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Modulation of urokinase plasminogen activator gene expression during the transition from quiescent to proliferative state in normal mouse cells

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We have investigated the regulation of urokinase (u-PA) mRNA in quiescent mouse fibroblasts and keratinocytes stimulated to divide by the addition of serum or epidermal growth factor (EGF), respectively. Serum stimulation of quiescent fibroblasts (BALB/c 3T3 or Swiss 3T3) results in an early and transient increase of u-PA mRNA level, which precedes by several hours the onset of DNA synthesis. A similar response is elicited by EGF stimulation of quiescent keratinocytes. The increase of u-PA mRNA parallels that of c-*myc* mRNA, does not require protein synthesis and is at least in part due to increase in template activity of the u-PA gene. Induction of terminal differentiation of mouse keratinocytes results in a decrease of u-PA mRNA which parallels the decrease of thymidine incorporation. In conclusion, variation in the level of u-PA mRNA is seen during G0/G1 transition and correlates with the proliferative state of these normal mouse cells.

Key words: urokinase plasminogen activator/mRNA regulation/ epidermal growth factor/serum stimulation

Introduction

Pro-urokinase is a surface-associated (Stoppelli *et al.*, 1986a) and secreted pro-plasminogen activator belonging to the serine protease gene family (Nagamine *et al.*, 1984; Patthy, 1985; Riccio *et al.*, 1985). The activator enzyme urokinase (u-PA), as the tissue-type plasminogen activate, catalyzes the conversion of the serum zymogen plasminogen into plasmin, a protease of broad specificity, which plays an important role in the dissolution of fibrin clot (Robbins *et al.*, 1973). Because of the regulatory role in the production of plasmin, plasminogen activators have been proposed to control the extracellular proteolysis required in those physiological and pathological processes that involve cell migration and tissue destruction (reviewed in Reich, 1978; Danø *et al.*, 1985). In addition, production of plasminogen activators, mainly u-PA, in cultured cells and *in vivo* is modulated by a variety of physiological and experimental stimuli (reviewed in Danø *et al.*, 1985) and several studies suggest that u-PA production might be linked to cell growth. In fact, some of the u-PA modulating effectors are also able to promote cell proliferation in the competent cellular system. These include hormones (Myra-y-Lopez *et al.*, 1983, 1985; Ossowski *et al.*, 1979), phorbol esters (Wigler and Weinstein, 1976; Belin *et al.*, 1984; Stoppelli *et al.*, 1986b), epidermal growth factor (EGF, Lee and Weinstein, 1978; Stoppelli *et al.*, 1986b) and colony stimulating factor (CSF, Hume and Gordon, 1984). Moreover, increased production of u-PA is associated with many spontaneous tumors (Mullins and Rohrlich, 1983; Danø *et al.*, 1985) and is an early transformation-dependent effect of tumor viruses (Ossowski *et al.*, 1973a; Unkeless *et al.*, 1973; Goldberg, 1974; Rifkin, 1980). Finally, a very close correlation has been observed between tumor growth and plasminogen activator activity (Myra-y-Lopez *et al.*, 1983, 1985). These data have led to the proposal that modulation of plasminogen activator activity, and more specifically of u-PA activity, is part of the cellular response to growth-modulating stimuli (Myra-y-Lopez *et al.*, 1983; Stoppelli *et al.*, 1986b). In line with this proposal, earlier data indicated that the level of plasminogen activator activity varies between quiescent and growing cells (Jaken and Black, 1979, 1980).

In this study we have investigated whether the production of u-PA in normal mouse cells is dependent upon their proliferative state by analyzing the temporal expression of u-PA mRNA in three types of cells that reach a quiescent state (G0) by depletion of growth factors and respond to the addition of mitogens by synchronously entering a new cell cycle. Recently it has been established that upon mitogen-induced transition a set of cellular mRNAs (Cochran *et al.*, 1983; Lau and Nathans, 1985) including the proto-oncogenes c-*myc* (Kelly *et al.*, 1983) and c-*fos* (Müller *et al.*, 1984; Greenberg and Ziff, 1984; Kruijer *et al.*, 1984) are differentially induced and turned off. Here we present evidence indicating that the u-PA gene is expressed at different levels in response to growth-stimulating or inhibiting stimuli.

Results

Induction of the u-PA mRNA level in mouse 3T3 fibroblasts

To investigate the expression of the u-PA gene in quiescent versus serum-stimulated 3T3 fibroblasts, confluent cultures of BALB/c 3T3 cells were exposed to 20% fetal calf serum (FCS) after 24 h of serum deprivation. This led to synchronous cell-cycle initiation as shown by [^{3}H]thymidine incorporation which starts after 8 h and peaks at 18 h (Figure 1A). Total RNA was extracted from serum-stimulated cells at different times, and the level of u-PA mRNA measured by dot-blot hybridization to a ^{32}P-labelled mouse u-PA probe. Figure 1B shows the time-dependent variation of u-PA mRNA in quiescent or serum-stimulated cells. The u-PA mRNA level increased 4-fold after serum stimulation. The induced level is maximal at 2 h and transient, since between 15 and 24 h u-PA mRNA level returns to that of quiescent cells. Northern analysis (Figure 1C) shows that the u-PA mRNA molecular species is indistinguishable in quiescent and serum-stimulated cells. We conclude from these experiments that quiescent BALB/c 3T3 cells have a low amount of u-PA mRNA which rapidly and transiently increases as cells switch to a proliferative state. As control, duplicate dot-blots were hybridized to β-2-microglobulin and c-*myc* ^{32}P-labelled probes. As expected (Kelly *et al.*, 1983) the level of β-2-microglobulin mRNA does not vary upon serum stimulation; c-*myc* mRNA level increases several-fold after 2 h exposure to serum, the induced level is maintained for the next 6 h and decreases by the time cells are in S-phase.

