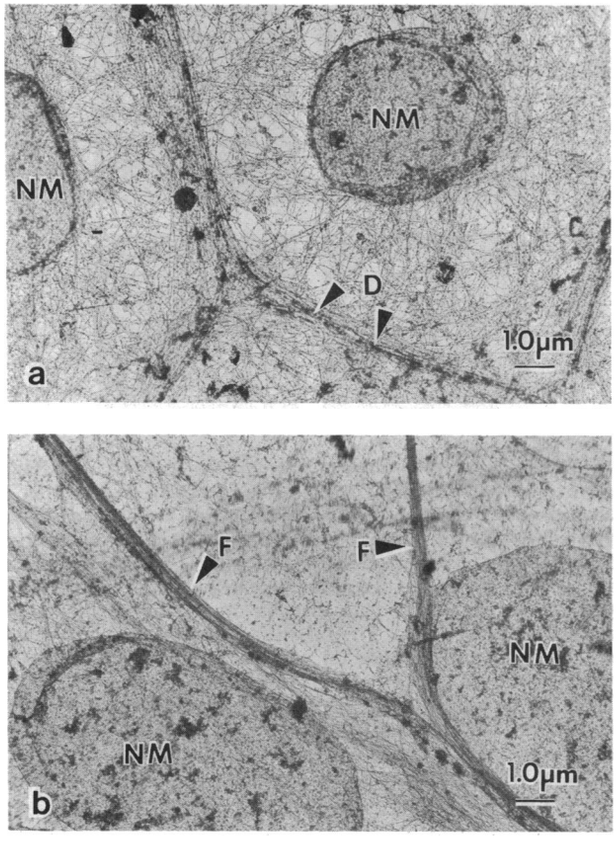

FIG. 2. Transmission electron micrographs of NM-IF scaffolds derived from whole-mount preparations of MDCK epithelial colonies. (*a*) Control MDCK colonies, depleted of soluble protein, phospholipid, and nucleic acids, retain cell borders with residual desmosomes (D). The nuclear matrices (NM) are located in the center of the cell and are associated with a fine web of intermediate filaments. (*b*) After exposure to TPA at 5 ng/ml for 2 hr, the nuclear matrices (NM) have become enlarged and distorted. The web of individual filaments has been replaced by dense filament bundles (F), which are closely associated with the nuclear matrix structures.

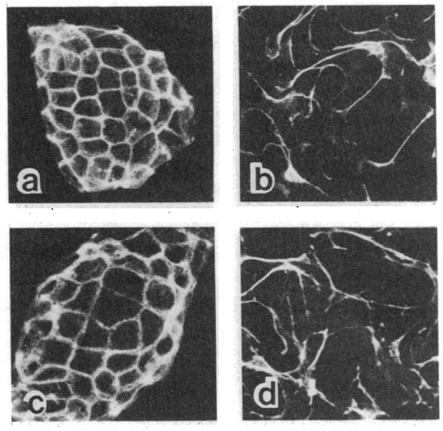

FIG. 3. Immunofluorescence micrographs of NM-IF scaffolds derived from MDCK colonies and stained for cytokeratins. Protein and RNA syntheses were inhibited for 30 min with cycloheximide at 50 μg/ml (*a*) or actinomycin D at 5 μg/ml (*c*) prior to extraction and fixation. Neither drug altered the epithelial morphology of MDCK colonies (compare with Fig. 1*e*). When TPA at 5 ng/ml was subsequently added to the MDCK colonies pretreated with cycloheximide (*b*) and actinomycin D (*d*) and incubated for 2 hr, the morphological signature characteristic of TPA treatment alone was observed (compare with Fig. 1*h*). (×200.)

distinguishable from control colonies (Fig. 1*e*). When colonies treated with either drug were subsequently exposed to TPA at 5 ng/ml, the characteristic morphological response to tumor promoters was observed (Fig. 3 *b* and *d*). Since this response to TPA is independent of gene expression, it is therefore distinguishable from those biochemical markers of tumor promotion that require protein and/or RNA synthesis (see *Discussion*).

