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ABSTRACT

Association of alteration in DNA methylation pattern in triggering 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced transcription of ornithine decarboxylase (ODC) gene in T24 cells was determined. In accord with our previous findings (Archiv. Biochem. Biophys., 262: 326–336, 1988), TPA treatment of T24 cells, cultured in serum-free medium, resulted in a dramatic (~15-fold) increase in ODC activity which was accompanied by a proportional increase in hybridizable amount of ODC mRNA. Data from nuclear run-off transcription assay revealed that TPA-induced accumulation of ODC mRNA is the result of increased transcription initiation. Since DNA hypomethylation has been proposed to be a mechanism involved in the regulation of transcription of some gene(s), we examined the changes in the methylation patterns in the ODC gene isolated from the vehicle (ethanol)- and TPA-treated T24 cells. The autoradiograms resulting from the Southern blot analysis of DNA cleaved with several methylation-sensitive restriction endonucleases [e.g., HpaII, MspI, cfoI (HinfI), SalI, XhoI] exhibited no difference in methylation pattern of ODC gene in T24 cells. Also, a single or chronic application of TPA to either noninitiated or 7,12-dimethylbenz(a)anthracene-initiated mouse skin failed to alter DNA methylation pattern of ODC gene. Furthermore, the hypomethylating agent 5-azacytidine failed to induce ODC mRNA in T24 cells. These results indicate that TPA does not affect the methylation status of ODC gene and hypomethylation may not be sufficient for TPA-increased ODC gene transcription in T24 cells.

INTRODUCTION

TPA, a component of croton oil, is a potent mouse skin tumor promoter (1). A precise molecular mechanism of tumor-promoting activity of TPA remains to be defined but overwhelming evidence indicates that TPA-induced mouse epidermal ODC activity and the resultant accumulation of putrescine are essential components of the mechanism of skin tumor promotion by TPA (2–4). ODC which decarboxylates ornithine to form putrescine is the first enzyme in the pathway of mammalian polyamine biosynthesis (5). Polyamines have been shown to play a decisive role in nucleic acid and protein synthesis, but their exact mode of action is not clear (5).

A further definition of signal(s) which leads to a dramatic increase in ODC activity in response to TPA treatment may provide clues of the mechanism of tumor promotion by TPA. We have shown both in intact mouse epidermis in vivo and in isolated epidermal cells that TPA-induced ODC activity involves both increased ODC mRNA and ODC protein possibly mediated by protein kinase C activation (6, 7). In order to further determine the mechanism of TPA-induced accumulation of ODC mRNA, we used serum-deprived quiescent T24 cells. Recently, we have reported that TPA-induced steady state levels of ODC mRNA in T24 cells are the result of increased transcription initiation (8). In this paper, we have examined the role of changes in methylation of ODC gene as a mechanism for TPA-induced ODC gene expression in T24 cells.

Recent evidence indicates an inverse relationship between the extent of DNA methylation and gene expression (9, 10). Also, DePaoli-Roach et al. (11) have shown that PKC can selectively phosphorylate human DNA methyltransferase in vitro. Alhoen-Hongisto et al. (12) have shown DNA methylation polymorphism in mouse and human ODC gene-amplified cell lines. Furthermore, Leinonen et al. (13) have reported that hypomethylation status in ODC gene may be associated with the accumulation of ODC mRNA in human myeloma cell line after chronic exposure to dexamethasone. However, we present here contrary data indicating that the extent of DNA methylation may not be crucial in TPA-induced ODC gene expression.

MATERIALS AND METHODS

Materials. TPA was purchased from Life System, Newton, MA. DL-[1-14C]Ornithine hydrochloride (49.9 mCi/mmol) and GeneScreen were purchased from New England Nuclear, Boston, MA. Nitrocellulose paper (type BA 85) was obtained from Schleicher and Schuell Inc., Keene, NH. [a-32P]dCTP (3000 Ci/mmol) and [a-32P]UTP (3000 Ci/mmoll) were obtained from Amersham Corporation, Arlington Heights, IL. Calf liver tRNA and 5-azacytidine were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. RNasin and RNase-free DNase I were obtained from Promega Biotech, Madison, WI. CsCl (optical grade), urea, λDNA-HindIII marker, poly(U) Sephadex and restriction endonucleases were purchased from Bethesda Research Laboratories, Gaithersburg, MD. pODC10/2H, a cDNA clone prepared from human ODC mRNA was a gift from Dr. O. A. Janne (14), Rockefeller University, New York, NY. pH84, a human DHFR cDNA plasmid derived from pBR322 (15), was a gift from Dr. B. J. Dolnick, Roswell Park Memorial Institute, Buffalo, NY. Actin 6, a Drosophila actin cDNA plasmid derived from pXm-A2 (16), was a gift from Dr. William F. Dove, McArdle Laboratory for Cancer Research, Madison, WI. The T24 cell line was purchased from American Type Culture Collection, Rockville, MD.

