**The EMBO Journal** vol.3 no.8 pp.1901–1906, 1984

# Tumor promoter PMA stimulates the synthesis and secretion of mouse pro-urokinase in MSV-transformed 3T3 cells: this is mediated by an increase in urokinase mRNA content

**D. Belin[1,2], F. Godeau[3] and J.-D. Vassalli[1]**

[1]Institut d'Histologie et d'Embryologie et [2]Département de Pathologie, Centre Médical Universitaire, 1 rue Michel-Servet, CH-1211 Geneva, Switzerland, and [3]Division of Cell Growth and Regulation, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115, USA

Communicated by R. Epstein

**In mouse MSV-3T3 cells the synthesis of the urokinase form of plasminogen activator was increased 10-fold after addition of the tumor promoter phorbol-12-myristate-13-acetate (PMA). PMA also stimulated the secretion of the protein into the culture medium, mostly in the form of enzymatically inactive pro-urokinase. When assayed by injecting RNA into *Xenopus laevis* oocytes, the concentration of functional urokinase mRNA was found to be 6- to 10-fold higher in the PMA-treated cells; a similar increase in urokinase mRNA content was measured by hybridisation with a mouse urokinase cDNA probe. Thus, the induction of plasminogen activator by PMA in MSV-3T3 cells is accounted for by an increased content of urokinase mRNA.**

*Key words:* mouse urokinase/mRNA/plasminogen activator/protein synthesis/tumor promoter

## Introduction

Plasminogen activators (PAs) are highly selective, trypsin-like serine proteases which convert plasminogen, an inactive zymogen present in most extracellular fluids, into plasmin, a neutral protease of broad specificity. Mammalian cells can produce two different PAs: urokinase and tissue activator (TA) (Danö *et al.*, 1980b; Rijken and Collen, 1981; Marotti *et al.*, 1982). A comparison of the deduced amino acid sequence of human TA (Pennica *et al.*, 1983) with that of human urokinase (Steffens *et al.*, 1982; Günzler *et al.*, 1982) shows that these proteins are the products of distinct genes.

In a number of cell types, the production of PAs was found to be under strict hormonal control (Reich, 1978). Experiments with inhibitors of macromolecular synthesis suggest that modulation of PA activity can occur at the level of the gene expression (Rifkin *et al.*, 1975). In human melanoma cells, the induction of PA activity by the tumor promoter phorbol-12-myristate-13-acetate (PMA) results from an increase in the intracellular level of functional TA mRNA (Opdenakker *et al.*, 1983). Similarly, the amount of urokinase mRNA in a porcine kidney cell line is increased by calcitonin (Nagamine *et al.*, 1983).

Here we describe an analysis of the effect of PMA on PA production in MSV-transformed 3T3 cells (MSV-3T3). PMA increased the synthesis of mouse urokinase and enhanced its release, mostly in the form of a proenzyme. The capacity of cellular RNA to direct the synthesis of mouse urokinase in *Xenopus laevis* oocytes was higher in PMA-treated cells. The relative abundance of mouse urokinase mRNA, as measured by hybridisation to a mouse urokinase cDNA probe, was similar to that detected by the oocyte assay. Thus, PMA causes an increase in the steady-state concentration of urokinase mRNA in mouse MSV-3T3 cells, and enhances the synthesis and release of the protein.

## Results

### *PMA stimulates PA production by MSV-3T3 cells*

We have used a cell line derived from MSV-transformed mouse 3T3 cells that secretes a PA which proved to be a mouse urokinase of mol. wt. 48 000 (Danö *et al.*, 1980a). As a preliminary to cloning mouse urokinase messenger sequences, we screened several cellular effectors known to enhance PA production in other cell types; one of these, the tumor promoter PMA, stimulated PA production by this line (Figure 1).

Cells grown to subconfluency in the presence of serum contained 1.5–3.0 Plough units of PA/mg of protein. In control cultures (Figure 1b), the amount of cell-associated PA increased linearly during further incubation in serum-free conditions. PA also accumulated in the culture medium; however, the amount of secreted enzyme was only a small fraction of that present in the whole culture. To evaluate the stability of secreted PA, we compared the amount of PA recovered after 20 h with the total secreted by a parallel culture the medium of which was changed every 4 h. No difference was detected (not shown); therefore, secreted PA was relatively stable under our culture conditions. We calculated the total apparent rate of enzyme production by control cells to be 100–140 mU/h per culture; this rate was constant over the 20 h period of the experiment.

In contrast with control cells, most of the PA produced by PMA-treated cells (Figure 1a) appeared in the culture

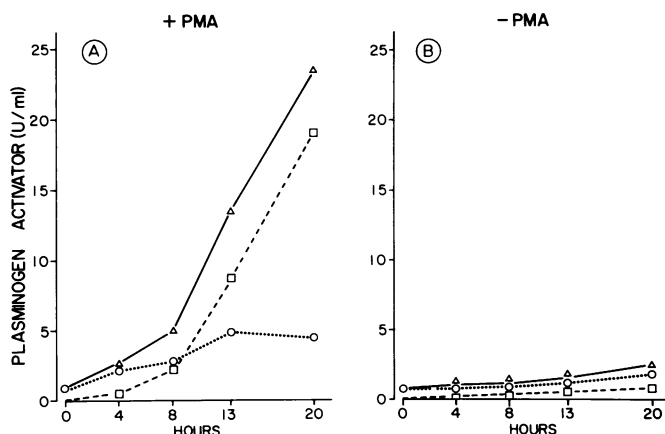


**Fig. 1.** Kinetics of PA production by PMA-treated and control MSV-3T3 cells. Cultures were seeded at a density of $10^5$ MSV-3T3 cells/35 mm dish. After 60 h in DMEM + 10% FBS, subconfluent cultures were washed and incubated in DMEM + AT-BSA (80 μg/ml) with (**A**) or without (**B**) PMA (20 ng/ml). At the indicated times, media (1 ml) (□- - -□) and cell extracts (1 ml) (○- - -○) were collected and assayed for plasminogen-dependent fibrinolytic activity. Total PA activity (△——△) is the sum of the activities in medium and cell extracts.

