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The tumor promoter phorbol 12-myristate 13-acetate induces a program of altered gene expression similar to that induced by platelet-derived growth factor and transforming oncogenes

(malignant transformation/growth factors/transcription regulation/lysosomal protein)

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ABSTRACT **Treatment of mouse NIH 3T3 cells with the phorbol ester tumor promoter, phorbol 12-myristate 13-acetate, results in altered transcription of several genes as measured in nuclear run-off experiments. The first set of genes, whose altered transcription occurs rapidly in the absence of protein synthesis, is typified by induction of c-*myc* and c-*fos* and decreased transcription of α_2 type I procollagen. This work demonstrates the existence of a second class of genes whose rapidly increased transcription requires prior protein synthesis, which is represented by the gene encoding a secreted lysosomal protein, MEP. Similar induction of MEP RNA is seen after treatment with platelet-derived growth factor or transformation with Kirsten sarcoma virus.**

Recent evidence has accumulated indicating that transformation of cultured cells by transforming oncogenes results in increased mRNA and protein levels for a variety of cellular proteins including the secreted protease, plasminogen activator (1, 2), the secreted lysosomal protein MEP (3, 4), class I major histocompatibility antigens (5), and increased mRNA levels for other genes whose protein product has not been determined (6–8). Expression of other genes such as the genes for the procollagens (9–11) and fibronectin (12–14) is decreased. Altered patterns of mRNA accumulation and protein synthesis similar to those seen in transformed cells are seen in cells treated with the tumor promoter, phorbol 12-myristate 12-acetate (PMA), or platelet-derived growth factor (PDGF) (15–23).

An increased level of a given RNA species must reflect either an increase in its rate of transcription, altered kinetics of intracellular transport, processing, localization, or a decrease in its rate of destruction. Control of gene expression at the level of transcription has been demonstrated for hemoglobin (24), fibronectin (14), collagen (14), c-*myc* and c-*fos* (25–27), and other proteins. Transcriptional-level control is not universal, however. Significant regulation of RNA at the level of RNA processing or degradation has been shown for a variety of growth-regulated gene products including c-*myc* (28) and dihydrofolate reductase (29).

Our laboratory has been studying regulation of expression of a major excreted protein (MEP) as a model for the positive control of gene expression by malignant transformation, growth factors, and the tumor promoter PMA. MEP is a phosphoglycoprotein of M_r 39,000 that contains the mannose 6-phosphate marker characteristic of lysosomal proteins (30). It is processed intracellularly into smaller forms of M_r 29,000 and 20,000 (31), both of which are found in lysosomes. In Kirsten sarcoma virus-transformed NIH 3T3 cell fibroblasts (KNIH 3T3 cells), MEP secretion is increased 50- to 100-fold compared to parental NIH 3T3 cells (3). MEP synthesis and secretion are stimulated in nontransformed cells by the addition of the growth-promoting agents PMA (19) and PDGF (16, 17). It has been shown recently that PMA and PDGF increase the levels of mRNA coding for MEP (4, 32).

In the course of identifying the level of control responsible for this increase in MEP mRNA, we have found that MEP falls into a previously undescribed class of genes regulated by growth factors and transformation, because rapid increases in MEP transcription require prior protein synthesis. For this study, we approached the control of MEP RNA levels with the technique of nuclear run-off transcription assays (14, 24). Isolated nuclei are incubated with [α-^{32}P]NTP and other unlabeled nucleotides under conditions that allow partial elongation of nascent RNA strands but no new transcription initiations. When this radioactive RNA is purified and hybridized on filters to specific DNA sequences, the relative amount of each RNA species reflects its relative transcription rate at the time the nuclei were harvested. Our data indicate that MEP induction by transformation or by growth factors is largely due to transcriptional regulation of the MEP gene. These data, therefore, provide direct evidence that the cellular response to certain growth factors or oncogenes involves rapid increases in transcription of genes such as those encoding MEP.

