Ornithine Decarboxylase Induction and DNA Synthesis in Hamster Embryo Cell Cultures Treated with Tumor-promoting Phorbol Diesters

Thomas G. O’Brien and Leila Diamond

The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

SUMMARY

The effects of tumor-promoting phorbol diesters on ornithine decarboxylase (ODC) activity and DNA synthesis in normal and chemically transformed hamster embryo fibroblasts (HEF) in culture were studied. Only those phorbol diesters with promoting activity in mouse skin induced ODC in HEF. ODC was induced in both cell types by 12-O-tetradecanoyl-phorbol-13-acetate (TPA); maximal induction occurred 4 to 6 hr after the addition of the promoter to the medium of confluent cultures and was greater in transformed cells than in normal cells. The extent of induction in transformed cells treated with 0.016 to 1.6 μM TPA was dose dependent. The cellular concentrations of the polyamines, particularly putrescine, also increased after TPA treatment.

The addition of TPA to confluent cultures of either normal or transformed HEF did not produce an increase in cell number or the percentage of [3H]thymidine-labeled nuclei and did not stimulate the incorporation of [3H]thymidine.

ODC was induced in both cell types by 12-0-tetradecanoyl-phorbol-13-acetate (TPA); maximal induction occurred 4 to 6 hr after the addition of the promoter to the medium of confluent cultures and was greater in transformed cells than in normal cells. The extent of induction in transformed cells treated with 0.016 to 1.6 μM TPA was dose dependent. The cellular concentrations of the polyamines, particularly putrescine, also increased after TPA treatment.

INTRODUCTION

In the 2-stage system of mouse skin carcinogenesis, the development of tumors after a single low dose of a carcinogen is dependent on subsequent treatment with frequent and multiple applications of a tumor promoter (6). The mechanism of promotion is unknown. The isolation of pure phorbol diesters with promoting activity from croton oil, the classic promoting substance first described by Berenblum (5), has stimulated research on the interactions of tumor promoters with various cells and tissues. In mouse epidermis, active promoters induce irritation, inflammation and hyperplasia, but the relationship of these properties to promotion is not understood (for reviews, see Refs. 7 and 26). At the molecular level, promoters stimulate macromolecular synthesis (3), enhance proteolytic activity (25), and interact with the adenylate cyclase system (4, 12).

It was reported recently that one of the earliest and largest responses of mouse epidermis to tumor promoters was the induction of ODC (EC 4.1.1.17) and subsequent accumulation of polyamines (18, 20), and it was proposed that this event is a specific and essential component of the 2-stage mechanism of skin tumor formation (18). It was also found that epidermal tumors produced by a 2-stage treatment protocol have permanently high levels of ODC in comparison to the very low levels in normal epidermis (18), which suggests that the control of ODC is altered during the progression of normal cells to malignancy during promotion.

Cell culture systems responsive to tumor promoters would be useful for studying the interactions of these compounds with biological systems at the cellular and molecular levels. Weinstein et al. (27) have reviewed the diverse effects that tumor promoters may have on cells in culture, including a 2-stage protocol for the malignant transformation of the mouse embryo fibroblast line C3H/10T1/2, in which carcinogenic hydrocarbons or UV are "initiators" and phorbol diesters are "promoters" (16, 17).

To extend our studies on the role of polyamines in carcinogenesis, we have examined the effects of tumor promoters on the induction of ODC and DNA synthesis in cultures of normal and transformed HEF. We have also studied the regulation of polyamine biosynthesis in these cells. If regulation of this metabolic pathway is involved in the control of cell growth, as has been suggested by several authors (1, 22), then an aberration(s) in control of polyamine biosynthesis may contribute to the alteration in growth control of malignant cells both in vivo and in culture.