The morphological signature induced in MDCK epithelia by TPA was characteristic for all tumor promoters examined. The micrographs shown in Fig. 4 compare the effects of a variety of agents. The nonpromoting or weak first-stage-promoting phorbol compounds 4α-phorbol 12,13-didecanoate and phorbol (19) had no detectable effect on MDCK morphology (Fig. 4 *a* and *b*). In addition the mitogen epidermal growth factor and the hyperplastic calcium ionophore A23187, both agents that mimic some biochemical aspects of promotion (20, 21), did not affect MDCK morphology (Fig. 4 *c* and *d*). In contrast, a wide variety of compounds, shown to be complete or second-stage promoters *in vivo* (19, 22–24), induced flattened morphologies with cytokeratin-rich processes that were indistinguishable from the morphological signature induced by TPA. The promoters examined were mezerein (Fig. 4*e*), aplysiatoxin (Fig. 4*f*), teleocidin (Fig. 4*g*), and benzoyl peroxide (Fig. 4*h*). These compounds constitute a broad range of structurally unrelated tumor-promoting agents, and all produced very similar lesions in NM-IF organization of these epithelial colonies.

To determine whether the microtubule or microfilament networks were involved in the altered morphology induced by tumor promoters, MDCK colonies were incubated in either colchicine or cytochalasin D for 2 hr. Colchicine treatment caused no change in epithelial colony organization (Fig. 5*a*). When TPA (5 ng/ml) was added after prior incubation in colchicine, the morphological signature characteristic of tumor promoters was observed (Fig. 5*b*). This observation indicates that the microtubule system is not directly involved in the morphological response to tumor promoters. The effect of cytochalasin D was somewhat more complex. After a 2-hr exposure to cytochalasin D, the morphology of the MDCK colonies was severely altered (Fig. 5*c*). However, the resultant morphology was clearly different from that produced by tumor promoters. The colonies remained in tight clusters; although there was a partial breakdown of intercellular association (25), the major characteristics of changes induced by tumor promoters (i.e., rapid cell spreading and the formation of long, cytokeratin-rich filament bundles) were not observed in response to cytochalasin D. When TPA was added to MDCK colonies previously exposed to cytochalasin D (Fig. 5*d*), both cell spreading and process formation were observed. The morphological signature that resulted from a combination of cytochalasin D and TPA was a striking stellate pattern clearly different from that induced by TPA alone. This result suggests that whereas TPA still induces the cytokeratin-rich process formation and cell spreading, associations with the microfilament network may influence the alteration of cytokeratin organization produced by TPA and other tumor promoters.

In the experiment described in Fig. 6, total cell protein was labeled with [^{35}S]methionine 24 hr before incubation with TPA. After incubation with TPA at 5 ng/ml for 2 hr, the MDCK colonies were sequentially extracted to produce the NM-IF structure. The proteins of the three structural fractions were analyzed on 10% polyacrylamide gels. The distribution of proteins among the structural subfractions was virtually unchanged by exposure to TPA (Fig. 6). This result suggests that morphological changes induced by TPA result from the reorganization rather than the disruption of cytoskeletal elements.

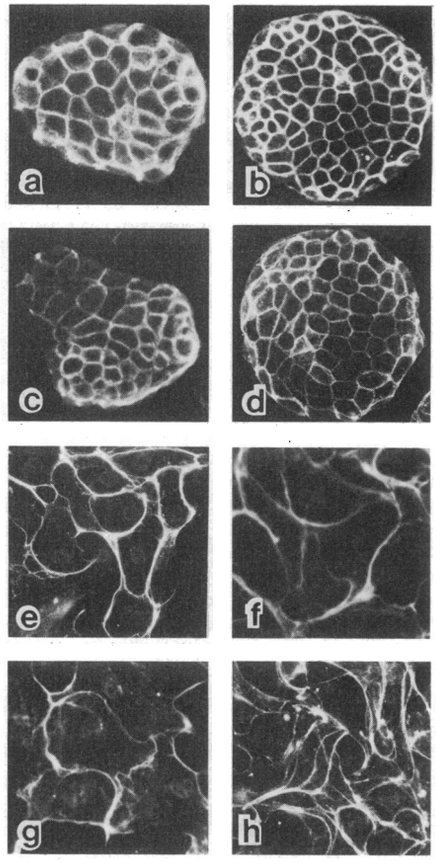

FIG. 4. Immunofluorescence micrographs of NM-IF scaffolds derived from MDCK colonies and stained for cytokeratins. MDCK colonies, treated with the following nonpromoting compounds for 2 hr, were fractionated to NM-IF scaffolds and stained with antikeratin: 4α-phorbol 12,13-didecanoate at 20 ng/ml (*a*), phorbol at 20 ng/ml (*b*), epidermal growth factor at 100 ng/ml (*c*), and the calcium ionophore A23187 at 1 μg/ml (*d*). None of these compounds caused any significant alteration of epithelial morphology. A morphological signature characteristic of that produced by TPA was produced by the addition of the following tumor promoters: mezerein at 5 ng/ml (*e*), aplysiatoxin at 5 ng/ml (*f*), teleocidin at 5 ng/ml (*g*), and benzoyl peroxide at 20 μg/ml (*h*). (×200.)

