REGULATION OF MELANOGENESIS IN B16 MOUSE MELANOMA CELLS BY PROTEIN KINASE C (PKC)

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Dedicated

to

my grandparents

Shri. & Smt. M. P. Kuppuswami Iyer

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PKC	protein kinase C
RAR	retinoic acid receptor
RXR	retinoic X receptor
RARE	retinoic acid response element
mi	microphthalmia
MITF	microphthalmia-associated transcription factor
bHLH.Zip	basic helix-loop-helix. leucine zipper
PDBu	phorbol dibutyrate
DMSO	dimethyl sulfoxide
TRP1	tyrosinase-related protein 1
TRP2	tyrosinase-related protein 2
cDNA	complementary DNA
mRNA	messenger RNA
AP1	associated protein-1
PBS	phosphate-buffered saline
TBS	Tris-buffered saline

INTRODUCTION

Melanin Biosynthesis

Melanogenesis is regulated by a variety of environmental and hormonal factors. In mammals, the functional melanocytes (pigment-producing cells) are found principally in three locations: the hair follicles, the basal cell layer of the epidermis and the eye, including the pigmented retina, iris and choroid. They are also found in the inner ear, the mucous membranes and the harderin gland of the mouse eye (12, 121). The pigmentation of most mammals and birds is determined by the melanocytes of hair and feather follicles, respectively, while humans are unusual in the importance of their basal epidermal melanocytes. Mammalian melanocytes are capable of producing many hues; they synthesize at least three chemically related classes of pigments (12). These are eumelanins (usually black to brown), pheomelanins (red to yellow) and trichochromes (red to yellow) (Greek: eu, well; melas, black; phaios, dun colored; trichos, hair; chroma, color).

Eumelanin is a polyquinonoid pigment synthesized from tyrosine (Fig 1). The first biosynthetic step, hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA), requires the enzyme tyrosinase, which also catalyzes the second step, oxidation of DOPA to DOPAquinone. Thus tyrosinase is both a monophenolase (EC 1.14.18.1) and a diphenolase (EC 1.10.3.1) (84,85). Since DOPA can spontaneously autooxidise

and give rise to melanin, tyrosinase was thought to be the only enzyme essential to this pathway. However, several factors have been described which influence the production of melanin in mammals either in a positive or a negative fashion. Genetic and biochemical studies have revealed at least two other enzymes (tyrosinase related proteins) that function in the synthesis of mammalian eumelanin (49). The mouse *brown* locus on chromosome 4 encodes a protein, tyrosinase-related protein 1 (TRP1), having 52% amino acid identity with tyrosinase (60,132). The mouse *slaty* locus on chromosome 14 encodes a protein, tyrosinase-related protein 2 (TRP2) that has about 40% amino acid identity with tyrosinase and TRP1 (143). TRP1 and TRP2 are thought to posess dihydroxy indole carboxylic acid (DHICA) oxidase (66) and DOPAchrome tautomerase (143) respectively, which accelerate reactions further downstream in the melanogenesis pathway (49). The rate-limiting enzyme in melanin biosyntheis, tyrosinase, is subject to both hormonal (82,150) and non-hormonal (80) regulation. The gene that encodes tyrosinase has been mapped to the mouse *albino* locus on chromosome 7 (79, 100, 141, 152). Both tyrosinase and TRP-1 are specifically expressed in melanin-producing cells, such as melanocytes and retinal pigment epithelium, which are derived from the neural crest and the optic cup of the brain, respectively.

Melanocytes are dendritic cells that develop from melanoblasts and originate in the neural crest (29, 30, 81, 96). They reside in the basal layer of the epidermis, the hair follicle and the eye and are responsible for the production of pigment melanin, which gives rise to hair, eye and skin color (133). Melanin biosynthesis occurs in specialized

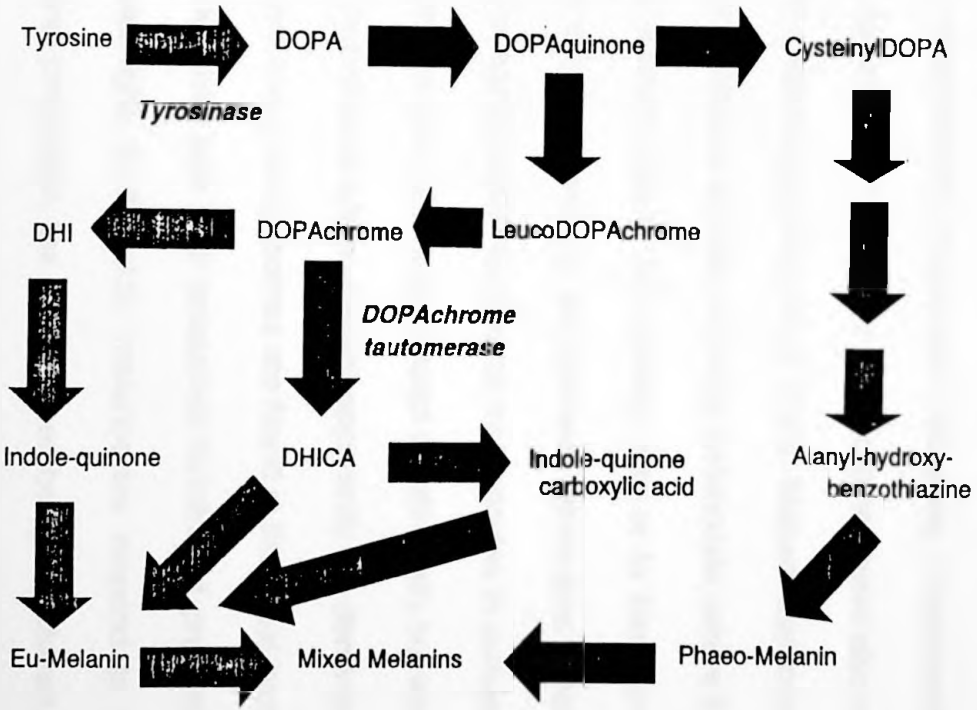


Figure 1: Schematic of melanin biosynthesis pathway

organelles, the melanosomes. The enzymes of melanin synthesis are contained in the membrane-bound melanosome, produced only by melanocytes. Tyrosinase and the two related proteins (TRP-1 and 2) have a transmembrane domain and since melanin accumulates inside the organelle, these proteins are thought to be located on the inner face of the melanosomal membrane. However, membraneous vesicles or "vesciglobular bodies" observed inside melanosomes have also been suggested as the location of melanosomal enzymes (121). Mature melanosomes are moved outward along the dendritic processes of the melanocyte, where they are transferred to the dividing keratinocytes of the growing hair or to basal keratinocytes of the epidermis. The transfer seems to be directed, rather than a result of nonspecific exocytosis and phagocytosis (44). Normal melanocytes in culture do not appear to release melanosomes into the medium except on cell death, but will transfer them into keratinocytes or other cells when present, apparently via dendrites (44, 77, 135). In human skin *in vivo*, most melanosomes are found in the basal keratinocytes, distal to their nuclei, in keeping with their presumed function of protecting stem-cell DNA against ultraviolet light. In the skin, melanocytes respond to ultraviolet light by increasing melanin production, the melanin then being transferred to the surrounding keratinocytes as protection against UV damage (30). Excessive exposure to UV irradiation can result in transformation of the melanocyte to a cutaneous malignant melanoma, a highly aggressive and increasingly common form of cancer.

Melanocyte and Melanoma

The study of the cellular genetics of melanoma is greatly helped by the availability of established mouse and human melanocyte and melanoma cell lines. The differences between the melanocytes and melanoma cell lines may provide clues to understanding the progression from the melanoblast to the melanocyte and from the melanocyte to malignant melanoma. Normal mouse and human melanocytes are highly pigmented (9). Melanoma cells are unpigmented or lightly pigmented at low pH, in which melanocytes by comparison remain pigmented (10). In contrast to malignant melanoma, normal melanocytes don't have the ability to grow in an anchorage-independent fashion. Melanoma cells generally will form tumors when implanted into nude (immunodeficient) mice or syngenic animals. In terms of the growth requirements, melanoma cells grow well in a standard culture medium with serum (125). However, melanocytes require a combination of growth factors, or serum plus an activator of protein kinase C, often the phorbol ester tumor promoter, tetradecanoyl phorbol acetate (TPA) (44, 125, 145).

Pigment Cell-specific Transcription

The molecular mechanisms controlling melanocyte differentiation are poorly understood. The most important characteristic of melanocyte differentiation is melanogenesis. Precise and coordinated control of gene expression is crucial for

normal growth, division and differentiation of cells. The identification of the transcriptional factors responsible for the regulation of tyrosinase, TRP1 and other pigment cell-specific genes should eventually enable several important questions to be answered; in particular, whether factors controlling the expression of the pigmentation genes also govern melanocyte differentiation.

The *microphthalmia (mi)* phenotype is associated with the mutant *mi* locus and characterized by small non-pigmented eyes, a lack of melanocytes in the skin and inner ear, a defect of mast cells and osteopetrosis, possibly caused by a dysfunction of the osteoclasts (52, 57). The coats of mice homozygous for mutant alleles of *mi* are white. These mice lack pigmentation due to the absence of melanocytes, unlike *albino* mice, which are incapable of producing pigment in structurally normal melanocytes. Reciprocal melanoblast transplantation experiments showed the cell autonomy of the defect and aggregation-chimera experiments attest to the inviability of *mi/mi* melanoblasts (133). It is hypothesized that *mi* is required for melanoblast differentiation and/or proliferation (140). The expression of the *mi* gene is detected in restricted tissues, such as melanocytes, heart, mast cells, and the outer layer of the retina (52). The *mi* gene encodes a member of the basic helix-loop-helix-leucine zipper (bHLH.zip) family of transcription factors. The three conserved motifs that constitute this family are: the basic region, which recognizes a canonical CANNTG DNA-binding sequence, the HLH motif and the Zip motif. The HLH and Zip motifs participate in protein dimerization, a prerequisite for DNA binding. Sequence relationships within the bHLH.Zip proteins show that mi is most related to the transcription factors TFEB,

TFEC and TFE3 (7, 52, 57, 158), all of which can form stable heterodimers with *mi* and with each other (50). The gene product of the *mi* gene is predicted to play a major role in pigment cell specific transcription.

The promoter regions of the mouse and human tyrosinase, TRP-1 and TRP-2 genes have been cloned (11, 60, 61, 79, 100, 132). The cell-type-specific promoter function of the mouse tyrosinase and TRP-1 genes has been well characterized. Specifically, the promoter function of the mouse tyrosinase gene was analyzed in transgenic mice, in which the introduced gene was expressed specifically in skin melanocytes and retinal pigment cells (8, 73, 142). Kluppel et al. (73) showed that the 270 bp 5' flanking region of the mouse tyrosinase gene is sufficient to direct its pigment cell specific transcription in transgenic mice as well as in cultured cells. An 11 bp sequence termed the M box (AGTCATGTGCT) was identified in the mouse TRP-1 promoter (90) and is conserved in TRP-1 and tyrosinase promoters of different species (61, 90). The M box contains a canonical sequence (CANNTG-E box motif) that is known to be a binding site for a class of transcription factors with bHLH motifs (67, 101). The products of the mouse *mi* and human (microphthalmia-associated transcription factor (*MITF)* genes (52, 57, 138), bHLH.Zip transcription factors (52, 57), were shown to transactivate the mouse and human tyrosinase as well as TRP-1 promoters (13, 37, 50, 108, 153, 154). Activation requires the presence of an E-box, corresponding to another CANNTG motif, closer to the start site in human as well as mouse tyrosinase (13, 154) and is enhanced when the M-box is also part of the promoter region (13, 37).

Role of PKC in Differentiation

Protein Kinase C (PKC) is a calcium and phospholipid dependent kinase which has been implicated as a key messenger in cellular signaling (70, 106). PKC is a family of proteins containing at least 11 isoforms divided into three major groups, classical, novel and atypical, based on their structure and mode of activation (70). The classical PKCs (α, β1, β2 and γ) require both calcium and phospholipid for activation, while PKCs belonging to the novel group (δ, ε, η and θ) do not require calcium (70, 109). The atypical isotypes (ι/λ, ζ) are independent of calcium and diacylglycerol (70). All isotypes except the atypical ones bind phorbol esters (70). PKC is also the major, if not the only receptor for phorbol ester tumor promoters (70, 103). Besides activating PKC, prolonged treatment with phorbol esters can result in down-regulation of the enzyme (3, 55).

PKC is involved in regulating the growth and differentiation of a variety of cell types in response to growth factors, neurotransmitters and hormones (36, 91, 126, 136, 148). PKC was originally found to be a kinase which was activated by diacylglycerol (DAG) and calcium (97). The importance of this enzyme became clear when it was shown that tumor promoters such as phorbol esters bind to PKC due to their steric similarity to DAG (123). PKC is the major intracellular receptor for phorbol esters (18, 103). The use of phorbol esters has provided evidence for the potential for PKC to regulate a plethora of biological events. Several studies have been carried out to

define the function of PKC in response to extracellular stimuli. When the cells are stimulated by phorbol esters, there is an initial activation of PKC. This is followed by down-regulation and degradation of PKC. It is suggested that the loss of PKC protein is due to a net increase in proteolysis without a change in the rate of synthesis (155). The mechanism(s) involved in this process is not clear.

Several lines of evidence suggest that PKC may play a positive role in cell growth by activating gene transcription. The expression of either c-fos, c-jun or both genes is induced by PKC (41, 47, 99, 106, 127). These proteins, which form heterodimers, bind to DNA sequences and activate gene expression (21, 45, 102, 124, 129, 130, 146). The DNA sequence to which these dimers bind was originally called the TPA response element, which is also referred to as the AP1 site (1). This region was called the TPA response element because it was identified as a sequence that was responsible for phorbol ester-induced gene expression.

Since each isoform has a distinct tissue specific pattern of expression, it has been proposed that each member of the PKC family has a unique cellular function. In addition, it has been proposed that this function may vary depending on the cell type. PKC α, β and γ are increased during HL60 granulocyte differentiation by RA or DMSO and also during monocytic differentiation by 1α, 23, dihydroxyvitamin D3 (134). PKCε has been shown to increase during the HMBA mediated induction of MELC differentiation (120). The same isoform is the only one which is decreased during thyrotropin-releasing hormone induction of GH4C1 rat pituitary cells (71).

Transfection of PKCβ-II into rat fibroblasts resulted in an increased rate of cell growth (53). However, when the β-II isoform was expressed in HT29 cells, a human colon cancer cell line, it caused a slowing of cell growth and the transfected cells were less tumorigenic in nude mice (22). Overexpression of PKCα in NIH 3T3 fibroblasts resulted in a slightly transformed phenotype (26). Overexpression of PKCα in B16 cells induced a differentiated phenotype (42). Thus it is clear that the effect a particular PKC species has on cell function depends upon the unique cellular environment found within that specific cell type.

Retinoids

More than 65 years ago Wolbach et al. (149) established that deficiency of vitamin A causes squamous metaplasia and keratinization of columnar epithelia of the body. Since then the crucial role of retinoids in controlling differentiation processes has become evident from studies conducted in a variety of *in vivo* and *in vitro* systems. Retinoic acid (RA) has been shown to play a critical role in the regulation of growth and differentiation of many different cell types (88, 89). The role of retinoids in the control of growth and differentiation of skin epithelium is well documented (23). From a clinical standpoint, RA has received widespread attention because of its effect in the treatment of keratinization (111), and pigmentation disorders (17, 115). Most of the *in vitro* studies investigating the effects of RA on epithelial differentiation have been

performed using epidermal keratinocytes. Differentiation of these cells is efficiently blocked by addition of vitamin A (33) or RA (4) in the culture medium. Thus, the modulation of keratinocyte differentiation by topical RA treatment observed *in vivo* is successfully reproduced in vitro. However, while the phenotypic control exerted by retinoids on keratinocytes is well documented, little information is available on the action of RA on melanocytes (115).

The similarity of the mode of action of retinoids to that of the steroid and thyroid hormones became evident with the discovery of nuclear receptors for retinoic acid (25, 39, 118). The physiological action of retinoic acid is thought to be mediated by nuclear retinoic acid receptors (RAR). There are three different subtypes of RAR (RAR α, β and γ) all of which share structural homology with the superfamily of steroid receptors (69, 157). Another related class of receptors, the RXR, which specifically bind 9-cis retinoic acid (86) has been recently described. This class of receptors also consists of three subtypes (RXR α, β and γ) (94) and can form heterodimers with RAR, as well as several other members of the steroid super family of receptors (72, 83). These receptors act as ligand-dependent transcriptional activators by binding as heterodimers to specific nucleotide sequences in the response elements of target genes. The heterodimer of RAR-RXR is thought to be the form which specifically binds to retinoic acid response elements (RARE) (156). Many genes under direct trasncriptional control by RA (43). Most of these genes share a common response

element (RARE) in their 5' flanking region consisting of a hexanucleotide direct repeat, usually separated by 5 base pairs (DR-5) (43, 74).

Retinoids, PKC and Melanin Biosynthesis

Phorbol esters have previously been shown to inhibit melanin production in B16 cells and tyrosinase activity and mRNA production in Cloudman S91 melanoma cells (35, 98). The ability of these compounds to interfere with melanogenesis correlated with their activity as tumor promoters. Since the major receptor for phorbol esters is protein kinase C (PKC) (106, 107, 117), these findings suggest that PKC plays a role in regulating melanogenesis. There are reports implicating PKC in the effect of UV irradiation (32, 40) and cytokines (59) on melanin production. It appears that certain human melanoma cells require PKCβ for melanogenesis (116).

Several groups have shown that RA-induced differentiation of various tumor cell types is accompanied by an increase in PKC expression (37, 93, 104, 126). In B16-F1 mouse melanoma cells, RA induces a 5-8-fold increase in PKCα message and protein (42, 104, 126). Since this increase in PKCα occurs relatively early during the differentiation program, Gruber et al. (42) investigated whether overexpression of PKCα in these cells could mimic some of the effects of RA treatment in these cells. Overexpression of PKCα was able to induce some of the phenotypic changes (i.e. decreased monolayer growth rate, elimination of anchorage independent growth and

increased melanin production) induced by RA administration (42). Melanin production is an important differentiation characteristic in these cells. The regulation of this pathway is complex with many gene products contributing to this process. The role of retinoids in tyrosinase expression and melanin production is controversial with various laboratories reporting that they either stimulate, inhibit or have no effect on this process (19, 28, 31, 42, 112, 113). RA was also found to be a potent inhibitor of hormone (melanocyte-stimulating hormone MSH) and drug (isobutyl-methylxanthine-IBMX, cholera toxin)-induced melanogenesis in mouse melanoma cells (19, 95, 112, 113).

Experimental Objective

The aim of the first part of the study was to determine the role of PKC in the phorbol ester-induced inhibition of melanogenesis. We found that chronic treatment of B16 mouse melanoma cells with phorbol dibutyrate (PDBu) down-regulated PKC and prevented the density-dependent induction of tyrosinase activity, tyrosinase protein and tyrosinase mRNA. This treatment also decreased the level of TRP-1 protein by 50%, but had no effect on TRP-2 protein level.

The next part of the study focussed on identifying *cis*-acting elements in the 5' flanking region of the mouse tyrosinase gene. Deletion analysis of the 270 bp 5' flanking region in B16 cells suggested that a 45 bp region colocalizing with the M-box region is essential for the transcription of the tyrosinase gene. To investigate these

findings further, we examined the effect of cell density and PKC activation/depletion on trancriptional activity and nuclear protein interaction with the M-box region. Our studies suggest that PKC can regulate promoter function and that one specific DNA-protein complex correlates with tyrosinase expression. The formation of this complex is regulated by PKC and antibody studies suggest that one of the components of this complex is MITF.

Studies on B16 mouse melanoma cells in our laboratory have shown that retinoic acid (RA) induced differentiation is accompanied by a large increase (5-8 fold) in the PKCα protein levels. Gruber et al. (42) have shown that overexpression of PKCα in these cells stimulated melanin production and increased tyrosinase activity. Studies also show that depletion of PKC in B16 cells leads to a marked decrease in RA-induced transcriptional activity. This effect was also reflected in a decreased ability of RARs from PKC-depleted cells to bind to RARE in electrophoretic mobility shift assays (EMSA) (unpublished observations). In the third part of the study we investigated whether RA mediates its effect on melanin production through its ability to regulate the tyrosinase promoter. We found that RA stimulated the expression of a tyrosinase promoter-luciferase plasmid transfected into B16 cells. EMSA revealed that the M-box region of the tyrosinase promoter bound both exogenous and endogenous RARs.

