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# Tumor Promoters Induce Changes in the Chick Embryo Fibroblast Cytoskeleton

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## Summary

We have examined the effect of the tumor promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA), on the actin-containing elements of the cytoskeleton of chick embryo fibroblasts (CEF). TPA at concentrations as low as  $7.3 \times 10^{-10}$  M induces a reversible change in the cytoskeleton as visualized by indirect immunofluorescence using anti-actin antibodies. Cells incubated with TPA lose the ordered actin-containing structures found in normal cells and resemble Rous sarcoma virus-transformed cells in that the immunofluorescent actin pattern is diffuse. The TPA effects are both dose- and time-dependent. Analogs of TPA which are inactive as tumor promoters do not induce cytoskeletal changes at the concentrations tested, while a second tumor promoter, PDD, is also able to cause alterations in actin-containing structures. The action of TPA requires *de novo* synthesis of both RNA and protein. The direct cytoskeletal changes are neither plasmin-dependent nor subject to inhibition by incubating the cells with high levels of protease inhibitors during the exposure to TPA. However, plasminogen does increase the sensitivity of cells to TPA.

## Introduction

Tumor promoters are molecules which, while not carcinogenic themselves, increase the incidence of tumors in animals pretreated with certain carcinogens (van Duuren, 1969; Boutwell, 1974; Hecker, 1975). This potentiating effect was first described by Rous and Kidd (1938) and later analyzed experimentally by Berenblum (1975), whose studies led to the proposal of the two stage model of carcinogenesis. In this model, the application of the initiator or carcinogen to a tissue is believed to result in the modification of target cells and their descendents. If low doses of carcinogen are used, this treatment is insufficient to cause the formation of malignant tumors. Subsequent application of promoting agents to the carcinogen-treated tissue, however, results in the development of tumors in the treated area.

The most effective tumor promoters known are the phorbol esters. These compounds are macrocyclic

diterpene esters extracted from the seeds of *Croton tiglium* and are the active agents in croton oil (Hecker, 1968), the material used by Berenblum in his studies on tumor promoters. It has recently been shown that the tumor-promoting phorbol esters have significant biological effects on cells in culture. These effects include biochemical changes such as the induction of ornithine decarboxylase (Yuspa et al., 1976), increases in 5' nucleotidase and sodium-potassium-ATPase levels (Sivak, Mossman and van Duuren, 1972), changes in lipid synthesis (Suss, Kreibich and Kinzel, 1972; Ohuchi and Levine, 1978; Brume et al., 1978), alternations in cell morphology (Diamond et al., 1974) and increases in cell growth (Sivak, 1972; Diamond et al., 1974). Moreover, the potent tumor promoter 12-O-tetradecanoyl phorbol-13-acetate, or TPA, is capable of causing the loss of fibronectin from the cell surface (Blumberg, Driedger and Rossow, 1976; Driedger and Blumberg, 1977), increasing the rate of sugar transport (Driedger and Blumberg, 1977), inducing the production of plasminogen activator (Wigler and Weinstein, 1976; Wigler, De Fes and Weinstein, 1978) and inhibiting both terminal differentiation and the synthesis of specific macromolecules associated with the differentiated phenotype in a variety of cells (Diamond, O'Brien and Rovera, 1977; Rovera, O'Brien and Diamond, 1977; Cohen et al., 1977; Yamasaki et al., 1977; Lowe, Pacifici and Holtzer, 1978). These changes also occur upon *in vitro* transformation of many cell types (Easton and Reich, 1972; Weber, 1973; Unkles et al., 1973; Fiszman and Fuchs, 1975; Hynes and Wyke, 1975; Levinson, Bhatnager and Liu, 1975; Holtzer et al., 1975; Abrogast et al., 1977; Boettiger et al., 1977; Muto et al., 1977; Okayama et al., 1977; Pacifici et al., 1977; Schwarz et al., 1978), and they may be related to the *in vivo* activity of tumor promoters.