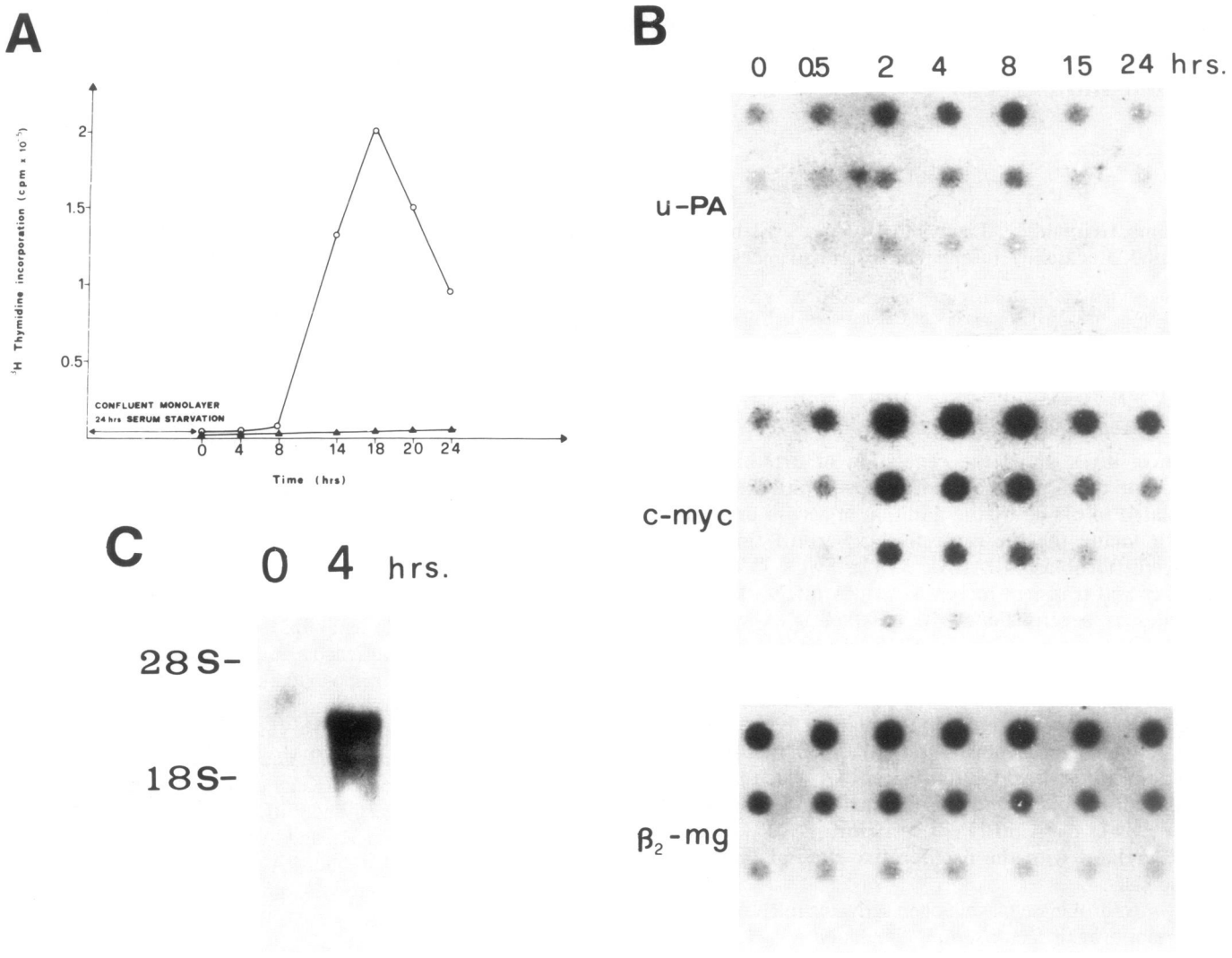

Fig. 1. Time course of u-PA expression in BALB/c 3T3 quiescent cells stimulated with FCS. **(A)** Thymidine incorporation following stimulation of quiescent cells. [Me-^{3}H]thymidine incorporated into TCA-precipitable counts is plotted against time after addition of DMEM-20% (○) or DMEM-0.25% FCS (▲). **(B)** Dot-blot analysis of the time course of u-PA, c-*myc* and β-2-microglobulin mRNA levels in quiescent or FCS-stimulated cells for the times indicated. Two-fold serial dilutions, starting from 5 μg of total RNA, was applied to nitrocellulose in triplicate for each time point and hybridized to ^{32}P-labelled probes as described in Materials and methods. Filters were exposed for 48 h. **(C)** Size of u-PA mRNA in quiescent or serum-stimulated cells. 30 μg of total RNA were applied to each lane, subjected to Northern analysis (described in Materials and methods) and hybridized to the u-PA probe. The estimated size of u-PA mRNA is 2300 nucleotides by comparison with the migration of rRNA markers.