MATERIALS AND METHODS

Cell Lines. NIH 3T3 mouse fibroblasts and KNIH 3T3 cells were obtained from C. D. Scher (Univ. of Pennsylvania). Their growth conditions have been described (3).

Nuclear Transcription Assays. Nuclei were isolated from fibroblasts by the technique of Groudine *et al.* (24). Nuclear transcription and RNA purification were done essentially as described (24) with the modifications of Tyagi (14). Reaction mixtures contained 20 mM Tris·HCl (pH 7.9), 30% (vol/vol) glycerol, 2.5 mM dithiothreitol, 5 mM $MgCl_2$, 110 mM KCl, and 500 μM each ATP, CTP, and GTP. [α-^{32}P]UTP (100 μCi; ≈3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) was used to label nascent RNA strands. Each reaction was 200 μl and was performed with 30–60 μl of nuclei (packed volume, an estimated 100–150 μg of DNA). Incubation of the transcription reaction was at 26°C for 45 min. Whole DNA plasmids were diluted in 1× SSC (1× SSC = 0.15 M NaCl/0.015 M Na citrate) and then treated with NaOH to a concentration of 0.1 M NaOH. The mixture was neutralized with monobasic sodium phosphate and applied to BA-85 nitrocellulose (Schleicher & Schuell) using a Minifold 2 (slot blot) apparatus (Schleicher & Schuell) and gentle suction. Each slot received 1.5 μg of plasmid DNA. Filters were

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; PDGF, platelet-derived growth factor; MEP, major excreted protein.

baked at 80°C for 2 hr *in vacuo*. Prehybridization, hybridization, and washing of filters were done essentially as described (24). Hybridization volumes of 3 ml included 5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate, and 10% (wt/vol) dextran sulfate. Hybridizations were continued for 4 days at 42°C. RNase A treatment (20 μg/ml in 2× SSC at 37°C for 30 min) was performed if background was excessive after routine washing. Filters were exposed to Kodak XAR-5 film with DuPont Cronex Lightning Plus intensifying screens at −70°C. DNA on the filters was in vast excess to the RNA in solution; it was demonstrated that the relative intensity of a given band was proportional to the amount of sample hybridized (data not shown). RNase treatment did not significantly change the relative intensities of different bands, which were measured by densitometry of the original autoradiograms.

Plasmid DNAs. Plasmid pMMEP-14 contains a 1.4-kilobase insert coding for part of mouse MEP; it was cloned by P.J.D. from cDNA as described (4). Human β-tubulin plasmid pDβ1 was the gift of N. Cowan (New York Univ.) (33). A collagen plasmid (pAZ1002) containing the first exon of the mouse α_2 type I procollagen gene was obtained from A. Schmidt (National Institutes of Health) (34). B. Paterson (National Institutes of Health) provided a chicken actin probe (33). The Ki-v-*ras* plasmid pDN82 was from D. Lowy (National Institutes of Health). The plasmid encoding human c-*fos* was from Oncor (Gaithersburg, MD) and mouse c-*myc* plasmid was from the American Type Culture Collection.

RESULTS

Increased Transcription of MEP in KNIH 3T3 Cells. It has been demonstrated previously that KNIH 3T3 cells produce more MEP and have higher levels of MEP mRNA than do NIH 3T3 cells (3, 4, 19). To determine whether increased MEP RNA reflects a greater rate of transcription, a run-off assay was performed on nuclei isolated from exponentially growing NIH 3T3 and KNIH 3T3 cells (Fig. 1). MEP transcription is increased ≈30-fold in KNIH 3T3 cells in this experiment. There is also an expected increase in Ki-v-*ras* expression, while actin and collagen signals are markedly diminished from NIH levels. Tubulin expression decreases a little. There was no binding of labeled RNA to pBR322. The finding of increased transcription of MEP in KNIH 3T3 cells has been made in five independent experiments.