One of the phenotypic changes which correlates well with the transformation of many cell types is the loss of ordered cytoplasmic actin and myosin (Pollack, Osborn and Weber, 1975; Edelman and Yahara, 1977; Tucker, Sanford and Frankel, 1978). Numerous reports document, by means of immunofluorescent techniques, changes in the state of these two proteins in cells transformed by a variety of agents. In general, it appears that the large bundles or cables of actin and myosin observed in normal cells are replaced in transformed cells by a diffuse intracellular fluorescence with little change in the total amount of either protein. Since TPA has the ability to induce a condition in cultured cells which mimics the transformed phenotype, we have used the technique of indirect immunofluorescence to examine the ability of TPA to induce a modification of the intracellular distribution of actin in normal chick embryo fibroblasts. Moreover, since we have previously shown that some of the

cytoskeletal changes observed in transformed fibroblasts may be the result of the production of plasminogen activator (Pollack and Rifkin, 1975), a protease produced in cell cultures exposed to TPA, we have correlated the TPA-mediated induction of plasminogen activator with changes in the cytoskeleton.

## Results

### Effect of TPA on Cytoskeletal Actin Pattern

The exposure of CEF to TPA in the range  $1.4\text{--}7.3 \times 10^{-9}$  M for 24–48 hr causes an alteration in morphology similar to that described by others (Driedger and Blumberg, 1977). The cells lose their normal orientation and display numerous overlaps, and many cells become long and thin and acquire many processes. A small increase in the growth rate takes place in cultures exposed to TPA, as reported by Driedger and Blumberg (1977) (data not shown).

When the actin in CEF exposed to TPA is examined by immunofluorescence, changes in the organization of the cytoskeletal elements are apparent (Figure 1). These changes are essentially identical to those observed upon Rous sarcoma virus transformation of CEF (Edelman and Yahara, 1977; D. Rifkin and R. Pollack, unpublished observations). The thickness and length of the anti-actin-decorated cables is reduced in the treated cells. In some cells this results in the replacement of cables by a diffuse fluorescence throughout the cytoplasm. It should be noted that when scoring cultures for the degree of cytoskeletal modification induced by TPA or other drugs, we have been careful to count only those cells which remained well spread and attached to the surface of the petri dish. Cells that were elongated were not scored, since the lack of a well defined cytoskeleton routinely observed in these cells may have been due to an artifact resulting from loss of available packing volume for actin filaments and/or increases in the depth of the cytoplasm.

### Relationship of Plasmin to TPA-Induced Changes in Cytoskeleton

A number of experiments with cells which produce PA have demonstrated that plasmin formed by the activation of plasminogen, present in the serum used in the culture medium, may have profound effects on a variety of properties associated with the transformed phenotype (Ossowski et al., 1973; Pollack et al., 1974; Pollack and Rifkin, 1975; Ossowski, Quigley and Reich, 1975; Whur et al., 1975). To determine whether TPA-induced cytoskeletal changes might result from plasmin formed by the TPA-induced PA, we tested the effect of TPA on the cytoskeletons of cells grown in the absence of plasminogen. The results (Table 1) indicate that cytoskeletal changes occur over a range of TPA concentrations, even in the absence of plasminogen.

Plasmin is capable, however, of enhancing the effect of TPA (Table 1). Small amounts of plasminogen added to the culture medium cause a significant increase in the loss of actin cables beyond that observed in the absence of plasminogen. The amount of plasmin required (Table 1) to affect the cytoskeleton is quite small, amounting to less than 5% of the plasminogen normally present in culture medium which contains 10% fetal bovine serum. We therefore conclude that the observed effects are not plasmin-dependent but can be enhanced by plasmin. All subsequent experiments were carried out in the absence of plasminogen.

### Characterization of the TPA Effect on Actin-Containing Elements of the Cytoskeleton

As shown in Table 2, the changes in actin pattern in CEF exposed to TPA show a typical dose versus time response. The effects of lower doses of tumor promoter take longer to be expressed and are not as extensive as those observed with the higher doses of TPA. Concentrations of TPA as high as  $1.9 \times 10^{-7}$  M also cause changes in the cytoskeleton, but the drug appears to be toxic at these concentrations, as determined by trypan blue exclusion and cell number (data not shown).