To establish whether the increase in the u-PA mRNA level, which follows the stimulation of BALB/c 3T3 quiescent cells, is a general response of resting fibroblasts to mitogenic signals, a similar experiment was carried out on quiescent Swiss 3T3 fibroblasts. Serum-stimulated Swiss 3T3 cells synchronously enter S-phase ~15 h after mitogen addition and reach maximal [^{3}H]thymidine incorporation by 24 h (Figure 2A). The temporal expression of u-PA, c-*myc* and β-2-microglobulin mRNAs is shown in Figure 2B. Also in this cell line the u-PA mRNA level (Figure 2B and C) increases upon mitogenic stimulation. Figure 3 (panels A, B) shows a quantitative analysis of the time-dependent change of u-PA and c-*myc* mRNA levels in both BALB/c and Swiss 3T3 cells, during the first 3 h of serum stimulation. The increase of u-PA mRNA is an early event (30 min) following serum stimulation in both cell lines and coincides with the induction of c-*myc*. A slight difference in the kinetics of u-PA mRNA induction is reproducibly observed between the two lines. In Swiss 3T3 cells the maximal induction of u-PA mRNA (8-fold) is higher and is reached later (3 h) than in BALB/c cells.

Since u-PA mRNA increase is an early event after serum stimulation, we studied whether this process is affected by the block of protein synthesis. As shown in Figure 4 treatment of both types of quiescent cells with cycloheximide alone increases the level of u-PA mRNA; the combined exposure of cells to cycloheximide and serum does not prevent the induction by serum. Thus, the increase of u-PA mRNA is an early response of 3T3 cells to mitogenic stimulation that does not require synthesis of new proteins.

Transcriptional activity of the u-PA gene during serum stimulation of quiescent 3T3 cells

To establish whether the variation of u-PA mRNA upon serum stimulation is due to changes in the transcriptional activity of the gene or in the stability of the mRNA, we examined the extent of transcription of the u-PA gene using a nuclear run-on assay