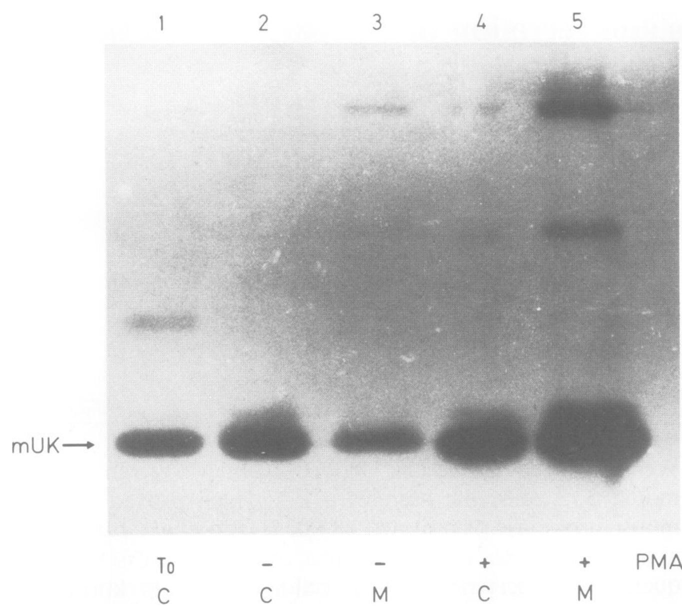


**Fig. 2.** Zymographic analysis of PA produced by PMA-treated and control MSV-3T3 cells. Cultures prepared as described in Figure 1. The cultures were then incubated in serum-free medium for 24 h without (**lanes 2** and **3**) or with (**lanes 4** and **5**) PMA (40 ng/ml). PA present in extracts of cells at the time of serum removal is shown in **lane 1**. Equivalent volumes of cell extracts (**C**) and media (**M**) were analysed as described in Materials and methods.

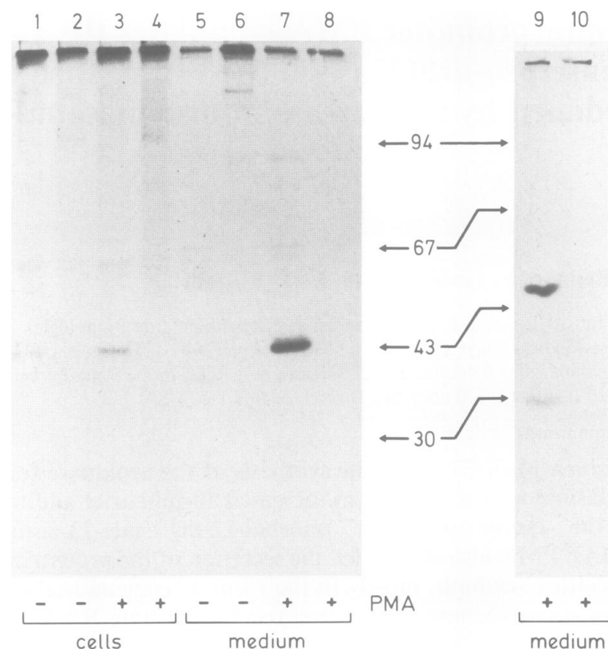


**Fig. 3.** Immunoprecipitation of [$^{35}$S]methionine-labeled mouse urokinase synthesised by PMA-treated and control cells. Cultures prepared in parallel with those analysed in Figure 2 were incubated for 24 h in modified DMEM containing [$^{35}$S]methionine (40 $\mu$Ci/ml) and one-tenth the normal amount of non-radioactive methionine. Cell-extracts (**lanes 1–4**) and culture media (**lanes 5–10**) were immunoprecipitated with anti-mouse urokinase (**lanes 1, 3, 5, 7** and **9**) or non-immune (**lanes 2, 4, 6, 8** and **10**) IgG (120 $\mu$g/ml). Immune complexes eluted from the protein A-Sepharose were electrophoresed under non-reducing (**lanes 1–8**) or reducing (**lanes 9** and **10**) conditions and revealed by fluorography. The migration of standard proteins is indicated with their mol. wt. The ratio of mol. wt. 48 000 and 29 000 mouse urokinase (**lane 9**) was determined by counting the radioactivity present in the bands (corrected for the methionine content of both proteins; Belin *et al.*, in preparation).

medium: following a 4–8 h lag, secreted PA accumulated at a constant rate and represented ~70% of the total PA present in the culture. The concentration of cell-associated enzyme followed different kinetics since a significant increase could already be detected 4 h after the addition of PMA. The overall rate of PA accumulation was 1–2 U/h per culture between 8 and 20 h after addition of PMA, a 10-fold increase over control cells.

*PMA increases the synthesis of mouse urokinase*

Several forms of mouse PA can be distinguished on the basis of their apparent mol. wts. (Granelli-Piperno and Reich, 1978). PMA could either increase the amount of 48 000 mol. wt. mouse urokinase which is secreted by the untreated cells, or induce the production of other form(s) of PA, or both. To distinguish between these possibilities, culture media and cell extracts of PMA-treated and control cells were analysed by a zymographic assay (Figure 2). In all samples, most of the activity migrated with an apparent mol. wt. of 48 000 identical to that of purified mouse urokinase. Using antibodies raised against mouse urokinase and human TA, we established that this PA was immunologically identical to the former (not shown). As expected from the above (Figure 1), both the cell-associated and the secreted 48 000 mol. wt. mouse urokinase were markedly increased in PMA-treated cultures. Bands with minor activities were also apparent in the zymogram (Figure 2). Some of these presumably resulted from the activity of complexes formed by the reaction of part of the mouse urokinase with protease inhibitors, either from the serum (Waller *et al.*, 1983) or of cellular origin (Baker *et al.*, 1980). In some experiments (e.g. Figure 2, lane 1), mouse TA was also detected in these cultures; however, it represented only a small fraction of the total PA activity and did not increase upon addition of PMA.