Doses of TPA which result in cytoskeletal changes induce the production of PA in CEF (Table 2). It should be noted that, at the lowest concentration of promoter tested ( $1.4 \times 10^{-9}$  M), a significant decrease in the percentage of cells with well defined actin-containing cytoskeletons was observed by 16 hr, while there was only a small increase in the level of PA. It should be noted that the effect of TPA ( $1.4 \times 10^{-9}$  M) on the cytoskeleton is more pronounced in this experiment than the effect observed at a similar concentration (Table 1). This variability may be related to subtle differences in the state of the cells at the time of exposure to TPA. In general, however, the responses of the cells to TPA in terms of PA production and cytoskeletal changes are similar. This parallelism is quite striking if one examines the 8 hr time points for  $3.6$  and  $7.6 \times 10^{-8}$  M TPA. At the higher drug concentration there is a diminution of the effect of TPA on the cytoskeleton. This decrease in the effectiveness of the TPA is also noted in the PA levels. The effect of high doses of tumor promoter on PA synthesis has been observed by other investigators but is not understood (Weinstein, Wigler and Pietropaolo, 1977; Goldfarb and Quigley, 1978). The increase in PA appears to occur more rapidly than the changes in the cytoskeleton, but this may be due to differences in the assays for the two phenomena.

The effect of TPA on the cytoskeleton is reversible although the time required for reversal is long (data not shown). Cells which have been exposed to  $1.4 \times 10^{-8}$  M TPA require up to 72 hr for the effect of the drug to be reversed, even with medium changes every