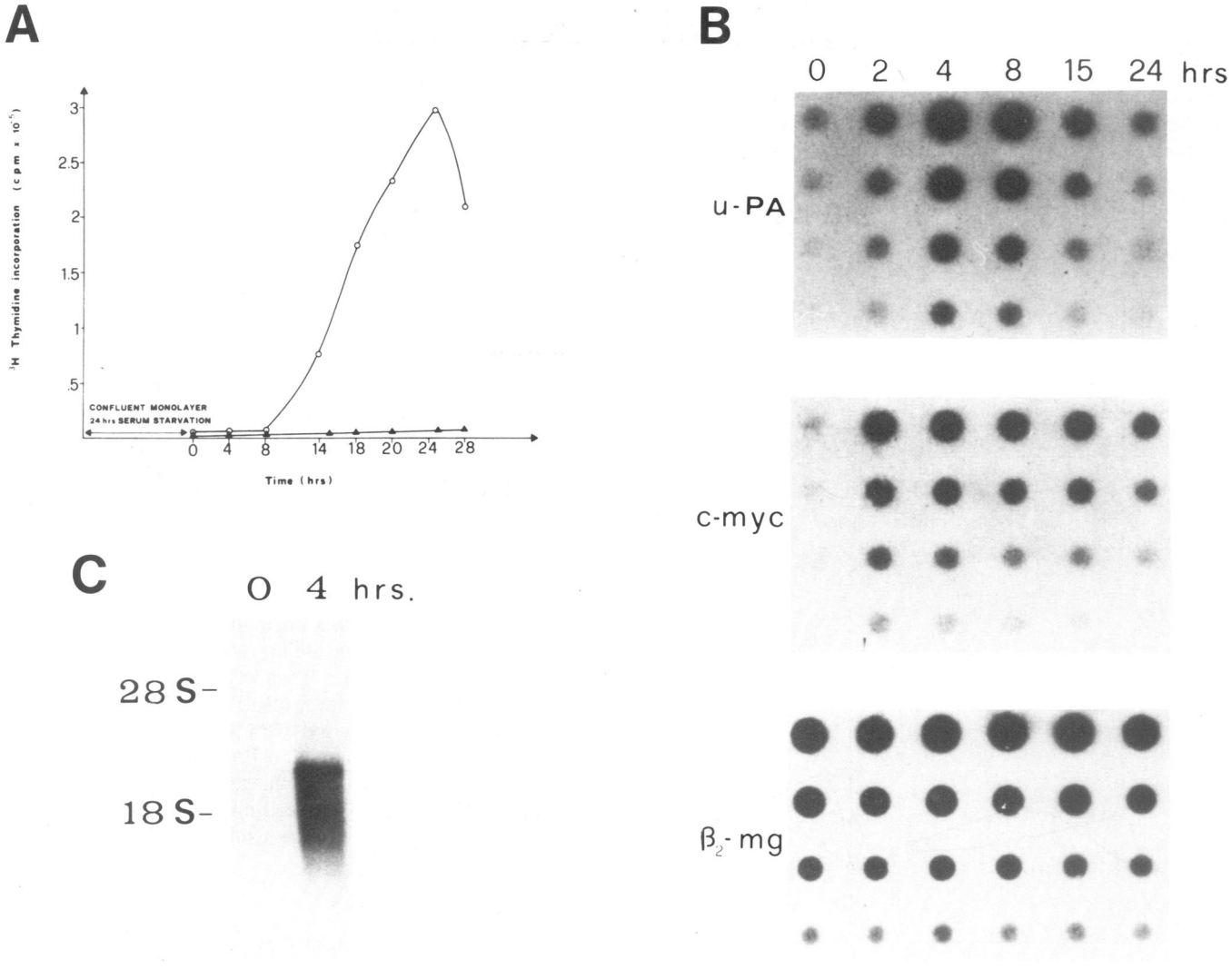

Fig. 2. u-PA mRNA expression following stimulation of quiescent Swiss 3T3 cells. **(A)** Thymidine incorporation following stimulation of quiescent Swiss 3T3 cell. [Me-^{3}H]thymidine incorporated is plotted against time after addition of DMEM-20% FCS (○) or DMEM-0.25% FCS (▲). **(B)** Temporal expression of u-PA, c-*myc* and β-2-microglobulin in FCS-stimulated quiescent cells. ^{32}P-Labelled probes and conditions for dot-blot analysis are described in Figure 1. Filters were exposed for 72 h. **(C)** Northern analysis of u-PA mRNA in quiescent and stimulated Swiss 3T3 cells. Conditions of Northern analysis, hybridization to the u-PA probe and mRNA size determination were as described in Figure 1.

(McKnight and Palmiter, 1979; Groudine *et al.*, 1981). Initiated transcripts from nuclei isolated from either BALB/c 3T3 or Swiss 3T3 cells between 0 and 4 h after serum stimulation were allowed to elongate *in vitro* in the presence of [^{32}P]UTP and the RNA was hybridized to several cloned DNA probes (i.e. u-PA, c-*myc*, β-actin and β-2-microglobulin). Figure 5 shows the results obtained with BALB/c 3T3 cells; similar data were obtained with Swiss 3T3 fibroblasts (not shown). A small increase (2-fold) in the transcriptional activity of the u-PA gene was obtained after 30 min of serum stimulation. A similar increase in c-*myc* transcription was reached after 2 h of serum stimulation; transcriptional activity of the β-actin gene was strongly increased at 30 min of serum stimulation and subsequently decreased in agreement with previous data (Greenberg and Ziff, 1984; Blanchard *et al.*, 1985). We did not detect hybridization to β-2-microglobulin DNA at any time, possibly because the specific activity and length of the probe was not sufficient to detect homologous transcripts under the conditions used. Figure 3 (panels C, D) shows a quantitation of the run-on data of both BALB/c and Swiss 3T3 cells. Comparison of the steady-state level (Figure 3A, B) with the synthetic rate (Figure 3C, D) of u-PA mRNA may suggest the presence of additional post-transcriptional control mechanisms.

Variations of the urokinase mRNA level during proliferation and differentiation of BALB/MK cells

BALB/MK cells are epidermal keratinocytes dependent on EGF for optimal growth. In addition, these cells undergo a complex pattern of biochemical changes that result in terminal differentiation when exposed to Ca^{2+} concentrations higher than 1 mM (Weissman and Aaronson, 1983). We took advantage of the properties of this cell line to investigate further the link between cellular proliferation and u-PA mRNA levels. BALB/MK cells deprived of EGF enter into a quiescent state within 48 h. Re-

addition of the growth factor leads to synchronous cell cycle initiation, with thymidine incorporation peaking at 20 h (not shown). Northern-blot analysis (Figure 6) shows that u-PA mRNA increases within 30 min reaching maximal induction within 2–4 h of EGF addition. Similarly to 3T3 fibroblasts, the increase is transient and the decrease coincides with entry of cells into S-phase, basal level being reached within 12 h. The level of c-*myc* increases in these cells with kinetics similar to those of u-PA mRNA and is maintained high throughout the S-phase (Di Fiore *et al.*, in preparation). Also in the BALB/MK cells, treatment with cycloheximide does not prevent induction of u-PA mRNA by EGF (Figure 6) indicating that newly synthesized proteins are not required for the induction to occur.