We quantitated the amount of newly synthesised mouse urokinase by immunoprecipitation of [$^{35}$S]methionine-labeled proteins with a specific IgG (Figure 3). From the culture medium of PMA-treated cells, most of the labeled mouse urokinase-related protein was recovered as a 48 000 mol. wt. band (Figure 3, lane 7), in amounts some 20-fold greater than from the medium of controls (Figure 3, lane 5). We also found more labeled mouse urokinase in the extract of PMA-treated cells (Figure 3, lane 3) than in that of controls (Figure 3, lane 1). Most of the newly synthesised mouse urokinase remained cell-associated in control cells; in contrast, the labeled protein accumulated preferentially in the medium of PMA-treated cells. This effect was selective since no changes in total amounts, or electrophoretic profiles of other secreted proteins were detected (not shown). Thus, the PMA-induced stimulation of PA production in MSV-3T3 cells could be completely accounted for by the increased synthesis of mouse urokinase, and changes of specific activity due to post-translational modifications did not appear to play a role.

*Mouse urokinase is synthesised and secreted as a proenzyme*

Purified mouse urokinase consists of two polypeptide chains of mol. wt. 29 000 and 17 000 linked by disulfide bond(s) (Marotti *et al.*, 1982; Skriver *et al.*, 1982). However, when [$^{35}$S]methionine labeled mouse urokinase was analysed under reducing conditions, 70–80% of the newly synthesised protein migrated as a single polypeptide chain of mol. wt. 48 000 (Figure 3, lane 9). Single-chain forms of human and mouse urokinase (Wun *et al.*, 1982; Skriver *et al.*, 1982) are inactive proenzymes which fail to react with diisopropylfluorophos-

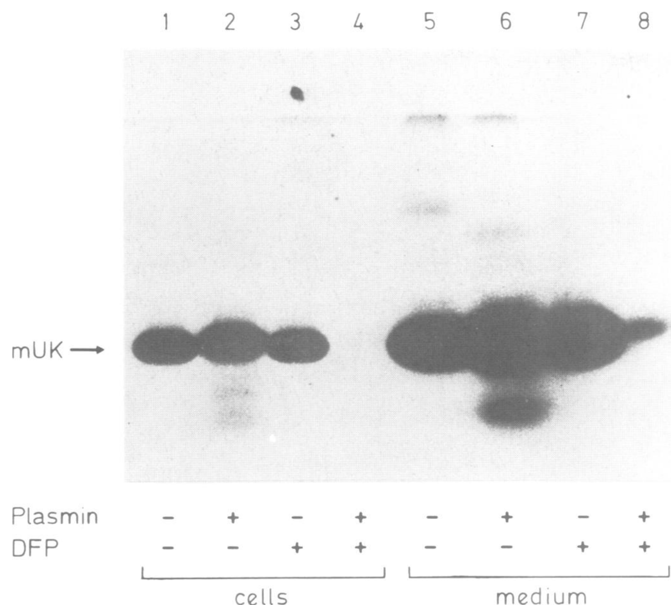


**Fig. 4.** Mouse pro-urokinase in cell extract and culture medium of PMA-treated MSV-3T3 cells. Samples were prepared as described in Figure 2. Plasmin-treatment was for 1 h at 37°C with 1 $\mu$g/ml of plasmin. Samples were made 50 mM Tris-HCl pH 8.1 and incubated twice for 30 min at 20°C with 10 mM DFP. Non-treated samples received the same amount of isopropanol. Zymographic analysis immediately followed the DFP treatment. Mouse urokinase (**lanes 2** and **6**) has a slightly higher apparent mol. wt. than the proenzyme (**lanes 1, 3, 5** and **7**), confirming the results of Skriver *et al.* (1982) with purified proteins. Small amounts of 29 000 mol. wt. mouse urokinase (Granelli-Piperno and Reich, 1978) were present in plasmin-treated samples (**lanes 2** and **6**).

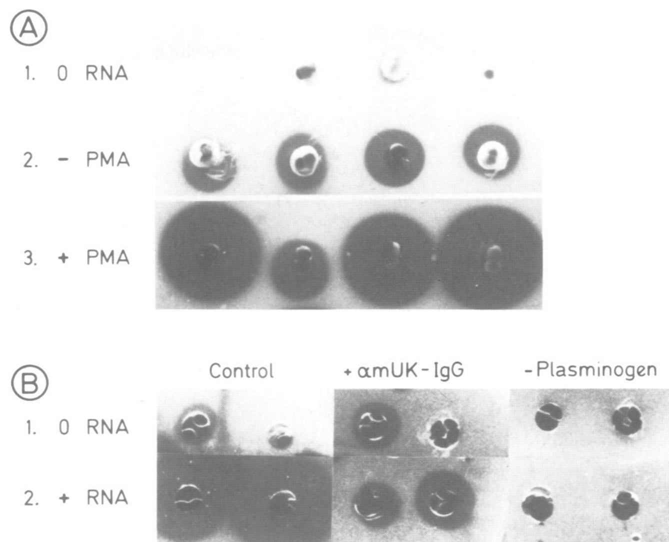


**Fig. 5.** Mouse urokinase production by *X. laevis* oocytes injected with RNA from MSV-3T3 cells. Oocytes were injected with 50 nl and incubated for 40 h at 23°C in casein-agar. **A (1)** injection buffer; **A (2,3)** total cytoplasmic RNA (10 mg/ml) from MSV-3T3 cells incubated 14 h without **(2)** or with **(3)** PMA (40 ng/ml). **B (1)** injection buffer; **(2)** poly(A)$^+$ RNA (350 $\mu$g/ml) from PMA-treated MSV-3T3 cells. The oocytes were incubated in casein-agar with plasminogen (65 $\mu$g/ml) (control), with plasminogen and anti-mouse urokinase IgG (120 $\mu$g/ml) (+ $\alpha$ mUK IgG), or without plasminogen (– plasminogen).