Materials and Methods

Cell Culture

B16 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated calf serum (Sterile Systems, Logan, UT), 2 mM L-glutamine, 2 mM sodium pyruvate, 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate. Cells were incubated at 37°C in a 6% CO_2/94% air humidified atmosphere. New cultures were initiated from frozen stock every 6 weeks in order to prevent phenotypic drift.

Treatment of Cells with Phorbol Esters, 8-bromocyclic AMP and RA

Phorbol, phorbol diacetate and phorbol dibutyrate (PDBu) were obtained from Sigma Chemical Co., St. Louis, MO. These compounds were dissolved in DMSO (Dimethyl sulfoxide- Me_2SO_4) and maintained at -20°C until used. Cells were seeded at the densities indicated and 24 h later were refed with growth medium containing DMSO (control) or various concentrations of phorbol esters or RA. At designated intervals cells were harvested and assayed as described below. When examining the effect of PDBu on 8-bromocyclic AMP induction of melanogenesis, cells were pre-incubated for 24 h with 1 μM PDBu or DMSO (control). 8-bromocyclic AMP (0.5 mM,

Sigma) + 1-methyl, 3, isobutylxanthine (0.2 mM, Aldrich Chemicals) were then added in either the absence or continued presence of PDBu. After an additional 24 and 48 h of incubation, cells were harvested and assayed for tyrosine hydroxylase activity and the amount of tyrosinase, TRP-1 and TRP-2 proteins by Western blotting as described below. To measure the effect of PDBu on cell growth, B16 cells were seeded at 2 X 10^5 cells /100 mm tissue culture dish. After allowing the cells to attach overnight, they were refed with or without 1 μM PDBu. Control cells received the solvent for PDBu (DMS0). At 16, 40, 64 and 88 h after this refeeding, triplicate dishes were harvested and the number of cells enumerated through the use of a hemacytometer.

Tyrosine Hydroxylase Enzyme Activity

Tyrosinase is an unusual enzyme in that it has both tyrosine hydroxylase and DOPA-oxidase activity. In this study we measured the tyrosine hydroxylase component by a modification of the method of Pomerantz (119). Cells were washed twice with cold saline and once with 10 mM soduium phosphate buffer (pH 7.2). After scraping the cells from the dishes into the phosphate buffer, they were disrupted by sonication for 30 s (in 10 s bursts) at power setting #3 (Heat Systems Sonifier, Plainview, NY). The reaction mixture consisted of 2 mM L-tyrosine, 2 μCi of L-[^{3}H]tyrosine (40-60 Ci/mmol, NEN-DuPont, Boston, MA), 10 mM sodium phosphate buffer, pH 6.8 and 50 μM L-DOPA in a total volume of 400 μl. The reaction was initiated by adding 50 μg of cell

protein and incubated at 37°C. The reaction was terminated by the addition of 75 µl of 45% TCA (trichloroacetic acid). The resultant precipitate was removed by centrifugation (4000 X g for 30 min) and the supernatant applied to a Dowex 50 column (0.4 x 8 cm). The void volume plus a 0.5 ml column wash was collected . Charcoal slurry (500 µl of stock 1.5 g in 20 ml H_2O) was then added to this solution and the samples vortexed intermittently over a 15 min period. The charcoal was pelleted by centrifugation (4000 X g, 10 min) and a 200 µl aliquot of the supernatant counted for the production of radioactive H_2O. It should be noted that this procedure measures soluble tyrosine hydroxylase activity *i.e.*, that which is not associated with melanosomes. However, in the PDBu dose-response experiment (Fig. 1), 1% NP-40 to the extraction buffer was added and obtained similar results.

Assay of PKC enzyme activity

Cells were lysed on ice with 20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride and 10 mg/ml aprotinin. Complete cell disruption was further ensured by three consecutive 10-sec sonications. The total cell lysate was centrifuged at 12,000 X g for 15 min. The supernatant was loaded onto a DEAE cellulose anion exchange column (Cellex-D, Biorad) previously equilibrated with column buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol). The column was washed with 15 volumes of column buffer. The PKC fraction was eluted with 2 volumes of column buffer

containing 100 mM NaCl and concentrated with a centricon-10 microconcentrator (Amicon). Protein concentrations of samples were determined by the Pierce BCA assay. Samples were diluted to equal protein concentrations, and assayed with a commercially available PKC assay system (Amersham) in the presence and absence of tetradecanoyl-phorbol-acetate (TPA) and phosphatidyl serine (PS). The degree of phosphorylation was determined by liquid scintillation counting. Enzyme activity was calculated from CPM taking into account the specific activity of the radioisotope and reaction time.

Western Blot Analysis for PKCα, Tyrosinase, TRP-1 and TRP-2

Cells were washed twice with cold PBS and harvested in 250 μl of lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 1% glycerol, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 50 mg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride). Harvested cells were lysed on ice by sonication as described above. Protein concentrations were determined by the BCA (Pierce) protein assay system. Crude samples were adjusted to equal protein concentration in SDS sample buffer, boiled for 4 min, centrifuged in a microfuge for 1 min at high speed and the supernatant proteins electrophoretically separated on 10% sodium dodecyl sulfate-polyacrylamide gels. Proteins were electrophoretically transferred to Hybond-C extra nitrocellulose membrane (Amersham). The membrane was incubated overnight in blocking solution (5% non-fat dry milk in Tris-buffered saline (TBS), pH 8.0, containing 0.2% Tween).

For detection of PKCα, a 1:25 dilution of monoclonal anti-PKCa antibody (Seigagaku, Inc and United Biochemical Corp.) was added for 2 h. For the detection of the *brown* locus, *albino* locus or *slaty* locus protein, anti-PEP1 (63, 64) at a dilution of 1:3000, anti-PEP7 (65) at a dilution of 1:500, or anti-PEP8 (143), at a dilution of 1:3000 were added to the blots for 2 h respectively. For all the antibodies, the solution was removed, and the blot washed several times in blocking solution followed by a 1 h incubation with a 1:3000 dilution of either rabbit anti-mouse IgG conjugated with horseradish peroxidase (Amersham) for anti-PKC or goat anti-rabbit IgG conjugated with horseradish peroxidase (Pharmacia) for anti-PEP1, anti-PEP7 or anti-PEP8. The blots were washed several times in blocking solution and then one final time in blocking solution lacking milk. Reactive bands were visualized by the enhanced chemiluminescence method (Amersham). All washes and incubations were performed at room temperature. All of the immunoblots were quantitated by a computerized laser scanning densitometer (Molecular Dynamics, CA) and the OD units of the peak area compared between control and experimental samples.

RNA Isolation and Northern blot Analysis

RNA was isolated by an acid phenol method. Cells were lysed on ice into Solution A (4M guinidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl and 100 mM β-mercaptoethanol) and transferred into 15 ml polypropylene tubes. For every 2 X 10^6 cells, 1 ml of solution A was used. The following reagents were added

sequentially with vigorous mixing after each addition: 0.1 volume 2 M sodium acetate (pH 4.0), 1 volume water-saturated phenol and 0.2 volume of chloroform:isoamyl alcohol (24:1). The samples were incubated on ice for 15 minutes and then centrifuged at 12, 000 X g for 30 minutes at 10°C. The supernatant was mixed with an equal volume of isopropanol and RNA was precipitated for at least 2 hours at -20°C. Samples were then centrifuged at 12,000 X g for 15 min and the resulting pellet was dissolved in autoclaved DEPC (Diethyl pyrocarbonate)-treated water. Total RNA in the resulting mixture was quantitated by measuring absorbance at 260 nm and stored at -70°C.

RNA samples were electrophoretically separated on a formaldehyde agarose gel and transferred onto Hybond-N (Amersham) nylon membrane by the downward displacement method as described in Sambrook et al. (128). A GAPDH cDNA clone was purchased from the American Type Culture Collection. The cDNA clone for mouse tyrosinase, TRP-1 and TRP-2 were obtained from Drs. Gunther Schutz (Germany), Shigeki Shibahara (Japan) and Ian J. Jackson (United Kingdom) respectively. cDNA fragments were labeled with [^{32}P]dCTP (duPont NEN) using the multiprime labeling kit from Amersham.

Hybridizations were carrried out in 6 X SSC, 2% SDS and 1 X Denhardt's solution at 60°C overnight after prehybridization with 20 mg/ml herring sperm DNA at 68°C for 2 h. Blots were sequentially washed in the following conditions until low background levels were achieved: 0.6 X SSC, 0.1% SDS, 2 X 15 min at room temperature; 0.6 X SSC, 0.1% SDS, 2 X 15 min at 42°C; 0.6 X SSC, 0.1% SDS, 2 X 15 min at 65°C; 0.1

X SSC, 0.1% SDS, 15 min at 42°C. Blots were exposed to Kodak X-ray film with an enhancing screen at -70°C. Autoradiograms were quantitated by densitometer (Molecular Dynamics, CA).

Construction of Fusion genes

pL1, a promoterless luciferase construct, was described previously (154). The construct pMTL1 contains the XbaI/BstNI fragment (positions -270 to +6) derived from the BaLB/c mouse tyrosinase gene cloned upstream from the luciferase gene (154) and was obtained from Dr. Shigeki Shibahara, Tohoku Univ., Sendai, Japan. The XbaI and BstNI sites have been converted to BamHI and XhoI sites, respectively, in the process of construction of the plasmid pMTL1. To construct pMTL2, pMTL1 was cut with BamHI and filled in using klenow enzyme, followed by digestion with HindIII. The BamHI(XbaI)/HindIII fragment (positions -270 to -82) was isolated and digested with RsaI. The restriction site, XbaI, shown within the parentheses, represent the original sequence found in the gene. The resulting RsaI/HindIII fragment (-127 to -82) was ligated to the larger fragment of the pMTL1 obtained after the HindIII digestion in the earlier step. For pMTL4, pMTL1 was digested with BamHI and HindIII, filled in using the Klenow enzyme, and self-ligated. For a construct with an internal deletion, pMTL1(delta)40, pMTL1 was linearized with HindIII, filled in using the Klenow enzyme, and then digested with BamHI. The larger fragment was ligated to the BamHI(XbaI)/RsaI fragment (positions -270 to -127).

Transient Transfections

B16 mouse melanoma and NIH3T3 mouse fibroblast cells, seeded at 70% confluence in a 6-cm dish, were transfected using the calcium phosphate precipitation method (128). The amounts of the DNA were 7 μg of the fusion gene (pMTL1, 2, 1(delta)40 or 4) and 1.4 μg of β-galactosidase expression vector pEF-β-gal(154). The duration of glycerol shock was 2-3 min. Transfected cells were then incubated for 27 h at 37°C and lysed with 100 μl of lysis buffer from the PicaGene luciferase assay system (Toyo Ink, Inc.). Luciferase activity was measured with a Lumat LB9501 luminometer (Berthold). The activity of β-galactosidase was measured following the protocol described in Sambrook et. al (128). The luciferase activity was normalized with the β-galactosidase activity, which was then divided by the value obtained with pMTL4 and shown as relative luciferase activity.

In some experiments with PDBu and RA-mediated regulation of the mouse tyrosinase promoter activity, B16 cells were cotransfected with 1.5 μg of pMTL1 together with 0.5 μg of a β-gal expression plasmid (to correct for transfection efficiency) using the lipofectin procedure according to the manufacturer's protocol (BRL Life Technologies, MD). After a 5 h incubation at 37°C the DNA-containing medium was removed and replaced with growth medium and the cells were returned to the tissue culture incubator for an additional 12 h. The transfected cells were treated with RA (10 μM) or PDBu (1 μM). For the experiments with PDBu the transfected cells were then divided into three groups: the first group was treated with DMSO (control),

the second with 1 μM PDBu for 24 h (chronic treatment) and the third group was treated for the last 1 h of incubation with 1 μM PDBu (acute treatment). All groups were harvested 48 h after transfection and assayed for luciferase activity using a kit supplied by Promega (Madison, WI). Light emissions were detected using a luminometer (Lumat, LB9501, Berthold Industries). The activity of β-galactosidase was measured as described in Sambrook et al. (128). Luciferase activity was normalized to β-galactosidase activity and the effect of PDBu or RA expressed as a ratio to the untreated control (set at 100%).

Preparation of nuclear extracts

B16 cells were washed with ice-cold PBS, scraped into PBS and collected by centrifugation (30 sec 1000 X g) in a microfuge. Cells were disrupted in NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$ and 1% NP-40). Nuclei were recovered by centrifugation (5 min at 500 X g) and washed twice with ice-cold NP-40 lysis buffer. The nuclear pellet was then resuspended in extraction buffer (0.05 M Tris-HCl, pH 7.4, 10% v/v glycerol, 0.01 M monothioglycerol, 0.0001 M Na_2EDTA, 0.0001 M PMSF, 0.6 M KCl and 2 μg/ml each of aprotinin, leupeptin and pepstatin), sonicated 3 X 10 s, incubated for 2-3 h at 4°C and centrifuged at 100,000 X g for 1 h to obtain a soluble nuclear extract.

Gel Mobility Shift Assay

Double stranded oligonucleotides corresponding to the wt1 region (Fig. 9A) containing the M box region of the mouse tyrosinase promoter or AP1 site of the collagenase promoter (1) or a 33 bp oligonucleotide containing the RARE element, corresponding to a region in the promoter of the human RARβ gene (137) were used as probes in gel shift analysis. The respective double-stranded oligonucleotides were labeled with [32^{P}]dCTP (3000 Ci/mmol) by end-filling of the 5' protruding termini with the Klenow fragment of DNA polymerase I. The labeled oligonucleotides were purified using a G25-Sephadex column. Gel shift reactions typically contained 10 µg of nuclear protein from B16 cells harvested at the indicated times with or without treatment with PDBu or RA. Gel shift reactions typically contained 10 µg of B16 extracts or sf9 cell extracts from recombinant hRARα1 baculovirus-infected cells (1 µg) (122). The extracts were incubated with 30,000 DPM of labeled oligonucleotides in a reaction mixture (total volume 25 µl) containing 5 µl of 5X incubation buffer (1 M Tris-HCl, pH 7.5, 0.5 M EDTA, pH 8.0, 3 M NaCl, 3.2 µl β-mercaptoethanol and 4% glycerol) and 1 µg poly dI-dC for 25 min at room temperature and separated on a 6% polyacrylamide gel (60:1 acrylamide:bis) in 0.25 X TBE (0.045 M Tris-borate, pH 8.0, 0.0001 M Na_2EDTA) at 20 mA at 23°C. For antibody supershift experiments, 1-2 µg (in 1-2 µl) of the appropriate antibody was added to the reaction mixture after the initial incubation of nuclear extract with the radioactive oligonucleotide. This mixture was then incubated for an additional 45 min before separation in polyacrylamide gels. The antibodies

against c-jun and c-fos were obtained from Santa Cruz Biotechnology Inc., CA. In experiments with MITF, 3 µl of antiserum specific for MITF (obtained from Dr. Tachibana, NIDCD, NIH) was used. The identity of shifted bands as RARs was accomplished by incubating the B16 nuclear extracts or sf9 extracts with 1 µg of unfractionated serum from the γ-IIIB polyclonal anti-RAR antibody which cross-reacts with all the RAR subtypes (122). This mixture was then incubated for an additional 45 min before separation in polyacrylamide gels. After separation, the gels were dried and autoradiographed to detect protein-DNA complexes.

PART I

ROLE OF PKC IN MELANOGENESIS

SUMMARY

Melanogenesis is regulated by a variety of environmental and hormonal factors. In this part of the study we showed that protein kinase C (PKC) plays a major role in regulating melanogenesis in B16 mouse melanoma cells. Chronic treatment of B16 cells with PDBu resulted in a concentration-dependent loss of density-dependent induction of tyrosine hydroxylase activity, which was positively correlated with a concentration-dependent loss of PKC enzyme activity. Different phorbol derivatives inhibited tyrosine hydroxylase activity and depleted cellular PKCα in a manner which reflected their reported tumor promoting activity. Western blotting analysis showed that PDBu decreased the amount of the *brown* locus gene product (TRP-1) by 50% and lowered the amount of the *albino* locus gene product (tyrosinase) to undetectable levels. None of the phorbol derivatives affected the level of the *slaty* locus protein (TRP-2). Northern analysis suggested that PKCα may regulate the steady state levels of the respective mRNA of tyrosinase and TRP-1. In addition to inhibiting the density-dependent increase in tyrosine hydroxylase activity, PDBu inhibited some, if not all of the 8-bromocyclic AMP-induced increase in tyrosine

hydroxylase activity. This was accompanied by a decrease in the amount of tyrosinase protein induced by 8-bromocyclic AMP. Although 8-bromocyclic AMP did not change the level of TRP-1, it did reverse the decrease in the amount of this protein induced by PDBu. The amount of TRP-2 was not altered by any of these agents. These data suggest that PKC regulates melanogenesis primarily by controlling the constitutive expression of tyrosinase and, to a lesser extent, TRP-1.

RESULTS

A: PKC and density-dependent melanogenesis

Effect of PDBu on tyrosine hydroxylase activity- During the course of a previous study (104), we observed that treatment of B16 cells with PDBu prevented the density-dependent increase in melanin production that normally occurs in these cells. To investigate this finding, we treated B16 cells with different concentrations of PDBu for 72 h, then harvested the cells and assayed for tyrosine hydroxylase activity. Addition of 0.06 μM PDBu resulted in an 80% reduction in tyrosine hydroxylase activity, while 0.1 μM decreased the activity by 96% when compared to the control cells (Fig 2 A). Increasing the concentration of PDBu to 1 and 5 μM further decreased tyrosine hydroxylase activity to a level that was barely above the background for this enzyme assay.

B16 cells were treated with 1 μM PDBu for different time periods and assayed for tyrosine hydroxylase activity (Fig 2 B). Control cells exhibited a time-dependent increase in tyrosine hydroxylase activity that correlated with an increase in cell density. The greatest rate of increase in enzyme activity occured between 48-56 h, with an additional increase at 72 h. At all times tested, tyrosine hydroxylase activity from cells treated with PDBu was barely above the background level for the assay.