MATERIALS AND METHODS

Cells. Primary hamster embryo cell cultures were prepared from 13-day-old Syrian hamster embryos (Lakeview...
Farms, Newfield, N. J.) as described (11). The cells were
grown in plastic flasks or Petri dishes in Eagle’s minimal
essential medium (Autopow, Flow Laboratories, Inc., Rock-
ville, Md.) containing an additional mixture of vitamins as
formulated for Eagle’s basal medium and 10% fetal bovine
serum (Reheis Chemical Co., Chicago, Ill., or Flow Labora-
tories, Inc.). Cells were routinely subcultured at a 1:4 ratio
twice weekly by trypsinization with 0.025% trypsin:0.02%
EDTA. Cells at the 2nd or 3rd passage level were used in
the experiments described.

The HE68BP cell line, developed in this laboratory by
treating secondary HEF with benzo(a)pyrene, was used at
passes 22 to 40. The cells produce tumors within 2
weeks after s.c. inoculation of 10⁸ cells into newborn
hamsters.

Solutions of TPA and other phorbol diesters to be added
to cell cultures were freshly prepared as follows. Stock
solutions (160 μM; 100 μg/ml) in acetone (kept at —20° in
the dark) were diluted with either serum-free medium or
medium “conditioned” by cells plated at the same time
and handled in the same manner as were the cultures to be
treated. A small volume of medium (usually 2 to 6% of the
volume already present) was then added directly to the
dishes without a change of medium.

Preparation of Cell Extracts. To harvest cells for ODC
determination, the cell monolayers were quickly washed 3
times in situ with cold phosphate-buffered saline (2.7 mM
KCl:1.5 mM KH₂PO₄;136 mM NaCl:806 mM Na₂HPO₄) and
then rapidly frozen in dry ice:ethanol. While the cells were
still frozen, a small amount of buffer (200 to 500 μl 50 mM
sodium phosphate, pH 7.2, containing 5 mM dithiothreitol
and 0.1 mM EDTA) was added to each dish, and the cells
were harvested by scraping the dish with a Teflon police-
man. Two additional cycles of freezing and thawing ensured
complete lysis of the cells. After centrifugation at 20,000 ×
g for 20 min, the supernatants were assayed for ODC
activity.

Determination of ODC Activity. Fifty- to 100-μl volumes
of cell extract were assayed for ODC activity in a final
volume of 200 μl in 15-mI Corex (Corning Glass Co.,
Corning, N. Y.) centrifuge tubes equipped with rubber-
stopped center well assemblies (Kontes Glass Co., Vine-
land, N. J.). The final concentrations of reagents in the
incubation mixture were: 50 mM sodium phosphate, pH 7.2;
200 μM pyridoxal phosphate; 1 mM EDTA; 5 mM
dithiothreitol; and 1 mM L-ornithine. After the cell extract
was preincubated for 5 to 10 min at 37°, approximately 0.5
μCi DL-[¹⁴C]ornithine (New England Nuclear, Boston,
Mass.; specific activity, 40 to 60 mCi/mmole) was added to
start the assay. After 60 min, 0.5 ml 2 M citric acid was
injected through the rubber stopper, and the released ¹⁴CO₂
was trapped in 0.2 ml ethanolamine:methoxyethanol (2:1)
contained in the center well. The tubes were shaken for
at least 2 hr, and usually overnight, to ensure complete liber-
ation of ¹⁴CO₂. The contents of the center wells were then
released into scintillation vials (Minivials, Amershamsarle
Corp., Arlington Heights, Ill.), containing 4 ml Triton X-100-
based scintillant (TT-21; Yorktown Research, Hackensack,
N. J.) and 0.5 ml absolute ethanol. Enzyme activities are
expressed as nmoles CO₂ liberated in 60 min per mg
protein. All values are corrected for blanks containing
buffer instead of cell extract. Enzyme activity was linear for
at least 60 min of incubation and was proportional to the
amount of extract added in the range of 50 to 300 μg
protein. Protein was determined by the procedure of Ross
and Schatz (21).

