Isolation and Characterization of Complementary DNA Clones Corresponding to Genes Induced in Mouse Epidermis in Vivo by Tumor Promoters

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ABSTRACT

Complementary DNA clones representing genes in SENCAR mouse epidermis, the expression of which is induced 4 h after one topical application of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) were isolated. Of 56 isolated complementary DNA clones, 32 were identified to be identical to either metallothioneins (MT-I and MT-II) or endogenous retroviral like (VL30) sequences. In situ hybridization and analysis of mRNA levels in cell fractions separated by density gradient centrifugation revealed that MT induction was restricted to keratinocytes in the basal cell layer. Immunohistochemistry and time-kinetic studies on mRNA levels in mouse epidermis showed that the increase in MT and VL30 RNAs coincide in time with a TPA-induced transient block in basal cell proliferation (3-12 h after TPA treatment). MT immunoreactivity and transcript levels had returned to control values at a time point (24 h after treatment) when epidermis is known to hyperproliferate. Treatment with other types of tumor promoters showed that MT-I and MT-II mRNAs were coordinately induced and indicated that se-1,2-diacetanoylglycerol, 12-O-retinolcomphorol-13-acetate, and mezerein MT to a lesser degree than TPA. The calcium ionophore A23187 induced mRNA levels for MTs as well as VL30. VL30 and MT mRNA levels were not found to be elevated in epidermal tumors whereas the mRNA level corresponding to glyceraldehyde-3-phosphate dehydrogenase was elevated in tumors and induced by TPA with time kinetics that correlate with a TPA-induced hyperproliferation. These complementary DNA clones provide useful tools in the study of the gene-regulating effects of TPA in a target tissue relevant for tumor promotion.

INTRODUCTION

TPA was first identified as a potent tumor promoter in the mouse skin model of multitarget carcinogenesis (1, 2). In this model the progression of a normal cell to a benign tumor (papilloma) may be viewed as a clonal expansion of an epidermal cell initiated by some genetic change, such as activation of the Harvey ras (c-Ha-ras) protooncogene (3-5). Exactly how TPA initiates a transformed enucleated squamous cell that is eventually shed (6). Proliferation in the basal cell layer of epidermis, migration to the surface during differentiation, and form terminally differentiated enucleated squamous cells that are eventually shed (6). Topical application of TPA on mouse skin elicits a strong pleiotropic response, including a transient inhibition of proliferation, followed by a hyperplastic response and inflammation (7-10). In addition, a subpopulation of keratinocytes shows an increase in the rate of terminal differentiation (11).

Induction of an altered program of gene expression by TPA

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The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; MT, metallothionein; A23187, calcium ionophore; RPA, RNA polymerase; poly(A)+ RNA, polyadenylated RNA; SSC, standard saline citrate (1× SSC is 0.15 M NaCl-0.015 M sodium citrate, pH 7.0).

MATERIALS AND METHODS

Animal Treatments and RNA Isolation. Female SENCAR mice, 6 to 9 weeks old, were purchased from Harlan Sprague-Dawley, Indianapolis, IN. The backs of the mice were shaved 48 h prior to topical treatment with 10 nmol of TPA (Pharmacia, Upplands, Sweden). Other chemical agents used were RPA (LC Services, Woburn, MA), mezerein (LC Services), 4-O-methyl-TPA (Pharmacia), calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO), and se-1,2-diacetanoylglycerol (Sigma). All agents (concentrations are given in "Results") were applied in 200 μl of acetone. Control mice were treated with 200 μl of acetone only. Mice were killed by cervical dislocation. Papillomas were induced by initiation with 100 nmol of 7,12-dimethylbenz(a)anthracene followed by promotion with TPA (3 nmol twice weekly) for 12 weeks. Carcinomas were derived from papillomas, induced as described above, but subsequently treated with 20 mg urethan i.p. once weekly to accelerate malignant conversion (22). Total RNA was isolated from papillomas (time points are given in "Results") and from pools of papillomas or carcinomas as previously described (15). Poly(A)+ RNA was isolated by one binding-elution cycle on oligo(dT)20-cellulose type T-2 (Collaborative Research, Inc., Lexington, MA).

cDNA Library Construction. A cDNA library was constructed using agt10 as the cloning vector essentially as described by Huynh et al. (23). The library was packaged in vitro using a commercially available...
extract system (Promega Biotec, Madison, WI). The resulting cDNA library contained 1 x 10^6 independent clones that were stored and screened without amplification.