Figure 2

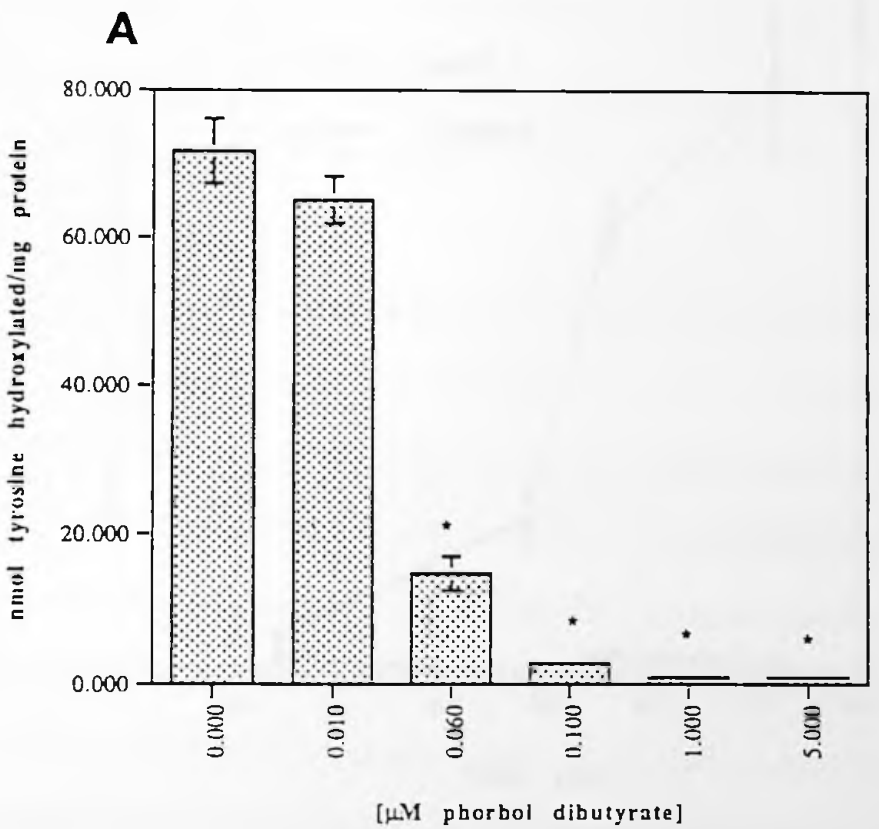
Concentration and time-dependent effect of PDBuon B16 mouse melanoma tyrosine hydroxylase activity

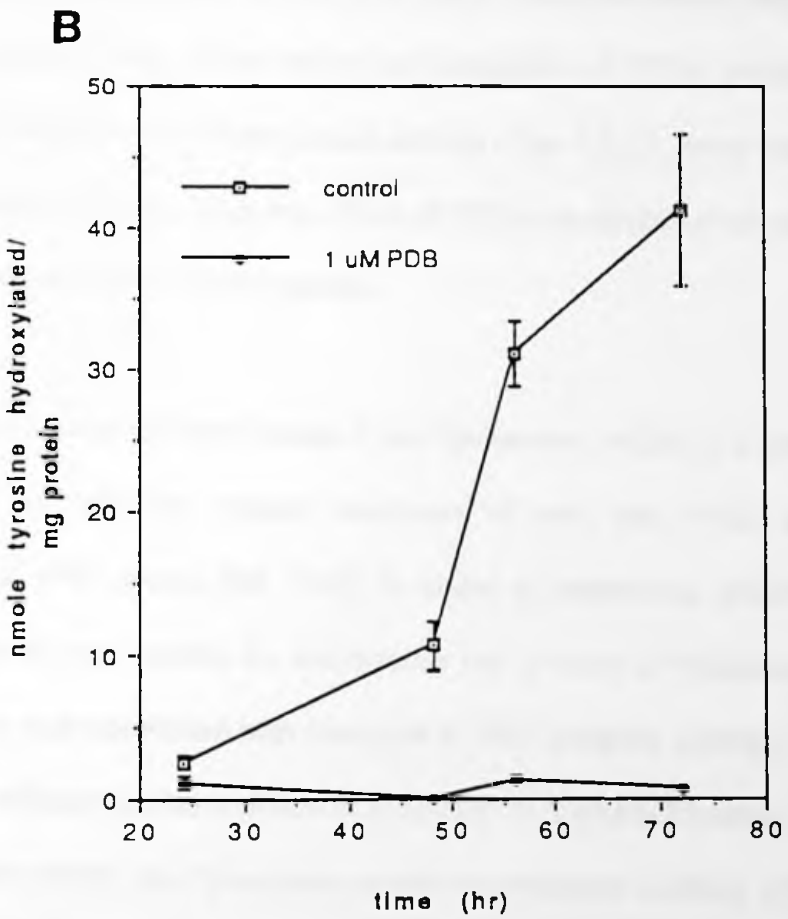
A. Concentration-dependent effect of phorbol dibutyrate on B16 mouse melanoma tyrosine hydroxylase activity.

B16 cells were treated for 72 h with the concentrations of phorbol dibutyrate listed. Control cells received 0.05% DMSO, the solubilization vehicle. At the end of the incubation, cells were harvested and protein content and tyrosine hydroxylase activity assayed as described in the methods section. The data are presented as mean +/- SEM (error bars) of three separate experiments. Samples without apparent error bars had errors too small to be plotted by the computer graphics program. The * indicates that the samples were significantly different form control (DMSO) at the $p<0.01$ confidence level.

B. Time-dependent change of tyrosine hydroxylase activity in control and phorbol dibutyrate-treated B16 mouse melanoma cells.

B16 cells were treated with either 0.05% DMSO, or 1 μM phorbol dibutyrate. At the indicated times, cells were harvested and assayed for protein content and tyrosine hydroxylase activity as described in the methods section. The data is presented as the mean +/- SEM (error bars) of three separate experiments.


A
80.000
60.000
40.000
20.000
0.000
nmol tyrosine hydroxylated/mg protein
0.000
0.010
0.060
0.100
1.000
5.000
[μM phorbol dibutyrate]


B
50
40
30
20
10
0
20
30
40
50
60
70
80
control
1 uM PDB
nmole tyrosine hydroxylated/
mg protein
time (hr)

Effect of PDBu on growth of B16 melanoma cells- Since the increase in tyrosine hydroxylase activity is density-dependent, one possible explanation for the effect of PDBu on inhibition of this enzyme would be an effect on cell proliferation. To test this possibility we treated B16 cells with 1 μM PDBu and measured its effect on the growth of these cells. At the same concentration of PDBu which was shown to drastically inhibit tyrosine hydroxylase activity (Figs 1 & 2), there was little effect on cell proliferation (Fig 3). Thus the effect of PDBu on inhibition of melanogenesis is not due to an inhibition of cell growth.

Relationship between protein kinase C and tyrosinase- PDBu is a potent activator of PKC (117). In addition, chronic treatment of cells with PDBu leads to down-regulation of PKC levels (55, 110). In order to determine whether PDBu was inhibiting tyrosinase activity by decreasing the amount of tyrosinase protein and whether this was correlated with changes in PKC enzyme activity, B16 cells were treated with different concentrations of PDBu for 72 h and cell lysates were tested for PKC enzyme activity and tyrosinase protein by Western blotting. PDBu induced a concentration-dependent decrease in tyrosinase protein level which was roughly correlated with a decrease in PKC enzyme activity (Fig 4A and B). These data suggest that PKC may be required for expression of tyrosinase and its depletion (by chronic PDBu treatment) leads to inhibition of melanogenesis.

Figure 3

Effect of phorbol dibutyrate on the growth of B16 mouse melanoma cells.

B16 cells were seeded in growth medium at 1 X 10^5 cells/100 mm tissue culture dish. After allowing the cells to attach overnight, they were refed with growth medium containing either 1 μM phorbol dibutyrate or DMSO (solvent for phorbol dibutyrate). At the indicated times, triplicate dishes from each treatment group were harvested and cell number determined by hemacytometer counting. The data are presented as the mean +/- SEM (error bars) of triplicate dishes. In cases where error bars are not shown, the SEM was too small to be represented in this computer drawn graphic curve.

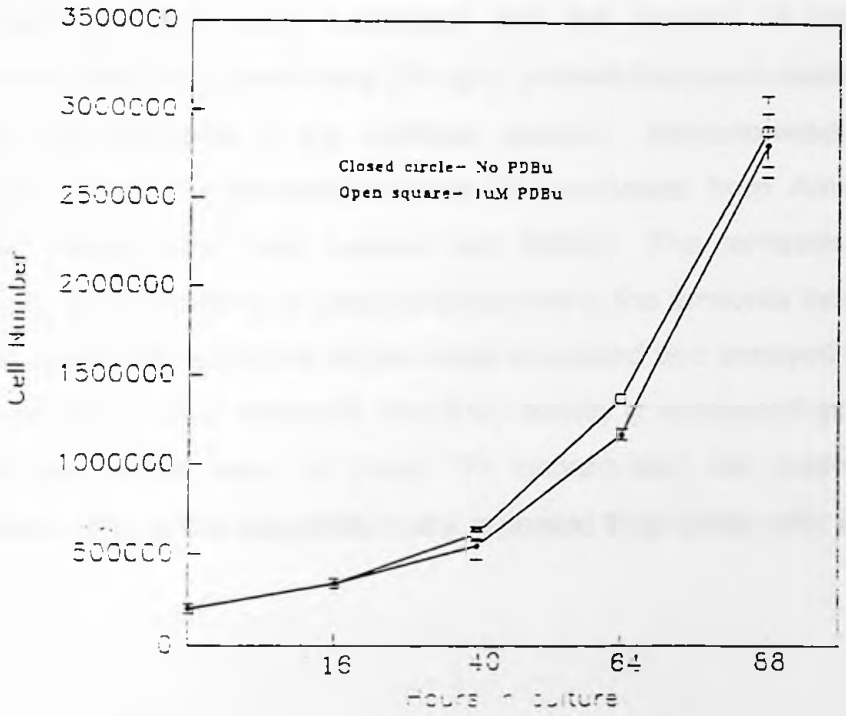
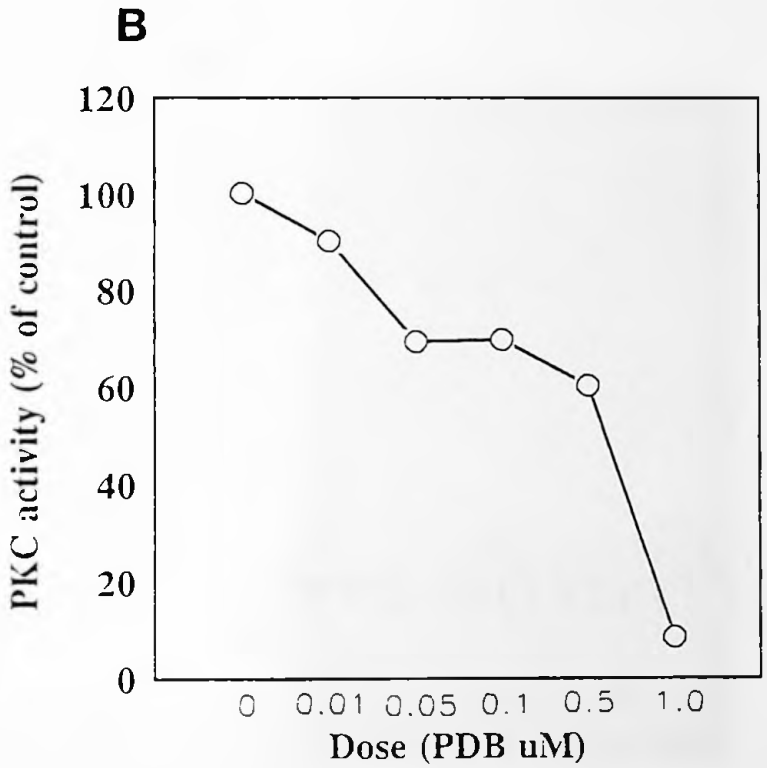
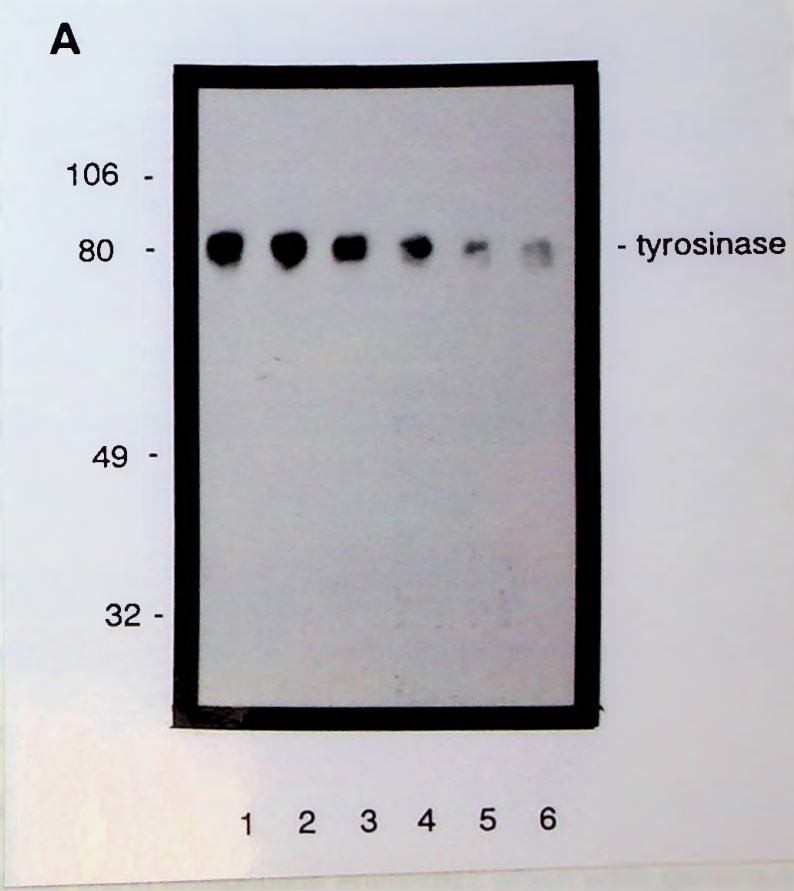

3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Cell Number
Closed circle- No PDBu
Open square- 1uM PDBu
16
40
64
88

Figure 4

Concentration-dependent changes in PKC enzyme activity and tyrosinase protein level induced by phorbol dibutyrate in B16 cells.

B16 cells were incubated for 72 h with the indicated concentrations of phorbol dibutyrate. (**A**) Cells were harvested and the amount of tyrosinase protein determined using immunoblotting (50 μg of protein from each sample) with a pep-7 antibody as described in the methods section. Immuno-reactive bands were visualized using a chemiluminescence kit purchased from Amersham. Lane 1 contains protein from cells treated with DMSO. The remaining lanes contain increasing concentrations of phorbol dibutyrate at the amounts indicated in the part B of this figure. (**B**) Replicate dishes were harvested and assayed for PKC enzyme activity as described in Methods. The PKC activity is expressed as a percent of the control level which was 1.8 pmol ^{32}Pi incorporated into peptide substrate/μg protein/min. The entire experiment was repeated three times with similar results.


B
120
100
80
60
40
20
0
PKC activity (% of control)
0
0.01
0.05
0.1
0.5
1.0
Dose (PDB uM)


A
106 -
80 -
49 -
32 -
- tyrosinase
1 2 3 4 5 6

Effect of phorbol derivatives on PKCα, tyrosinase activity and protein level, TRP-1 and TRP-2 protein levels- In order to obtain further evidence for the involvement of PKCα in the regulation of tyrosinase, we measured the ability of different phorbol derivatives to inhibit tyrosine hydroxylase enzyme activity, down-regulate PKCα, and alter the level of tyrosinase, TRP-1 and TRP-2 proteins. Among the compounds tested, phorbol treatment resulted in a small but reproducible increase in tyrosine hydroxylase activity, while both phorbol diacetate and PDBu treatment of cells consistently decreased tyrosine hydroxylase activity, with the latter compound giving the greatest inhibition (Fig 5 A).

Next, we examined the ability of these phorbol derivatives to downregulate PKCα (Fig 5 B). A 72 h treatment with phorbol resulted in a small reduction in PKCα levels. In contrast, PKCα protein in phorbol diacetate-treated cells was reduced by 83%, while PDBu treated cells had only 6% of the amount of PKCα found in control cells (determined by scanning densitometry).

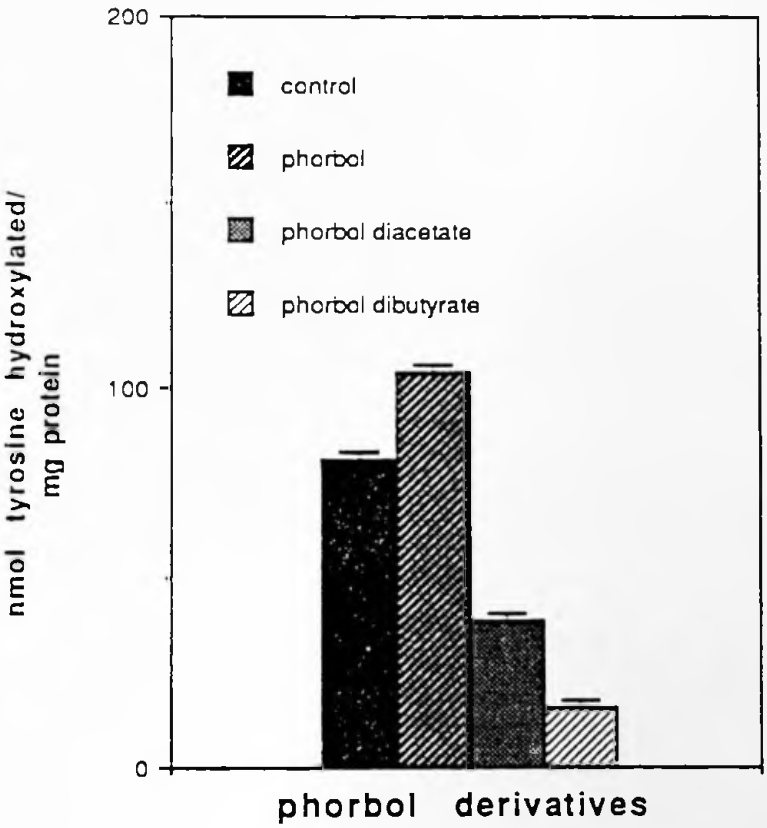
We examined the level of three proteins thought to be involved in the regulation of melanogenesis, using anti-peptide antibodies which were generously provided by Dr. Hearing (NIH). TRP-1 is a protein encoded by the *brown* locus gene. Although it has some tyrosine hydroxylase and DOPA oxidase activity (66), its exact role in melanogenesis is yet to be defined. As previously reported (63), B16 cells express a 70-72 kDa protein recognized by TRP-1 specific antibody. We found that phorbol

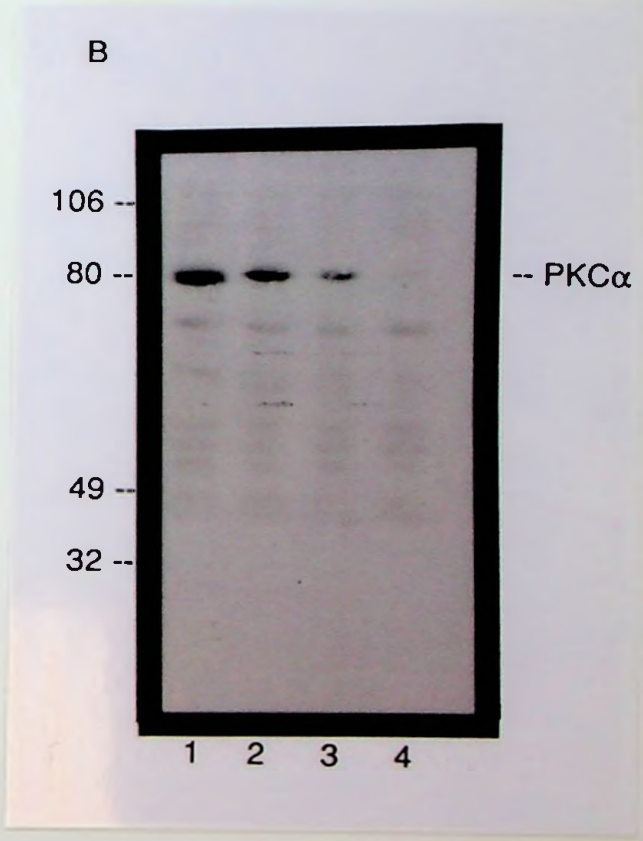
Figure 5

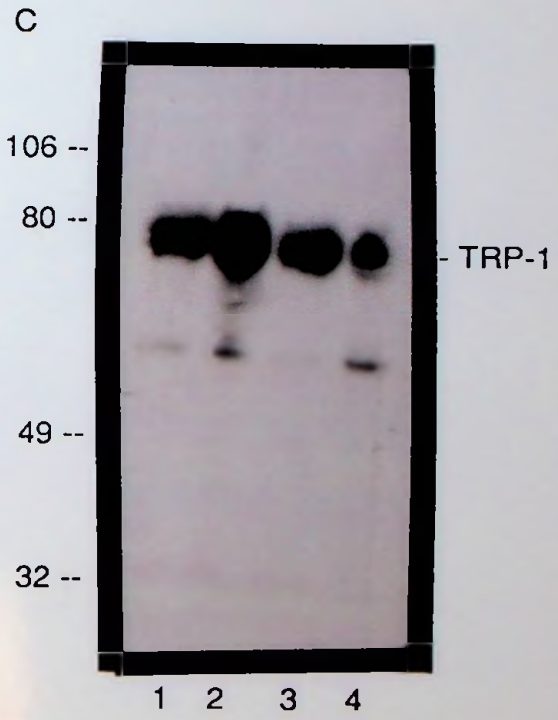
The effect of different phorbol derivatives on tyrosine hydroxylase activity, PKCα levels, TRP-1, tyrosinase and TRP-2 protein levels in B16 mouse melanoma cells.