Polyamine Analysis. The fluorometric method of Seiler
and Weichmann (23) was used to estimate the cellular
polyamine concentrations. Cell pellets were sonically dis-
rupted in 400 μl cold 0.2 M HCIO₄ with a Kontes cell
disintegrator equipped with a 4.5-inch probe. The suspen-
sions were centrifuged at 1000 × g for 10 min at 2°, and
200-μl volumes of the supernatants were analyzed by deri-
vatization with 5-(dimethylamino)-1-naphthalenesulfonyl
chloride (dansyl chloride) as described by Heby et al. (13).
After separation of the dansyl polyamines by thin-layer chro-
matography on 0.25-mm Silica Gel G plates (Analtech, Inc.,
Newark, Del.), each individual polyamine spot (correspond-
ing to cell extracts or known amounts of standards) was
scraped onto a centrifuge tube and eluted with 5 ml ben-
ze-ne:triethyamine (19:1). The silica gel was removed by
centrifugation, and the fluorescence intensity of the extract
was determined in a Perkin-Elmer MPF-4 spectrophotofluo-
rometer with an excitation wavelength of 365 nm and an
emission wavelength of 500 nm. The polyamine concentra-
tions were determined by comparing the fluorescence in-
tensity of cell extracts with standard curves obtained by
spotting known amounts (50 to 200 pmoles) of each polya-
mine on the thin-layer chromatography plates. The fluores-
cence of each polyamine was linear in the range of 10 to
200 pmoles and directly proportional to the number of
amino groups in the polyamine. Results are expressed as
nmoles polyamine per mg DNA [as measured by the diphen-
ylamine reaction (9)].

Incorporation of [³H]Thymidine. Cultures were labeled
for 60 min with [³H]thymidine (New England Nuclear; 2
μCi/ml, 1 1LM), and the incorporation of the precursor into
the acid-insoluble fraction was determined essentially as
described by Wiebel and Baserga (28). Aliquots of the final
HC1O₄ extract were counted by liquid scintillation, and the
amount of DNA was determined by the diphenylamine
reaction. Results for [³H]thymidine incorporation are ex-
pressed as dpm/mg DNA.

Labeling Index. Cells were seeded in 60-mm Petri dishes
containing two 9- x 22-mm coverslips. At various times after
seeding, [³H]thymidine was added to the dishes to give a
final concentration of 1 μCi/ml (0.5 1LM). After 60 min, the
coverslips were washed quickly 3 times in cold phosphate-
buffered saline, the cells were fixed in absolute methanol,
and they were processed for autoradiography according to
the procedure of Cristofalo and Sharf (10). At least 400
cells in random fields were scored to determine the per-
centage of cells with labeled nuclei (>10 grains/nucleus).

Chemicals. TPA and 4α-PDD were obtained from Consol-
disated Midland Corp., Brewster, N. Y. We gratefully ac-
knowledge the gift of PDD, PDB, and PDA from Dr. R. K.
Boutwell, University of Wisconsin, Madison, Wis.

Cycloheximide, actinomycin D, putrescine dihydrochlo-
ride, and spermidine trihydrochloride were purchased from
Calbiochem, San Diego, Calif. 5-(Dimethyl-amino)-1-nap-
thalenesulfonyl chloride (dansyl chloride) was obtained from
Pierce Chemical Co., Rockford, Ill., as an acetone

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solution. Spermine tetrahydrochloride was from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Effect of Tumor Promoters on ODC Activity. ODC activity is induced when cultures of normal HEF or HE68BP cells are subcultured or are refed with fresh medium (19). Therefore, for determination of the effects of tumor promoters on ODC activity and DNA synthesis, the compounds were added without any fresh medium to confluent cultures 4 to 5 days after cells were seeded. As will be seen, the cellular responses to promoters added under these conditions can be very different from the responses to these compounds added at the time of plating the cells or of changing the medium.

When the potent tumor promoter TPA was added to confluent cultures of low-passage HEF at a final concentration of 0.16 mM, ODC activity increased rapidly, reaching a peak value at 4 hr that was approximately 6 times greater than that of 0-hr control cultures (Chart 1). The basal levels of ODC were approximately the same in normal and transformed HE68BP cells, but TPA caused a much greater induction of ODC in the transformed cells, with a maximal increase at 6 hr that was 30-fold higher than the basal level (Chart 1). We studied 3 other malignantly transformed HEF cell lines and several different isolates of low-passage HEF with similar results: ODC activity after addition of TPA to the medium was always much greater in transformed than in normal HEF (data not shown).