PMA Enhances MEP Transcription in NIH 3T3 Cells. PMA treatment of NIH 3T3 cells results in increased levels of MEP RNA and of MEP protein (4, 19). To examine the effect of PMA on transcription, eight roller bottles of NIH 3T3 cells were grown in parallel. At various times prior to harvesting, PMA was added to individual cultures (final concentration, 30 ng/ml). Cultures were harvested in staggered fashion over a period of 1 hr and kept on ice. Run-off transcription (a typical experiment is shown in Fig. 2) reveals that MEP transcription increases at least 10-fold within 30 min after exposure to PMA, is maximal from 1–4 hr from onset of exposure, and is diminishing by 8 hr. Actin, c-*fos*, and c-*myc* show some enhancement soon after exposure to PMA, in general agreement with the findings of Greenberg and Ziff (25). The changes they saw were of larger magnitude, but their experiments utilized quiescent BALB/c 3T3 cells, whereas our experiments used growing NIH 3T3 cells. Procollagen transcription is decreased by a factor of >10 by PMA within 30 min and remains reduced throughout the time course of this experiment, in agreement with the decrease in procollagen mRNA levels previously observed following PMA treatment (21). It is noteworthy that after 24 hr of exposure to PMA, MEP transcription and collagen transcription are close to baseline levels.

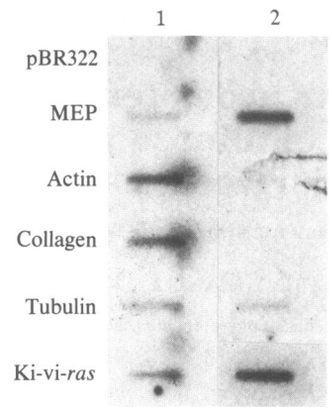

FIG. 1. Comparison of transcription of MEP, actin, collagen, tubulin, and Ki-v-*ras* in nontransformed NIH 3T3 and KNIH 3T3 cells. Growth of cells, isolation of nuclei, and transcription run-off were done in parallel as described in *Materials and Methods*. Lane 1, NIH 3T3 nuclei; lane 2, KNIH 3T3 nuclei.

PMA Effect on MEP Requires Protein Synthesis. The increase in MEP transcription and peak rates of transcription with PMA occur later than early transcripts such as *fos*, which peaks at 15 min (25). This delay suggested that a time-consuming event such as new protein synthesis might be necessary to effect an increase in MEP transcription. To test this hypothesis, NIH 3T3 cells were treated with PMA (30 ng/ml) for 2 hr with or without pretreatment with cycloheximide (10 μg/ml), which inhibits >95% of protein synthesis in mouse 3T3 cells (30) (Fig. 3). Cycloheximide blocks the 8-fold increase in MEP transcription seen here, but it does not prevent the decrease by a factor of 3 in transcription of collagen by PMA. Treatment with cycloheximide without PMA allows baseline transcription of MEP. These data suggest that the effect of PMA on MEP transcription is mediated through new protein synthesis.

PDGF Increases MEP Transcription. PDGF treatment increases MEP mRNA and MEP protein synthesis in BALB/c 3T3 cells, which is blocked by cycloheximide (32). To determine whether this accumulation reflects transcriptional control, BALB/c 3T3 cells were grown to confluence in roller bottles. They were serum-starved in 0.5% calf serum overnight, and then one-half of the cells received 3000 units of PDGF per bottle. After 6 hr, all nuclei were harvested. PDGF effects an 8-fold increase in MEP transcription (Fig. 4), which is comparable to the increase seen in MEP mRNA levels after PDGF treatment (32). Actin transcription is also enhanced; this is consistent with a demonstration in previous studies of increased actin transcription in PDGF-treated cells (25).