B16 mouse melanoma cells were treated for 72 h with phorbol, phorbol diacetate, phorbol dibutyrate or DMSO (control). All phorbol compounds were used at a concentration of 1 μM. At the end of the incubation, cells were harvested in extraction buffer for (**A**) tyrosine hydroxylase assay, and aliquots were taken for immunoblot assay of (**B**) protein kinase Cα (100 μg protein), (**C**) TRP-1 (50 μg protein), (**D**) tyrosinase (50 μg protein) and (**E**) TRP-2 (50 μg protein) levels. Details of tyrosine hydroxylase enzyme assay and immunoblotting are described in the methods section. In panels B-E; lane 1- control, lane 2- phorbol, lane 3- phorbol diacetate, lane 4- phorbol dibutyrate. Tyrosine hydroxylase activity is expressed as the mean +/- SEM (error bars) of three separate experiments.

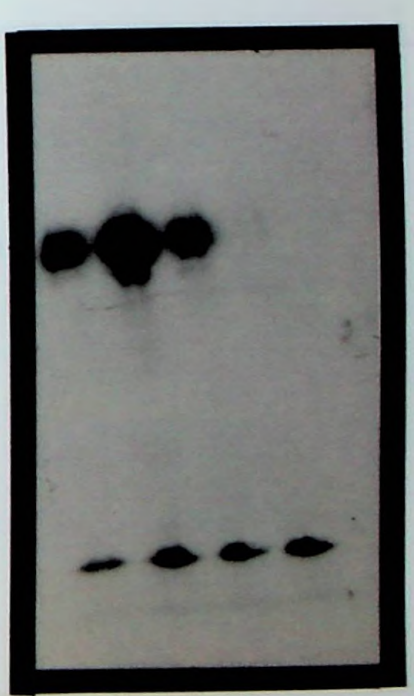
A

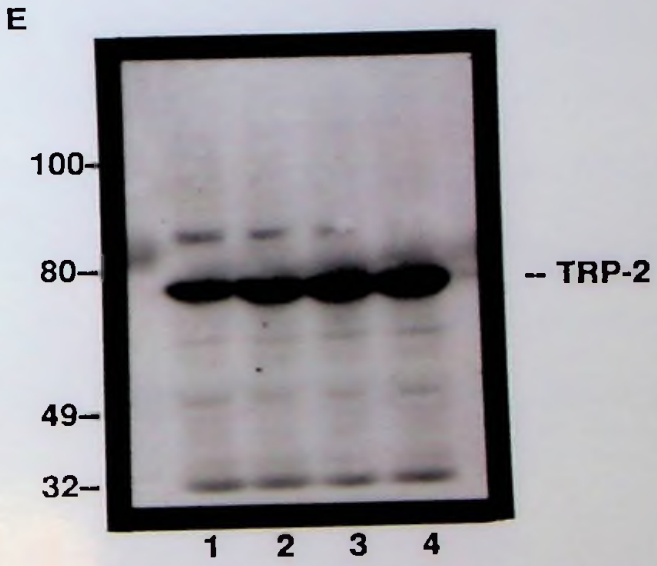

200
100
0
control
phorbol
phorbol diacetate
phorbol dibutyrate
nmol tyrosine hydroxylated/
mg protein
phorbol derivatives


B
106 --
80 --
-- PKCα
49 --
32 --
1
2
3
4


C
106 --
80 --
49 --
32 --
TRP-1
1 2 3 4

D




E
100
80
49
32
-- TRP-2
1
2
3
4

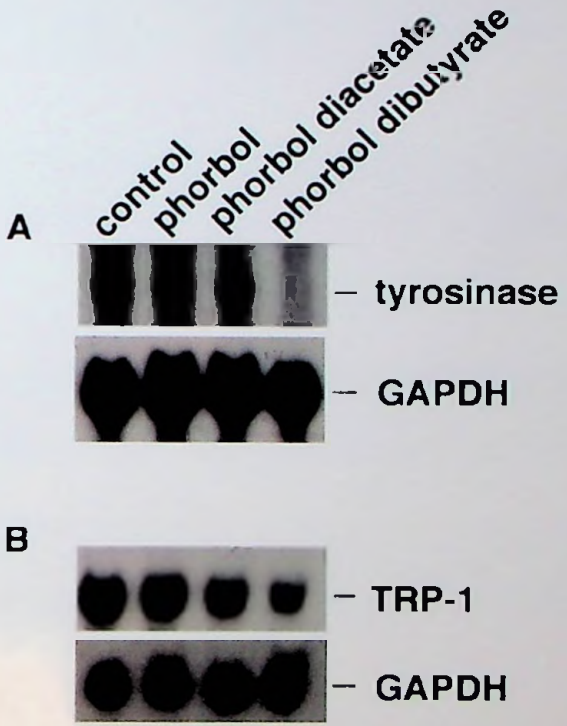
consistently increased TRP-1 levels by roughly 2-fold, while PDBu consistently decreased (40-60% depending on the experiment) TRP-1 levels (Fig 5C). Tyrosinase is now thought to be encoded by the *albino* locus gene. Using antibodies specific for this protein, we detected a 68-70 kDa protein expressed in B16 cells (Fig 5 D). Shorter exposures of the autoradiograms revealed this protein to be a doublet. As in the case of TRP-1, treatment with phorbol led to a consistent 3-fold increase in tyrosinase protein, however in contrast to TRP-1, treatment of B16 cells with PDBu resulted in the complete loss of immunoreactive tyrosinase protein. TRP-2 maps to the mouse *slaty* locus on chromosome 14 and has recently been shown to encode DOPAchrome tautomerase (143). B16 cells express a protein of 79 kDa which reacts with peptide-antisera specific for TRP-2 (Fig 5E). In contrast to the effect of the various phorbol compounds on TRP-1 and tyrosinase, phorbol esters had little effect on the amount of TRP-2. In some experiments, there was a small increase in TRP-2 (20-37%) in PDBu-treated cells.

Effect of phorbol derivatives on tyrosinase, TRP-1 and TRP-2 mRNA levels- To determine if phorbol ester-induced changes in tyrosinase, TRP-1 and TRP-2 levels were reflected in changes in their respective mRNA levels, we extracted total RNA from cells treated for 72 h with DMSO (control), phorbol, phorbol diacetate or PDBu. The relative amount of the appropriate mRNA was determined by Northern analysis. Tyrosinase mRNA levels were reduced approximately 50% by phorbol diacetate and are not detectable in cells treated with PDBu (Fig 6 A). TRP-1 mRNA levels were

Figure 6

The effect of different phorbol derivatives on mRNA level of tyrosinase and TRP-1

B16 cells were treated for 72 h with DMSO (control), phorbol, phorbol diacetate and phorbol dibutyrate for 72 h. All phorbol compounds were used at a concentration of 1 μM. At the end of the incubation cells were harvested and RNA extracted and purified. 20 μg of RNA from each sample was fractionated on formaldehyde agarose gels, blotted onto nylon membranes (Amersham) and hybridized with the appropriate ^{32}P-labeled cDNA plasmid as described in the Methods. The blots probed for (**A**) tyrosinase and (**B**) TRP-1 were also assayed for GAPDH mRNA.


control
phorbol
phorbol diacetate
phorbol dibutyrate
A
tyrosinase
GAPDH
B
TRP-1
GAPDH

reduced by about 30% with phorbol diacetate treatment of cells, but were reduced by 75% in cells treated with PDBu (Fig 6B). Lastly,TRP-2 mRNA levels were reduced by about 60% only in cells treated with PDBu (data not shown). Thus the changes in mRNA for these melanogenic factors induced by phorbol esters roughly parallels changes in protein levels.

B: PKC and cAMP-induced melanogenesis

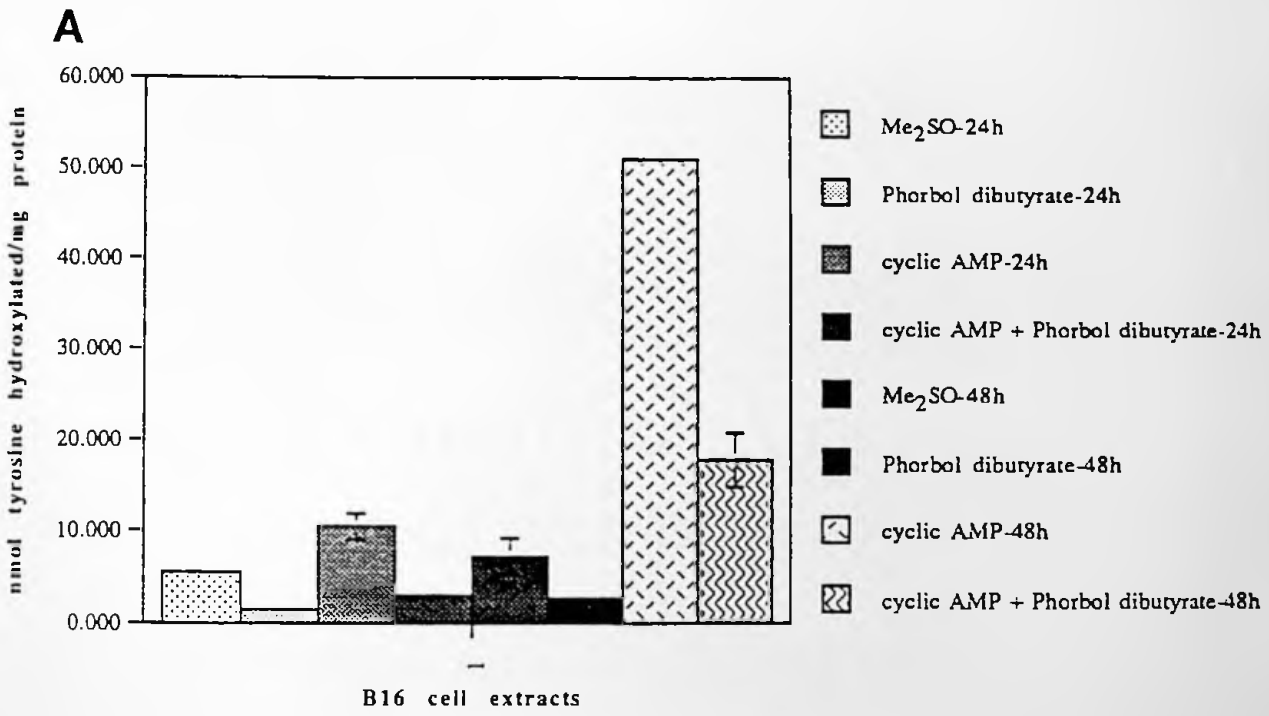
Effect of PDBu on 8-bromo-cyclic AMP-induced melanogenesis: All of the experiments reported thus far, have examined the ability of phorbol esters to inhibit density-dependent melanogenesis (78). Melanocyte-stimulating hormone (MSH) increases melanin production in B16 cells even at low cell density, where "spontaneous" melanogenesis is minimal. MSH stimulates cyclic AMP production (82, 150), which mediates the action of MSH on melanin production. Therefore, we examined whether PDBu can also inhibit hormone-induced melanogenesis, using 8-bromo-cyclic AMP as a surrogate for MSH-induced increase in cyclic AMP.

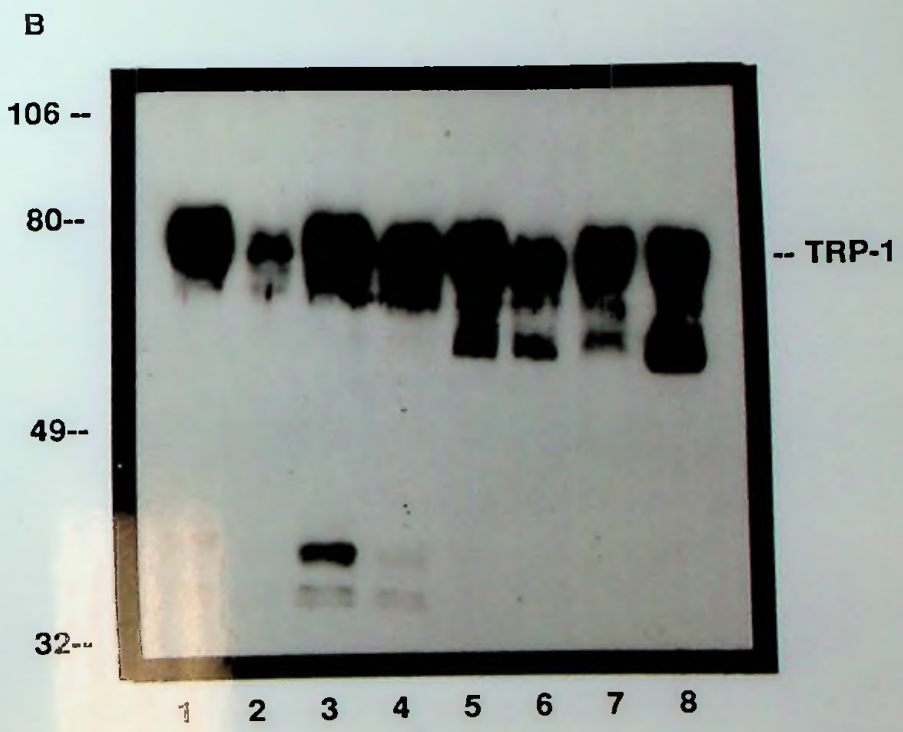
B16 cells were preincubated for 24 h with DMSO (control) or 1 μM PDBu. Each of these groups were then incubated with 0.5 mM 8-bromo-cyclic AMP + 0.2 mM 1-methyl, 3-isobutylxanthine (cyclic AMP phosphodiesterase inhibitor) for an additional 24 or 48 h. Cells harvested at these time points were examined for tyrosine hydroxylase activity, TRP-1, tyrosinase and TRP-2 protein levels. 8-bromo-cyclic

Figure 7

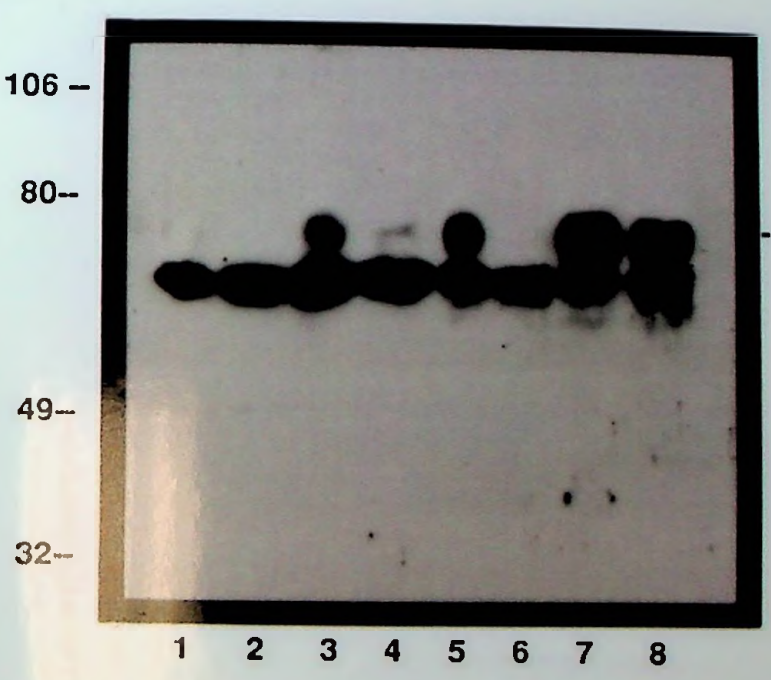
Effect of 8-bromo-cyclic AMP with or without phorbol dibutyrate on B16 mouse melanoma tyrosine hydroxylase activity, and TRP-1, tyrosinase and TRP-2 levels.

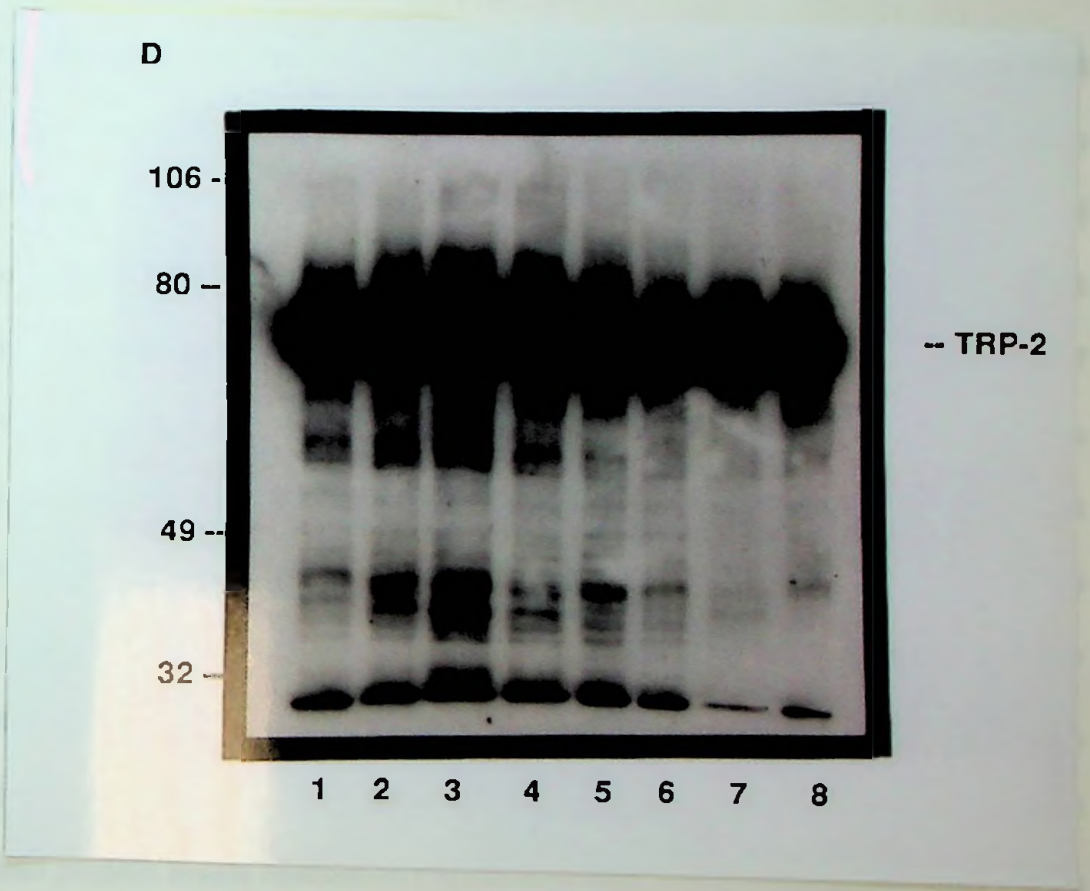
B16 cells were incubated for 24 h with or without 1 μM PDBu. At this time replicate dishes were refed with 0.5 mM 8-bromo-cyclic AMP + 0.2 mM 1, methyl, 3 isobutylxanthine in the absence or continued presence of 1 μM PDBu for an additional 24 or 48 h. Control cells received DMSO, the solubilization vehicle for PDBu. At the indicated times cells were harvested and assayed for protein content and (**A**) tyrosine hydroxylase activity, (**B**) TRP-1, (**C**) tyrosinase and (**D**) TRP-2 protein levels as described in the text. The tyrosine hydroxylase activity in panel A is expressed as the mean +/- SEM (error bars) of three separate experiments. Samples lacking apparent error bars had errors too small to be plotted by the computer graphics program. In panels B-D; lane 1- 24 h control (DMSO), lane 2- 24 h PDBu,lane 3- 24 h 8-bromo-cyclic AMP, lane 4- 24 h PDBu + 8-bromo-cyclic AMP, lane 5- 48 h control (DMSO), lane 6- 48 h PDBu, lane 7- 48 h 8-bromo-cyclic AMP and lane 8- 48 h PDBu + 8-bromo-cyclic AMP.


