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THE INDUCTION OF ORNITHINE DECARBOXYLASE BY THE TUMOR PROMOTER TPA IS CONTROLLED AT THE POST-TRANSCRIPTIONAL LEVEL IN MURINE SWISS 3T3 FIBROBLASTS

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The expression of ornithine decarboxylase (ODC) mRNA after treatment of murine Swiss 3T3 cells with the tumor promoter TPA was studied. The induction of ODC mRNA was detectable after 20-40 min, peaked after 60-120 min and declined within 24 hrs. Using an in vitro nuclear transcription assay, we found that the polymerase II density on the ODC gene is not affected by TPA treatment. Additionally, we were able to detect stable ODC mRNAs in cycloheximide pretreated fibroblasts. These two different experimental approaches lead us to the interpretation that in Swiss 3T3 cells TPA controls ODC expression predominately at the post transcriptional level by prolonging the half-life of ODC-mRNA.

Ornithine decarboxylase (ODC, E.C. 4.1.1.17) is the first and rate limiting enzyme in polyamine synthesis. It catalyses the conversion of ornithine to putrescine. The activity of this enzyme in vivo undergoes drastic and rapid changes. High levels of enzyme activity are frequently associated with rapidly dividing cells. Stationary cells exhibit little or no ODC activity (for reviews see refs. 1,2). Depending on the cell type the induction of ODC can be achieved by administration of various drugs such as growth hormones, cAMP analogues, phosphodiesterase inhibitors, steroid hormones (1). The tumor promoter TPA has been shown to be one of the most potent inducers of ODC activity in mouse skin (3,4), in rat liver (5) and in Swiss 3T3 fibroblasts (6). The exact molecular mechanism of skin tumor promotion by TPA is still unknown, but there is accumulating evidence that induction of epidermal ODC activity is one of the essential components in the induction of epidermal hyperplasia which is an essential condition of tumor promotion by TPA (4,7,8).

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The mechanism by which TPA induces ODC is unknown. In hamster fibroblasts induction of ODC by TPA involves changes at the mRNA and protein level (9). The object of our experiments was to define the level at which TPA controls the induction of ODC in vitro. This information may help to better understand how phorbol esters modify gene expression in the course of tumor promotion.

Methods

2.1. Cell culture; Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Medium was changed routineously 24 hrs prior to treatment or harvesting. Serum starved cells were grown to 80% confluence in 10% serum and subsequently grown 2-3 days in 0.5% serum.

2.2. Northern blot analysis; Cells were washed three times with ice cold PBS, and lysed in 50 mM Tris pH 8.4, 2% SDS, 1% Diethyl Pyrocarbonate. The cell lysate was extensively extracted with phenol, phenol/chloroform and chloroform, precipitated in 4 M LiCl at 4°C overnight. The pellet was resuspended in H_2O at a concentration of 1 mg/ml. RNA (5 µg) was heated to 65°C for 10 min in 50% formamide, 20 mM MOPS, 5 mM Na-acetate, 1 mM EDTA, 2.2 M formaldehyde, prior to gel electrophoresis in 1% agarose, containing 2.2 M formaldehyde, 20 mM MOPS, 5 mM Na-acetate, 1 mM EDTA at 40 V overnight. The gel was stained with 0.5 µg/ml ethidium bromide, destained, photographed and transferred to GeneScreen plus membranes (NEN) according to suppliers instructions. The filters were prehybridized at 65°C for 1 hr in 10% dextran sulfate, 1 M NaCl, 1% SDS and hybridized in the same solution with a nicktranslated probe. The probe was the isolated and purified 750 bp PST I fragment of pOD48 supplied by Dr. P. Coffino (10), the specific activity was 5 x 10^8 cpm/µg DNA.

2.3. In vitro nuclear transcription assay; nuclei preparation and in vitro transcription was essentially done as described in (11). Briefly, the cell pellet was resuspended in 10 ml lysis buffer (11), pelleted at 4500 rpm for 4 min, resuspended in 5 ml lysis buffer and spun through 3.5 ml 30% sucrose in lysis buffer. The nuclei were resuspended in nuclei storage buffer (11) and frozen at -70°C. The transcription reaction was performed in the presence of 330 µM ATP, UTP, GTP, 10 µM CTP and 25 µCi ^{32}P-CTP (410 Ci/mmol) for 10 min at 26°C. The nuclei were treated with DNAse I, proteinase K for 30 min and for 15 min with SDS at 37°C. The RNA was phenol/chloroform extracted and TCA precipitated and counted. Equal amounts of radioactivity were used for hybridization. The RNA was incubated at 65°C for 5' prior to hybridization to GeneScreen plus (NEN) filters with immobilized pOD48, pBR322 and GAPDH DNA on it: Hybridization was performed in 50% formamide, 10% dextran sulfate, 1 M NaCl and 1% SDS at 42°C for 72 hrs. The washing procedure was the same as described in (12).