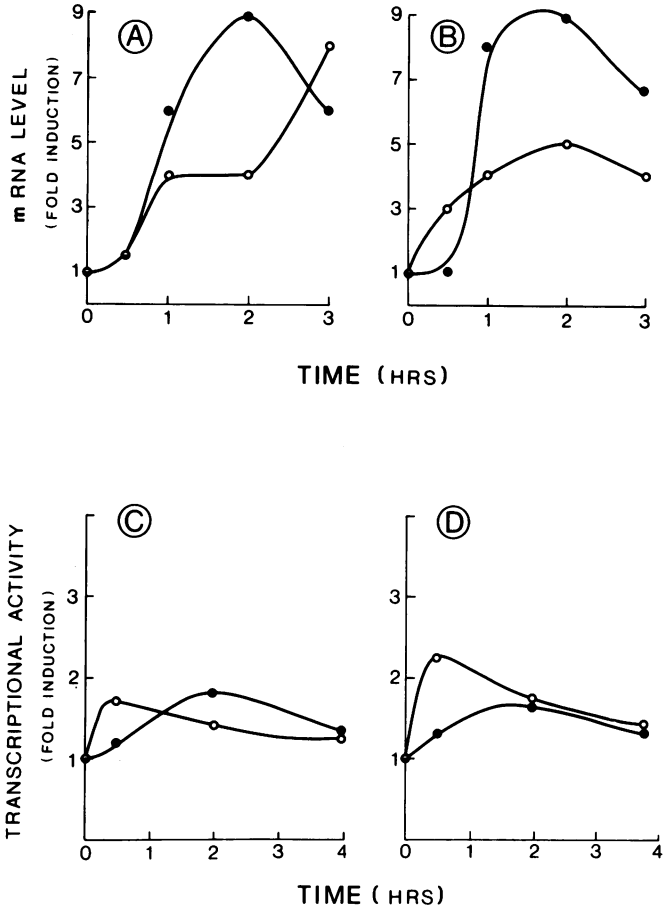

Fig. 3. Quantitation by film-scanning of the time-dependent changes of steady-state **(A,B)** and transcriptional rate **(C,D)** levels of u-PA (○ – ○) c-*myc* (● – ●) mRNA. In all cases the RNA levels at time 0 have been set equal to 1.0 and the data at subsequent times expressed as fold induction. **A,C:** BALB/c 3T3 cells; **B,D:** Swiss 3T3 cells.

Further correlation between the proliferative state of cells and the level of u-PA mRNA is assessed by the experiment shown in Figure 7. BALB/MK cells treated with 1.5 mM Ca^{2+} undergo a complex pattern of biochemical and morphological differentiation (Weissmann and Aaronson, 1983, 1986) and stop proliferating by 72 h even in the presence of EGF. Analysis of the u-PA mRNA after 24, 42 and 72 h of exposure of cells to Ca^{2+} shows that the level decreases as cells differentiate and stop proliferating (Figure 7A). Calcium treatment, in fact, causes a 75, 90 and 95% inhibition of thymidine incorporation after 24, 48 and 72 h of treatment, respectively (data not shown). The link between u-PA induction and cellular proliferation is further suggested by the results obtained with two mutant cell lines, BALB/MK EGF^I and BALB/MK Ca^R. Although the mutants were selected either for EGF independence (BALB/MK EGF^I) or for survival to calcium (BALB/MK Ca^R), they both display the same phenotype, i.e. they do not differentiate and do not stop proliferating upon calcium addition (Di Fiore *et al.*, in preparation). As shown in Figure 7B and C, addition of calcium had little or no effect on the level of u-PA mRNA in either of the two mutant lines over a period of 72 h. In conclusion, the results indicate that the reduction of u-PA mRNA in Ca^{2+}-treated BALB/MK keratinocytes is not due to a direct effect of calcium, but appears to be part of the cell differentiation response correlated with the block of DNA synthesis.

Discussion

In this paper we have presented evidence indicating that the expression of u-PA, one of the two cellular plasminogen activators that regulate the production of plasmin, is modulated by factors that influence cell growth in normal murine cells. In two fibroblast cell lines, BALB/c 3T3 and Swiss 3T3, and in epidermal keratinocytes, BALB/MK, we have observed a similar response of the u-PA mRNA expression following stimulation of quiescent cells by the competent mitogenic agent(s). Thus the relationship bet-

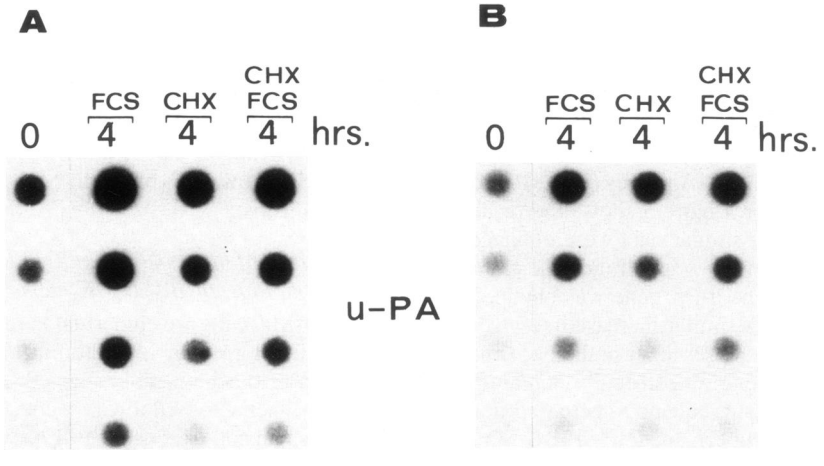

Fig. 4. Induction of u-PA mRNA following treatment of quiescent cells with cycloheximide and FCS. Quiescent Swiss 3T3 **(A)** and BALB/c 3T3 **(B)** cells were exposed to FCS (20%) or cycloheximide (20 μg/ml) for 4 h or pre-treated for 30 min with cycloheximide followed by FCS for 4 h. Filters were exposed for 72 h. Conditions of dot-blot analysis are described in Figure 1.