Differential Probe. Poly(A)^+ RNA prepared from TPA-treated mouse epidermis was used as template for synthesis of [32P]-labeled cDNA. The conditions used were the same as for the library construction except that the reaction mixture contained 10 μM [α-32P]dCTP, [α-32P]dGTP, and [α-32P]dATP (Amersham Solna, Sweden; specific activity, 600 Ci/mmol). [32P]-labeled cDNA was freed from its template RNA by incubation in 0.3 M NaOH-10 mM EDTA (2 h, 37°C), neutralized (0.5 volume 1 M acetic acid), and passed over a Sephadex G-50 M column (Pharmacia). Single-stranded cDNA (500 ng) was hybridized to poly(A)^+ RNA (25 μg) prepared from aceto-treated epidermis in 8 μL of 0.5 M sodium phosphate buffer (pH 7.5)-1 mM EDTA-0.1% sodium dodecyl sulfate, overlaid with paraffin oil, and incubated for 20 h at 68°C (R^2 4500). After dilution in preheated (60°C) 0.12 mM phosphate buffer (pH 7.4), separation of single- and double-stranded nucleic acids was performed on a 1-mL (bed volume) hydroxylapatite (DNA grade, Pharmacia). The resulting cDNA was performed on a 1-ml (bed volume) hydroxylapatite (DNA grade, Pharmacia). The resulting cDNA was performed on a 1-ml (bed volume) hydroxylapatite (DNA grade, Pharmacia). The resulting cDNA was performed on a 1-ml (bed volume) hydroxylapatite (DNA grade, Pharmacia). The resulting cDNA was performed on a 1-ml (bed volume) hydroxylapatite (DNA grade, Pharmacia). The resulting cDNA was performed on a 1-ml (bed volume) hydroxylapatite (DNA grade, Pharmacia).

Screening Procedure. The differential single-stranded cDNA obtained (approximately 5% of the starting amount of cDNA) was used as a probe in the initial screening of 16,000 clones. The screening was performed by plaque hybridization (24) at a density of 4 x 10^5 plaque-forming units/15-cm diameter plate. The filters (colony/plaque screen; NEN/DuPont, Boston, MA) were hybridized to 0.5 x 10^6 cpm/ml of the differential cDNA probe under conditions recommended by the supplier. Five hundred plaques giving hybridization signals were picked and plaque purified. Amplification of recombinant λ-phages in microtitre dishes and binding of RNA to nylon filters (Genescreen; NEN/DuPont) using a Manifold apparatus (Schleicher & Schuell, Keene, NH) was carried out according to the method of Love and Minton (25). Differential rescreening was performed using [32P]-labeled cDNA probes (1 x 10^5 cpm/ml, 1 x 10^5 cpm/ml) prepared from poly(A)^+ RNA from TPA- and acetone-treated epidermis, respectively. The probe length (400-3000 base-pairs) as estimated by agarose gel electrophoresis was similar for both probes.

RNA Analysis. Total RNA was fractionated by electrophoresis through formaldehyde agarose gel. 1.1% gels and transferred to Hybond-N filters, which were hybridized and washed as recommended by the supplier (Amersham). In addition to subcloned λB3 and λB10 cDNA, the following nick-translation (24) plasmids were used as probes: pODC16 (26), v-fos (27), M, 27, 000 keratin (K1, p4-2, Ref. 21) and GAPDH (28). Complementary synthetic oligonucleotides specific for MT-I (5'-GACGCTGGTGGTCCGA-3') and MT-II (5'-GCTAGGCTTCTACATGTT-3') were synthesized on an Applied Biosystems Oligonucleotide Synthesizer (Foster City, CA). Oligonucleotides were labeled by T4 polynucleotide kinase (Boehringer-Mannheim, Bromma, Sweden) as described (24). Hybridization temperatures were 45°C for the MT-I probe and 50°C for the MT-II probe under conditions described by Zeff et al. (29).