Cell Culture and ODC Induction. T24, a transitional epithelial cell line, was derived from a human bladder carcinoma. The generation time of this cell line is 20 h in MEM containing 10% FBS. For ODC induction, cells were cultured for 48 h in MEM containing 10% FBS at a plating density of 6 × 10³ cells/100-mm petri dish. Cells were washed twice with Hank’s balanced salt solution without Ca²⁺, Mg²⁺, and then were starved for 48 h in serum-free MEM. ODC activity was determined by measuring the release of 14CO₂ from DL-[1-14C]-ornithine hydrochloride (17).

RNA Isolation. For isolation of total cellular RNA from T24 cells, cells were trypsinized and the cell pellet was then frozen in a −70°C environment.
DNA METHYLATION PATTERN AND ODC GENE TRANSCRIPTION

Total cellular RNA was prepared from the frozen cells by phenol-chloroform extraction and the CsCl gradient centrifugation method of Ross (18) with minor modification. The RNA pellet was resuspended in Tris-phenol buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS, 5% phenol) and was reextracted with phenol-chloroform. Finally, RNAs were precipitated with 2.5 volume of 100% ethanol and 1/10 volume of 5 M NaCl. The RNA was then washed, desiccated, dissolved in sterile distilled water, and stored at −70°C. The yields of total cellular RNA were about 15 µg/10⁶ cells as quantified spectrophotometrically by using 50 µg RNA/A₂₆₀ unit. Poly(A)+ RNA was isolated by affinity chromatography on poly(u) Sephadex. In order to determine the integrity of mRNA preparation from each treatment, we used 28S and 18S RNAs as internal standards and found that the ratio of the two RNAs was constant in each treatment (19). All the samples contained the same amount of total polyadenylate-containing RNA determined using poly(U)-¹HJuridylic acid (20).

Dot Blot Analysis of Cellular RNA. ODC mRNA was quantified by the dot blot method of Kafatos et al. (21). After denaturation (22), a serial dilution of total cellular RNA was spotted on GeneScreen filter presoaked in 1.5 M NaCl 0.015 M sodium citrate (pH 7.0) by using a Bethesda Research Laboratories Hybri-Dot manifold to apply the RNA in 3-mm circles. Then the GeneScreen was baked for 2 h at 80°C and prehybridized. The filter was hybridized with a nick-translated ³²P-labeled probe (1.5 x 10⁹ cpm/µg) (23). The resulting autoradiogram was scanned by soft-laser densitometry.

Northern Blot Analysis. Species of RNA containing regions of ODC mRNA homology were identified by modification of the Northern blot procedures (24). RNA was fractionated by electrophoresis in a denaturing formaldehyde agarose gel. The transfer to GeneScreen was performed at 4°C for 24 h, which allows complete transfer. The filter was baked for 2 h at 80°C and prehybridized. The filter was then hybridized with a nick-translated ³²P-labeled probe. ³²P-labeled λDNA-HindIII was included in the gels as a molecular weight standard and as a marker for transfer efficiency during Northern blot procedures (25). RNA bands containing ODC mRNA homology were made visible by exposing Kodak X-Omat AR film to the GeneScreen filter at −70°C with intensifying screens.

DNA-Excess Filter Hybridization Technique. The method was essentially as described by Maniatis et al. (26). Plasmid DNA was denatured and immobilized on a nitrocellulose filter. These filters were hybridized with ³²P-labeled RNA dissolved in the hybridization buffer. Calf liver tRNA (100 µg/ml) was included in the hybridization buffer. After hybridization, the filters were washed and incubated for 90 min at room temperature with ribonuclease A (20 µg/ml) (27). Finally, the filter was washed, dried, and the associated radioactivity was determined by autoradiography. The linearity of the DNA-excess filter hybridization technique was determined and 10 µg plasmid DNA was found to be excess.