A
60.000
50.000
40.000
30.000
20.000
10.000
0.000
nmol tyrosine hydroxylated/mg protein
B16 cell extracts
Me2SO-24h
Phorbol dibutyrate-24h
cyclic AMP-24h
cyclic AMP + Phorbol dibutyrate-24h
Me2SO-48h
Phorbol dibutyrate-48h
cyclic AMP-48h
cyclic AMP + Phorbol dibutyrate-48h


B
106 --
80--
49--
32--
-- TRP-1
1
2
3
4
5
6
7
8

C




D
106 -
80 –
49 --
32 –
-- TRP-2
1 2 3 4 5 6 7 8

AMP increased tyrosine hydroxylase activity by 2 fold following 24 h of treatment, and five-fold after 48 h of incubation. PDBu inhibited tyrosine hydroxylase activity relative to the control cells at both 24 and 48 h of treatment. This phorbol ester also reduced the degree of the 48 h 8-bromo-cyclic AMP-mediated stimulation of enzyme activity by 65%, but did not eliminate all of the 8-bromo-cyclic AMP induced tyrosine hydroxylase activity (Fig 7 A).

We also examined the level of TRP-1, tyrosinase and TRP-2 in the different treatment groups (Fig 7 B-D). PDBu decreased TRP-1 levels as compared to control cells at both 24 and 48 h of incubation. 8-bromo-cyclic AMP treatment did not result in any consistent increase or decrease in TRP-1 levels at either 24 or 48 h of treatment. However, 8-bromo-cyclic AMP did reverse the PDBu inhibition of TRP-1 expression at both 24 and 48 h treatment times (Fig 7 B; compare lane 2 vs lane 4 and lane 6 vs lane 8). PDBu also induced a complete loss of tyrosinase protein following 24 and 48 h of incubation (a faint band was visible at 24 h in the control cells on the original autoradiogram). Treatment with 8-bromo-cyclic AMP for 24 h resulted in a large increase in tyrosinase protein relative to untreated cells, while a 48 h treatment with 8-bromo-cyclic AMP gave a further 4-fold increase in tyrosinase protein levels relative to the control cells. Cells treated with both PDBu and 8-bromo-cyclic AMP for 24 h still contained very low amounts of tyrosinase protein. At 48 h of incubation with both agents tyrosinase protein was decreased by 33% compared to cells treated only with 8-bromo-cyclic AMP (Fig 7 C; compare lanes 7 vs 8). Interestingly, there was a lower MW band that strongly reacted with

the antiserum and was present in all the samples. Lastly we examined the amount of TRP-2 (Fig 7D). There was no consistent difference in the level of TRP-2 among the different treatment groups at 24 h of incubation. At 48 h of incubation, however, there appeared to be a slight decrease in the amount of TRP-2 in all groups when compared to the 24 h levels.

DISCUSSION

The regulation of melanogenesis in cultured melanoma cells is complex. Hormones (82, 150), cell density (78, 147) and pH (80) can all influence melanin production. We have provided evidence that PKC plays an important role in the regulation of melanogenesis in B16 mouse melanoma cells. This conclusion is supported by the loss of tyrosinase activity (Fig 2 A and B) and melanin production (visual observation) in cells depleted of PKCα by chronic treatment with PDBu. In addition, we found that there was a rough correlation between the concentration-dependent ability of PDBu to down-regulate PKC and to decrease tyrosinase protein levels (Fig 4 A & B). The correlation is not perfect because PKC levels may have to decrease below a certain critical level before tyrosinase expression is affected. Also, cells that overexpressed PKCα had higher levels of tyrosinase activity and produced more melanin (42). The combined weight of these data strongly suggest that PDBu inhibits tyrosinase via its ability to down-regulate PKC, rather than by constant stimulation of PKC enzyme activity. We believe that PKCα is the isoform involved in the regulation of melanogenesis, since it is the only conventional PKC expressed in B16 cells (126). Although we have detected mRNA for PKCε and PKCζ, the amount was extremely low and neither form was increased by retinoic acid (unpublished), which we have shown to induce PKCα (104) and to stimulate melanogenesis (42). Several other investigators have

recently reported that PKC may be involved in the stimulation of melanogenesis in cultured melanoma cells and melanocytes by UV irradiation (32, 40) and cytokines (59).

Different phorbol ester derivatives have varying abilities to promote carcinogenesis in the mouse skin model (6). The tumor-promoting ability has previously been correlated with their ability to inhibit melanogenesis (98). With the exception of phorbol, the ability of these compounds to down-regulate PKCα correlated with their effect on inhibition of tyrosine hydroxylase activity and their reported skin-tumor promoting activity. Similar results were reported by Park et al. (116) who found that the level of PKC correlated with the degree of melanin production by melanocytes from various sources.

The mechanism by which phorbol esters, *via* their ability to down-regulate PKCα, inhibit tyrosine hydroxylase activity and melanin production does not appear to involve the increased production or activation of tyrosinase inhibitors (20, 68), since mixing extracts of control and PDBu-treated cells, gave the predicted additive enzyme activity (data not shown). This finding is in contrast to that of Park et al. (116) who reported that PKCβ restored tyrosinase activity in human amelanotic melanoma cells by "activating" the enzyme. They found that tyrosinase protein levels were similar in both amelanotic and melanotic cells.

The function of TRP-1 in melanin production is not known. The protein does have some tyrosine hydroxylase and DOPA oxidase activity, but its specific activity

is considerably lower than tyrosinase. Thus the residual tyrosine hydroxylase activity remaining in PDBu-treated cells (Fig 5 A), in spite of complete absence of tyrosinase protein (Fig 5 D), may be due to TRP-1. The positive effect of phorbol on tyrosine hydroxylase activity is probably the result of the increased level of both TRP-1 and tyrosinase protein induced by phorbol.

In 8-bromo-cyclic AMP-treated cells, there was not a strict temporal or quantitative correlation between tyrosinse hydroxylase activity and tyrosinase protein levels. These findings may be explained in part by reports that suggest that the 8-bromo-cyclic AMP-induced increase in tyrosinase activity involves an increase in the catalytic activity of the enzyme (34, 63), as well as increases in the synthesis of the protein. In contrast to these findings, Martinez-Lierte et al (95), reported that MSH and 8-bromo-cyclic AMP decreased dopachrome tautomerase activity (TRP-2) in B16 cells. Since we failed to detect any decrease in TRP-2 levels, this result suggests that these agents do not decrease dopachrome tautomerase activity by altering the amount of TRP-2, but rather affect enzyme activity, either by directly modifying TRP-2 by phosphorylation, or by increasing the amount or activity of an inhibitory molecule.

Interestingly, 8-bromo-cyclic AMP, which did not alter TRP-1 levels by itself, reversed the decrease in TRP-1 induced by PDBu. This result suggests that cyclic AMP may interfere with the ability of PDBu to deplete the cells of PKC. PDBu was not able to completely inhibit the 8-bromo-cyclic AMP-induced increase in tyrosinase protein (Fig 7C). These data correlate with the ability of PDBu to

reverse some, if not all of the 8-bromo-cyclic AMP-induced increase in tyrosine hydroxylase enzyme activity (Fig 7 A). Similar results were found by Fuller et al. (35), in Cloudman S91 melanoma cells. They reported that tetradecanoyl phorbol acetate (TPA) was not able to completely reverse the stimulation of tyrosine hydroxylase activity by 8-bromo-cyclic AMP.

Lastly, the presence of a protein that reacted strongly with the antiserum to tyrosinase (Fig 7C) is intriguing. Two observations argue against this being a non-specific protein. First, it was not present when pre-immune serum was used in place of the antiserum (data not shown), and second, this protein was only observed in Western blots using proteins from lower density B16 cells in experiments examining cyclic AMP-regulated melanogenesis. The same MW protein was not found where density-dependent melanogenesis was present (Fig 5 D). The amount of this cross-reacting protein did not correlate with the amount of tyrosinase, although it still remains a possibility that it could be a proteolytic product of tyrosinase, which retains the antigenic epitope. The identification of this protein will require further investigation.

These results strongly suggest that in B16 mouse melanoma cells PKC regulates melanogenesis by controlling the expression of both tyrosinase and TRP-1.

PART II

REGULATION OF MOUSE TYROSINASE PROMOTER FUNCTION

SUMMARY

In the first part of the study we showed that depletion of protein kinase C (PKC) by chronic treatment of B16 mouse melanoma cells with PDBu prevented cell density-dependent melanogenesis. This was accompanied by a lack of induction of tyrosinase protein and mRNA. In this part of the study, we identified a 45 bp region that is critical for the efficient transcription of the tyrosinase gene by deletion analysis. The central portion of this region co-localizes with the "M-box" that is conserved in melanocyte promoters and is flanked by an AP-1-like site. We investigated the effect of cell density and PDBu on the functional activity of the mouse tyrosinase promoter by a reporter gene assay and the binding of nuclear proteins from B16 cells. Acute PDBu treatment of B16 cells transfected with a mouse tyrosinase promoter-luciferase construct resulted in increased reporter gene activity, while chronic treatment inhibited reporter gene activity. Using an oligonucleotide containing the "M-box" and its flanking residues in electrophoretic mobility shift assays, we found a density-dependent change in the pattern of DNA-protein complexes. One complex was found to be negatively regulated by chronic treatment. Competition experiments with

various mutated oligonucleotides demonstrated that both the M-box and flanking residues are important for nuclear protein binding. The complex whose formation was inhibited by chronic treatment was shown to contain the basic helix-loop-helix leucine zipper (bHLH.Zip) protein, microphthalmia-associated transcription factor (MITF). Acute treatment of B16 cells with PDBu (which stimulated mouse tyrosinase promoter activity) resulted in the formation of an additional complex containing c-fos. These results suggest that tyrosinase gene expression is controlled by a number of different transcription factors. One of these factors, MITF, is likely to be regulated by PKC.

RESULTS

A: Transcriptional regulation

Identification of cis-acting elements in the transcriptional regulation of the mouse tyrosinase promoter: It has been previously found that a 270 bp fragment containing the mouse tyrosinase promoter is sufficient to confer pigment cell specific expression of the gene in transgenic mice. To confirm this finding in our cell culture model, we transfected a plasmid pMTL1 containing the 270 bp promoter region cloned upstream of the firefly luciferase reporter gene into B16 cells and compared the reporter activity obtained in non-pigmented NIH 3T3 cells. The fusion gene showed low levels of activity in NIH 3T3 cells confirming that this region mediates preferential expression of the tyrosinase gene in pigmented cells (data not shown).

Within the 270 bp promoter region of pMTL1, several potential binding sites for transcription factors were identified by searching the transcription factor database (Fig 8 A). This included three CANNTG motifs (nucleotide positions -214 to -209, -104 to -99 and -12 to -7). To identify potential *cis*-acting elements responsible for transcription of the tyrosinase gene, we performed deletion analysis of its promoter region in B16 mouse melanoma cells (Fig 8 B). In this series of experiments, the relative luciferase activity is shown as the ratio to the normalized values obtained

Figure 8

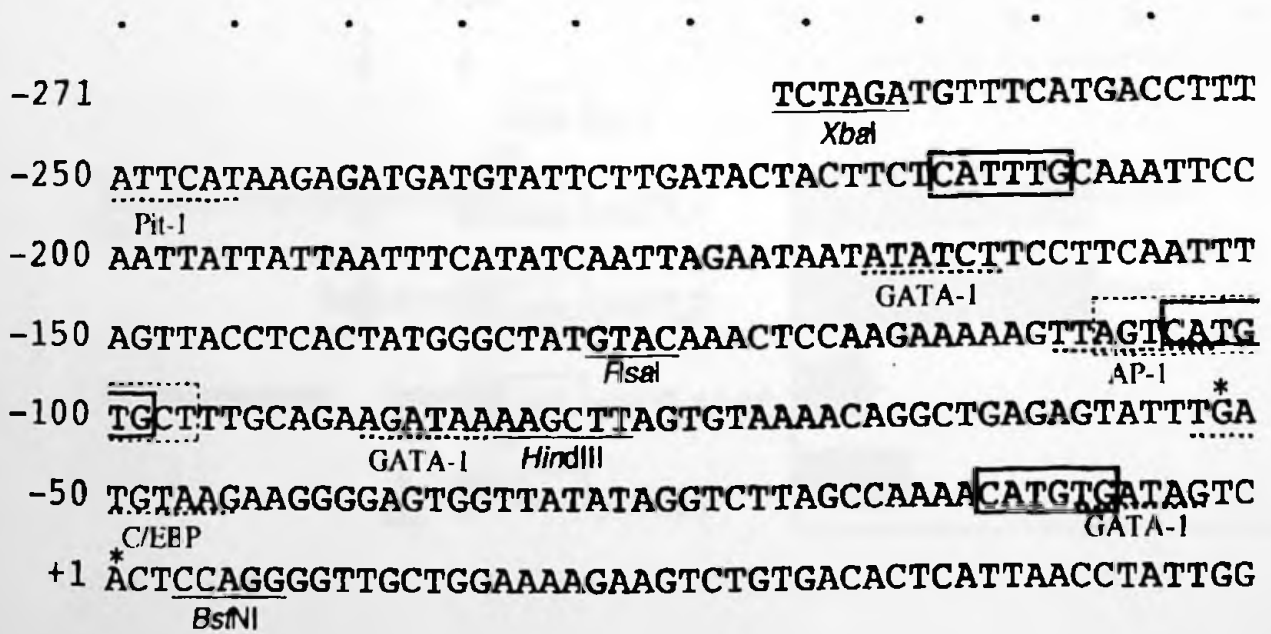
Nucleotide sequence and functional analysis of the -270 bp region of the mouse tyrosinase promoter.

A. Schematic of the mouse tyrosinase promoter. The coding strand is shown and is numbered from the transcriptional start site. The two transcriptional start sites are indicated by asterisks. The CANNTG motifs are open-boxed. The M box region (-107 to -97) is enclosed in a dashed open box. The 5' flanking AP-1 like site is underlined (dashed).

B. Localization of the *cis*-acting elements responsible for transcription of the mouse tyrosinase gene. The mouse tyrosinase promoter regions used for construction of the fusion genes are shown to the left. A construct pMTL1(delta)40 contains the internal deletion of the RsaI/HindIII fragment (positions -127 to -82). B16 mouse melanoma cells were cotransfected with each fusion gene and a β-galactosidase construct (pEF-βgal). Luciferase activity was normalized with β-galactosidase activity (an internal control), the value of which was then divided by the normalized value obtained with pMTL4, shown as relative luciferase activity to the right. The data shown are means +/- standard deviations for three independent experiments.

C. Effect of acute and chronic phorbol dibutyrate treatment on reporter gene activity in B16 cells transfected with the plasmid pMTL1. B16 cells were co-transfected with pMTL1 and β-galactosidase expression plasmids as described in Methods. Luciferase and β-galactosidase activities were determined and after correcting for transfection efficiency, the effect of PDBu treatment is expressed relative to the untreated control cells where the amount of luciferase activity is set at 100%.

A


-271
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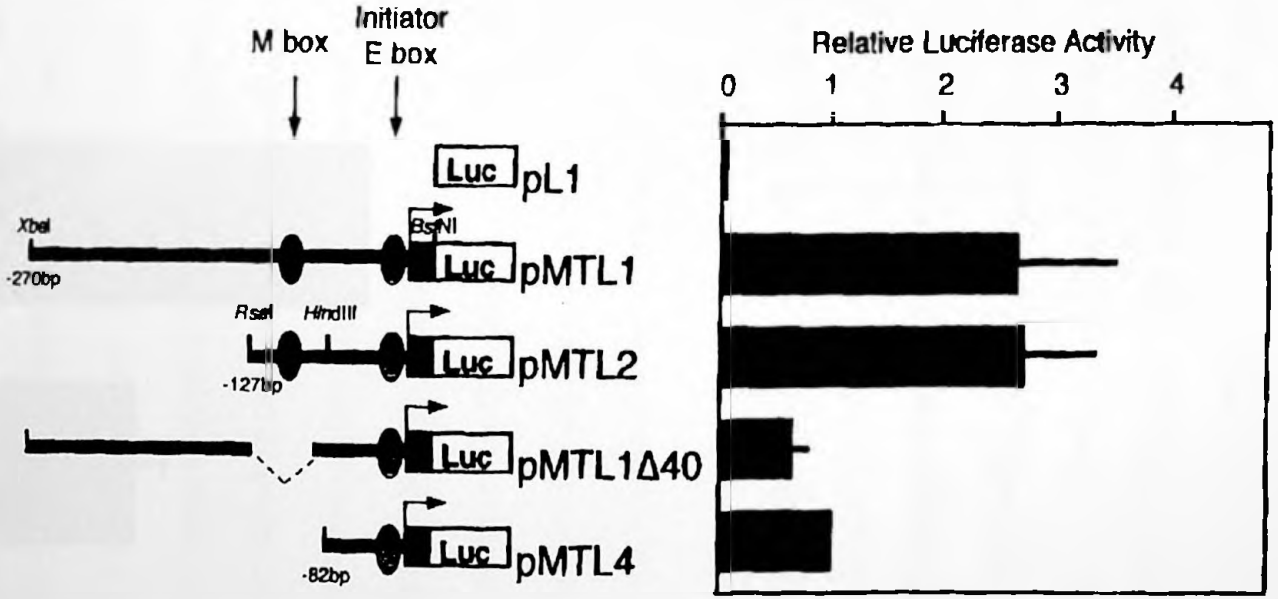
XbaI
-250 ATTCATAAGAGATGATGTATTCTTGATACTACTTCTCATTTGCAAATTCC
Pit-1
-200 AATTATTATTAATTTCATATCAATTAGAATAATATATCTTCCTTCAATTT
GATA-1
-150 AGTTACCTCACTATGGGCTATGTACAAACTCCAAGAAAAAGTTAGTCATG
HsaI
AP-1
-100 TGCTTTGCAGAAGATAAAAGCTTAGTGTAAAACAGGCTGAGAGTATTTGA
GATA-1
HindIII
-50 TGTAAGAAGGGGAGTGGTTATATAGGTCTTAGCCAAAACATGTGATAGTC
C/EBP
GATA-1
+1 ACTCCAGGGGTTGCTGGAAAAGAAGTCTGTGACACTCATTAACCTATTGG
BstNI

B


M box
Initiator
E box
Luc
pL1
XbaI
-270bp
BspNI
Luc
pMTL1
RsaI
HindIII
-127bp
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Luc
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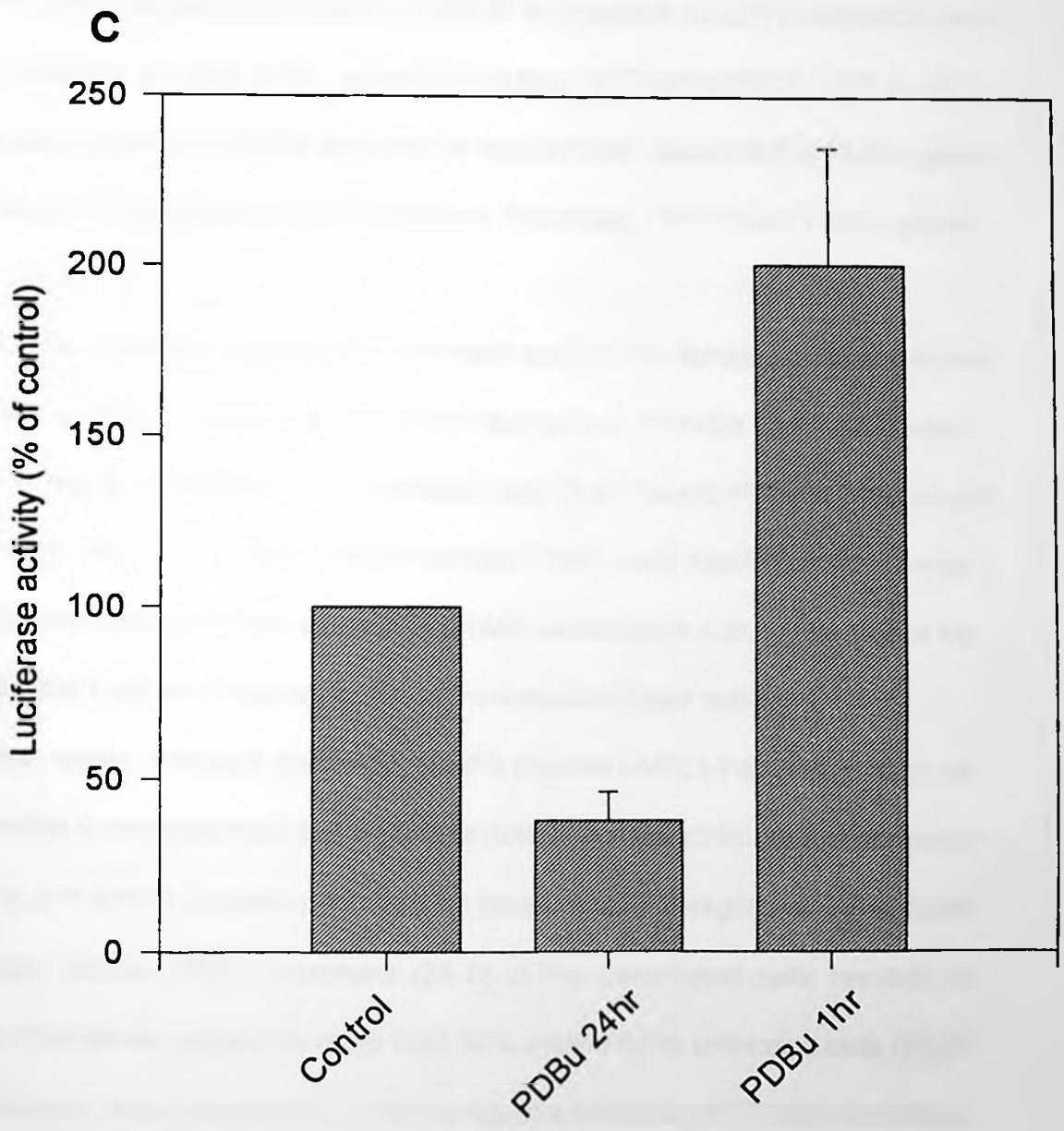
pMTL4
Relative Luciferase Activity
0
1
2
3
4


C
250
200
150
100
50
0
Luciferase activity (% of control)
Control
PDBu 24hr
PDBu 1hr

with pMTL4. The fusion gene pMTL2, containing the 127 bp promoter region, showed promoter activity comparable to pMTL1. A construct pMTL1(delta)40, lacking the RsaI/HindIII fragment (-127 to -82), gave a significantly lower expression compared to the values obtained with pMTL1 and pMTL2. The activity of pMTL1(delta)40 was similar to that with pMTL4 containing the 82-bp promoter region (Fig 8 B). The RsaI/HindIII fragment (-127 to -82) deleted in pMTL1(delta)40 and pMTL4, contains a 11 bp motif, termed the M-box, AGTCATGTGCT (-107 to -97). The M-box motif was originally identified in the promoter region of the TRP-1 gene and is conserved between mouse and human tyrosinase, TRP-1 and TRP-2 genes.