phate (DFP), an irreversible inhibitor of serine proteases including PAs (Danö and Reich, 1979). To confirm that the single-chain mouse urokinase stored and secreted by PMA-treated MSV-3T3 cells was a proenzyme, we determined its susceptibility to inactivation by DFP (Figure 4). A comparison of control (Figure 4, lane 1) and DFP-treated (Figure 4, lane 3) cell extracts showed that most of the PA was resistant to DFP prior to zymography. As a control we verified that plasmin could quantitatively convert the proenzyme into DFP-sensitive mouse urokinase (Figure 4, lanes 2 and 4). Similar results were obtained with culture media (Figure 4, lanes 5–8) and there was no difference between PMA-treated and control cells (not shown). Thus, MSV-3T3 cells contained and secreted mostly the pro-urokinase, and PMA did not alter significantly the ratio of proenzyme to enzyme.

*The concentration of mouse urokinase mRNA is increased by PMA*

*X. laevis* oocytes translate injected mRNAs into functionally active proteins, including PAs (Miskin and Soreq, 1981). The production of mouse urokinase by injected oocytes was first detected by the appearance of lytic zones in a casein-agar medium. Oocytes injected with total cytoplasmic RNA extracted from PMA-treated cells produced much larger lytic zones than did those injected with RNA from control cells (Figure 5A). This proteolytic activity was mainly due to mouse urokinase because: (i) there was no lysis in the absence of plasminogen; (ii) the area of lysis was strongly reduced when blocking anti-mouse urokinase IgG was included in the agar (Figure 5B). Small lytic zones were often detected around control oocytes injected with saline. This activity, which was not affected by the specific antibody, was due to endogenous *X. laevis* PAs.

The above assay was only semi-quantitative because the PA secreted by individual oocytes fluctuated, and because of endogenous *X. laevis* PAs. To quantitate more accurately the activity of mouse urokinase mRNA, we analysed the culture medium and cell extracts from pools of injected oocytes by the zymographic assay, which discriminates between mouse urokinase and the *X. laevis* PAs (Figure 6). The 48 000 mol. wt. PA produced by oocytes injected with MSV-3T3 mRNA co-migrated with purified mouse urokinase and could be immunoprecipitated specifically with anti-mouse urokinase IgG (not shown). When increasing amounts of mRNA from PMA-treated cells were injected, the amounts of both secreted and oocyte-associated mouse urokinase were directly proportional to the quantity of injected RNA. Oocytes injected with 40 ng of mRNA from PMA-treated cells synthesised ~0.6 mU/oocyte/24 h, of which 60–80% was recovered from the culture medium.

The production of mouse urokinase was also detected after injection of oocytes with mRNA from control MSV-3T3 cells (Figure 6). However, the concentration of functional urokinase mRNA was ~10-fold lower than in PMA-treated cells. In contrast, when both mRNAs were translated in wheat germ extracts, the total incorporation of amino acids and the general electrophoretic pattern of translation products were indistinguishable (not shown). Thus, the increase in functional mouse urokinase mRNA is not likely to be the result of a general effect of PMA on the abundance, stability or translational efficiency of all cellular mRNAs.

The mouse urokinase mRNA from both PMA-treated and control cells sedimented in sucrose gradients with a coefficient of 21S. As expected, oocytes injected with 21S mRNA from PMA-treated cells produced ~10 times more mouse urokinase than those injected with an equivalent amount of control 21S mRNA (not shown). We have used this 21S mRNA from PMA-treated cells to construct a cDNA library. The library was screened with a pig urokinase cDNA probe

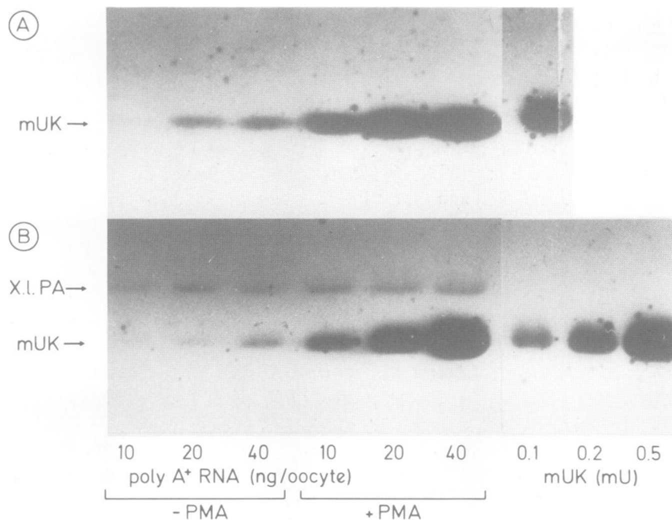


**Fig. 6.** Concentration of functional mouse urokinase mRNA in PMA-treated and control MSV-3T3 cells. Oocytes were injected with 50 nl containing the indicated amounts of poly(A)$^+$ RNA isolated from the cytoplasmic RNAs used in the experiment of Figure 5. Pools of 20 injected oocytes were incubated for 24 h. Oocyte extracts (**A**) and culture media (**B**) were analysed by zymography together with the indicated amounts of mouse urokinase. The zymograms were incubated for 40 h (**A**) or 18 h (**B**) at 37°C.