Nuclear Run-off Assay. The rate of transcription of ODC gene from the isolated nuclei was determined by the nuclear run-off assay as described by Greenberg and Ziff (28). At appropriate times after treatment of T24 cells with TPA or the vehicle ethanol, the cells were harvested and the nuclei were isolated. The nuclei (1 x 10⁸) were resuspended in 50 mM Tris-HCl, pH 7.8, 2.5 mM MgCl₂, 150 mM KCl, 0.5 mM EDTA, and TPA was added to 0.05%.

**Table:**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 h</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>6 h</th>
<th>14 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>0.09 ± 0.001</td>
<td>0.23 ± 0.01</td>
<td>0.40 ± 0.06</td>
<td>0.49 ± 0.01</td>
<td>0.76 ± 0.09</td>
<td>0.72 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>0.31 ± 0.21</td>
</tr>
<tr>
<td>TPA</td>
<td>0.14 ± 0.05</td>
<td>0.34 ± 0.03</td>
<td>1.07 ± 0.26</td>
<td>3.00 ± 1.00</td>
<td>10.3 ± 1.93</td>
<td>18.58 ± 3.53</td>
<td>2.68 ± 0.66</td>
<td>0.35 ± 0.16</td>
</tr>
</tbody>
</table>

**Fig. 1.** Effect of TPA on the induction of ODC and ODC mRNA in T24 cells. T24 cells were treated with the indicated concentrations of TPA or the vehicle ethanol (final concentration 0.05%); ODC activity and the level of ODC mRNA was determined from the same culture plates at either 6 h (A) or at the indicated times (B) after TPA or ethanol treatment. For Northern blot analysis (C), total cellular RNA was isolated at 6 h after ethanol or 50 nM TPA. A: a, autoradiogram of dot blot; B, kinetics of TPA-induced ODC and ODC mRNA. E, a, autoradiogram of dot blot; b, kinetics of TPA-induced ODC and ODC mRNA. C: autoradiogram of Northern blot; lanes E, RNA from ethanol treated cells and T, RNA isolated from TPA-treated cells.

### ODC activity (nmol CO₂/mg protein/30 min) at the following treatment times:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>0.5</th>
<th>5.0</th>
<th>10.0</th>
<th>50.0</th>
<th>500.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA</td>
<td>0.72 ± 0.01</td>
<td>2.16 ± 0.08</td>
<td>9.24 ± 0.99</td>
<td>12.09 ± 1.05</td>
<td>18.58 ± 3.53</td>
<td>7.65 ± 0.65</td>
</tr>
</tbody>
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4252

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0.05 mM EDTA, 25% glycerol in a volume of 1 ml. The assay mixture contained 0.4 mM each of ATP, GTP, CTP, and 100 µCi of [α-32P]-UTP (3000 Ci/mnmole), 2.5 mM MnCl₂, 1.0 mM DTT, 150 µg/ml heparin, 5.0 mM MgCl₂, 0.5 units/µl RNasin and 3 x 10⁷ nuclei. The reaction mixture was incubated at 37°C for 15 min, and then 20 units RNase-free DNase I was added and further incubated at 37°C for 10 min; then 20 µg Escherichia coli tRNA was also added and ethanol precipitated. The precipitates were dissolved in 1% SDS, 0.2 M NaCl, 2 mM EDTA, 0.02 M Tris-HCl, pH 7.4, 1 mg/ml proteinase K, and incubated at 37°C for 1 h. 32P-labeled RNA was extracted by phenol-chloroform method (18). Specific ODC mRNA was quantified by using DNA-excess filter hybridization.

Southern Blot Analysis. High molecular DNA was purified essentially by the procedure described by Davis et al. (29). DNA fragments were analyzed by the Southern blot analysis (30). An equal amount of high molecular weight DNA was digested with restriction endonucleases, and was fractionated by electrophoresis in a 0.8% agarose gel containing ethidium bromide. After transfer to GeneScreen paper, the filter was baked for 2 h at 80°C and prehybridized at 42°C overnight, and then hybridized with nick-translated 32P-labeled probe. For autoradiography, the filter was exposed to Kodak X-Omat AR film at −70°C with intensifying screens.