Phorbol ester regulation of tyrosinase promoter activity: The flanking residues of the M box in the mouse tyrosinase promoter resemble an AP1-like site (underlined - dashed in Fig 8 A). Based on our previous data that chronic PDBu treatment of mouse B16 melanoma cells down-regulated PKC and blocked cell density-dependent induction of tyrosinase mRNA, protein and enzyme activity, we tested the possibility that PKC may regulate mouse tyrosinase promoter activity.

As seen earlier, transient transfection of the plasmid pMTL1 into B16 melanoma cells resulted in a large amount of luciferase activity compared to the promoterless plasmid pL1 in which luciferase activity was barely above background levels (data not shown). Chronic PDBu treatment (24 h) of the transfected cells resulted in reduction of luciferase activity by more than 50% compared to untreated cells (Fig 8 C). Conversely, acute treatment (1 h) of the transfected cells with PDBu stimulated

luciferase activity by two fold (Fig 8 C). Thus it seems likely that PKC regulates tyrosinase expression by influencing the function of its promoter.

B: Promoter protein-DNA interactions:

Protein complexes associated with the M-box region of the mouse tyrosinase promoter: *Cis*-acting elements bind specific nuclear proteins producing a complex that interacts with the basal transcriptional machinery to stimulate or stabilize assembly of a pre-initiation complex. We synthesized oligonucleotides spanning the M-box region to investigate nuclear proteins which bind to this region and whether their binding was affected by PDBu treatment of the B16 cells. The oligonucleotide wt1 contains a 21 bp region corresponding to the central region of the 46 bp RsaI/HindIII fragment (-127 to -82) (Fig 9 A). The oligonucleotide mt1 has a mutation in the CATGTG motif of the M-box and mt2 has a mutation in the flanking region leaving the CATGTG motif in the M-box intact (Fig 9 A).

Using radiolabeled wt1 as a probe, nuclear extracts from highly pigmented B16 cells, were found to produce five major specific DNA-protein complexes (Fig 9 B). A 100-fold excess of unlabeled wt1 was able to compete all of these complexes, while the mt1 oligonucleotide at the same concentration did not compete any of the complexes.

Figure 9

Effect of PDBu treatment on nuclear protein binding to a region of the mouse tyrosinase promoter encompassing the M-box and flanking residues.

A. Schematic of the synthetic oligonucleotides wt-1, mt-1 and mt-2 and their relative position within the mouse tyrosinase promoter. The base changes introduced in the mt-1 and mt-2 oligonucleotides are denoted by lower case letters.

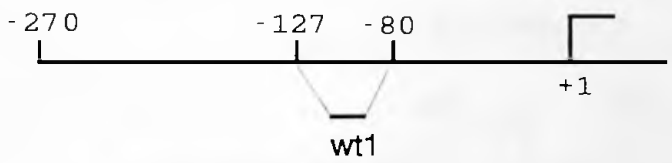
B. Binding of nuclear protein complexes at the M-box region. The DNA binding activity of endogenous proteins was analyzed by electrophoretic mobility shift assay (EMSA). The probe was radiolabeled wt-1 double stranded oligonucleotide, containing the M-box and its flanking residues from the mouse tyrosinase promoter. Unlabeled excess of wt-1 and mt-1 oligonucleotides were added in 10, 50 and 100-fold excess to check for the specificity of binding of the complexes to the wt-1 DNA.

C. Effect of phorbol dibutyrate on nuclear protein binding and localization of the putative regions where the complexes may be formed. Nuclear protein binding to the M-box was compared using extracts (10 μg) from control and B16 cells treated for 72 h with 1 μM phorbol dibutyrate. Unlabeled excess of wt-1, mt-1 and mt-2 oligonucleotides were included at a 200-fold excess in the reaction mixture containing nuclear proteins from control cells to localize binding regions of the complexes on the DNA.

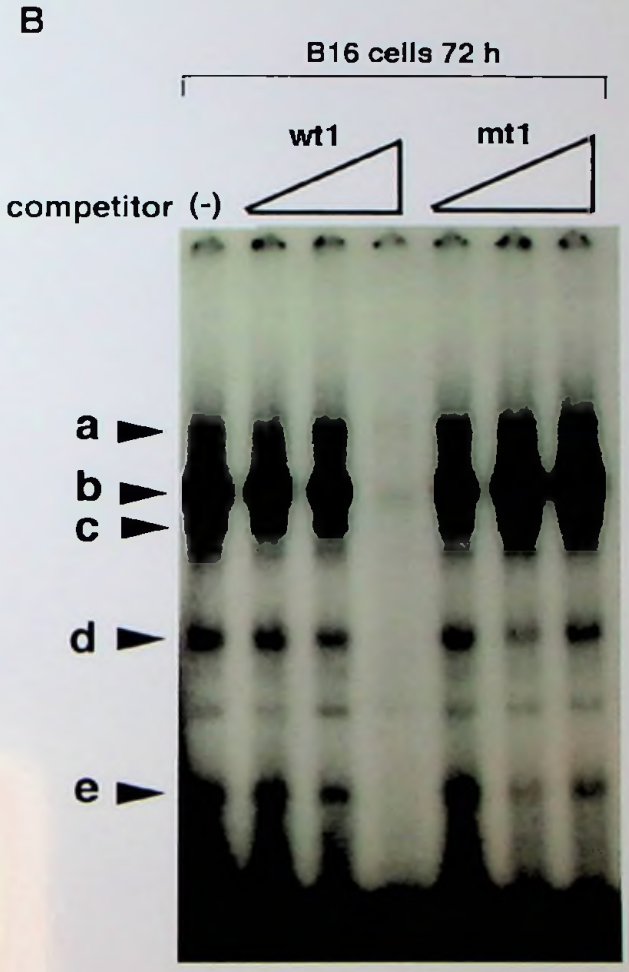
D. Effect of chronic treatment of B16 cells with phorbol dibutyrate on PKCα protein level. B16 cells were treated with either DMSO or 1 μM phorbol dibutyrate. At the indicated times cells were harvested as described under "Materials and Methods". 50 μg of total protein was loaded in all lanes and the levels of PKCα was determined by Western blotting using antibodies obtained form Seigagaku Inc.

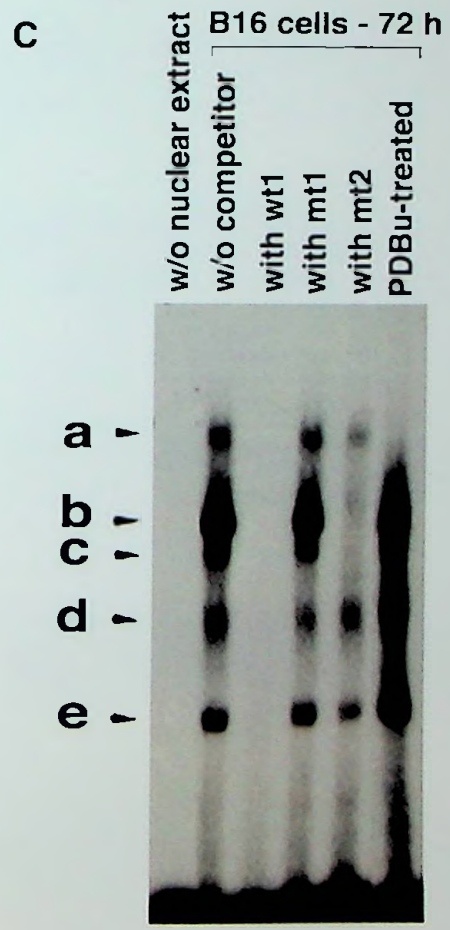
A

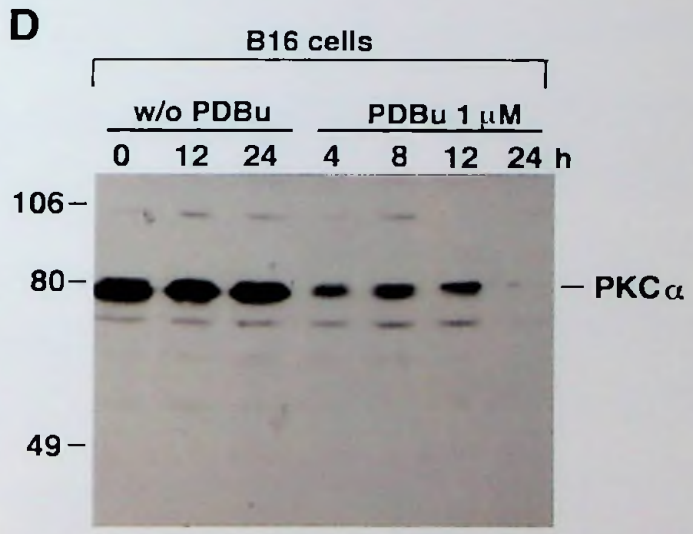
mouse tyrosinase 5' flanking region



wt1:	GATCCAAAAGTTAGTCATGTGCTTTGA
	GTTTTCAATCAGTACACGAAACTTCGA
mt1:	GATCCAAAAGTTAGTacTGTGCTTTGA
	GTTTTCAATCAtgACACGAAACTTCGA
mt2:	GATCCAAAAGgTgGTCATGTGCTTTGA
	GTTTTCcAcCAGTACACGAAACTTCGA


B
B16 cells 72 h
wt1
mt1
competitor (-)
a
b
c
d
e


c
B16 cells - 72 h
w/o nuclear extract
w/o competitor
with wt1
with mt1
with mt2
PDBu-treated
a
b
c
d
e


D
B16 cells
w/o PDBu
PDBu 1 μM
0 12 24 4 8 12 24 h
106
80
49
PKCα

Localization of the PKC-mediated regulation of complex formation: Having identified the protein complexes which bind to this region of the mouse tyrosinase promoter, we then examined the effect of PDBu treatment (72 h) of B16 cells on complex formation (Fig 9 C). The major change in PDBu-treated cells, which induces depletion of PKC (see Fig 9 D), was a complete loss of the slowest mobility complex (Fig 9 C, complex a). We verified again (see panel B) that unlabeled wt1 oligonucleotide at a 200-fold excess competed all of the complexes, while mt1, also at a 200-fold excess did not compete any of the complexes. Interestingly, mt2, which has a mutation in the flanking residues, leaving the CATGTG motif of the M-box intact (Fig 9 C) was able to compete complexes b and c, but not complex d. This oligonucleotide weakly competed complexes a and e compared to mt1, with which there was essentially no competition. It is likely that complexes b and c bind the CANNTG motif within the M box. Based on these results, we conclude that the other complexes likely span both the M-box and the 5' flanking residues, since they are not competed by either mutated oligonucleotide.

D:Cell density is a key regulator of tyrosinase expression:

Cell density dependent changes of tyrosinase, PKCα and nuclear protein interaction with the M box region of the tyrosinase promoter: B16 cells seeded at low density do not produce tyrosinase mRNA or protein (Part I). As the cells reach a certain critical density, tyrosinase mRNA is induced followed by an increase in tyrosinase activity

and visible melanin production (Part I). We previously found that there is also a density-dependent increase in PKCα protein, which roughly correlates with the increase in melanin production (105). These density dependent changes can be prevented by chronic treatment with PDBu. In order to determine whether these changes were reflected in DNA-protein interactions at the M-box region, we prepared nuclear extracts from B16 cells at different culture periods and analyzed their DNA interaction by EMSA. We also determined the protein level of tyrosinase and PKCα in whole cell extracts at the same time points by Western blotting.

Tyrosinase protein increased greatly between 18 and 30 h in culture and was maximal at 48 h after seeding of the cells (Fig. 10 A). In agreement with our previous work (126), PKCα was the major PKC isoform expressed in B16 cells. The amount of PKCα increased dramtically at 48 h in culture and was maintained at this level in the last time point examined (Fig. 10 B). Several changes were noted in nuclear protein binding to the oligonucleotide spanning the M-box region. At the earliest time point (18 h) only complexes b and c exhibited strong binding (Fig. 10 C). All remaining time points had five protein complexes (a-e). There was an additional band a', which is more easily observed in shorter exposures of the autoradiogram, seen only in cells at 48 h in culture. The appearance of this DNA-protein complex correlates with the dramatic increase in PKCα.

Figure 10

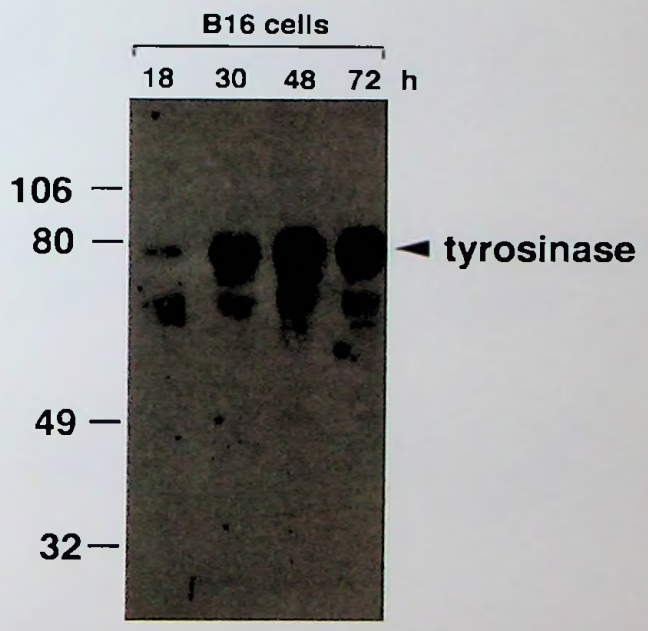
Cell density-dependent changes in tyrosinase protein, PKCα protein and nuclear protein complexes at the M-box region. Total protein and nuclear extracts were prepared simultaneously from B16 cells at different times in culture as described in Methods.

A. Western blotting analysis of tyrosinase protein level. Total protein (25 μg) was loaded in each lane and tyrosinase protein detected using anti-peptide polyclonal antibodies (anti-pep7) provided by Dr. V. Hearing, NCI, Bethesda, MD.

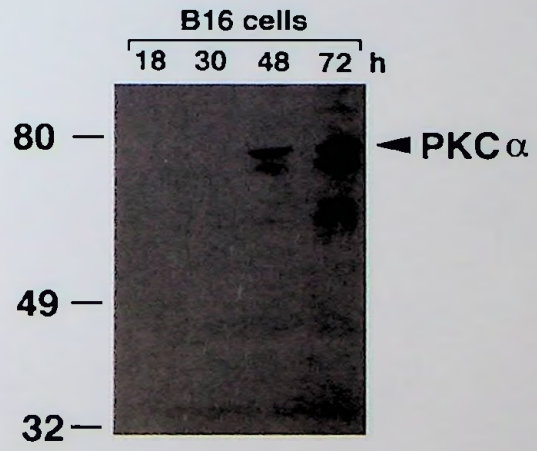
B. Western blotting analysis of PKCα protein. Total protein (50 μg) was loaded in each lane and PKCα protein detected using a monoclonal PKCα specific antibody from Upstate Biotechnology Corp.

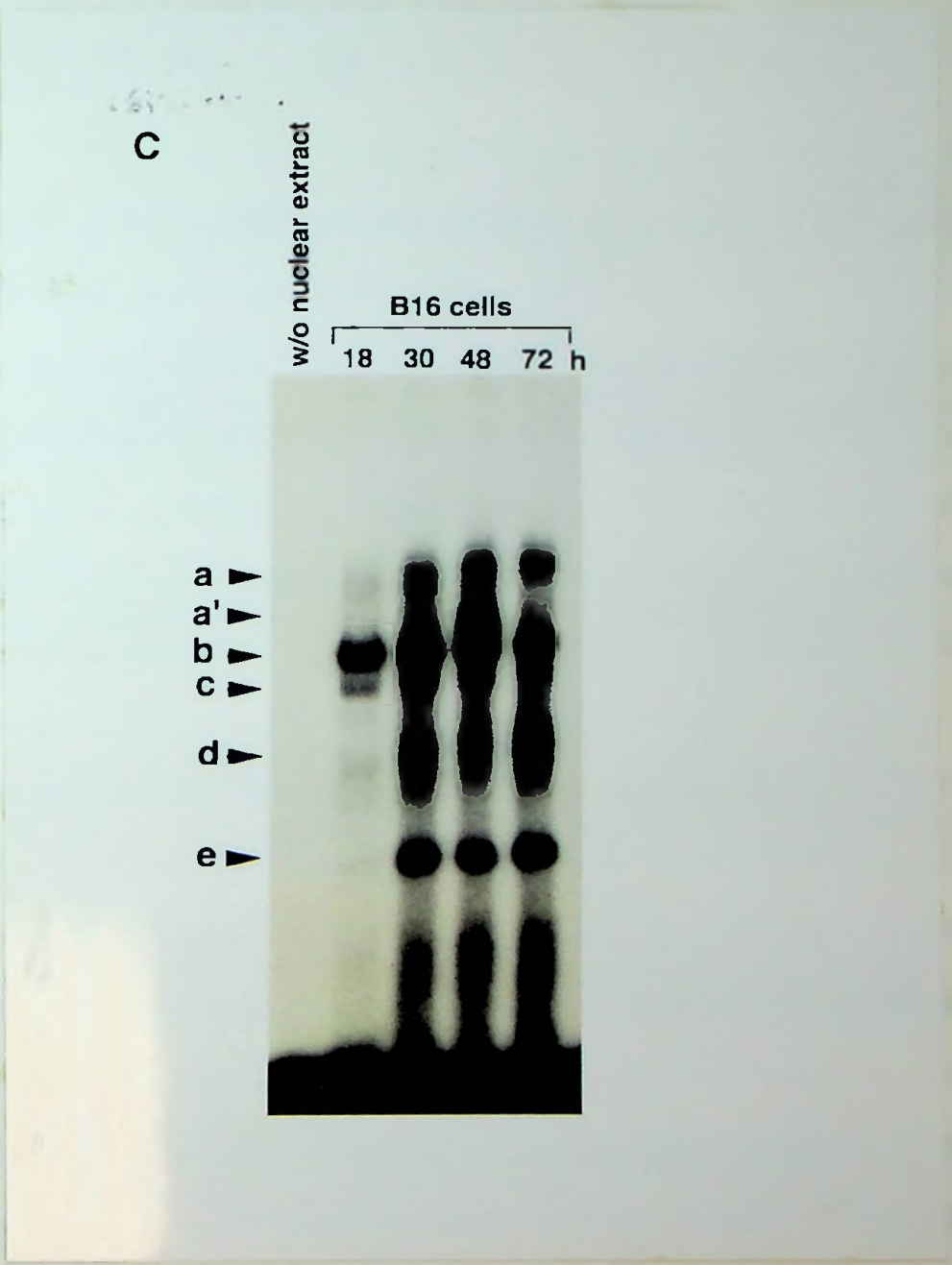
C. EMSA. Nuclear extracts (10 μg) from B16 cells at different times in culture were incubated with labeled wt-1 oligonucleotide. Specific protein complexes are indicated on the left of the figure (a-e).