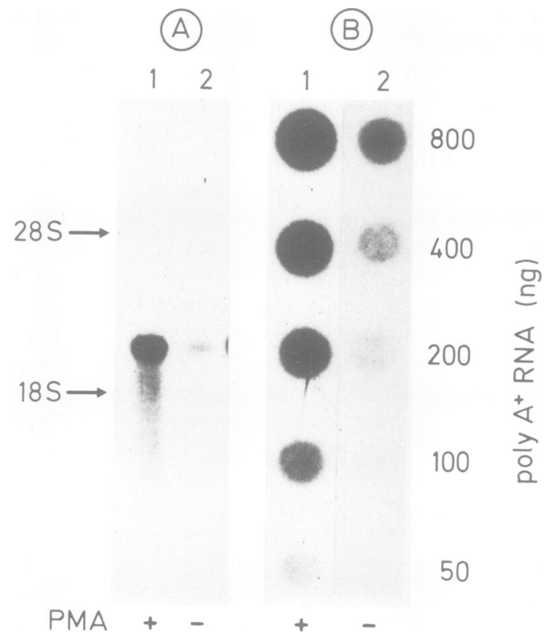


**Fig. 7.** Mouse urokinase mRNA abundance in PMA-treated and control MSV-3T3 cells. (**A**) The poly(A)$^+$ RNAs analysed functionally (Figure 6) were electrophoresed (1 $\mu$g/lane) in a 1.4% agarose gel and transferred to nitrocellulose. Cytoplasmic RNA (10 $\mu$g) was electrophoresed in an adjacent lane and stained with ethidium bromide to determine the position of 18S and 28S rRNAs. (**B**) The same RNAs were serially diluted and the indicated amounts were spotted on nitrocellulose. Both filters were hybridised to a nick-translated mouse urokinase cDNA probe and autoradiographed. The spots were cut and counted (Cerenkov radiation) to confirm the visual evaluation of relative mouse urokinase mRNA amounts.

(Nagamine *et al.*, 1983); several cDNA clones hybridised to this probe and were shown to contain mouse urokinase coding sequences by the following criteria: (i) the amino acid sequence predicted by the DNA sequence bears a strong homology to that of human urokinase; (ii) the bacteria harboring one of these plasmids synthesise a PA catalytically and immunologically indistinguishable from mouse urokinase (Belin *et al.*, in preparation).

We have used one of these cDNA clones to identify the mouse urokinase mRNA in Northern blots (Figure 7A), and to quantitate its relative amount in both mRNA preparations (Figure 7B). These results confirmed that the amount of mouse urokinase mRNA in PMA-treated cells was 6- to 10-fold greater than that in control cells. Since no size difference was detected between the specific mRNA present in both preparations, it appeared that PMA did not induce the synthesis of a new species of mouse urokinase mRNA, but increased the steady-state concentration of the mRNA normally present in MSV-3T3 cells.

## Discussion

Since the original report that tumor promoters stimulate PA production by cells in culture (Wigler and Weinstein, 1976), this observation has been extended to a large spectrum of different cell types (Vassalli *et al.*, 1977; Granelli-Piperno *et al.*, 1977; Christman *et al.*, 1978; Jaken and Black, 1981; Opdenakker *et al.*, 1983). In most instances, however, the description of the phenomenon was limited to measurements of overall plasminogen-dependent fibrinolytic activity.

The MSV-3T3 cell line is known to secrete the urokinase form of PA under normal conditions (Danö *et al.*, 1980b). As we have shown here, the stimulation of PA production by PMA is due to an increase in mouse urokinase synthesis, and not to an induction of mouse TA production. An analysis of several hamster cell lines indicated that PMA increased PA production only in lines producing urokinase, suggesting that TA production was unaffected by PMA (Christman *et al.*, 1978). However, PMA increased TA production both in human HeLa cells (Wigler and Weinstein, 1976) and melanoma cells (Opdenakker *et al.*, 1983). Thus, the expression of both PA genes shows a cell- and/or species-specific sensitivity to PMA. In this context, it might be interesting to study the effect of PMA on mouse-human somatic cell hybrids which produce both mouse urokinase and human TA (Kucherlapati *et al.*, 1978).

Several factors can affect estimates of PA protein based on PA activity measurements. First, urokinase is found in culture medium either in an active form (Danö *et al.*, 1980a) or as an inactive proenzyme (Wun *et al.*, 1982; Skriver *et al.*, 1982). Second, many cells secrete protease-nexin (Baker *et al.*, 1980) and other PA inhibitors (Vassalli *et al.*, 1984), which will not react with proenzymes. Inhibitor production is itself modulated by tumor promoters and growth factors (Eaton and Baker, 1983). In our experiments, the PMA-stimulated increase in PA activity was not due to an altered pattern of inhibitor production (D. Belin and J.-D. Vassalli, unpublished data). Both PMA-treated and control cells were also shown to contain and secrete mouse urokinase mostly in the proenzyme form, and PMA did not alter the proenzyme's activation pattern. Thus, the increased PA activity was totally accounted for by an increase in pro-urokinase synthesis.

In addition, PMA also increased the secretion of mouse urokinase. This suggests that mouse urokinase is associated with a saturable cellular compartment, the capacity of which could be exceeded by the enhanced rate of enzyme synthesis. Alternatively, PMA might stimulate mouse urokinase secretion directly; a similar effect of PMA on prolactin secretion

by a rat pituitary cell line has been reported (Osborne and Tashjian, 1981).