Results and Discussion

The addition of TPA (0.1 µM) to Swiss 3T3 cells growing in 10% fetal calf serum (fcs) leads to very rapid and transient increase in ODC messenger RNA

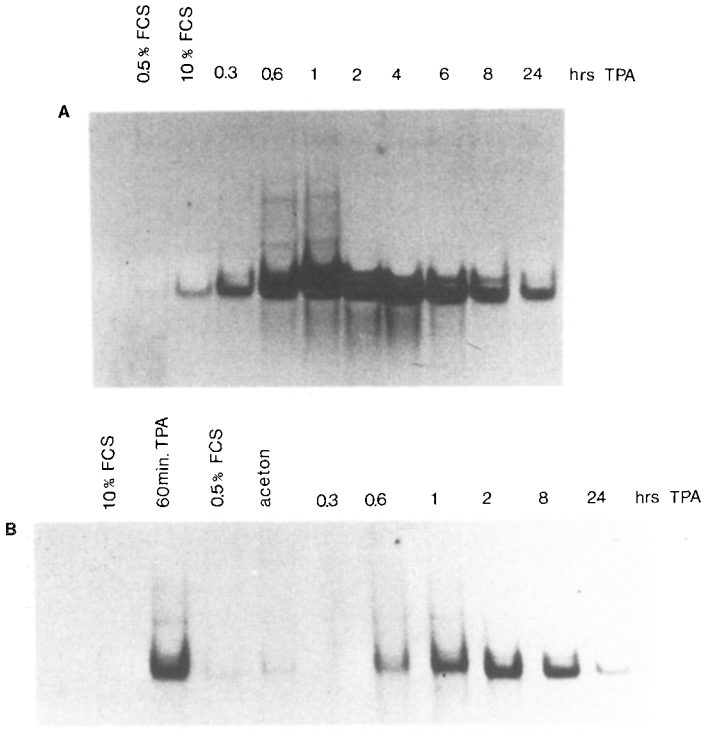

Figure 1. Northern blot hybridization of RNA extracted from 3T3 cells treated with TPA (0.1 µM).
A: cells grown in 10% fetal calf serum, B: cells grown in 0.5% fetal calf serum.

as detected by Northern blot hybridization. The induction is detectable after 20-40 min, the peak is at 1-2 hrs. After 24 hrs the ODC mRNA level returns to values close to the control level (Fig. 1A). This induction of ODC-mRNA is somewhat more rapid than previously described (6,9). Differences might be due to different cell systems used or to the fact that other authors did not determine ODC induction earlier than 3 hrs after TPA. Fig. 1, panel B shows the same experiment as in panel A but with serum starved cells (see experimental for details). For direct comparision RNA from cells treated with TPA in 10% fcs for 1 h has been applied to the same gel. Altogether the induction in 0.5% serum seems to be somewhat lower than in 10% serum but it follows the same time course.

Fig. 2 shows an in vitro transcription assay of nuclei isolated from cells grown in 10% serum and treated with 0.1 µM TPA for 0, 0.5, 2, 4 and

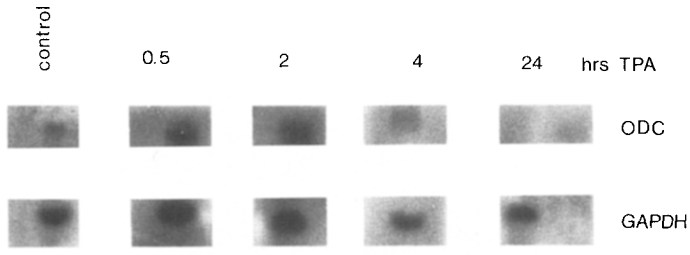

Figure 2. Hybridization of ^{32}P-run-on transcripts to c-DNA clones of GAPDH and ODC. Nuclei were prepared after treatment with TPA (0.1 μM) for the times indicated in the figure.

24 hrs. In an in vitro nuclear transcription assay, transcripts that are initiated in vivo are elongated in vitro using ^{32}P-labelled ribonucleotide triphosphate. The hybridization of labelled RNA to complementary DNA immobilized on nitrocellulose is a measure of the level of transcription at the time of nuclei preparation since new RNA transcription is not initiated in vitro (12,14,15). As an internal standard we used the transcription of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) which is supposed to remain constant because in all tissues examined the expression of GADPH is regulated at the post-transcriptional level (13). In fact, the amount of hybridizable GAPDH-RNA remains constant throughout the experiment. The hybridized ODC signal has been quantitated by densitometry. The relative rate of transcription was 1, 2.8, 3.6, 2.4, and 1 for TPA treatments 0, 0.5, 2.4, and 24 hrs respectively. In comparison with Fig. 1A it becomes clear that differences in the transcriptional rate cannot explain the at least 20 to 50-fold increase in ODC-mRNA seen after TPA treatment. Since there is only a slight transcripitional induction of ODC-RNA by TPA but a very strong increase in hybridiz-able ODC mRNA, the ODC induction by TPA must be regulated predominantly at the post transcriptional level.