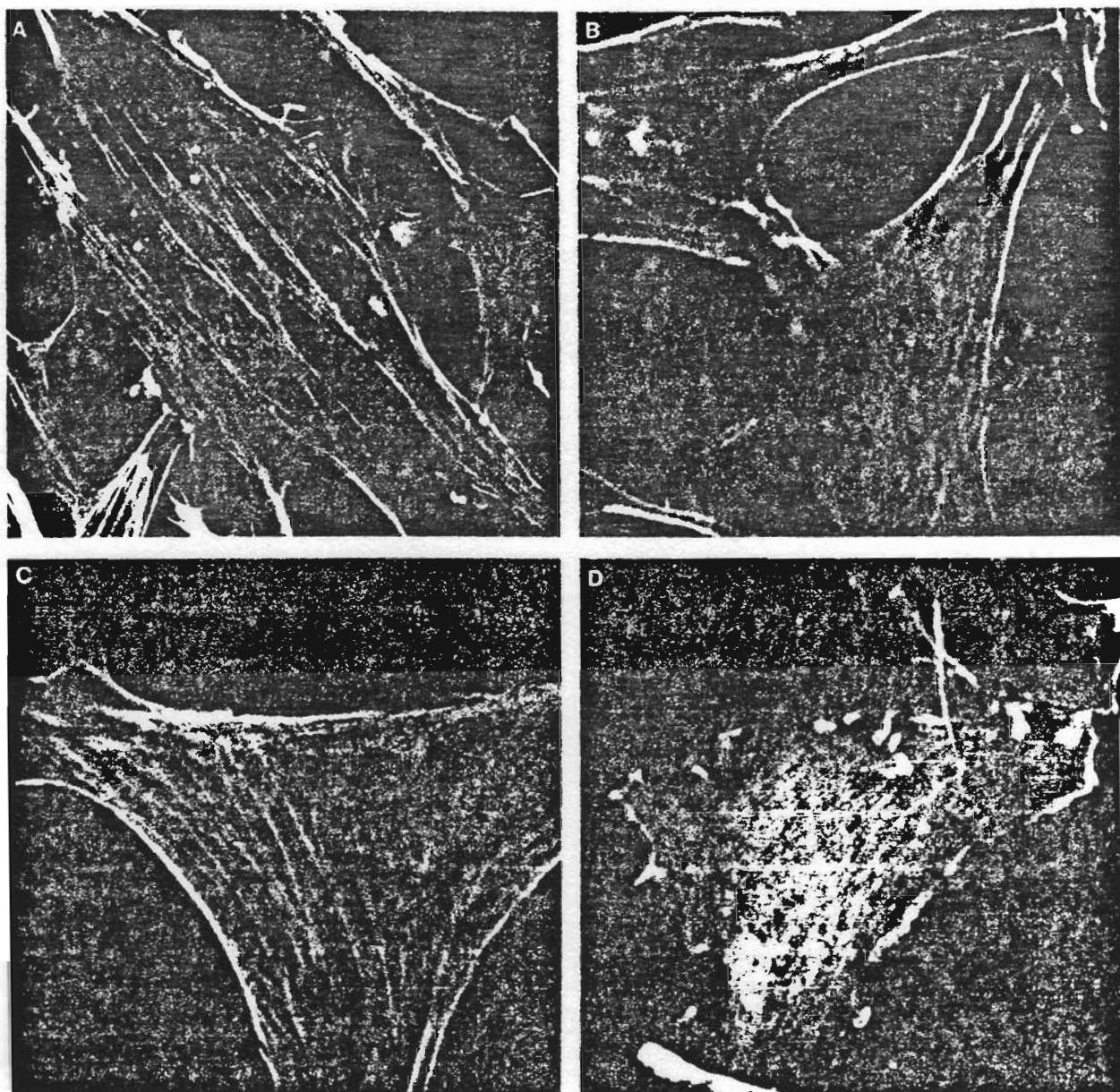


Figure 1. Actin Patterns in Tertiary Chick Fibroblasts

After 2 days in 1% fetal bovine serum depleted of plasminogen, coverslip cultures ( $2 \times 10^5$  cells per 60 mm dish) were incubated in growth medium for 16 hr at 37°C in the presence of TPA and/or plasminogen as indicated. Coverslips were fixed and stained with anti-actin antibody, and patterns were visualized as described in Experimental Procedures.

(A) No additions; (B)  $1.4 \times 10^{-6}$  M TPA; (C) 20  $\mu$ g/ml plasminogen; (D) 20  $\mu$ g/ml plasminogen and  $1.4 \times 10^{-6}$  M TPA. Bar = 10  $\mu$ .

12 hr. Thus the drug does not induce permanent changes in the cytoskeleton.

We have also tested the ability of analogs of TPA, including active and inactive tumor promoters, to cause alterations in the cytoskeleton (Hecker, 1978). PDD, another potent tumor promoter, effectively induced changes in actin organization (Table 3). 4-O-Me TPA, which is a very poor tumor promoter in vivo, produced only a small change in anti-actin-decorated structures. 4 $\alpha$ -PDD, which is completely inactive as

a tumor promoter, induced no detectable cytoskeletal changes. The levels of PA induced by these compounds are also shown in the table. The parallelism observed earlier (Table 2) between cytoskeletal changes and PA induction was maintained.

We have attempted to determine whether the TPA-induced cytoskeletal alteration requires the synthesis of RNA or protein. The results of experiments in which either actinomycin D or cycloheximide was added to cultures together with TPA (Table 4) indicate that both

Table 1. Effect of TPA on Actin Structures in Chick Embryo Fibroblasts

Additions		Cells Containing Ordered Actin Bundles (%)
TPA	Plasminogen	
None	None	92
$7.3 \times 10^{-10}$ M	None	79
$1.4 \times 10^{-9}$ M	None	67
$1.4 \times 10^{-8}$ M	None	15
$7.3 \times 10^{-8}$ M	None	15
$1.4 \times 10^{-9}$ M	0.4 $\mu$ g/ml	34
$1.4 \times 10^{-9}$ M	4 $\mu$ g/ml	17
$1.4 \times 10^{-9}$ M	20 $\mu$ g/ml	1

Chick embryo fibroblasts were grown in medium containing 1% fetal bovine serum which was depleted of plasminogen. The cells were exposed to the indicated additions for 16 hr, fixed and stained for actin as described in Experimental Procedures.