Relative Quantification. Signal intensities on Northern blot autoradiograms were measured densitometrically. To overcome any nonlinear response that may be inherent in the X-ray film and/or due to use of intensifying screens, the density values were converted to cpm using a standard curve where cpm values (determined using a Beckman liquid intensifying screens, the density values were converted to cpm using a standard curve where cpm values (determined using a Beckman liquid...
of 500 clones (~3% of the screened clones). One clone hybridized to the ODC probe, thus validating the approach taken since an accumulation of ODC mRNA is known to take place in mouse epidermis 4 h after TPA treatment (15). Identical filters of dot-blotted λDNA from these clones were then re-screened several times using complex cDNA probes generated from TPA or acetone-treated mouse epidermis (Fig. 1). In this way 56 cDNA clones showing consistent differential hybridization were isolated, corresponding to 0.35% of the clones initially screened.

Clones showing strong differential hybridization were selected for further study. Two groups of differentially expressed cDNAs were found to account for more than 50% of the isolated cDNA clones indicating that accumulation of mRNA for these genes is a major early response to TPA. TPA induced transcripts recognized by the clones λB3 (0.5-kilobase transcript) and λB10 (5.6-kilobase transcript), representing the two groups are shown in Fig. 2A. Of the 56 isolated cDNA clones 10 cross-hybridized to λB3 and 22 to λB10.

The cDNA inserts (ranging from 0.85 to 2 kilobases) of clones cross-hybridizing to λB10 were found to have a high sequence similarity (>90%) to the LTR region of several members of the endogenous retrovirus-like VL30 gene family (37). Southern blot hybridization confirmed that λB10 (850-base pair cDNA insert) recognizes a multigene family (Fig. 2B).

The sequence of λB3 was identical to a full length mouse MT-II cDNA (data not shown). In the mouse there are two metallothionein genes known, which have been referred to as MT-I and MT-II (38). MT-I and MT-II are transcribed from genes located proximal to each other on chromosome 8 (38). It has been previously shown that both MTs are induced together in response to agents such as lipopolysaccharide, cadmium, and dexamethasone (38). However, it is not known whether MT-I is induced by TPA. To answer this question we used synthetic oligonucleotide probes specific for MT-I and MT-II. Of the 22 cDNA clones that cross-hybridized to clone B3, 14 corresponded to MT-II and 8 to MT-I. The identification of MT-I was confirmed by partial sequencing of one of the clones that hybridized to the MT-I oligonucleotide.

Kinetics of MT-I, MT-II, VL30, ODC, c-fos, and GAPDH Induction. As shown in Fig. 3 an elevated steady-state level of c-fos mRNA was detected 1.5 h after one topical application of 10 nmol of TPA, confirming a previous observation by Rose-John et al. (16). MT-I, MT-II, VL30, and ODC mRNA reached their maximal levels 4 h after treatment and returned to control levels after 12–24 h. At 4 h the relative increase was approximately 30-fold for MT-I, 40-fold for MT-II, 20-fold for VL30, and 15-fold for ODC. In the Northern blot analysis we used the GAPDH mRNA levels as a control for the amount of RNA loaded on the gel. This was, however, possible only at early time points (<4 h) (Figs. 2 and 3). At later time points a marked increase in GAPDH mRNA level was observed reaching a
maximum (10-fold induction) at 24 h.

Regulation of VL30, MT-I, and MT-II mRNA Levels. To ascertain whether MT-I, MT-II, and VL30 might be differentially regulated with respect to various tumor promoters (Fig. 4), we chose a time point (4 h) when these genes were maximally induced by TPA. Mezerein (10, 39) and RPA (40) are PKC activators that have the capacity to enhance the formation of tumors in SENCAR mouse epidermis. Both agents, applied at a dose of 10 nmol, did induce MT-I and MT-II albeit to a lesser degree than TPA. The induction pattern for VL30 was found to be slightly different. RPA was almost as potent as TPA and mezerein more potent than TPA, in inducing VL30 expression.