Our observation of an increased concentration of functional mouse urokinase mRNA in PMA-treated cells correlates well with two recent reports of increased PA production together with elevated levels of functional PA mRNA (Opdenakker *et al.*, 1983; Nagamine *et al.*, 1983). We have also observed a good correlation between the functional concentration of mouse urokinase mRNA and its chemical abundance, as measured by hybridisation with a cDNA probe; thus, the increased steady-state concentration of mouse urokinase mRNA induced by PMA appears sufficient to account for the increased synthesis of the enzyme. We did not detect a difference in the size of urokinase mRNA from PMA-treated and control cells. However, even if PMA were to induce a different mRNA, the *in vivo* translation efficiency of both species should be equivalent. Similar results were obtained in an analysis of porcine urokinase mRNA in calcitonin-treated $LLCPK_1$ cells (Nagamine *et al.*, 1983). Although the induction of PA activity has been correlated in these two systems with an increased abundance of a specific PA mRNA, it is unclear whether this reflects an increased transcription of the PA genes or stabilisation of the PA mRNAs.

PMA has been reported to stimulate the activity of a protein kinase (Castagna *et al.*, 1982), to induce DNA synthesis and cell proliferation (Berenblum and Armuth, 1981), and to cause changes in the pattern of protein synthesis (Laskin *et al.*, 1981; Hiwasa *et al.*, 1982). Increased levels of mRNAs for proteins whose synthesis is stimulated by PMA have also been described (Hoffman-Liebermann *et al.* 1981; Hiwasa *et al.*, 1982). In addition to mouse urokinase cDNA clones, we have also isolated several recombinant plasmids containing PMA-induced sequences (Belin *et al.*, in preparation). Comparisons of nucleotide sequences in this set of genes might detect common elements involved in the induction of their expression by tumor promoters. Since elevated urokinase secretion is associated with the invasive properties of malignant tumors (Ossowski and Reich, 1983; Markus *et al.*, 1983), this analysis may help dissect the cellular events involved in tumor promotion.

## Materials and methods

*Materials*

*X. laevis* females were purchased from South African Snake Farm. DMEM (H21) and Leibovitz (L15) media were obtained from Gibco. To prepare modified L15 medium, Leibovitz medium was made 30 mM in Hepes, supplemented with gentamycin (50 μg/ml), adjusted to pH 7.7 with NaOH and diluted 2-fold in water. Fetal bovine serum (FBS) was from Reheis Chemical. Unless otherwise stated, we used Falcon tissue culture plastics.

Human plasminogen was purified from human plasma according to the procedure of Deutsch and Mertz (1970). Plasmin was prepared and stored in 50% glycerol according to Danö and Reich (1979). The purification of mouse urokinase and rabbit anti-urokinase IgG has been described (Marotti *et al.*, 1982). Human urokinase was purchased from Leo Pharmaceutical. Protein A-Sepharose and standard proteins (LMW Kit) were obtained from Pharmacia. Proteinase K was obtained from Merck. Trasylol and bovine serum albumin (A-7511) were from Sigma. Stock solutions of albumin were acid-treated (AT-BSA) (Loskutoff, 1978).

PMA, Nonidet-P40 and Triton X-100 (TX-100) were obtained from Sigma; the protein assay reagent from Bio-Rad; [$^{35}$S]methionine (sp. act. = 400 Ci/mmol) and En$^3$Hance were from New England Nuclear. SDS was from British Drug House; DFP from Merck; RNase-free sucrose from Schwarz-Mann; yeast tRNA from Boehringer; glutathione and oligo(dT)-cellulose type 77F from PL-Biochemicals; thymidine-3',5'-diphosphate from Calbiochem; nitrocellulose (BA85) from Schleicher and Schuell.

*Cell culture*

The MSV-3T3 cell line was obtained from Dr. L. Ossowski (Rockefeller University). Cells were grown and maintained in DMEM supplemented with 10% FBS, pyruvate (110 mg/l), streptomycin (200 mg/l) and penicillin ($5 \times 10^4$ U/l). Cultures were seeded at a density of $10^5$ cells per 35 mm dish (Falcon No. 3001) and grown for 48 – 60 h in the presence of serum. For the preparation of cellular RNAs, $2.5 \times 10^6$ cells were seeded per 800 ml flask (Nunclon No. 156.502) and grown for 72 h. Subconfluent cultures were used in all experiments since the response of MSV-3T3 cells to PMA was reduced at confluency. At the time of serum removal, the cultures were washed three times with phosphate-buffered saline (PBS) and incubated in serum-free medium as indicated. Preliminary dose-response experiments showed that the stimulation of PA production could be detected with 3 ng/ml of PMA and was maximal with 20 – 100 ng/ml.

*Plasminogen activator assays*

Culture media were used directly or after dilution in 0.1 M Tris HCl pH 8.1, 1 mM EDTA and 80 μg/ml AT-BSA. MSV-3T3 cell extracts were prepared by resuspension of the monolayers in 0.1 M Tris-HCl pH 8.1, 0.5% TX-100. After a low-speed centrifugation (5 min, 750 *g*), the supernatants were collected and stored at –20°C. Protein content was measured according to the method of Bradford (1976). Subconfluent cultures contained 0.35 – 0.40 mg of TX-100-soluble proteins per 35 mm dish. The plasminogen-dependent fibrinolytic activity of the samples was assayed according to the method of Ossowski and Reich (1980), and compared with that of human urokinase standards (1 – 10 Plough mU) assayed in parallel. All assays were incubated at 37°C for 2 or 3 h.

*Gel electrophoresis and zymography of PA*

Samples were electrophoresed under non-reducing conditions in 10% SDS-polyacrylamide slab gels using the buffer system of Laemmli (1970). After electrophoresis, the gels were soaked for 20 min in 2.5% TX-100, washed twice for 10 min in water (Granelli-Piperno and Reich, 1978), and layered on casein-agar underlays containing 2% casein, 1.2% agar, and plasminogen (30 – 100 μg/ml) in 0.1 M Tris-HCl pH 8.1 (Vassalli *et al.*, 1977). The underlays were incubated at 37°C in humidified chambers and photographed under dark-field illumination.