DISCUSSION

In these studies, we have used the secreted lysosomal protein MEP (3, 31) as a monitor of the response of certain cellular genes to the tumor promoter, PMA, the growth factor PDGF, and the viral oncogene v-*ras*. We have recently found that MEP is an acid-activated protease distinct from previously described cathepsins (S. Gal and M.M.G., unpublished work). While the precise role of this acid protease activity in normal or cancerous cells remains to be demonstrated, it is clear that in transformed mouse fibroblasts MEP production occupies a major segment of each cell's metabolic effort. In KNIH 3T3 cells, >1% of total protein synthesis is MEP. It also seems clear that increased synthesis of MEP is an early event specifically associated with transformation and the action of certain growth factors. MEP synthesis is stimulated by PMA, an agent that facilitates but is not sufficient for transformation, and by PDGF, a competence-inducing factor

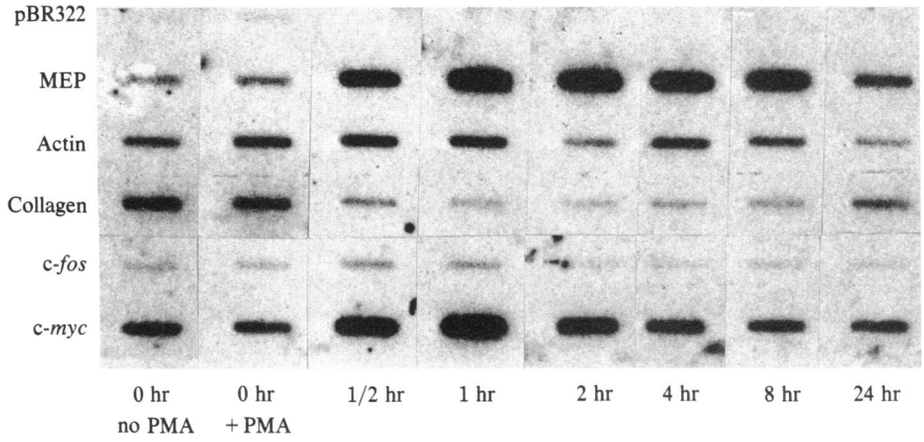

FIG. 2. Time course of PMA effect on gene expression: Left to right, increasing length of exposure to PMA. The first lane received no PMA; the second lane received PMA just prior to harvesting. The filter was RNase-treated and exposed to film for 1 week. Cell culture and run-off experiments were done as described in the legend to Fig. 1.

that is not sufficient to initiate new DNA replication (18, 32). Other growth factors, such as epidermal growth factor, somatomedin C, and insulin, required for progression through the cell cycle, do not increase MEP RNA levels (32).

We have presented evidence that enhanced MEP synthesis by KNIH 3T3 cells is due largely to an increased rate of transcription of the gene for MEP compared to NIH cells. Although the transcriptional effects we found in nuclear run-off experiments seem large enough to explain the observed increase in MEP RNA synthesis (20-fold or more) (4), we have also been studying the relative stability of MEP RNA in KNIH 3T3 and NIH 3T3 cells and have found no major effect of cell transformation on MEP RNA half-life. We exposed cells to a high dose of actinomycin D for various lengths of time, and we measured the survival of MEP RNA by RNA blot analysis (data not shown). MEP RNA was comparably long-lived in both KNIH 3T3 and NIH 3T3 cells, with a half-life of 6–8 hr, while the half-life of tubulin used as a control was estimated as 1–2 hr, consistent with other previous findings (D. W. Cleveland, personal communication). Actinomycin D experiments are fraught with pitfalls, among them the myriad toxic effects of the drug and the possibility that the mRNA half-life is overestimated because of loss of a short-lived nuclease. Another technique that can be used to measure RNA stability is to follow the rate of approach to equilibrium of radioactive MEP RNA by using [^{3}H]uridine (36), but we have found that this method is hampered by the toxicity of tritium at the levels and exposure time necessary to detect transcripts of long half-life. The apparent long half-life of MEP RNA is in contrast to the demonstrated short half-life of c-*myc* RNA, another PMA- and PDGF-inducible transcript (28), and the assumed short half-life of c-*fos* RNA (37, 38). This contrast suggests that MEP itself is not a regulatory protein, but rather a protein species whose sustained synthesis might be needed to allow cell growth.