ween cellular proliferation and elevated u-PA mRNA level appears to be independent of the cell type and origin. The profile of induction of u-PA mRNA during the transition from quiescent to proliferative state is very similar in the three cell types and coincides in time with the elevation of c-*myc* (Kelly *et al.*, 1983; Müller *et al.*, 1984; Kruijer *et al.*, 1984 and Figures 1, 2 and 3). The u-PA mRNA increase is an early cellular response to growth stimulation, already evident within 30 min and peaking at 1 or 3 h, i.e. during the period when cells become competent to synthesize DNA (Pledger *et al.*, 1977). Whether the increse of u-PA mRNA in serum-stimulated 3T3 cells is mediated by the competence-inducing platelet-derived growth factor (PDGF) and/or by other factors, remains to be established. In EGF-dependent BALB/MK cells the increase of u-PA mRNA is mediated by EGF or by the combined action of EGF and serum factors.

Increased expression of u-PA mRNA is at least in part due to increased transcriptional activity of the gene, as shown by run-on experiments (Figure 5), and is likely directed by intracellular signals that do not require newly synthesized proteins as treatment with cycloheximide does not prevent the induction (Figures 4 and 6).

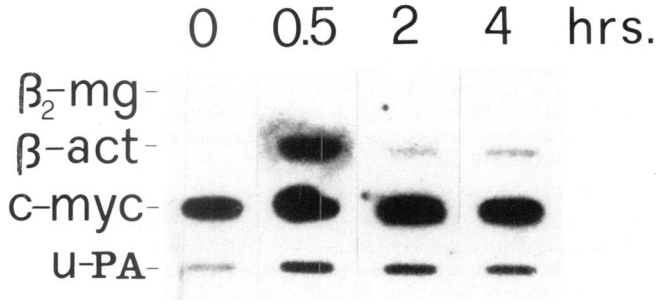

Fig. 5. Transcriptional activity of the u-PA gene following stimulation of quiescent BALB/c 3T3 cells. Linear, cloned DNAs bound to nitrocellulose were hybridized to ^{32}P-labelled run-on transcripts from nuclei isolated from quiescent or FCS(20%) stimulated BALB/c 3T3 cells. Linearized DNAs (3 μg) were immobilized onto nitrocellulose as described (Greenberg and Ziff, 1984). DNA probes, isolation of nuclei, elongation of RNA, hybridization and washing conditions are described in Materials and methods. Filters were exposed for 4 days.

The relationship between u-PA induction and cell growth is further strengthened by the following data: (i) even in the presence of all factors that elevate the u-PA mRNA (e.g. EGF and serum), when BALB/MK cells are exposed to Ca^{2+}, which counteracts the EGF action on quiescent cells, the level of u-PA mRNA decreases concomitantly with the proliferative block (Figure 5); (ii) in the two mutant cell lines BALB/c MK Ca^{R} and BALB/c MK EGF^{I}, that do not differentiate upon exposure to Ca^{2+}, the u-PA mRNA level does not decrease upon calcium treatment (Figure 7B and C). Interestingly, u-PA mRNA induction by EGF is direct, not requiring protein synthesis, but calcium is able to counteract it. Thus calcium must either act independently of EGF through a parallel regulatory mechanism epistatic to that of EGF or prevent the effect of EGF interfering with regulatory steps that do not require protein synthesis. Altogether the data indicate that modulation of the u-PA mRNA level is part of the mechanism of control of proliferation in these cells.

The elevation of u-PA mRNA is transient and is restricted to the G0-G1 phase. Whether the increase in u-PA mRNA has a functional effect on the progression of cells through the G1 phase remains to be established. It must be recalled that quiescent cells can be stimulated to proliferate by mild protease treatment (reviewed in Reich *et al.*, 1975), and that proteases potentiate the action of growth factors, including serum, possibly by increasing the efficiency of their utilization. In addition, the data of Cohen *et al.* (1981) indicate that u-PA might possess a proliferation stimulating activity of its own. Alternatively, a regulated increase of u-PA might negatively modulate cell surface receptor turnover (Miskin *et al.*, 1978; Hatzfield *et al.*, 1982). In fact, in EGF-treated human carcinoma cells A431, increased u-PA activity parallels down-regulation of the EGF-receptor (Gross *et al.*, 1983). Whatever the possible role of u-PA in cell proliferation, the recent discovery of a specific cell membrane-associated u-PA receptor both in human (Stoppelli *et al.*, 1985, 1986a;