Table 2. Effect of Dose and Time on TPA-Induced Changes in Actin-Containing Elements of the Cytoskeleton

Additions	Cells Containing Ordered Actin Bundles (%)	
	After 8 Hr	After 16 Hr
None	76 (5)	80 (3)
TPA ( $1.4 \times 10^{-9}$ M)	65 (14)	9 (10)
TPA ( $7.3 \times 10^{-9}$ M)	39 (73)	7 (87)
TPA ( $1.4 \times 10^{-8}$ M)	10 (70)	8 (87)
TPA ( $3.6 \times 10^{-8}$ M)	7 (61)	13 (79)
TPA ( $7.3 \times 10^{-8}$ M)	22 (46)	15 (70)

Chick embryo fibroblasts were grown on coverslips in medium containing 1% fetal bovine serum depleted of plasminogen. The cells were exposed to the indicated drugs for either 8 or 16 hr, and fixed and stained for actin as described in Experimental Procedures. The cells remaining on the petri dish were then assayed for total plasminogen activator. The results, given as the percentage of radioactive fibrin removed from the surface of an  $^{125}$ I-fibrin-coated well, are indicated by the numbers in parentheses.

DNA-dependent RNA synthesis and protein synthesis are required for the modification of the actin cables, since the inhibition of either process protects against TPA-induced cytoskeletal changes. These results distinguish the cytoskeletal modifications from several other early effects of TPA, such as changes in phospholipid metabolism (Kinzel et al., 1979), which do not require the synthesis of protein and/or RNA.

Several of the effects of tumor promoters both in vivo and in vitro can be prevented by protease inhibitors (Troll, Klassen and Janoff, 1979; Hozumi et al., 1972; Belman and Troll, 1974; Kinsella and Radman, 1978; Nagasawa and Little, 1979). To determine whether the effect of TPA on the cytoskeleton could be inhibited by these agents, cells were exposed to high levels of a variety of protease inhibitors before

Table 3. Effects of Derivatives of TPA on Actin-Containing Structures

Treatment	Cells Containing Ordered Actin Bundles (%)	Plasminogen Activator (%)
None	86	0
TPA ( $7.3 \times 10^{-9}$ M)	39	2
( $7.3 \times 10^{-8}$ M)	21	39
PDD ( $5.4 \times 10^{-9}$ M)	6	51
( $5.4 \times 10^{-8}$ M)	15	45
4 $\alpha$ -PDD ( $5.4 \times 10^{-9}$ M)	79	1
( $5.4 \times 10^{-8}$ M)	85	0
4-O-Me TPA ( $7.2 \times 10^{-9}$ M)	76	4
( $7.2 \times 10^{-8}$ M)	61	2

Chick embryo fibroblasts were grown on coverslips in medium containing 1% fetal bovine serum depleted of plasminogen. The cells were exposed to the indicated drugs for 16 hr, and fixed and stained for actin as described in Experimental Procedures. The cells remaining on the petri dish were then assayed for plasminogen activator. Plasminogen activator activity is expressed as the percentage of  $^{125}$ I solubilized by the sample compared to the total radioactivity removed by trypsin.

Table 4. Requirements for Macromolecular Synthesis

Treatment	TPA	Cells Containing Ordered Actin Bundles (%)
None	0	79
None	$3.6 \times 10^{-8}$ M	14
Actinomycin D (1 $\mu$ g/ml)	0	74
Actinomycin D (1 $\mu$ g/ml)	$3.6 \times 10^{-8}$ M	81
Cycloheximide (5 $\mu$ g/ml)	0	ND
Cycloheximide (5 $\mu$ g/ml)	$3.6 \times 10^{-8}$ M	85

Chick embryo fibroblasts grown in medium containing 1% fetal bovine serum depleted of plasminogen were exposed to drugs as indicated, and 16 hr later the cells were fixed and stained for immunofluorescence as described in Experimental Procedures. ND = not done.