Changes in cellular polyamine concentrations after TPA treatment were determined in transformed HEF. As shown in Table 1, TPA produced more than a 7-fold increase in the putrescine concentration at 9 hr after addition, and elevated putrescine levels persisted for at least 24 hr. The cellular concentrations of spermidine and spermine did not change appreciably after TPA treatment.

![Chart 1. The effect of TPA (0.16 μM) on ODC activity in normal and transformed HEF. Without changing the medium TPA was added to 5-day-old cultures of tertiary HEF or HE68BP cells, and the cells were harvested for enzyme determinations at the indicated times thereafter. Each point is the average enzyme activity in 2 dishes assayed separately. •, normal HEF; ■, HE68BP.](chart1.png)

Table 1

<table>
<thead>
<tr>
<th>Time (hr) after TPA addition</th>
<th>Polyamine concentration (nmoles/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Putrescine</td>
</tr>
<tr>
<td>Control</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>4</td>
<td>105 ± 3</td>
</tr>
<tr>
<td>6</td>
<td>157 ± 14</td>
</tr>
<tr>
<td>9</td>
<td>211 ± 31</td>
</tr>
<tr>
<td>16</td>
<td>157 ± 22</td>
</tr>
<tr>
<td>24</td>
<td>86 ± 10</td>
</tr>
</tbody>
</table>

Table 2

The induction of ODC by TPA

Without changing the medium TPA was added to 6-day-old cultures of HE68BP cells to give the final concentrations indicated. Four hr later, the cells were harvested for ODC determinations as described in "Materials and Methods." The control value is the mean ± S.E. of 3 dishes assayed separately, and the values for TPA-treated cultures are the average ± range of 2 dishes.

<table>
<thead>
<tr>
<th>TPA concentration (μM)</th>
<th>ODC specific activity (nmoles CO₂ in 60 mm/ mg protein)</th>
<th>Treated/control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.54 ± 0.15</td>
<td>27.0</td>
</tr>
<tr>
<td>1.6</td>
<td>14.5 ± 0.1</td>
<td>23.7</td>
</tr>
<tr>
<td>0.8</td>
<td>12.8 ± 0.1</td>
<td>16.6</td>
</tr>
<tr>
<td>0.16</td>
<td>8.96 ± 0.16</td>
<td>10.2</td>
</tr>
<tr>
<td>0.08</td>
<td>5.50 ± 0.37</td>
<td>2.7</td>
</tr>
<tr>
<td>0.016</td>
<td>1.44 ± 0.13</td>
<td>1.8</td>
</tr>
<tr>
<td>0.0016</td>
<td>1.00 ± 0.20</td>
<td></td>
</tr>
</tbody>
</table>

Within the phorbol diester series, TPA is the most potent tumor promoter in mouse skin; PDD is slightly less active, PDB is moderately active, and PDA and 4α-PDD are completely inactive (2, 14, 15). The induction of ODC by these phorbol diesters was studied in detail in the HE68BP cell line. Concentrations of TPA in the range of 0.016 to 0.16 μM increased ODC activity 3- to 27-fold over the basal level (Table 2). The increase in enzyme activity was completely inhibited by the simultaneous addition of actinomycin D (2.5 μg/ml) or cycloheximide (1.5 μg/ml) to cultures (Table 3), which indicates that new protein and RNA synthesis are required for increased enzyme activity.

The comparative ability of different phorbol diesters to induce ODC in HE68BP cells is shown in Table 4. The basal (control) level of ODC, especially in cultures of transformed cells, may vary from experiment to experiment, probably depending on the age of the culture, passage level of the cells, and various culture conditions. However, the changes in ODC activity after addition of the different phorbol diesters were consistently observed and the results of 2 representative experiments are shown. At a concentration of 0.16 μM, only TPA and PDD induced ODC activity. At a 10-fold higher concentration, PDB induced ODC 17-fold, but 4α-PDD and PDA had no effect.