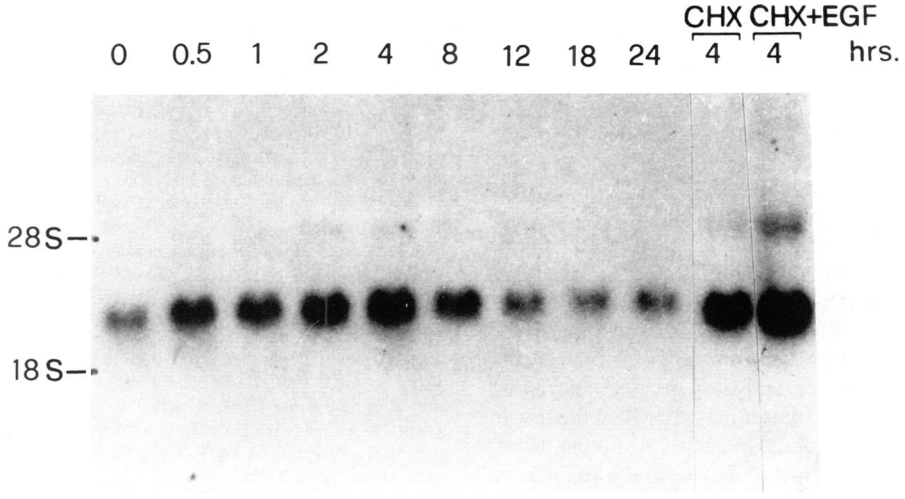

Fig. 6. Northern blot analysis of u-PA mRNA induction in EGF-stimulated quiescent BALB/MK keratinocytes. 20 μg total RNA were applied in each lane. The numbers on top indicate the time (h) after addition of EGF (4 ng/ml). 18S and 28S markers indicate the migration of rRNA. The last two lanes were loaded with RNA extracted from quiescent cells treated for 4 h with cycloheximide 10 μg/ml (CHX) or cycloheximide in the presence of 4 ng/ml EGF (CHX + EGF). Labelling of the DNA probe and hybridization and washing conditions are described in Materials and methods. Thymidine incorporation was measured at each time point as described in Materials and methods. Incorporation started ~7–8 h after EGF addition and peaked at 20 h, being ~50-fold over background. Filters were exposed for 12 h.

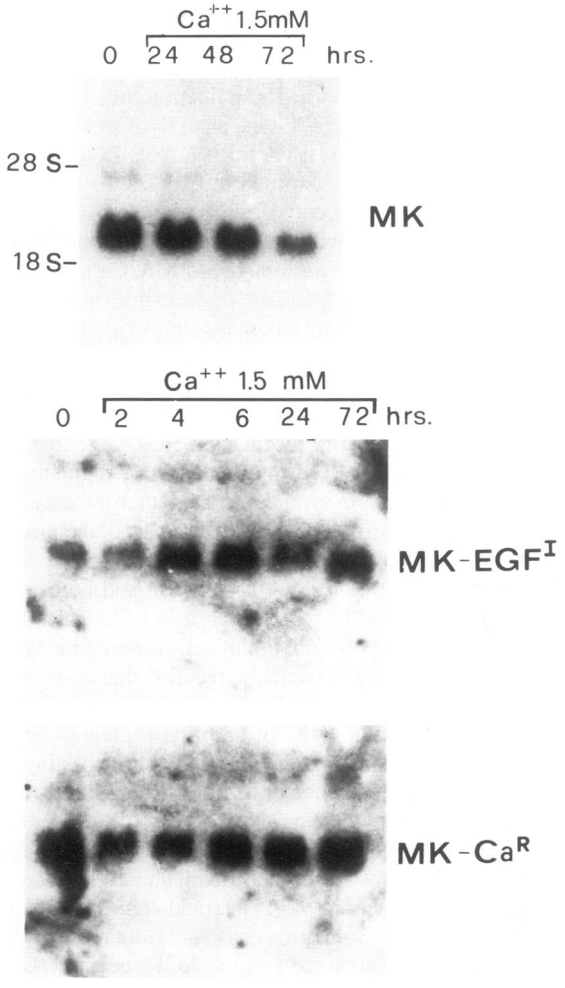

Fig. 7. Expression of u-PA mRNA following exposure of BALB/MK cells and BALB/MK mutants to Ca^{2+}. Northern blot analysis. **(A)** Time course of u-PA mRNA level in BALB/MK cells untreated or treated with 1.5 mM Ca^{2+} for the indicated times. **(B** and **C)** Time courses of the u-PA mRNA expression in BALB/MK EGF^I and BALB/Mk Ca^R, respectively, after exposure for the indicated times to 1.5 mM Ca^{2+}. Northern analysis and hybridization to the u-PA probe are the same as in Figure 6 and are described in Materials and methods.

Vassalli *et al.*, 1985) and mouse cells (Vassalli and Belin, personal communication) may indicate a possible site of u-PA action. This suggestion is in agreement with previous data on the plasminogen activator activity of quiescent versus serum-stimulated fibroblasts showing that although the overall enzyme activity is higher in quiescent than proliferating cells (Rohrlich and Rifkin, 1977; Loskutoff and Paul, 1978), the molecular species (Jaken and Black, 1980) and the cellular compartmentalization (Jaken and Black, 1979) of the enzymatic activity changed. In particular the plasminogen activator of quiescent Swiss 3T3 cells has a mol. wt of 75 kd, thus it may represent the tissue-type plasminogen activator, and is present in soluble form. Serum-stimulated cells, conversely, have a 49-kd plasma membrane-associated plasminogen activator which we interpret today as being receptor-associated pro-u-PA (Stoppelli *et al.*, 1986a).