SH-l,2-Dioctanoylglycerol is a synthetic analogue to endogenous diacylglycerols known to activate PKC and to be active as a tumor promoter (41). Treatment with sw-l,2-dioctanoylglycerol at a dose 1000 times higher (10 μmol) than that used for TPA resulted in a slight induction of the steady-state mRNA levels for the genes investigated. 4-O-methyl-TPA is an incomplete tumor promoter and unable to activate PKC, although at the dose used (0.4 nmol), it is as potent as TPA in inducing hyperproliferation in mouse skin (9). This treatment did not cause any alteration of the mRNA levels for MTs and VL30.

The calcium ionophore A23187 was used at a dose (0.49 μmol) chosen to give a hyperplastic response comparable to that of 10 nmol of TPA (42). This treatment resulted in an increased expression of all three genes.

It has recently been established that treatment of mouse skin with certain compounds (i.e., TPA, mezerein, or A23187) before the initiation phase of chemical carcinogenesis results in an enhancement of promoting activity of certain weak or incomplete promoters such as mezerein (39). One experiment was performed to investigate if repetitive treatments with TPA result in increased mezerein induction of MT or VL30 expression. Mice were treated twice with 10 nmol of TPA with a 3-day interval followed by one treatment with 10 nmol of mezerein. No further increase in mezerein-induced mRNA levels was detected; rather a reduced response was observed, as compared to a single mezerein treatment. A finding most easily explained by down-regulation of PKC (43) leading to a decreased responsiveness.

In papillomas and carcinomas MT and VL30 mRNA levels were not significantly increased whereas GAPDH mRNA levels were increased 5–10-fold. High levels of ODC mRNA were also found in tumors (data not shown) in agreement with earlier reports (15).

Localized Induction of MT mRNA. The localization of mRNA expression before and after treatment with 10 nmol TPA was studied using in situ hybridization. In acetone-treated skin, a low level of MT mRNA was observed in cells of the interfollicular epidermis (Fig. 5c) and in some hair follicle cells (Fig. 5b). TPA-induced MT expression in cells in the basal compartment of the interfollicular epidermis (Fig. 5a) and to a low degree, in dermal fibroblasts. In order to confirm that it was the basal cells that had responded to TPA with increased MT expression, total RNA from Percoll-separated epidermal cell fractions were analyzed by Northern hybridization (Fig. 6). The buoyant densities of keratinocytes decrease as they terminally differentiate. Consequently, Percoll gradient centrifugation separates keratinocytes into populations that differ in their stage of terminal differentiation. The fraction

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Fig. 3. Time course of induction of c-fos, MT-I, MT-II, VL30, ODC, and GAPDH mRNA. Northern blot hybridization of RNA from mouse epidermis at different times after a single topical treatment with TPA (10 nmol). Twenty μg total RNA were loaded in each lane. The plasmid probes used are described in "Materials and Methods." kb, kilobases.

Fig. 4. Analysis of mRNA levels corresponding to MT-I, MT-II, VL30, and GAPDH in mouse epidermis 4 h after a single topical treatment with various tumor promoters. The doses and abbreviations used are: 200 μl acetone-treated control, Cont; 10 nmol 12-O-retinoylphorbol-13-acetate, RPA; 10 nmol mezerein, Mez; 0.49 μmol calcium ionophore, A23187; 10 μmol sn-1,2-dioctanoylglycerol, DOG; papillomas, Pap; carcinomas, Car; and two treatments with TPA (10 nmol) followed by one treatment with mezerein (10 nmol) (treatments were performed with an interval of 3 days), 2x TPA + Mez. Twenty μg of total RNA, isolated 4 h after treatment, were loaded in each lane. Locations of 28S and 18S tRNA are indicated.
Fig. 5. Localization of MT mRNA in mouse skin before and 4 h after a single TPA treatment (10 nmol) by in situ hybridization. Formalin-fixed sections were hybridized with a 35S-labeled MT-II riboprobe (subcloned λB3 cDNA insert; see “Materials and Methods”). Exposure time was 10 days and all sections were counterstained with hematoxylin. a, TPA induction of MT mRNA in the basal cell layer of the interfollicular epidermis; b, TPA induction of MT mRNA in the hair follicle; c, control section (4 h after acetone treatment) (arrow, MT expression in some basal cells); d, base of a hair follicle from a control section (arrow, MT expression in the dense part of the follicle); e, negative control, skin section from a TPA-treated mouse hybridized to a 35S-labeled MT sense riboprobe. X 490.