and during exposure to TPA. The inhibitors used are active against a wide spectrum of proteases including those of the active serine, sulfhydryl and carboxyl classes of proteases. The data presented in Table 5 indicate that the inclusion of protease inhibitors in the culture medium has no effect upon the direct TPA-induced alteration of the cytoskeleton. However, those inhibitors known to be active against plasmin, such as leupeptin, soybean trypsin inhibitor and benzamide, are capable of inhibiting the plasmin-dependent enhancement of the cytoskeletal changes (data not shown). Thus these inhibitors were active against extracellular serine proteases (such as plasmin) under the conditions tested. These results strongly suggest that, in the absence of plasminogen, the direct effect of TPA on the cytoskeleton is not caused by traces of plasminogen, and that this action of TPA on the cy-

Table 5 Effects of Protease Inhibitors on TPA-Induced Cytoskeletal Changes

Treatment	Protease Inhibitor	Cells Containing Ordered Actin Bundles (%)
None	None	79
TPA ( $3.6 \times 10^{-8}$ M)	None	12
TPA ( $3.6 \times 10^{-8}$ M)	Benzamidine (2 mM)	8
TPA ( $3.6 \times 10^{-8}$ M)	Leupeptin (300 $\mu$ g/ml)	11
TPA ( $3.6 \times 10^{-8}$ M)	Antipain (100 $\mu$ g/ml)	13
TPA ( $3.6 \times 10^{-8}$ M)	E-amino caproic acid (2 mM)	15
TPA ( $3.6 \times 10^{-8}$ M)	Nitrophenyl guanidinobenzoate (10 mM)	12
TPA ( $3.6 \times 10^{-8}$ M)	Pepstatin (100 $\mu$ g/ml)	15
TPA ( $3.6 \times 10^{-8}$ M)	Benzamidine (2 mM) + leupeptin (300 $\mu$ g/ml) + antipain (100 $\mu$ g/ml) + soybean trypsin inhibitor (200 $\mu$ g/ml)	12
None	Benzamidine (2 mM)	73
None	Leupeptin (300 $\mu$ g/ml)	74
None	Antipain (100 $\mu$ g/ml)	73
None	E-amino caproic acid (2 mM)	82
None	Nitrophenyl guanidinobenzoate (10 mM)	78
None	Pepstatin (100 $\mu$ g/ml)	76

Chick embryo fibroblasts were grown for 24 hr on coverslips in Eagle's medium containing 1% fetal bovine serum depleted of plasminogen. The cells were then exposed to the indicated compounds for 16 hr, and fixed and stained for actin as described in Experimental Procedures.

toskeleton is not mediated through an extracellular protease.

### Discussion

In this paper we have demonstrated that tumor promoters such as TPA are capable of causing reversible changes in the pattern of cytoplasmic actin in CEF. We have also found similar effects on the pattern of myosin in CEF exposed to TPA (D. Rifkin and R. Pollack, unpublished observations). The concentration of tumor promoter required to induce such an alteration is related to its efficacy of tumor promotion: the highly active promoters induce cytoskeletal changes at low concentrations, while the inactive or poorly active promoters do not alter the cytoskeleton at the same concentrations. Both DNA-dependent RNA synthesis and protein synthesis are required for the TPA-induced changes to occur. The effect of TPA on the cytoskeleton is not plasminogen-dependent, although in the presence of plasminogen there is an enhancement of the effect of the drug. The plasmin-

ogen-independent change is not prevented by high concentrations of several protease inhibitors, suggesting that the alterations in actin organization are not mediated by an extracellular protease.

This result was unexpected, since extracellular proteases have been shown to be capable of affecting the cytoskeleton (Pollack and Rifkin, 1975) and most of the TPA-induced changes in the cytoskeleton are mirrored in the level of plasminogen activator produced under the same conditions. These include the dose-response curve, the activities of active and inactive analogs of TPA, and the requirements for both RNA and protein synthesis. However, we have been unable to demonstrate a direct relationship between PA production and cytoskeletal changes since the TPA-induced alterations in actin pattern were not prevented by the inclusion of PA inhibitors in the growth medium. In this regard, it should be noted that we have selected conditions such that a significant effect of TPA on the cytoskeleton is observed in a relatively short time. It may be that under these conditions the amount of protease inhibitor present is insufficient to prevent the observed change. Our own data (Table 2) indicate that large changes in cytoskeletal organization may occur with only small increments in PA.