RESULTS

TPA-induced ODC Activity Correlated with the Accumulation of ODC mRNA Regulated at the Level of Transcription Initiation. The effect of TPA concentration on the induction of both ODC activity and ODC mRNA is shown in Fig. 1A. In this experiment, T24 cells were treated with 0.5, 5, 10, 50, or 500 nM TPA in serum-free medium and the levels of ODC activity and ODC mRNA was determined 6 h after TPA treatment. A dramatic increase in ODC activity and ODC mRNA was observed at 50 nM TPA concentration and the 500 nM TPA concentration was inhibitory. At all TPA concentrations, the extent of the induction of ODC activity was proportional to the hybridizable amount of ODC mRNA (Fig. 1A).

We also determined a time course of the effect of TPA on the induction of ODC activity and ODC mRNA in T24 cells. Again, at all the time points examined, TPA-induced ODC activity correlated with the steady-state levels of ODC mRNA (Fig. 1B). These results are in accord with our previous findings with the isolated epidermal cells (7) and in intact mouse skin...
in vivo (6). Also, Northern blot analysis of poly(A⁺) RNA indicated that the majority of hybridizable ODC mRNA is polyadenylated (Fig. 1C). Recently, we have shown that TPA-induced steady state levels of ODC mRNA is the result of increased synthesis of ODC mRNA rather than the effect of TPA on the turnover of ODC mRNA (8). In a slightly more detailed time course, we further investigated the effect of TPA on the transcription of ODC gene in T24 cells. In this experiment, nuclei were isolated at 1, 4, and 24 h after treatment of T24 cells and the rate of transcription was determined by the nuclear run-off transcription assay (28). As shown in Fig. 2, the rate of ODC gene transcription was increased about 7- to 10-fold above ethanol control at 4 h after TPA treatment; ODC mRNA synthesis decreased to the basal levels at 24 h after TPA treatment. The RNA polymerase II inhibitor α-amanitin inhibited TPA-induced synthesis of ODC mRNA implying that RNA transcripts were newly synthesized. It is also noteworthy in the same experiment illustrated in Fig. 2 that TPA increased the synthesis of ODC mRNA but slightly affected the synthesis of α-actin mRNA.

TPA Treatment Failed to Alter DNA Methylation Pattern in T24 Cells. In this experiment, T24 cells were treated with 50 nM TPA or the vehicle ethanol and genomic DNA was isolated 1, 2, or 3 h after treatment. Genomic DNA was digested with several DNA methylation-sensitive restriction endonucleases (Table 1) and the DNA methylation pattern was determined by the Southern blot analysis. As shown in Figs. 3 and 4, MspI digestion generated two major bands of 2.3- and 3.4-kilobase sizes but the other methylation-sensitive enzyme HpaII failed to cleave DNA isolated from either ethanol or TPA-treated T24 cells. Both MspI and HpaII cleave at CCGG sequences but HpaII is unable to digest the sequence when the internal cytosine is methylated.

To explore the possibility that methylation pattern may occur in DNA sequences other than CCGG, genomic DNA was digested with other methylation-sensitive restriction endonucleases (e.g., CfoI, SalI, and XhoI) and results are shown in Fig. 4. Digestion with CfoI generated one major band of about 9.6-23.9 kilobases while SalI and XhoI digestion did not generate any detectable band at any of the time points after ethanol or TPA treatment.

We also analyzed DNA methylation after double digestion of T24 cell DNA. In this experiment, T24 cells were treated with ethanol vehicle or 50 nM TPA and high molecular DNA was isolated 3 h after treatment. Southern blot analysis of DNA digested with BamHI and HpaII or BamHI and MspI indicated that TPA treatment did not alter methylation pattern of ODC gene (Fig. 5).

The effect of the hypomethylation agent 5-azacytidine on ODC gene expression in T24 cells was also determined. As shown in Fig. 6, 5-azacytidine treatment resulted in decreased levels of methylation of ODC gene but failed to affect the induction of ODC mRNA or ODC activity (Table 2). In the same experiment, 5-azacytidine alone did not induce the steady state levels of DHFR mRNA in T24 cells but potentiated TPA-induced levels of DHFR mRNA (Table 2). Similar results have been observed with β-globin gene in C3H 10T½ cells (31).

We also determined the effect of chronic applications of TPA to mouse skin on the methylation pattern of ODC gene of mouse skin genomic DNA. In these experiments, mouse skin DNA was isolated after single or twice weekly applications (11 applications) of 10 nmol of TPA to DMBA-initiated or non-initiated female CD-1 mouse skin. Mouse skin genomic DNA was cleaved with MspI and HpaII restriction endonucleases (Fig. 6). We failed to detect alterations in DNA methylation patterns under the conditions that result in dramatic increase in ODC gene expression (6, 7).