Fig. 6. Levels of MT transcripts in cellular subfractions of acetone (CONT) and TPA-treated mouse epidermis. The epidermal cells were fractionated on Percoll gradients into three fractions. Fraction I (FI) contained cells from the differentiated cell layers of epidermis. Fraction II (FII) contained small basal cells and cells from the differentiated cell layers. Fraction III (FIII) was composed predominantly of small basal cells. Total RNA was extracted from the fractions (4 h after the treatments). A, Northern blot hybridized to a 32P-labeled MT probe (λB3 cDNA insert). B, the same Northern blot hybridized to a probe recognizing the M, 67,000 (67 KD) keratin subunit. KI, confirming the integrity of the RNA. KI is known to be expressed at different levels in all epidermal layers, as shown by in situ hybridization (62).

with the lowest density (FI, 1.05 g/ml) contained nucleated polygonal cells and cells with a high cytoplasmic:nuclear ratio. These morphologies are typical for the differentiated layers of epidermis. Fraction II (FII, 1.075 g/ml) was the most heterogeneous, consisting of small basal type cells and cells with high cytoplasmic:nuclear ratio. In fraction III (FIII, 1.08 g/ml) the small basal cells predominated. In acetone-treated epidermis MT-I and -II were expressed in all fractions isolated. TPA treatment caused an elevation of MT expression in both the FII and the FIII fractions. The highest MT induction was observed in the basal cell fraction having the highest buoyant density (FIII).

Localization of MT Immunoreactivity in Mouse Skin. A polyclonal rabbit antiserum raised against rat liver MT-I was used to localize MT protein in mouse skin. This antibody prepara-

tion recognized both mouse MT-I and MT-II protein as determined by Western blot analysis (data not shown). In untreated epidermis only a limited number of keratinocytes showed weak staining. Immunoreactivity in the interfollicular epidermis and hair follicles could be seen 4–16 h after TPA treatment (Fig. 7a). The immunoreactivity at 24 (Fig. 7b) and 48 h after treatment was comparable to that of untreated mouse skin.

DISCUSSION

Previous investigators have utilized the technique of differential hybridization to obtain cDNA clones of genes that are induced by TPA. These studies were performed using cultured cells, either density-arrested fibroblast cell lines (44, 45) or growing primary skin fibroblast (46). In the present study we sought to characterize early (4 h) effects of TPA on gene expression in intact mouse epidermis, where TPA classically functions as a potent tumor promoter. The identification of such genes may provide a useful system for the investigation of cellular events induced by TPA in epidermis, such as block in proliferation, hyperproliferation, hyperplasia, and terminal differentiation.

Two gene families, the metallothioneins and the endogenous VL30 retroviral sequences, were found to account for more than 50% of the isolated cDNA clones, indicating that accumulation of mRNA for these genes is a major early response to TPA. This is also confirmed by Northern blot analysis showing a 30–40-fold induction of MT mRNA and a 20-fold induction of VL30 mRNA.