DISCUSSION

In this report a number of structurally unrelated compounds known to promote tumors *in vivo* are shown to induce a rapid breakdown of the organization of epithelial cells that results in a characteristic morphological signature in MDCK epithelial colonies. The epitheloid nature of these cuboidal cell colonies is altered, and individual cells become motile, flattened, and elongated. The predominant aspects of altered morphology in these cells after exposure to tumor promoters are reflected in the distribution of cytokeratins in the NM-IF scaffold, a structure depleted of nucleic acids, phospholipid, and 95% of the total cell protein. The morphological alterations observed in intact cell colonies (Fig. 1 *a–d*) are preserved with great fidelity by the cytokeratin filaments in the extracted NM-IF structures (Fig. 1 *e–h*). Examination of whole mounts of the NM-IF scaffolds by whole-mount transmission electron microscopy (Fig. 2) indicates that the elongation and flattening of MDCK cells after exposure to tumor promoters is reflected by a breakdown of cell borders and bundling of the cytokeratin filaments along the axis of cell elongation. The generality of this morphological response to tumor promoters is demonstrated in Fig. 4. Five structurally unrelated compounds, all known to be complete or second-stage tumor promoters (19, 22–24), are shown to induce identical morphological signatures after exposure to the promoting agent for 2 hr. Putative first stage promoters or nonpromoting analogues to TPA (19) have no effect on MDCK morphology.

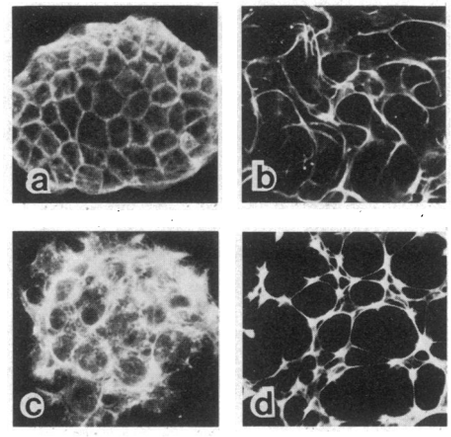

FIG. 5. Immunofluorescence micrographs of NM-IF scaffolds derived from MDCK colonies and stained for cytokeratins. MDCK colonies were treated for 2 hr with colchicine at 20 μg/ml (*a*) or cytochalasin D at 5 μg/ml (*c*). Colchicine had no effect on the organization of cytokeratins in the NM-IF structure, whereas cytochalasin D treatment resulted in a diffuse staining pattern. This effect of cytochalasins on intermediate filament organization has been reported (25). When TPA at 5 ng/ml was added for 2 hr to cells previously treated with colchicine (*b*), the cytokeratin pattern observed was characteristic of cells treated with TPA alone (Fig. 1*h*). When TPA (5 ng/ml) was added for 2 hr to cells exposed to cytochalasin D (*d*), a striking stellate keratin fluorescence is observed. This pattern displays the formation of keratin bundles and cell spreading characteristic of tumor promoters, but the signature is clearly different from that induced by TPA alone. (×200.)

Fig. 3 demonstrates that the morphological changes induced by tumor-promoting agents occur with no change in rate or extent in the absence of both protein and RNA synthesis. Two generally accepted markers for tumor-promoting activity *in vitro* are the induction of plasminogen activator and prostaglandins. Previous studies have shown that both