*Immunoprecipitation of mouse urokinase*

Culture media and cell extracts prepared as described above were adjusted to 0.2 M NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, 1% TX-100, 0.2% SDS, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 100 KIU/ml of trasylol. The samples were first pre-incubated 30 min at 20°C with non-immune rabbit IgG (120 μg/ml) and protein A-Sepharose (10 μl of settled resin/sample), and then incubated 2 – 12 h at 4°C with the indicated IgG. Immune complexes were collected on protein A-Sepharose, washed 6 times with 1 ml of 0.5 M NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.1, 1% TX-100, 0.2% SDS, and once with 200 μl of 0.5 M NaCl, 50 mM Tris-HCl pH 8.1, 0.1% SDS. The immune complexes were eluted twice with 25 μl of sample buffer with or without 2-mercaptoethanol as indicated. After electrophoresis, the gels were stained with Coomassie Blue to determine the migration of standard proteins included in a separate lane of each gel, treated with En$^3$Hance and dried. The gels were fluorographed at –80°C using Kodak SB-5 films and according to the method of Bonner and Laskey (1974).

*RNA extraction*

At the indicated times, the cultures were washed once with ice-cold PBS, scraped off the plastic surface, collected by centrifugation (10 min, 750 *g*) and resuspended at $1 - 3 \times 10^7$ cells/ml in 0.15 M NaCl, 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 0.15 M sucrose containing yeast tRNA (20 μg/ml), reduced glutathione (3 mM) and thymidine-3',5'-diphosphate (0.3 mM) (Gough and Adams, 1978). The cells were lysed with Nonidet-P40 (0.5% w/v) and the NaCl concentration raised to 0.3 M. After centrifugation of the nuclei (10 min, 1000 *g*), the cytoplasmic extracts were made 2.5% in SDS, 10 mM in EDTA, digested for 30 min at 37°C with proteinase K (300 μg/ml), extracted three times with phenol:chloroform:isoamylalcohol (50:48:2 v/v/v) and precipitated twice with ethanol. Cytoplasmic RNAs were resuspended in water at 10 mg/ml. The average RNA yield was 1 mg/$5 \times 10^7$ MSV-3T3 cells. Poly(A)$^+$ RNA was purified by batchwise fractionation using oligo(dT)-cellulose fines (Nagamine *et al.*, 1983). The poly(A)$^+$ RNAs, which represented 2 – 4% of the cytoplasmic RNA, were resuspended in water at 1 mg/ml.

*PA production by injected oocytes*

The production of mouse urokinase by RNA injected *X. laevis* oocytes was detected using procedures similar to those described by Miskin and Soreq (1981). Oocytes were prepared by collagenase digestion of dissected ovaries (Younglai *et al.*, 1982). Oocytes were injected with 50 nl of injection buffer

(80 mM NaCl, 1 mM KCl, 5 mM Hepes pH 7.7) containing the indicated amounts of RNA. Injected oocytes were either incubated directly in holes punched in casein-agar gels containing 2% casein (in 20 mM $Na-PO_4$ pH 7.7), 1% agar and plasminogen (50 – 100 μg/ml) in modified L15 medium, or cultivated in microtiter wells (20 – 30 oocytes/well) containing 5 μl/oocyte of modified L15 medium supplemented with AT-BSA (80 μg/ml) and trasylol (200 KIU/ml). After 24 h, the medium was collected and the oocytes extracted with 20 mM Tris-HCl pH 7.4, 4 mM EDTA, 0.5% TX-100 and trasylol (400 KIU/ml).

*Northern blot hybridisations*

RNAs were denatured with glyoxal, electrophoresed in 1.4% agarose gels and transferred to nitrocellulose according to Thomas (1980). Alternatively, RNAs were heated 2 min at 60°C, diluted in water and applied (2 μl per spot) onto moist nitrocellulose soaked in 20 x SSC. Filters were baked 4 h at 80°C and pre-hybridised for 3 h at 60°C in 0.9 M NaCl, 50 mM Na-Pipes pH 6.8, 10 mM EDTA, 0.2% SDS, 4 x Denhardt's solution and 100 μg/ml heat-denatured salmon sperm DNA. Hybridisation mixtures were filtered through nitrocellulose, pre-heated and degassed before use. Filters were then hybridised with fresh hybridisation mixture (50 $\mu l/cm^2$) containing 10 ng/ml of heat-denatured recombinant plasmid pDB15 (Belin *et al.*, in preparation) nick-translated to a specific activity of $0.6 - 1.4 \times 10^5$ c.p.m./ng. After 36 – 40 h, the filters were washed twice at 60°C with 3 x SSC, 2 x Denhardt's solution, twice at 20°C with 0.1 x SSC, 0.1% SDS and 0.1% $Na_4P_2O_7$, and finally twice at 37°C with the same solution. The filters were exposed to Kodak XAR-5 films at –80°C between Dupont Cronex ParSpeed intensifying screens.

## Acknowledgements

This work was initiated while one of us (D.B.) was a Damon Runyon-Walter Winchell Cancer Fund post-doctoral fellow in the Laboratory of Chemical Biology, The Rockefeller University. The authors wish to thank Ed Reich for his interest and support in the initial phase of the work, and for carefully reading the manuscript. We thank C. Combépine for excellent technical assistance; L. Ossowski for the MSV-3T3 cell line; Y. Nagamine for a porcine urokinase cDNA clone; P. Suter for help in analysing pro-urokinase; P. Rapin for preparing the illustrations; E. Waller, L. Andrus and A. Grabowska for helpful discussions; and P. Prentki for a critical reading of the manuscript. Supported by the Fonds National Suisse de la Recherche Scientifique, grant No. 3.159-0.81.