Lawrence et al. (1979) recently described an FSH-induced change in the shape of granulosa cells which was accompanied by the induction of PA. The change in cell morphology clearly preceded the induction of this protease. After several attempts we have been unable to show an alteration in the cytoskeleton preceding PA production. Thus we cannot unambiguously rule out a role for PA in this process of cytoskeletal reorganization.

Quigley (1979) has recently presented evidence for a direct effect of plasminogen activator on the morphology of transformed chick embryo fibroblasts. These data agree with our finding of a lack of a requirement for plasminogen in TPA-induced changes in the cytoskeleton.

It is not clear how tumor promoters induce the observed changes in actin and myosin patterns. The effect is obviously not direct, since both RNA and protein synthesis are required. Moreover, unlike in vivo tumor promotion, the effect of tumor promoters on the cytoskeleton is not prevented or even diminished by rather high concentrations of protease inhibitors. These data indicate that the observed effect requires the synthesis of new protein(s) but not a protease whose activity is limited to the extracellular space. While we do not know what this protein(s) might be, a report by McClain, Maness and Edelman (1978), demonstrating that extracts of RSV-transformed CEF are capable of causing a change in the normal intracellular pattern of actin when microinjected into CEF, provides a method for identifying the factor or factors responsible for cytoskeletal changes in TPA-treated cells.

The observation that TPA induces alterations in the cytoskeleton similar to those found in virally transformed cells is another demonstration that TPA is capable of producing changes associated with the transformed phenotype in normal cells. Some of the other observed phenotypic alterations induced by TPA in chicken embryo fibroblasts include stimulation of sugar transport (Driedger and Blumberg, 1977), increased growth (Driedger and Blumberg, 1977), morphological alterations (Driedger and Blumberg, 1977), loss of LETS protein (Blumberg et al., 1976; Driedger and Blumberg, 1977) and increased synthesis of plasminogen activator (Wigler and Weinstein, 1976; Goldfarb and Quigley, 1978; Wigler et al., 1978)—all of which are properties associated with *in vitro* transformation of chick embryo fibroblasts.

TPA does not, however, create a precise copy of the transformed phenotype. Certain properties such as growth without anchorage are not induced by the phorbol esters (Weinstein et al., 1977), while other properties, which are induced in chick cells, are similar but not identical to those in their transformed counterparts. For example, the TPA-induced cell volume changes described by Driedger and Blumberg (1977) are not nearly as extensive as those observed in RSV-infected cells. Similarly, the morphological effects of TPA on CEF are distinct from those observed in most virally transformed cultures (Driedger and Blumberg, 1977; D. Rifkin and R. Pollack, unpublished observations). The loss of LETS protein from TPA-treated cells occurs with kinetics much slower than those observed for ts-RSV-CEF cells in shifts from nonpermissive to permissive temperatures. Under these conditions LETS loss occurs in one day, while the TPA-induced loss requires 3 days. Even the induction of PA in CEF by TPA may not occur by the same mechanism as in virally transformed cells. Several investigators have shown that the effects of transformation and TPA on PA levels in CEF are not equivalent (Weinstein et al., 1977; Goldfarb and Quigley, 1978). This may indicate that the two agents act through similar but nonidentical pathways, each of which is capable of stimulating PA production to an intermediate level and both of which, in concert, yield PA levels higher than either state alone.

Recent data on the nature of the src gene product in RSV-transformed CEF suggest a possible explanation for these effects. Collett and Erickson (1978) have presented strong evidence that the src gene product in these cells is a specific protein kinase. Untransformed CEF have a host protein (endogenous sarc) which is apparently encoded by a sarc-like DNA sequence similar to that of viral src (Collett, Brugge and Erikson, 1978; Oppermann et al., 1979). Endogenous sarc is also a protein kinase (Oppermann et al., 1979). TPA and other phorbol esters may induce a related kinase which may produce changes similar but not identical to those observed with transforming

viruses. A more detailed analysis of both the properties of transformed and TPA-treated cells and the level of cellular protein kinases under different conditions may be useful in testing this hypothesis.