DISCUSSION
Several actively transcribing cellular genes, such as chicken ovalbumin gene (32), rabbit globin gene (33), DHFR gene (34), DNA of adenovirus in transformed cells (35), have been shown to be undermethylated in the expressed regions, while silent regions are highly methylated. In most higher organisms, 2-7% of the cytosine in DNA is modified to 5-methylcytosine shortly after DNA replication; 5-methylcytosine is predominantly found at the sequence 5-CpG (36). Further evidence indicating the role of DNA methylation in regulating gene expression is the finding that 5-azacytidine which induces hypomethylation status of DNA sequences alters specific gene expression (37-39). In view of the above evidence, we investigated whether TPA-induced ODC gene expression (Figs. 1 and 2 and Ref. 8) is associated with alteration in methylation pattern

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**Table 1 Recognition sites for the methylation-sensitive restriction endonucleases**

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Recognition sequence</th>
<th>Cleaved</th>
<th>Not cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>CfoI</td>
<td>GCGG</td>
<td>GCGG</td>
<td></td>
</tr>
<tr>
<td>HpaII</td>
<td>CCGG</td>
<td>CCGG</td>
<td></td>
</tr>
<tr>
<td>MspI</td>
<td>CCGG or CCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SalI</td>
<td>GTCGAC</td>
<td>GTCGAC</td>
<td></td>
</tr>
<tr>
<td>XhoI</td>
<td>CTCGAC</td>
<td>CTCGAC</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3.** Effect of TPA treatment of T24 cells on the DNA methylation pattern in ODC gene. T24 cells were treated with ethanol or 50 nM TPA for 1, 2, or 3 h. High molecular DNA (10 μg) from T24 cells was digested with either MspI (M) or HpaII (H) at 37°C overnight. DNA fragments were analyzed by electrophoresis on 0.8% agarose gel. After transfer, the filter was prehybridized, and then hybridized with nick-translated, 32P-labeled pODC 10/2H. Shown are the autoradiograms of the Southern blot (exposure time, 2 days). R.E., restriction endonuclease.

**Fig. 6.** Effect of 5-azacytidine treatment resulted in decreased levels of methylation of ODC gene but failed to affect the induction of ODC mRNA or ODC activity (Table 2). In the same experiment, 5-azacytidine alone did not induce the steady state levels of DHFR mRNA in T24 cells but potentiated TPA-induced levels of DHFR mRNA (Table 2). Similar results have been observed with β-globin gene in C3H 10T½ cells (31).
DNA METHYLATION PATTERN AND ODC GENE TRANSCRIPTION

Fig. 4. Effect of TPA treatment on the DNA methylation pattern in ODC gene of T24 cells. DNA was isolated 3 h after ethanol or TPA treatment. High molecular DNA (10 µg) was digested with HpaII (H),MspI (M),CfoI (C) (autoradiogram A, exposure time 7 days), or SalI (S),XhoI (X) (autoradiogram B, exposure time 8 days) at 37°C overnight. R.E., restriction endonuclease.

Table 2 Effect of 5-azacytidine on the transcription of ODC and DHFR genes

T24 cells were treated with the indicated concentrations of 5-azacytidine. 5-Azacitidine was removed from the medium 72 hr after plating, then T24 cells were treated with TPA or the vehicle ethanol. At three hr after ethanol or TPA treatment, the medium was removed and the cells were washed with PBS and pelleted. ODC activity was determined and total cellular RNA was isolated from the same cell pellet. For dot blot analysis, filters were hybridized with nick-translated pODC10/2H or pH84.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TPA (50 nm)</th>
<th>5-Aza* (µg/ml)</th>
<th>ODC activity (nmol CO2/mg protein/30 min)</th>
<th>ODC mRNA</th>
<th>DHFR mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>0.0</td>
<td>0.14 ± 0.05</td>
<td>1.0 ± 0.5</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>+</td>
<td>5.0</td>
<td>0.21 ± 0.01</td>
<td>1.0 ± 0.5</td>
<td>ND*</td>
<td>ND</td>
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<tr>
<td>+</td>
<td>2.5</td>
<td>0.19 ± 0.08</td>
<td>0.7 ± 0.6</td>
<td>0.9 ± 0.3</td>
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<tr>
<td>+</td>
<td>1.0</td>
<td>0.10 ± 0.07</td>
<td>0.6 ± 0.7</td>
<td>0.5 ± 0.7</td>
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<tr>
<td>+</td>
<td>0.2</td>
<td>0.13 ± 0.02</td>
<td>0.9 ± 0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>0.0</td>
<td>1.63 ± 0.32</td>
<td>8.2 ± 0.1</td>
<td>4.0 ± 0.6</td>
<td>ND</td>
</tr>
<tr>
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<td>1.54 ± 0.25</td>
<td>10.6 ± 0.7</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>2.5</td>
<td>1.76 ± 0.25</td>
<td>10.8 ± 1.0</td>
<td>16.2 ± 1.0</td>
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<tr>
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<td>1.0</td>
<td>1.33 ± 0.71</td>
<td>6.5 ± 0.5</td>
<td>8.2 ± 0.7</td>
<td>ND</td>
</tr>
<tr>
<td>+</td>
<td>0.2</td>
<td>1.70 ± 0.20</td>
<td>6.2 ± 0.9</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* 5-Aza, 5-azacytidine.
* ND, not determined.