The mouse VL30 gene family consists of 100–200 dispersed copies of DNA sequences having many retroviral characteristics (37). A recent sequence analysis of a complete VL30 element suggests the absence of any coding potential in these elements and a closer relationship to retrotransposons than to retroviruses (47). VL30 sequences can be reverse transcribed and integrated into the genome (48). This suggests that VL30 could act as an insertional mutagen. We were unable to detect VL30 transcripts of other sizes than 5.6 kilobases (49), which would have been indicative of hybrid transcripts between VL30 and
The TPA-induced expression of the metallothionein genes is localized to the basal cells while differentiating suprabasal cells are essentially nonresponsive. No differential expression was found in untreated epidermis and it has been shown that TPA-induced transforming growth factor \( \beta \) expression is confined to differentiating suprabasal cells (19). This indicates that the differentiation state of the target cell is an important determinant of the response to TPA. The underlying molecular mechanisms are at present not understood but differential expressions of trans-acting factors and/or proteins involved in signal transduction are likely to be decisive factors. In fact, a heterogeneity of TPA binding in primary mouse keratinocytes at different stages of maturation has been reported (52).

The kinetic analysis of mRNA steady-state levels in mouse epidermis after TPA treatment suggests a sequence of events where the early increase in expression of the \( \text{fos} \) (and most likely the \( \text{jun} \)) gene family may in turn be an important factor behind the subsequent increase in expression of a second group of genes such as MT-I, MT-II, VL30, and ODC. In these studies it was also discovered that the gene for the glycolytic enzyme GAPDH belongs to another group of genes where the peak of expression coincide in time with the induction of DNA synthesis that reaches a maximum 18–24 h after TPA treatment (7–9). GAPDH expression was also found to be elevated in both benign and malignant tumors where keratinocytes are known to exhibit an enhanced rate of proliferation (53). The GAPDH expression is therefore most likely associated with an increased rate of cell proliferation. Other genes showing a pattern of expression similar to that of GAPDH include the \( M_{55,000} \) (K14) proliferation-associated keratin subunit (21) and two tumor-associated sequences mal-1 and mal-2 (54).

MTs are ubiquitous low molecular weight proteins that are characterized by a selective capacity to bind heavy metal ions. Aside from the role of MTs in metal detoxification MTs are thought to play a central role in maintaining cellular Zn\(^{2+}\) homeostasis and thereby to be indirectly involved in the regulation of a variety of Zn\(^{2+}\)-dependent processes, e.g., transcription, DNA replication, DNA repair, and protein synthesis (55).

The MT expression in mouse epidermis after TPA treatment may be more associated to the transient block in DNA synthesis than to enhanced proliferation. The block of DNA synthesis, 3–9 h after TPA treatment (7–9), coincides in time with the MT induction. When DNA synthesis reaches a maximum, 18–24 h after TPA treatment MT protein is undetectable by immunohistochemistry and the MT mRNAs have returned to basal levels. Furthermore, 4-O-methyl-TPA does not induce MT and does not cause an initial depression of DNA synthesis while inducing nearly the same proliferative response as TPA (9). Finally, MT expression in both papillomas and carcinomas is similar to that in untreated epidermis. The identification of human MT-II\(_a\), among genes induced in primary skin fibroblasts after a transient inhibition of cell growth caused by TPA (46) is in agreement with these findings. In addition, MTs were not present among genes showing increased expression during a TPA-induced G_s-G_m transition of density-arrested fibroblasts (44, 45), or among genes induced after serum stimulation of quiescent fibroblasts (56). Considering the localized induction of MT expression in the basal cell fraction, it is tempting to speculate that the induction is restricted to actively growing keratinocytes and that enhanced MT synthesis could result in a transient depletion of Zn\(^{2+}\) essential for enzymes involved in DNA synthesis. Csermely et al. (57) have shown a redistribution of Zn\(^{2+}\) from the nucleus to the cytosol in thymocytes incubated

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flanking cellular genes. It is known that the TPA responsiveness is an intrinsic property of some VL30 genes (48) and that some isolated VL30 LTRs contain functional enhancer sequences (50). The VL30 transcript cloned in this study (B10) contains LTR sequences that can mediate transcriptional induction by TPA in keratinocytes. This makes it possible that VL30 LTRs may activate the transcription of cellular genes from distance as has been shown for retroviruses.