A



B




C
w/o nuclear extract
B16 cells
18
30
48
72
h
a
a'
b
c
d
e

E. Identification of factors that bind to the M-box region:

Binding of AP-1 transcription factors: In light of our previous results on the role of PKC and the fact that the flanking residues of the M box resemble an AP-1-like site, we tested whether the region binds AP-1 transcription factors. One of the mechanisms by which PKC is thought to mediate its effect is through increasing AP-1 transcriptional activity. We tested whether complex a, shown to be absent in cells chronically treated with PDBu (Fig 9 C), contained either c-jun, c-fos or both proteins. Nuclear extracts from B16 cells treated with or without PDBu for 72 h were incubated with wt1 oligonucleotide and antibodies against c-jun or c-fos. We were unable to detect a super-shift or inhibition of binding of any of the complexes (Fig 11 A). The flanking residues of the CATGTG motif in the M-box regions overlap an AP-1 site (Fig 8 A). To test if activation of PKC might increase binding in this region, we incubated nuclear extracts from B16 cells treated with PDBu for 1 h with a consensus AP-1 oligonucleotide corresponding to the AP-1 site of the collagenase promoter (1). Compared to the untreated cells there was a large increase in binding activity of nuclear proteins from PDBu-treated cells to this site. Inclusion of antibody to c-fos in the incubation resulted in a decrease in the intensity of the major band and the appearance of two new slower migrating bands (Fig 11 B-arrows). Addition of c-jun antiserum to the reaction mixture did not decrease or supershift any of the

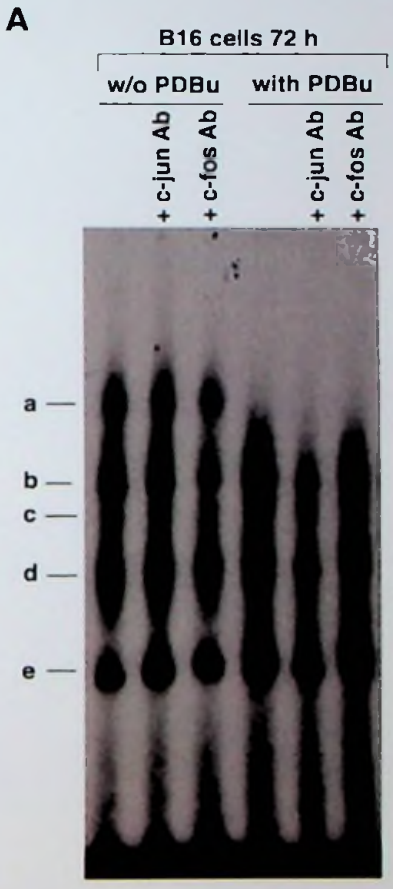
Figure 11

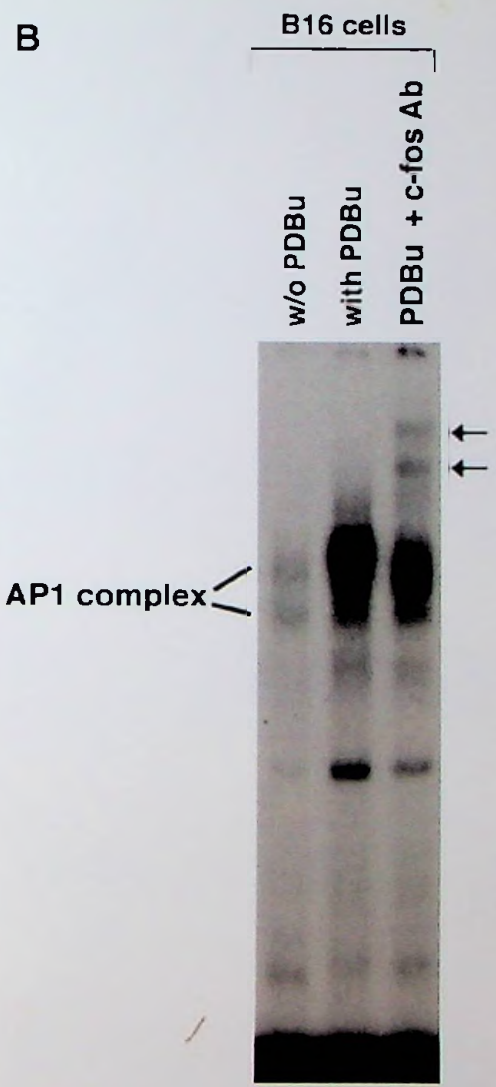
Determination of endogenous c-fos and c-jun binding to wt-1 and consensus AP-1 oligonucleotides.

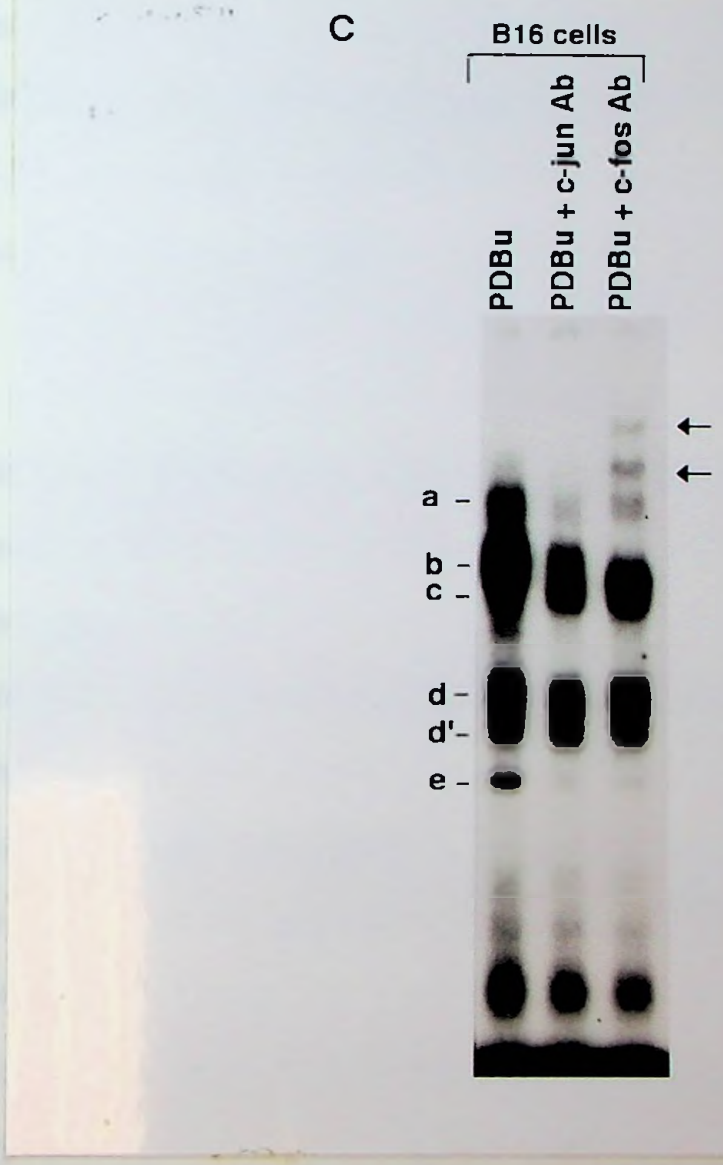
A. Nuclear extracts (10 μg) from B16 cells treated with or without 1 μM PDB (72 h chronic treatment) were incubated with wt-1 oligonucleotide and antibodies against c-fos and c-jun (Santa Cruz Biotechnology Inc., CA). 1.5 μg of the antibody in a volume of 1.5 μl was added to the reaction mixture containing the B16 nuclear extract and the probe for 45 min. Reaction mixtures were pre-incubated for 20 min before addition of the antibodies.

B. Nuclear extracts from B16 cells treated with or without 1 μM PDB (1 h acute treatment) were incubated with a labeled oligonucleotide corresponding to a consensus AP-1 site (1) in the collagenase promoter (CGCATGAGTCAGACA with Bgl-II and Mlu-I restriction sites on the 5' end of the upper and lower strand respectively) and antibodies against c-fos (Santa Cruz Biotechnology Inc., CA) as described above.

C. Nuclear extracts from B16 cells treated for 1 h with 1 μM PDBu were incubated with radiolabeled wt-1 oligonucleotide and antibodies against c-fos and c-jun as described above.


A
B16 cells 72 h
w/o PDBu
with PDBu
+ c-jun Ab
+ c-fos Ab
+ c-jun Ab
+ c-fos Ab
a
b
c
d
e


B
B16 cells
w/o PDBu
with PDBu
PDBu + c-fos Ab
AP1 complex


C
B16 cells
PDBu
PDBu + c-jun Ab
PDBu + c-fos Ab
a
b
c
d
d'
e

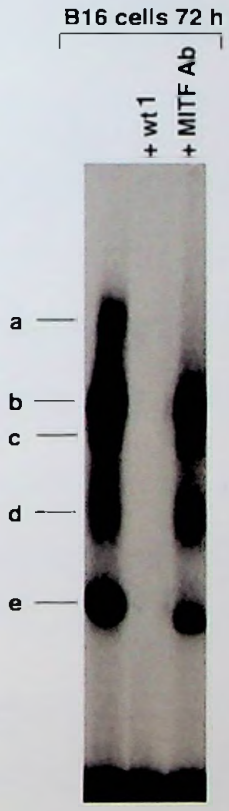
complexes (data not shown). Incubation of these same nuclear extracts with the wt1 oligonucleotide resulted in a clearly visible additional band (d'), compared to the five complexes found in untreated cells, and either a much stronger signal for complex b or another new complex with slightly slower mobility than complex b (Fig 11 C) (shorter exposures of the autoradiogram were not able to distinguish between these two possibilities). Addition of antibodies to c-jun or c-fos decreased the binding of complexes a, b and e. In addition antibodies to c-fos induced the appearance of two supershifted bands (Fig 11 C-arrows).

MITF is a component of complex a: Recent studies suggest that MITF, with a characteristic bHLH.Zip structure may be an important regulator of tyrosinase and TRP-1 expression (108, 139, 153, 154). The M-box region of the mouse tyrosinase promoter was shown to bind MITF (154). It was also suggested that the flanking regions of the M-box may be important for protein binding. We tested the possibility that complex a, which binds to the region containing the M-box of the mouse tyrosinase promoter may contain an MITF-like protein. Nuclear extracts from B16 cells were incubated with or without antibody to MITF and labeled wt1 oligonucleotide. Fig 12 shows that this antibody inhibited the appearance of complex a and also decreased the intensity of complex e. These effects were likely specific to the antibody against MITF since antibodies to mouse S-laminin has no effect on any of the complexes that bound to the wt1 oligonucleotide (data not shown).

Figure 12

Evidence that MITF is a component of complex a.

Radiolabeled wt-1 oligonucleotide corresponding to the M-box and flanking regions of the mouse tyrosinase promoter was incubated with or without a 200 fold excess of wt-1 oligonucleotide or 3 μl of antiserum specific for MITF as described under "Materials and Methods".


B16 cells 72 h
+ wt 1
+ MITF Ab
a
b
c
d
e

DISCUSSION

In the first part of the work, we have presented evidence that chronic PDBu treatment, through its ability to down-regulate PKC, inhibits expression of tyrosinase protein and mRNA and induces an amelanotic phenotype (92). In this part of the study we show that acute PDBu treatment, which activates PKC, stimulates the tyrosinase promoter when transfected into B16 cells as judged by a reporter gene assay. Conversely, chronic PDBu treatment, which depletes the cells of PKC, inhibits the reporter gene activity (Fig 8 C). Thus it seems likely that PKC regulates tyrosinase expression by influencing the function of its promoter.

A highly conserved region within the 270 bp segment of the tyrosinase promoter called the M-box is thought to be important for transcription of pigment cell-specific genes (13, 37, 38, 154). A 45 bp region containing this M-box element was found to be critical for the efficient transcription of the tyrosinase gene (154). Using a 31 bp oligonucleotide containing the M-box plus flanking residues, we found that five specific protein-DNA complexes were formed (Fig 9 B and C) when the oligonucleotide was incubated with nuclear extracts from B16 mouse melanoma cells actively producing pigment (as determined by visual observation of the cells and culture media). In nuclear extracts from B16 cells which were chronically treated with PDBu, and thus rendered amelanotic (92), the slowest migrating complex was absent (Fig 9 C). Using oligonucleotides that span this M-box region from the mouse TRP-1 gene, the mouse tyrosinase and the human tyrosinase, other laboratories

have found one (37), two (90) or four (13) protein-DNA complexes when these probes were reacted with nuclear extracts from B16 cells. To investigate this apparent discrepancy, we examined the intensity and number of protein-DNA complexes formed with the wt- oligonucletide when nuclear extracts were taken from cells at different times and densities in culture. The amount of tyrosinase and PKCα protein present in these cells was dependent on the length of the culture period. We found that cells which were expressing minimal amounts of tyrosinase had only two protein complexes associated with the oligonucleotide which spanned the M-box region. Thus, there are many cell density-dependent changes which occur in B16 melanoma cells and the varying results of other investigators (13, 37, 90) may be due, at least in part, to the density at which cells were harvested for analysis. Supporting this conclusion is the recent observation that ectopic expression of MITF in NIH-3T3 cells results in a cell density-dependent expression of tyrosinase and TRP-1 (139). Our experiments also revealed that a new DNA-protein complex (a') appeared at the time PKCα levels were substantially increased (Fig 10 C). The possibility that this new complex represented AP-1 transcripton factor, containing c-jun homodimer or c-jun/c-fos heterodimer was explored.

To address the question of what regions of the 31 bp oligonucleotide (wt1) were bound by the different complexes, we perfomed competition experiments with different amounts of wt-1 and two different mutant oligonucleotides (mt-1 and mt-2). An oligonucleotide mt-1, which contains a mutation in the M-box was not able to compete any of the five complexes (Fig 9 C). A different oligonucleotide, mt-2, which

contains a mutation in the 5' flanking residues of the M-box competed two of the five complexes (b and c) (Fig 9 C). Hence, it is likely that complexes b and c bind to the CATGTG motif within the M-box. The other complexes likely span both the CATGTG motif and the 5' flanking residues of the M-box, since they are not competed by two mutated oligonucleotides. Our results are consistent with the conclusion of Yasumoto et al. (154) that the flanking residues of the CATGTG motif are important for the assembly of protein complexes at the M-box region.

In light of our previous results on the role of PKC in the expression of tyrosinase (Part I) and the fact that the flanking residues of the M-box resemble an AP-1 like site (Fig 8 A), we determined whether any of the complexes contained AP-I transcription factor. In contrast to our expectations, none of the complexes were diminished in intensity or supershifted when nuclear extracts from cells producing melanin were incubated with anitbodies to c-fos or c-jun (Fig 11 A). Based upon our previous observation that a new complex (a') appeared at a time in culture when there was a large increase in PKC (Fig 10 B and C), we treated the B16 cells briefly (1 h) with PDBu to activate PKC and determined if this produced complex a'. Nuclear extracts from these cells were incubated with either wt1 oligonucleotide or a nucleotide corresponding to the AP-1 site in the collagenase promoter (1). Nuclear extracts from PDBu-treated cells had a large increase in binding to the authentic AP-1 site and antibody incubations demonstrated that this complex contained c-fos (Fig 11 B). We have found that acute PDBu treatment increases c-fos mRNA and protein in B16 cells (Desai and Niles, unpublished data). This increase in c-fos probably accounts

for the increased binding to the AP-1 site. Antibody against c-jun did not decrease or supershift any of the bands (data not shown). Since the assay is performed under non-denaturing conditions, one possibility for this result is that the antigenic site recognized by the antibody is not available for binding under these conditions.

Using the wt1 oligonucleotide a new complex (d'), was found in nuclear extracts from PDBu-treated cells. Also, either the intensity of complex b was increased, or a new complex with somewhat slower mobility than b was formed (Fig 11 C). Unfortunately, shorter exposures of the autoradiogram did not allow us to distinguish between these two possibilities. Complexes a and e were decreased in nuclear extracts incubated with antibodies to either c-jun or c-fos. Additionally, the c-fos antiserum induced the appearance of two super-shifted bands (Fig 11 C-arrows). It is not clear if these supershifted bands came from complexes a, b or e. These results suggest that AP-1 family members may form additional complexes in this region of the tyrosinase promoter and/or dimerize with other transcription factors to generate the band pattern found in nuclear extracts from PKC-activated cells. These changes may account for the increased tyrosinase promoter activity found in B16 cells acutely treated with PDBu (Fig 8 C).

It has been shown that ectopic expression of MITF can transactivate the tyrosinase promoter and induce melanocytic differentiation (108, 139, 153). Furthermore, mutations in the mouse *mi*, and the human MITF result in defective differentiation of melanocytes (50, 108, 140). Therefore these factors are most likely key regulators of the pigment cell-specific expression of tyrosinase. We found that

the presence of the slowest migrating complex (a) which specifically bound to the wt1 oligonucleotide spanning the M-box was absent when nuclear extracts from B16 cells were incubated with an antibody to MITF (Fig 12). This same complex is absent in cells at low culture density (Fig 10 C) and in cells chronically treated with PDBu (Fig 9C). Both of these conditions result in amelanotic cells where there is little or no detectable tyrosinase (92). Thus it is likely that MITF-like protein, which is a component of this complex, is crucial for tyrosinase expression in B16 melanoma cells. Also, since down-regulation of PKC inhibits the appearance of this complex, we speculate that the expression and/or function of this MITF-like protein is regulated by PKC. Indeed the predicted amino acid sequence of MITF has consensus PKC phosphorylation sites (52) and it is well established that phosphorylation of transcription factors plays a major role in their function (15, 56, 58, 87, 114). Future studies will determine if this MITF-like protein is phosphorylated either directly or indirectly by PKC and if so, whether this affects its ability to dimerize to other transcription factors, bind to its response element and/or activate transcription. In summary, correlation of PKC and tyrosinase expression in melanoma cells suggest that the tyrosinase promoter is regulated by PKC. The binding of a specific sequence in the tyrosinase promoter with melanoma cell nuclear protein which contains MITF is also regulated by PKC.

PART III

ROLE OF RETINOIC ACID IN TYROSINASE PROMOTER FUNCTION

SUMMARY

We previuosly found that RA increases melanin production in B16 cells. Experiments were initiated to determine whether RA mediates its effect on melanin production through its ability to regulate the tyrosinase promoter. The results demonstrated that RA stimulated the expression of a tyrosinase promoter-reporter gene construct transfected into B16 cells. Subsequently, we have identified a region in the 5' flanking region of the mouse tyrosinase gene that binds RARs and functions as an RARE in its binding characteristics. This RARE site shares homology with a half-site of a canonical RARE (DR-5) and binds all RAR subtypes. Endogenous RARs were also found to bind to this site.