## References

Baker,J.B., Low,D.A., Simmer,R.L. and Cunningham,D.D. (1980) *Cell*, **21**,. 37-45.
Berenblum,I. and Armuth,V. (1981) *Biochim. Biophys. Acta*, **651**, 51-63.
Bonner,W.M. and Laskey,R.A. (1974) *Eur. J. Biochem.*, **46**, 83-92.
Bradford,M. (1976) *Anal. Biochem.*, **72**, 248-254.
Castagna,M., Takai,Y., Kaibuchi,K., Sano,K., Kikkawa,U. and Nishizuka, Y. (1982) *J. Biol. Chem.*, **257**, 7847-7851.
Christman,J.K., Copp,R.P., Pedrinan,L. and Whalen,C.E. (1978) *Cancer Res.*, **38**, 3854-3860.
Danö,K. and Reich,E. (1979) *Biochim. Biophys. Acta*, **566**, 138-151.
Danö,K., Möller,V., Ossowski,L. and Nielsen,L.S. (1980a) *Biochim. Biophys. Acta*, **613**, 542-555.
Danö,K., Nielsen,L.S., Möller,V. and Engelhart,M. (1980b) *Biochim. Biophys. Acta*, **630**, 146-151.
Deutsch,D.G. and Mertz,E.T. (1970) *Science*, 170, 1095-1096.
Eaton,D.L. and Baker,J.B. (1983) *J. Cell. Biol.*, **97**, 323-328.
Gough,N.M. and Adams,J.M. (1978) *Biochemistry (Wash.)*, **17**, 5560-5566.
Granelli-Piperno,A. and Reich,E. (1978) *J. Exp. Med.*, **148**, 223-234.
Granelli-Piperno,A., Vassalli,J.D. and Reich,E. (1977) *J. Exp. Med.*, **146**, 1693-1706.
Günzler,W.A., Steffens,G.J., Otting,F., Kim,S.M.A., Frankus,E. and Flohé,L. (1982) *Hoppe-Seyler's Z. Physiol. Chem.*, **363**, 1155-1165.
Hiwasa,T., Fujimura,S. and Sakiyama,S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1800-1804.
Hoffman-Liebermann,B., Liebermann,D. and Sachs,L. (1981) *Int. J. Cancer*, **28**, 615-620.
Jaken,S. and Black,P.H. (1981) *J. Cell. Biol.*, **90**, 721-731.
Kucherlapati,R., Tepper,R., Granelli-Piperno,A. and Reich,E. (1978) *Cell*, **15**, 1331-1340.
Laemmli,U.K. (1970) *Nature*, **227**, 680-685.
Laskin,J.D., Mufson,R.A., Piccinini,L., Engelhardt,D.L. and Weinstein, I.B. (1981) *Cell*, **25**, 441-449.
Loskutoff,D.J. (1978) *J. Cell. Physiol.*, **96**, 361-370.
Markus,G., Camiolo,S.M., Kohga,S., Madeja,J.M. and Mittelman,A. (1983) *Cancer Res.*, **43**, 5517-5525.
Marotti,K.R., Belin,D. and Strickland,S. (1982) *Dev. Biol.*, **90**, 154-159.
Miskin,R. and Soreq,H. (1981) *Nucleic Acids Res.*, **9**, 3355-3363.
Nagamine,Y., Sudol,M. and Reich,E. (1983) *Cell*, **32**, 1181-1190.
Opdenakker,G., Ashino-Fuse,H., Van Damme,J., Billiau,A. and De Somer, P. (1983) *Eur. J. Biochem.*, **131**, 481-487.
Osborne,R. and Tashjian,A.H. (1981) *Endocrinology*, **108**, 1164-1170.
Ossowski,L. and Reich,E. (1980) *Cancer Res.*, **40**, 2300-2309.
Ossowski,L. and Reich,E. (1983) *Cell*, **35**, 611-619.
Pennica,D., Holmes,W.E., Kohr,W.J., Harkins,R.N., Vehar,G.A., Ward, C.A., Bennett,W.F., Yelverton,E., Seeburg,P.H., Heynecker,H.L., Goeddel,D.V. and Collen,D. (1983) *Nature*, **301**, 214-221.
Reich,E. (1978) in Berlin,R.D., Hermann,M., Lepow,I.H. and Tanzer,J.M. (eds.), *Molecular Basis of Biological Processes*, Academic Press, NY, pp. 155-169.
Rifkin,D.B., Beal,L.P. and Reich,E. (1975) in Reich,E., Rifkin,D.B. and Shaw,E. (eds.), *Proteases and Biological Control*, Cold Spring Harbor Laboratory Press, NY, pp. 841-847.
Rijken,D.C. and Collen,D. (1981) *J. Biol. Chem.*, **256**, 7035-7041.
Skriver,L., Nielsen,L.S., Stephens,R. and Danö,K. (1982) *Eur. J. Biochem.*, **124**, 409-414.
Steffens,G.J., Günzler,W.A., Otting,F., Frankus,E. and Flohé,L. (1982) *Hoppe-Seyler's Z. Physiol. Chem.*, **363**, 1043-1058.
Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201-5205.
Vassalli,J.D., Hamilton,J. and Reich,E. (1977) *Cell*, **11**, 695-705.
Vassalli,J.D., Dayer,J.M., Wohlwend,A. and Belin,D. (1984) *J. Exp. Med.*, in press.
Waller,E.K., Schleuning,W.D. and Reich,E. (1983) *Biochem. J.*, **215**, 123-131.
Wigler,M. and Weinstein,I.B. (1976) *Nature*, **259**, 232-233.
Wun,T.C., Ossowski,L. and Reich,E. (1982) *J. Biol. Chem.*, **257**, 7262-7268.
Younglai,E.V., Godeau,F., Mulvihill,B. and Baulieu,E.E. (1982) *Dev. Biol.*, **91**, 36-42.

*Received on 16 April 1984; revised on 25 May 1984*