Mouse MT-II and human MT-II\(_a\) share common TPA response elements such as AP-1 and AP-2 binding sites in their promoter regions. Mouse MT-II is therefore most likely induced by TPA via mechanisms identical to those described for human MT-II\(_a\) (12). We report for the first time that also MT-I expression is induced by TPA. No obvious TPA response element is present in the published 300-base pair 5'-flanking sequence. Further experiments are needed to determine if the TPA-dependent accumulation of MT-I is primarily due to increased transcription and, if so, by which mechanism. Our results are consistent with the idea that mouse MT-I and II could be coordinately regulated in view of their location within 6 kilobases of each other (38). This is further supported by analysis of DNAse I hypersensitive sites (51) and similarities in the response to various inducers such as RPA, mezerein, calcium ionophore, diacylglycerol (this study), cadmium, dexamethasone, and lipopolysaccharide (38).

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Fig. 7. Immunolocalization of epidermal metallothionein after a single topical treatment with 10 nmol of TPA. The primary antibody used was a rabbit antiserum against rat liver MT-I that exclusively recognizes both mouse MT-I and MT-II (determined by Western blot analysis; data not shown). The antiserum was preabsorbed by incubation with minced skin from an untreated mouse. Sections were counterstained with hematoxylin. A, immunoreactivity 8 h after TPA treatment; B, immunoreactivity 24 h after TPA treatment. Omission of primary antibody, use of preabsorbed normal rabbit serum, or blocking with purified MT resulted in no staining (data not shown). × 263.
for 90 min in the present of TPA; this could be driven by elevated MT levels.

Our results imply that induction of VL30 and MTs is associated with tumor promotion by PKC activators. However, for mouse skin tumor promoters of other types, e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin, this association is not observed, indicating the existence of mechanistically separate pathways through which tumor promoters may act. It is possible that the induction of MTs is a good indicator of promotion potency of PKC activators since sn-1,2-diocanoylglycerol, RPA, and mezerein induced MT to a lesser degree than TPA. This correlation was not observed for VL30, indicating that VL30 and MT are regulated by different mechanisms in mouse epidermis. We show that the calcium ionophore A23187 induced all three genes. Cytosolic free Ca++ thus may be another signal transduction pathway used to modulate the expression of these genes.

Ha-ras-transformed fibroblasts in vitro exhibit an increased VL30 and MT expression (58). This may be explained by increased levels of diacylglycerols (59) that activate PKC. A ras response element in the polyoma enhancer is identical to the AP-1 (fos/jun) binding site (60) and one theory raised is that enhanced transcriptional activation via this response element is a critical event in transformation (61). We were unable to detect elevated levels of VL30 and MT mRNA in either benign or malignant skin tumors. These tumors were produced by induction with DMBA followed by TPA promotion. Using this protocol it is known that approximately 90% of the tumors contain an activated c-Ha-ras gene (4). Possible explanations for this finding may be the existence of cell type-specific differences in gene regulation between fibroblasts and keratinocytes or that cells harboring an activated c-Ha-ras oncogene are sensitized to growth factors (PKC activators) present in the serum, resulting in an increased MT and VL30 expression in vitro, whereas cells in epidermal tumors are not continuously exposed to such factors, unless repetitively treated with TPA.

In summary the present study has described the isolation of cDNA clones corresponding to genes induced by TPA in mouse epidermis and the identification of MT-1, MT-2, and VL30 among these clones. The MT induction was restricted to keratinocytes in the basal cell layer indicating that the maturation status of the target cell is important for TPa effect on gene regulation. TPA induction of MTs and VL30 in epidermis coincides in time with a block in proliferation. Neither MTs or VL30 were overexpressed in skin tumors whereas the glycolytic enzyme GAPDH showed elevated expression both in tumors and during hyperproliferation in epidermis. The functional importance of these findings for the complex process of tumor promotion remains to be determined. However, these genes provide a valuable system to study the mechanisms through which TPA can alter mRNA levels in a target tissue in vivo.

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