RESULTS

A: Regulation of mouse tyrosinase promoter function by RA:

RA-mediated regulation of tyrosinase promoter activity: RA-induced differentiation of B16 mouse melanoma cells results in increased melanin production. In light of these results, we tested the possibility that RA may regulate the mouse tyrosinase promoter activity. Transient transfection of pMTL1 containing 270 bp of the mouse tyrosinase promoter upstream of a luciferase reporter gene into B16 mouse melanoma cells resulted in a strong enhancement of luciferase activity when compared to the promoterless plasmid pL1 in which the luciferase activity was barely above the background levels (data not shown). Treatment of the transfected cells with 10 μM RA for 24 h stimulated the luciferase activity by 2.5 fold (Fig. 13 A).

Effect of Retinoic acid on protein-DNA complex formation at the M-box region: We have previously shown that incubation of nuclear extracts from B16 cells that were producing melanin with a radiolabeled oligonucleotide wt1 (Fig 13 B) resulted in 5 DNA-protein complexes in electrophoretic mobility shift assays. Since RA increased the expression of a reporter gene driven by a tyrosinase promoter containing the wt1

Figure 13

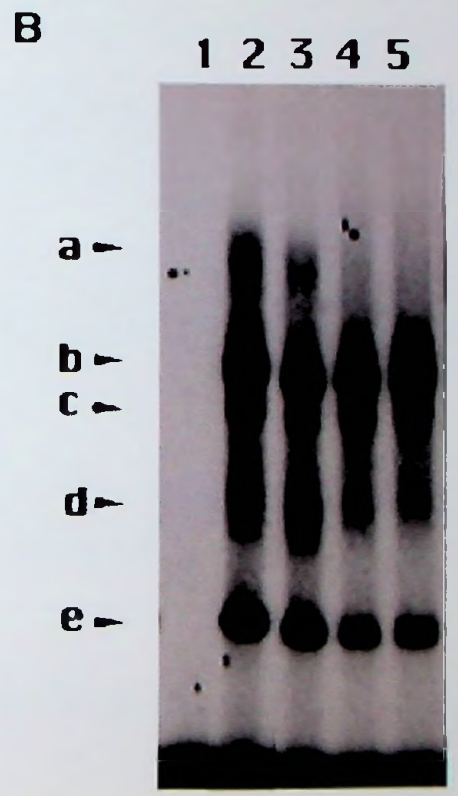
Effect of RA on mouse tyrosinase promoter function

A. Effect of RA treatment on reporter gene activity in B16 cells transfected with the plasmid pMTL1. B16 cells were co-transfected with pMTL1 and β-galactosidase expression plasmids as described in Methods. Luciferase and β-galactosidase activities were determined and after correcting for transfection efficiency, the effect of phorbol dibutyrate treatment is expressed relative to the control cells where the amount of luciferase activity is set at 100%.

B . Effect of RA on nuclear protein binding and determination of endogenous RARs binding to the M-box region. Nuclear extracts (10 μg) from B16 cells treated with or without 10 μM RA (48 h) were incubated with wt-1 oligonucleotide and antibodies against RARs. 1.5 μl of the antiserum RAR γ-III-b that reacts with all RARs (122) was added to the reaction mixture containing the B16 nuclear extract and the probe for 45 min. Reaction mixtures were pre-incubated for 20 min before addition of the antibodies.

Lane 1:	labeled wt1 alone
Lanes 2, 4:	B16 extracts
Lanes 3, 5:	RA-treated B16 extracts
Lanes 4, 5:	RAR-γ-IIIb

A
Luciferase activity (% of control)
300
250
200
150
100
50
0
Control
RA


B
1 2 3 4 5
a
b
c
d
e

region, we investigated the possibility that one of the complexes contained an RAR. Nuclear extracts from B16 cells treated with or without 10 μM concentration of RA (48 h) were incubated with wt1 probe, which contains the M box region and analyzed for protein-DNA complex formation by EMSA. We found the same profile of complexes in control and RA-treated cells except that complex a was slightly reduced in intensity in RA-treated cells compared to the untreated control cells (Fig 13 B- lanes 2, 3). The nuclear extracts were also incubated with polyclonal antibody (γ-III-b) that reacts with all the RARs RAR α , β and γ) (122). This treatment led to loss of complex a and a decrease in the intensity of complex d in both RA-treated and untreated B16 cells (Fig 13 B- lanes 4, 5).

B. The M-box region binds RAR:

Identification of a putative RARE-half site in the flanking region of the M box: Computer analysis of the 31-bp oligonucleotide (wt1) used in the gel shift experiments described above revealed a near consensus RARE half-site. To confirm whether this region binds RARs, we compared the binding of exogenous RAR α, β and γ to the wt-1 oligonucleotide versus their binding to a synthetic β-RARE (DR-5). Extracts from baculovirus-infected sf9 cells that express RAR α, β or γ were incubated with radiolabeled β-RARE (Fig 14 A- lanes 1 to 9) or wt-1 (Fig 14 A-lanes 10 to 18) oligonucleotides. The identity of the DNA-protein complexes was confirmed

Figure 14

The M-box region binds RARs and functions as a RARE in its binding characteristics

A. Gel shift analysis: The DNA binding activity of RARs to the wt1 oligonucleotide was analyzed by gel shift assay. As a positive control labeled β-RARE was used and the identity of the complexes were determined by incubating with antibodies against RARs.

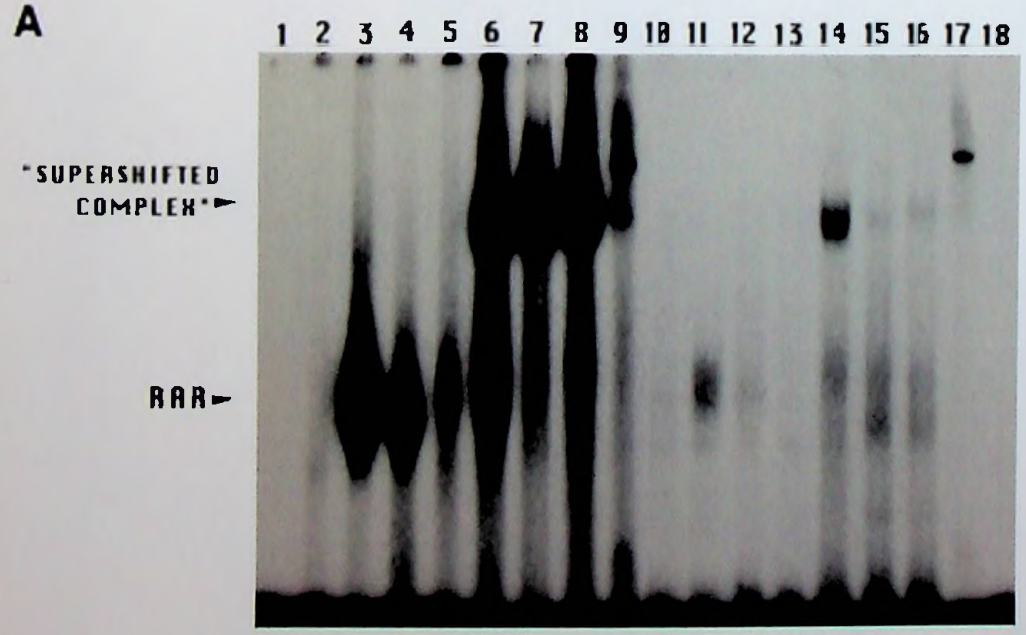
Lanes 1-9	labeled β-RARE
Lanes 10-18	labeled wt1
Lanes 2,10	sf9 wild-type extract
Lanes 3,11	sf9 RARα extract
Lanes 4,12	sf9 RARβ extract
Lanes 5,13	sf9 RARγ extract
Lanes 6,14	RARα+RAR γ-III-b
Lanes 7,15	RARβ+RAR γ-III-b
Lanes 8,16	RARγ+RAR γ-III-b
Lanes 9,17	RARγ-III-b (w/o sf9 extract)

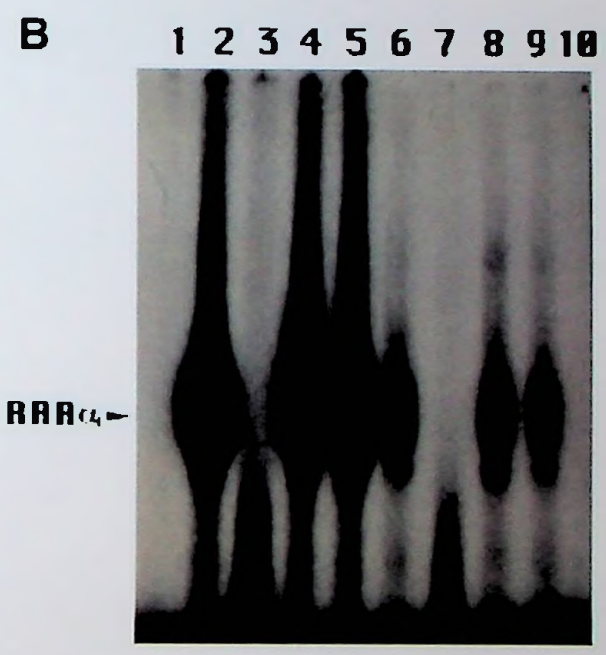
B. wt1 binds RARs as a composite element

Lanes 1-5	labeled β-RARE
Lanes 6-10	labeled wt1
Lanes 2-9	sf9 RARα extract
Lanes 3,7	sf9 RARα extract + wt1 comp.(200 fold)
Lanes 4,8	sf9 RARα extract + mt1 comp.(200 fold)
Lanes 5,9	sf9 RARα extract + mt2 comp.(200 fold)

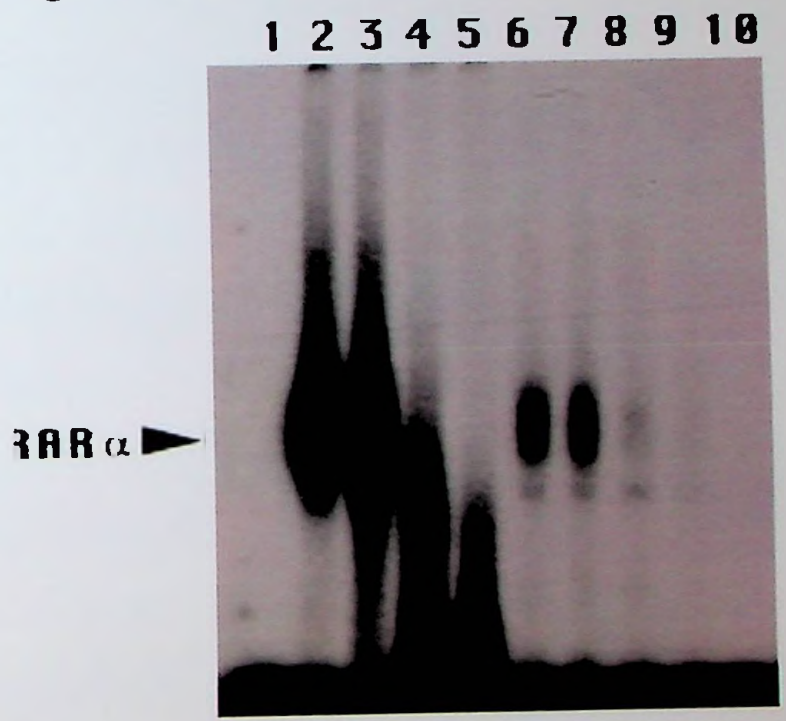
C. Mutual competition of RARα binding to β-RARE and wt1 oligonucleotides

Lanes 1-5	labeled β-RARE
Lanes 6-10	labeled wt1
Lanes 2-9	sf9 RARα extract
Lanes 3-5	with inc. conc. of wt1 oligonucleotide (10, 50, 100-fold)
Lanes 7-9	with inc. conc. of β-RARE oligonucleotide (10, 50, 100-fold)


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
"SUPERSHIFTED COMPLEX"
RAR


B
1 2 3 4 5 6 7 8 9 10
RRRα4

C



by incubating the extracts and labeled oligonucleotides with antibodies against RARs. These antibodies were able to "supershift" the specific RAR complexes binding to the β-RARE as well as the wt1 oligonucleotide (lanes 6 to 8 and 14 to 17). The wt1 oligonucleotide was able to bind all RAR subtypes (binding of RARγ was seen in longer exposure of the gel to the autoradiogram). Incubation of the RAR polyclonal antibody with the labeled β-RARE and wt1 show a small amount of non-specific binding (Fig 14 A- lanes 9 and 17).

In order to determine where RARα binds within the wt-1 oligonucleotide, we incubated RARα with radiolabeled β-RARE and wt-1 oligonucleotides with +/- equimolar quantities of the oligonucleotides wt-1, mt-1 and mt-2 shown in Fig 9 A. Neither mt-1 nor mt-2 was able to compete the binding of RARα to either β-RARE or wt-1 region (Fig 14 B). At 200-fold excess wt1 oligonucleotide was able to compete the binding of RARα to both β-RARE as well as to wt1.

To confirm that the binding of RARs to the wt-1 oligonucleotide was specific, reciprocal and non-reciprocal competition experiments were performed with unlabeled excess of β-RARE and wt-1 oligonucleotides. Unlabeled excess of wt-1 and β-RARE was able to compete the binding of RARα to either β-RARE or wt1 in non-reciprocal competition experiments (data not shown). As shown in Fig 14 C, unlabeled excess of β-RARE and wt-1 oligonucleotides were able to compete each others' binding of RARα, to either radiolabeled oligonucleotides.

DISCUSSION

The cellular effects of RA are mediated by nuclear receptors. RA is thought to mediate its effect by acting as a ligand and binding to its cognate nuclear receptors RARs and RXRs (69, 86, 157). These receptors are divided into two groups: the RARs and RXRs. The latter receptors bind only 9-cis RA, while the former receptors can bind both 9-cis and all-trans RA . These receptors have been shown to bind to specific response elements (RAREs-retinoic acid response elements) in the promoter regions of genes (156). Our laboratory has previously shown that the B16 cells constitutively express RARα and RARγ and RARβ is induced only upon RA treatment (151). We have also shown that depletion of PKC by chronic PDBu treatment leads to a marked decrease in the ability of the RARs to bind to β-RARE (DR-5) (unpublished observations).

A 270 bp promoter region of the mouse tyrosinase gene has been shown to be sufficient to elicit expression of the tyrosinase gene *in vivo* as well as in cultured cells (73). Using the 270 bp upstream region of the tyrosinase promoter in reporter gene assays, we found that RA increases the promoter activity (Fig 13 A). This region contains an 11 bp motif, termed the "M-box", that is conserved in promoter regons of tyrosinase, TRP-1 and TRP-2 genes ( ). We have shown that the flanking residues of the CATGTG motif in the M-box plays an important role in the assembly of transcriptional complexes at this region (Part II). The flanking residues of the CATGTG motif resemble a half-site of an RARE. Using an oligonucleotide, wt1,

spanning the M-box and the flanking residues , we found that this region may be an RARE in that it is able to bind all RAR subtypes (Fig 14 A). We also found that the binding of RARs to this region is specific in that they are competed by increasing concentrations of both wt1 and β-RARE (Fig 14 C).

In addition to increasing melanin production, RA also decreases the monolayer growth rate in B16 cells. The expression of tyrosinase is regulated as a function of cell density and therefore, we are currently exploring the effect of RA on the expression of tyrosinase at the mRNA and protein level, normalizing for the effect that RA has on the monolayer growth rate. We are also investigating whether retinoid analogs which have relative specificity for binding and activating individual subtypes of RARs may indicate which of the RARs regulates tyrosinase expression.

CONCLUSION

Mammalian melanin synthesis is a complex process, with many different gene products contributing to the pathway. We have been studying the regulation of density-dependent melanogenesis in B16 mouse melanoma cells. In this process, cells seeded at low density do not produce tyrosinase mRNA, protein or melanin (92). As cells reach a certain critical density, tyrosinase mRNA is expressed, followed by increased tyrosinase activity and the production of melanin. This process can be completely inhibited by incubating the cells with phorbol dibutyrate (PDBu). We have obtained evidence that this inhibition is due to the depletion of PKC. Using different phorbol derivatives with varying abilities to downregulate PKCα, we correlated PKC expression with density dependent induction of tyrosinase and TRP1 (Part I). Therefore we conclude that PKC is absolutely required for density-dependent melanogenesis.

The mouse and human tyrosinase genes have been cloned and regions of the promoter which confer melanocyte specificity identified (the so called M-box). Linkage of this promoter region to a heterologous gene resulted in the melanocyte-restricted expression of the heterologous gene in transgenic mice. We found that deletion of the M-box region leads to a marked reduction in pigment cell-specific transcription of the mouse tyrosinase gene. The transcription factor(s) which result in

melanocyte-specific tyrosinase gene expression are still unknown. Using an oligonucleotide which spans the M-box region from the tyrosinase promoter, we found 5 specific protein-DNA complexes in gel shift analysis with nuclear extracts from B16 cells which were actively expressing tyrosinase (Part II). The slowest migrating complex was absent when nuclear extracts from PDBu-treated cells (no tyrosinase expression) were used in gel shift assay. We also found that antibodies against MITF and RAR inhibit the formation of this complex (Part II and III). Additionally, evidence was obtained, that transcription factors directly regulated by PKC (AP1- jun and fos) may constitute a component of complex(s) formed at the M-box region (Part II). Thus we conclude that complex a may be critical for efficient transcription of the mouse tyrosinase gene.

In summary, using RA and PDBu as opposing agents to regulate PKC levels, we found that tyrosinase expression is controlled by a complex interplay of intracellular signaling mechanisms, possibly mediated through the *trans*-acting factors regulating its transcription. In related studies, showing a cross-talk between signaling pathways Schule et al. (131) demonstrated that jun-fos and the receptors for RA and vitamin D recognize a common element in the human osteocalcin gene. It has been shown that fos can heterodimerize with a bHLH.Zip factor, termed fos-interacting protein (FIP) (14). The FIP stimulated the transcription of an AP-1 responsive promoter when co-transfected with c-fos. It would be worthwhile to determine whether MITF can heterodimerize with c-fos in regulating the transcription of the tyrosinase gene.

The manner in which PKC regulates tyrosinase gene expression is unknown. It remains to be seen whether the AP1 factors, MITF or RARs require phosphorylation for optimal function. All these factors contains putative PKC-phosphorylation sites in their respective amino acid sequences. We hypothesize that assembly and/or DNA binding of transcriptional complexes at the M-box region is regulated either directly or indirectly by PKC. It would be interesting to determine whether MITF, RARs and AP-1 factors can interact with each other despite the apparent differences in their structures and whether formation of such complexes is required for tyrosinase expression.

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REGULATION OF MELANOGENESIS IN B16 MOUSE MELANOMA CELLS BY PROTEIN KINASE C (PKC)

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ABSTRACT

The pigment cell-specific expression of tyrosinase and TRP1 has been shown to be important for the production of melanin in pigmented cells. Using a pigmented cell line, B16 mouse melanoma, we obtained evidence that PKC plays a major role in regulating melanogenesis. Chronic treatment with phorbol dibutyrate (PDBu) leads to downregulation of PKC activity and protein levels. This is accompanied by a loss of pigmentation which is correlated with a 50% reduction and a complete loss of TRP1 and tyrosinase respectively. Similar results were obtained with Northern and Western blotting indicating that PKC may regulate the steady state levels of these melanogenic proteins. Deletion analysis of the mouse tyrosinase promoter region showed that a 45 bp region ~100 bp from the transcriptional start site is essential for efficient transcription of the gene. The central part of this region co-localizes with an 11-bp motif termed the M-box. This part of the promoter *i.e.* the M-box is conserved in the promoter regions of melanocyte-specific genes. Using Retinoic acid (RA) and PDBu as opposing agents in regulating PKC levels and melanin biosynthesis, we demonstrate a major role for PKC in transcription of the tyrosinase gene as determined by reporter gene assays. RA increased and PDBu decreased the transcriptional activity of the pigment cell-specific tyrosinase promoter. We also found that the assembly of nuclear protein complexes at the M-box region varies with cell density. One of the complexes was found to be negatively regulated by chronic PDBu treatment. Antibody-supershift experiments showed that this PKC-regulated complex may contain microphthalmia-associated transcription factor (MITF) and retinoic acid receptors (RARs). We conclude that tyrosinase gene expression is regulated by a complex interplay of signaling mechanisms, regulated either directly or indirectly by PKC.