The effects on ODC activity of 2 inducers, TPA and fresh medium, added either singly or in combination, were deter-
Four-day-old cultures of HE68BP cells were treated with TPA (final concentration, 0.16 μM) or TPA plus actinomycin D or cycloheximide. Six hr later, the cultures were harvested for ODC determinations as described in "Materials and Methods." Each value is the average ± the range of 2 dishes assayed separately.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ODC specific activity (nmoles CO₂ in 60 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.72 ± 0.12</td>
</tr>
<tr>
<td>TPA</td>
<td>12.0 ± 0.8</td>
</tr>
<tr>
<td>TPA + actinomycin D</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>(2.5 μg/ml)</td>
<td></td>
</tr>
<tr>
<td>TPA + cycloheximide</td>
<td>1.00 ± 0.19</td>
</tr>
<tr>
<td>(1.5 μg/ml)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3

Effect of actinomycin D and cycloheximide on the induction of ODC by TPA

In Experiment 1, 5-day-old cultures of HE68BP cells were treated with the phorbol diesters at a final concentration of 0.16 μM. In Experiment 2, the phorbol diesters were added at a final concentration of 0.16 or 1.6 μM. Control dishes were not treated. In both experiments, dishes were harvested 6 hr later; cell extracts were prepared and ODC activity was determined as described in "Materials and Methods." Values represent the mean enzyme activity ± the range of 2 dishes for each compound and concentration tested.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ODC specific activity (nmoles CO₂ in 60 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.81 ± 0.26</td>
</tr>
<tr>
<td>TPA</td>
<td>24.8 ± 2.2</td>
</tr>
<tr>
<td>4α-PDD</td>
<td>24.7 ± 1.5</td>
</tr>
<tr>
<td>0.16 μM</td>
<td></td>
</tr>
<tr>
<td>PDB</td>
<td>0.97 ± 0.57</td>
</tr>
<tr>
<td>0.16 μM</td>
<td>2.55 ± 0.46</td>
</tr>
<tr>
<td>PDA</td>
<td>2.29 ± 0.75</td>
</tr>
<tr>
<td>Control</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>TPA</td>
<td>4.88 ± 0.45</td>
</tr>
<tr>
<td>0.16 μM</td>
<td>3.42 ± 0.21</td>
</tr>
<tr>
<td>PDB</td>
<td>2.83 ± 0.20</td>
</tr>
<tr>
<td>0.16 μM</td>
<td>0.18 ± 0.14</td>
</tr>
<tr>
<td>PDA</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>0.16 μM</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>

Table 4

Effect of various phorbol diesters on ODC activity

Table 5

Effect of TPA and fresh medium on ODC activity in normal and transformed hamster embryocells

Five-day-old cultures of tertiary HEF or 6-day-old cultures of HE68BP cells were either left untreated, or TPA (final concentration 0.16 μM) was added directly to the spent medium, or the medium was replaced with fresh medium with or without TPA (0.16 μM). Four hr later, the cells were harvested for ODC determinations. Each value is the mean ± S.E. of 3 dishes (HEF) or the mean ± the range of 2 dishes (HE68BP cells).

<table>
<thead>
<tr>
<th>Cell</th>
<th>Treatment</th>
<th>ODC specific activity (nmoles CO₂ in 60 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal HEF</td>
<td>Control</td>
<td>0.87 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
<td>1.91 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>Fresh medium</td>
<td>6.14 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>Fresh medium + TPA</td>
<td>9.91 ± 0.32</td>
</tr>
<tr>
<td>Transformed</td>
<td>Control</td>
<td>0.39 ± 0.04</td>
</tr>
<tr>
<td>HE68BP</td>
<td>TPA</td>
<td>3.99 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Fresh medium</td>
<td>12.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Fresh medium + TPA</td>
<td>47.0 ± 0.8</td>
</tr>
</tbody>
</table>

Effect of TPA on Cell Growth and DNA Synthesis. Since the induction of ODC is an early event in many growth-stimulated systems (22), our results with HEF could indicate that the rate of cell division was increased after the addition of tumor promoters to the culture medium. The following observations, however, suggest that this is not so.