We have shown that PMA stimulates MEP RNA synthesis in a time-dependent manner. MEP transcription has already increased 30 min after PMA exposure, and it peaks at 1–4 hr before subsiding. The cycloheximide treatment experiment shows that this effect of PMA on MEP transcription is indirect, requiring synthesis of at least one protein intermediate to enhance MEP transcription. The increase of MEP RNA levels by PDGF also requires new protein synthesis (32). The cycloheximide experiment also suggests that other characteristic early changes induced by PMA are dissociable from effects on MEP—e.g., the decrease in collagen RNA transcription induced by PMA is unaffected by inhibition of protein synthesis.

The sequence of events associated with increased MEP RNA transcription after PMA or PDGF treatment has not yet been defined. PMA is known to activate the plasma membrane-associated protein kinase C (39), but a direct role of this kinase in increasing transcription has not yet been demonstrated. The PDGF receptor is a PDGF-dependent tyrosine protein kinase (15), but tyrosine kinase activity itself is not sufficient to increase MEP RNA transcription since the epidermal growth factor- and insulin-activated receptor kinases have little or no effect on MEP RNA levels (32). The identity of the early protein necessary for increased MEP transcription is unknown. c-*fos* is transcribed within 10–15

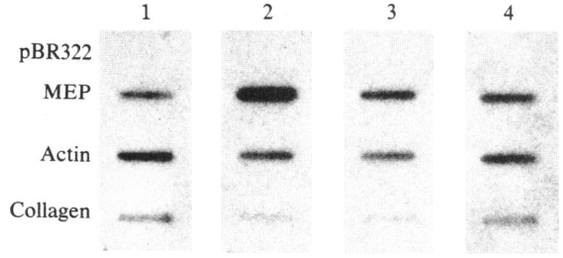

FIG. 3. The effect of cycloheximide on PMA-induced transcription changes. All nuclei are from NIH 3T3 cells. When cycloheximide was added (lanes 3 and 4) (10 μg/ml), it was at time 0. When PMA was added (lanes 2 and 3), this was done at 45 min. Lane 1, no additions. All nuclei were harvested at 2 hr 45 min.

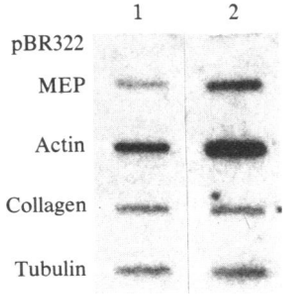

FIG. 4. Effect of PDGF on nuclear run-off of BALB/c 3T3 cells. Lane 1, untreated A-31/714 cells; lane 2, cells treated with PDGF for 6 hr. BALB/c 3T3 A-31/714 cells were from B. Peterkovsky (National Institutes of Health). They were starved in 0.5% serum overnight prior to addition of 3000 units of purified PDGF (gift of C. Scher, Univ. of Pennsylvania Medical School) per roller bottle (30 units/ml; 1×10^{-4} units per cell).

min of PMA stimulation (25). However, this transcription is not sustained for more than 1–2 hr, and the *fos* protein appears to have disappeared by 3–4 hr after stimulation (37, 38). These kinetics make c-*fos* an unlikely continuous positive regulator of MEP, whose increased levels of transcription persist for at least 8 hr. The early stimulation of the MEP transcript, which requires protein synthesis after PMA and PDGF treatment, suggests that there are other positive regulators in addition to c-*myc* and c-*fos* that might play an important role in initiating the program of transcriptional changes associated with malignant transformation or stimulation of cell growth. MEP is a member of a new class of proteins positively regulated by these putative regulatory proteins. These proteins may be needed early as competence factors, and their sustained synthesis may carry a cell through the initial phases of preparation for growth.

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