#### Experimental Procedures

##### Cells

Chick embryo fibroblasts were prepared from COFAL negative eggs as described previously (Rifkin and Reich, 1971). All experiments were performed on third to fifth passage cells. Cells were grown in Eagle's minimum essential medium containing 10% fetal bovine serum. To initiate an experiment, cells were trypsinized and plated at a density of approximately  $2 \times 10^5$  cells per 60 mm dish. Each petri dish contained three or four sterile 12 mm round glass coverslips. The cells were allowed to attach overnight, and the next day the medium was removed and replaced with fresh Eagle's minimal essential medium containing 1% fetal bovine serum which had been depleted of plasminogen (Deutch and Mertz, 1979; Quigley, Ossowski and Reich, 1974). Plasminogen-depleted serum contained less than 0.05% of the original plasminogen. The cells were allowed to grow for an additional 2 days in this medium. After this incubation, the medium was removed and replaced with medium containing the drug to be tested at the appropriate concentration.

##### Fixation and Antibody Reaction

After an appropriate incubation, the coverslips were removed and the cells were fixed in 3.8% formaldehyde in PBS for 25 min. At the end of this period, the coverslips were immersed in 1% NP40 in PBS for 25 min to extract membranes. The coverslips were rinsed twice in PBS for 1 min. The individual coverslips were then placed on a piece of moist filter paper in a 15 cm petri dish and 8  $\mu$ l of anti-actin antibody (Burrige, 1976) were placed on each coverslip. The covered petri dish was then placed in a 37°C humidified incubator for 45 min. At the end of this period, the coverslips were washed by dipping individual coverslips three times into each of three breakers of PBS. The coverslips were blotted after each series of three washes. 10  $\mu$ l of rhodamine-conjugated goat anti-rabbit serum (Cappel Labs) were placed on each coverslip, and a second incubation at 37°C for 45 min was performed. At the end of this period, the coverslips were again washed by three successive immersions in each of three PBS solutions followed by blotting after each third immersion. The coverslips were placed cell-side down on a drop of Aquamount (Lerner Laboratories) on microscope slides, and the mounting agent was allowed to harden overnight.

##### Immunofluorescence

Coverslips were observed with a Leitz photomicroscope using epifluorescence and a 63X Zeiss oil immersion objective. 100 cells on each coverslip were scored as containing organized or disorganized cytoskeletons as described previously (Pollack and Rifkin, 1975). The observer did not know which treatment the cells had received until after all counts were made. Only those cells which remained flat and attached to the surface of the dish were counted. Round or elongated (because of exposure to TPA) cells were not counted. In general, the morphology and size of cells scored in experimental cultures were similar to those of control cells.

##### Plasminogen Activator Assays

Assays for PA were performed on the cells that remained on the petri dishes after the coverslips were removed. The cells were washed with cold PBS, scraped from the dish in cold PBS and pelleted, and the cell pellet was lysed in 0.5% Triton in 0.1 M Tris-HCl (pH 8.1). Assays for plasminogen activator levels in the cell lysates were carried out as described by Rifkin and Pollack (1977). No plasminogen-independent activity was detected.

##### Materials

TPA and TPA derivatives were purchased from Consolidated Midlands Co. Leupeptin, antipain and pepstatin were gifts from W. Troll and

were supplied by the US-Japan Cooperative Cancer Research Program. Anti-actin antibody was a gift from K. Burridge (Cold Spring Harbor). All other chemicals were purchased from Sigma.

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Note Added in Proof

After this paper was submitted for publication, it was brought to our attention that Bissell et al. [*Proc. Nat. Acad. Sci.* (1979) 76, 348-352] predicted that TPA would induce cytoskeletal changes in chick embryo fibroblasts.