When TPA was added at a final concentration of 0.16 μM to confluent cultures of either normal HEF or transformed HE68BP cells, there was no difference in cell number between treated and untreated cultures at any time up to 4 days after addition. If the spent medium in confluent cultures was replaced with fresh medium, the cell number was increased by 60 to 100% in both cell types, but the inclusion of TPA in the fresh medium produced no further increases (data not shown).

The percentage of normal cells labeled with [³H]thymidine was not increased by the addition of TPA to the medium of confluent cultures (Chart 2). Although refeeding with fresh medium produced a marked increase in the labeling index, with a peak around 16 hr, the inclusion of TPA in the fresh medium did not increase the peak value of the labeling index (Chart 2).

TPA did not stimulate the incorporation of [³H]thymidine.

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in culture. In each system, the induction is rapid, large, and relatively short lived. The ability of promoters to induce ODC in these systems, which differ in species, cell type, and growth characteristics, suggests that this event may be a universal cellular reaction to promoter treatment in responsive cells and tissues.

Treatment of transformed HEF with TPA dramatically increased the cellular concentration of putrescine, the product of the ODC reaction. The level of this polyamine was increased 4-fold at 4 hr after TPA treatment and reached a maximal elevation of 7.5-fold at 9 hr, in agreement with the kinetics of ODC induction in these cells. There was, however, little change in the levels of spermidine and spermine.

The nature of the groups esterified at positions 12 and 13 of the phorbol molecule modifies the tumor-promoting activity of the diesters (14). Of the phorbol diesters that we have tested in HEF, only those with promoting activity in mouse skin induced ODC. Similar correlations between tumor-promoting activity and the ability to induce ODC have been observed in other systems (18, 29), and it has been suggested that the induction of ODC in these systems may be a marker for promoting activity. This study indicates that hamster cells, the HE68BP cell line in particular, can discriminate between active and inactive tumor promoters by their ability to induce ODC. Therefore, this cell system might be useful to screen for chemicals with promoting activity.

Treatment with tumor promoters stimulates both ODC and DNA synthesis in mouse epidermis and mouse epidermal cells in culture (3, 20, 29). Sivak (24) has reported that TPA induces cell division in contact-inhibited, quiescent monolayers of BALB/c 3T3 mouse fibroblasts. However, Boynton et al. (8) found no effect of TPA on DNA synthesis in BALB/c 3T3 cells under apparently similar conditions. In this study, DNA synthesis and cell division were not affected in either normal or transformed HEF exposed to tumor promoters under our treatment conditions, so that the enhanced ODC activity and putrescine accumulation were not simply events preceding a burst of promoter-induced cell proliferation. The close association between the induction of ODC and subsequent stimulation of growth in most of the systems studied previously has made it difficult to assess the role of ODC and polyamines in tumor promotion. It may be possible in HEF or in other cells in culture [e.g., the C3H/10T1/2 cell line in which a 2-stage transformation protocol has been reported (16)] to separate the effects of tumor promoters on cell growth from those effects that are specifically involved in promotion and the expression of malignancy. However, the effects of TPA and other tumor promoters on growth and other cellular properties probably depend on many factors, including the growth control mechanisms of the cells, the culture conditions, and the experimental protocol.

After cells were treated with tumor promoters, the ODC induction was greater in transformed HEF than in normal cells, which suggests that malignant cells may, in general, be more sensitive to the effects of promoting agents than are normal cells. An additional point is that, in transformed, but not normal, HEF, TPA potentiated the induction of ODC by fresh medium, with the level of enzyme activity

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**DISCUSSION**

The induction of ODC by tumor-promoting phorbol diesters has been demonstrated in mouse epidermis in vivo (20), in mouse epidermal cells in vitro (29) and, in the present study, in normal and chemically transformed HEF...
being much greater than the optimal levels obtained with either inducer alone. This suggests that malignant HEF differ from normal HEF in their regulation of ODC (19). The significance of such an alteration for the growth of these cells or their expression of the malignant phenotype is not known. However, an understanding of how normal, proneoplastic, and neoplastic cells differ in their response to the tumor-promoting agents in their environment will undoubtedly be important for an understanding of the mechanisms of tumor promotion and carcinogenesis.

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