To support this assumption we treated 3T3 cells with the transscriptional inhibitor actinomycin D and the translation inhibitor cycloheximide. The results of this experiment are shown in Fig. 3. A 1 h pretreatment with actinomycin D abolishes completely the induction of ODC-mRNA by TPA.Actinomycin alone and 2.5 hrs cycloheximide and

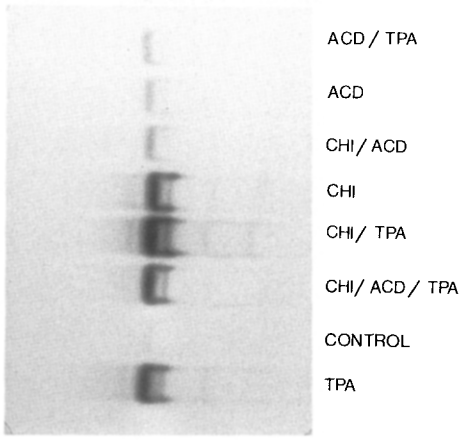

Figure 3. Northern blot hybridization of RNA extracted from 3T3 cells treated with actinomycin (ACD; 5 µg/ml), cycloheximide (CHI,10 µg/ml) and TPA (0.1 µM) for the times indicated in the text.

actinomycin fail to induce ODC-mRNA. But 2.5 hrs pretreatment with cycloheximide or 4 hrs pretreatment with cycloheximide plus 2 hrs pretreatment with actinomycin lead to a strong indution of ODC mRNA which is still even surpassed by the combination of cycloheximide 2.5 hrs and TPA (2 hrs). An explanation for this results is that cycloheximide leads to an induction of ODC mRNA by extending the half life of existing RNAs without inducing new transcription of ODC (lanes 8,9). TPA together with cycloheximide leads to a stronger induction suggesting that TPA also slightly enhanced transcription of ODC(comp. Fig. 2). Interestingly, Olsen and Spizz (19) reported that cycloheximide induced transcription of ODC mRNA and had no significant effect on the stability of ODC mRNA in the BC_3H1 cell line. In this cell line ODC mRNA induction by fetal calf serum seems to be regulated at the transcriptional level, whereas in 3T3 fibroblasts the transcriptional component of the TPA induction in 10 % serum is very small compared with the posttranscriptional component (Fig. 2, Fig. 3). Accordingly, the effect of serum and cycloheximide in BC_3H1 cells is synergistic and cycloheximide induction is much more pronounced than serum induction. In 3T3 fibroblasts induction of ODC mRNA by cycloheximide and TPA is identical and not additive (Fig. 3). Furthermore,

in BC_3H1 cells cycloheximide shows only an effect after 8 hrs whereas in 3T3 cells it induces ODC mRNA after 2 hrs. These differences may indicate that the effects fo cycloheximide in BC_3H1 cells and 3T3 fibroblasts are brought about by different mechanisms. It may be proposed that the main effect of TPA on ODC induction is 3T3 fibroblasts consists of an increase of the half life of ODC-mRNA. It is tempting to speculate that a phosphorylation event catalyzed by protein kinase C leads to a rapid inactivation of a specific RNAase which normally is responsible for the short half life of the ODC message. TPA has been shown to be a potent activator of protein kinase C by mimicking the effect of the second messenger diacylglycerol (20). Most of the biological effects of the phorbol ester, including ODC induction (21), can probably be explained by PKC activation. This hypothesis is supported by the finding of Shaw and Kamen (22) that many transiently expressed genes contain AT rich regions at their 3' end with the core sequence ATTTA. These regions are shown to decrease the stability of the mRNAs. Furthermore they show that TPA increases the stability of such mRNAs. Mouse ODC mRNA contains one ATTTA sequence motif within its 3' end.

Recently it has been published that TPA can also affect the stability of collagenase mRNA in rabbit synovial cells (20). Moreover, there is accumulating evidence that genes involved in the regulation of cell proliferation like the oncogene c-myc are regulated at the posttranscriptional level (17). In 3T3 cells c-myc has been found to be induced by TPA (16). Whether ODC induction by TPA in mouse skin in vivo follows a similar mechanisms as shown here for 3T3 cells remains to be established.

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