Fig. 5. Southern blot analysis of ODC sequences in T24 cell DNA digested with nonmethylation and methylation sensitive restriction endonucleases. T24 cells were treated with none (lanes 1–3), ethanol (lanes 4 and 5), or 50 nm TPA (lanes 6 and 7). High molecular weight DNA was isolated and was subjected to Southern blot analysis, Lane 1, BamHI digestion; lane 2, MspI digestion; lanes 3, 4, 6, BamHI digestion followed by MspI digestion; lanes 5, 7, BamHI digestion followed by HpaII digestion.

of ODC gene. We present that methylation status of ODC gene did not alter under the conditions which induced ODC gene expression in T24 cells.

As shown in Fig. 3, digestion with MspI resulted in 2.3- and 3.4-kilobase bands in T24 cells. Treatment of T24 cells with TPA did not affect the methylation pattern of CCGG sequences in ODC gene (Fig. 3). Further experiments involving use of other methylation specific endonucleases (CfoI, SalI, XhoI) failed to elicit detectable changes in the methylation pattern of CpG sequences in T24 ODC gene after TPA treatment (Fig. 4). These results indicate that changes in the methylation pattern of ODC gene does not associate with TPA-induced ODC gene expression but these results should be interpreted with caution. It is possible that the methylation pattern may change in different CpG sequences which were not recognized by the restriction endonucleases used in this study. Furthermore, evidence also exists which indicates that changes in the methyla-
Fig. 6. Effect of 5-azacytidine on DNA methylation pattern in ODC gene of T24 cells. T24 cells were treated for 72 h with the indicated concentrations of 5-azacytidine; cells were washed to remove 5-azacytidine then treated with 50 nm TPA or ethanol for 3 h and high molecular DNA was isolated. For Southern blot analysis, 10 μg DNA was digested with either MspI (M) or HpaII (H) at 37°C overnight. Shown are the autoradiograms of the Southern blots (exposure time, 5 days). A, ethanol; B, TPA. R.E., restriction endonuclease; 5-AZ, 5-azacytidine.

Fig. 7. Effect of application of TPA on DNA methylation pattern of mouse skin ODC. Female CD-1 mice were initiated by application of nmol of DMBA to skin. Two weeks after initiation, 10 nmol TPA in acetone or acetone was applied to skin twice weekly. Mice were killed 4 h after the last treatment. Skin DNA was analyzed for the methylation pattern in ODC gene by Southern blot analysis using pODC 10/2H, a human cDNA clone. HpaII (H); MspI (M), A, noninitiated skin; B, DMBA-initiated skin; R.E., restriction endonuclease.
tiated mouse skin did not affect the methylation pattern of ODC gene in mouse skin (Fig. 6).

In summary, TPA treatment did not alter the methylation state of ODC gene in T24 cells. However, the hypomethylation agent 5-azacytidine induced an extensive decrease in the methylation of ODC gene but failed to affect its transcription. Also, the methylation pattern remained unaltered in ODC gene after chronic applications of TPA to mouse skin. We conclude that methylation of DNA does not appear to play a major role in the mechanisms involved in TPA-induced ODC gene transcription. Recently, TPA-responsive enhancer sequences and TPA-inducible trans-acting factors (e.g., Ap1 family) have been shown in several studies (41-43). It is highly likely that some specific trans-acting factor(s) (PKC-mediated phosphorylated proteins) may play a role in the regulation of TPA-induced ODC gene transcription.

REFERENCES


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