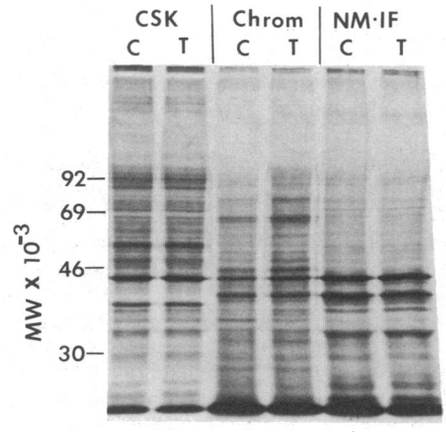

FIG. 6. Polyacrylamide gel analysis of proteins obtained after fractionation of MDCK colonies. MDCK colonies were prelabeled with [^{35}S]methionine (15 μCi/ml) for 24 hr. Cells were washed with unlabeled medium and incubated in 0.1% dimethyl sulfoxide (lanes C) or 5 ng of TPA per ml in dimethyl sulfoxide (lanes T) for 2 hr. The cells were fractionated as described into cytoskeleton (CSK), NM-IF, and chromatin (Chrom) fractions and compared on a 10% NaDodSO$_4$/polyacrylamide gel. The patterns of proteins obtained in each fraction are virtually unchanged by TPA treatment. The results of this experiment suggest that the morphological alterations observed in response to TPA are not due to a breakdown of structural elements.

of these effects require continued protein and RNA synthesis (6, 26). The results presented here suggest that the morphological changes resulting from exposure to tumor promoters are independent of either plasminogen activator release or prostaglandin stimulation. These results are in contrast to studies on fibroblasts that indicate that dispersal of actin cables by TPA requires RNA and protein synthesis. The relevance of the fibroblast results to those reported here is not clear because actin bundling is a very different manifestation of skeletal organization from cytokeratin architecture. A third biochemical marker normally associated with tumor promotion is the release of fibronectin from cell surfaces. Since this protein is either absent or present only in minute amounts in both normal and transformed epithelial cells (27), the relevance of this marker to promotion in this system is unclear. Ornithine decarboxylase is another biochemical marker for tumor promotion, although its significance is questionable (28, 29). Ornithine decarboxylase induction by tumor promoters is inhibited by colchicine (30), a compound that does not block the morphological alterations induced by tumor promoters. Whereas it is possible to separate tumor promotion from mitogenesis in selected cell systems (31), both tumor promoters and mitogens have been shown to elicit similar results in a number of systems (20, 32, 33). However, epidermal growth factor (Fig. 4*c*) and other growth hormones (unpublished data) cause no morphological alterations. These agents are distinguishable from tumor promoters in their effects on epithelial cytoarchitecture.

We propose that the cytokeratin intermediate filament system is a likely target for tumor promoter activity on cytoskeletons. This is suggested by the experiments shown in Fig. 5.

Depolymerization of microtubules has no effect on MDCK colony morphology or their subsequent response to TPA. Cytochalasin D does have a pronounced effect on cytokeratin organization. The bundling of cytokeratins and the extensive cell spreading, characteristic of tumor promoters, still occur in response to TPA. This suggests that at least part of the cytokeratin reorganization is independent of microfilament function.

These results apparently contradict a previous report (14) in which a significant reduction in the TPA effect in the presence of colchicine and cytochalasin was observed. The source of this discrepancy is unknown, but it may be significant that confluent cell sheets were used in the earlier study (14). We have found that confluent cell sheets are significantly less responsive to promoting agents than are the subconfluent colonies used in this study.

We observed (Fig. 6) that the distribution of proteins in the structural compartments is largely unaltered by tumor-promoting agents. This result suggests that the action of tumor promoters does not involve a breakdown of structural elements but rather a spatial reorganization of cellular elements whose connectedness remains unchanged.

Further insight into the mechanisms of chemical carcinogenesis may be afforded by the observation (to be reported elsewhere) that the active benzo[*a*]pyrene diol epoxide at nanogram levels induces a morphological signature very similar to that induced by tumor promoters. The result of this experiment suggests that such ultimate carcinogens have a promoting activity in addition to their genotoxic action and this may be essential in their mode of action.

The results presented in this study suggest that tumor promoters may all act by a common mechanism involving the reorganization of the NM-IF network. These observations provide a rapid and reliable assay for tumor promoters in an epithelial system and begin to establish a basis from which the mechanism of tumor-promoter action on cytoskeletal elements may be more completely understood.

We are grateful to Gabriela Krochmalnic for expert assistance in electron microscopy and Patricia Turner for preparing the manuscript. This work was supported by National Institutes of Health Grant CA08416 and National Science Foundation Grants 8004696-PCM and 8309334-PCM.

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