The finding that the expression of u-PA is regulated by factors that affect the control of the growth state of cells has an interesting counterpart in the increased level of u-PA found in most tumor or in *in vitro* transformed cells (reviewed in Danø *et al.*, 1985). Higher expression of u-PA may result from loss of growth-regulatory control. The finding that some human carcinoma cells which have elevated u-PA and normal EGF-receptor level do not respond to EGF by increasing u-PA mRNA and protein (Stoppelli *et al.*, 1986b), is in line with this possibility.

In several cells, plasminogen activators play a role in cell migration (Ossowski *et al.*, 1973b); antibodies to human u-PA block the metastatic activity of a human carcinoma cell line in an experimental system (Ossowski and Reich, 1983). The demonstration of growth-dependent regulation of the u-PA gene, the product of which is a surface-associated extracellular regulatory protease (Stoppelli *et al.*, 1986a), provides a link between cell growth and cell migration, i.e. the two properties whose deregulation are the most distinctive properties of malignancy.

Materials and methods

Cell lines and cultures

BALB/c 3T3 clone A31-714 (Kakunaga, 1973) was obtained from Dr Edward Kuff. Swiss 3T3 cells (Todaro and Green, 1963) were from the American Type Culture Collection. BALB/c keratinocytes (BALB/MK) have been described (Weissmann and Aaronson, 1983). 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% FCS (Flow Laboratories, Milan, Italy). BALB/MK cells were maintained in low-calcium (0.05 mM) MEM supplemented with 10% dialyzed FCS (Gibco) and 4 ng/ml EGF (Collaborative Research Lexington, MA, USA). BALB/MK EGF^I and BALB/MK Ca^R mutants (Di Fiore *et al.*, in preparation) were grown in the same low-calcium medium as parental BALB/MK cells.

Induction of proliferation and differentiation

BALB/c 3T3 and Swiss 3T3 cells were allowed to grow to confluence. The spent medium was removed and replaced for 24 h with DMEM containing 0.25% FCS. The cells were then stimulated to leave quiescence by the addition of fresh DMEM containing 20% FCS. Early passage cells were used for all the experiments.

BALB/MK cells were grown to confluence, spent medium was removed and replaced with low-calcium MEM, 10% FCS, but no EGF. After 48 h EGF was re-added (4 ng/ml), cells harvested at different times thereafter and the RNA extracted. In different experiments, BALB/MK cells were induced to differentiate by calcium addition. In this case subconfluent plates were incubated in high calcium (1.5 mM) MEM, 10% FCS, 4 ng/ml EGF. Cells were harvested and the RNA extracted at various times thereafter.

Thymidine incorporation

DNA synthesis was monitored by incorporation of [Me-^{3}H]thymidine (Amersham 84 Ci/mM), as described by Pardee (1974). Duplicate samples were assayed for each time point.

RNA analysis

Total cellular RNA was isolated by a modification of the guanidine hydrochloride extraction method (Adams *et al.*, 1977) or by SDS-proteinase K treatment followed by centrifugation through a step gradient of CsCl, as previously described (Riccio *et al.*, 1985). Total RNA was analyzed by quantitative dot-blot (Kafatos *et al.*, 1979) or by Northern analysis (Thomas, 1980). Dot-blots were prepared applying 2-fold serial dilutions of denatured total RNA starting from 5 μg onto nitrocellulose using the Shleicher & Schuell (Keene, NH, USA) dot-blot manifold. Hybridization of blots was carried out at 45°C in 50% formamide, 6 × SSC, 2 × Denhardt, 50 mM phosphate buffer, pH 7.0, 0.1% SDS, 200 μg/ml salmon sperm DNA and 2 × 10^5 c.p.m./ml of ^{32}P-labelled probes. The specific activity of nick-translated probes was 2−5 × 10^8 c.p.m./μg of DNA.

Cloned DNA probes

The following DNA probes were used in this study. Plasmid pDB9 carries part of the mouse u-PA cDNA (Belin *et al.*, 1985); plasmid pS107, carries exon II and III of mouse *myc* DNA (Kirsch *et al.*, 1981); a 540-bp *Sac*I − *Kpn*I fragment encompasses exon II of mouse β-2-microglobulin gene (Parnes and Seidman, 1982); plasmid pHFBA-3'UT, codes for the 3'-untranslated region of human β-actin (Ponte *et al.*, 1983).

Nuclear transcription assays

Preparation of nuclei, RNA elongation and isolation were performed as described (Vannice *et al.*, 1984); 10^7 nuclei were used for each assay. 5 × 10^6 c.p.m. incorporated into RNA were used for hybridization. Conditions of hybridization were: 50% formamide, 6 × SSC, 2 × Denhardt, 50 mM phosphate buffer pH 6.5, 1% SDS, 50 μg/ml yeast RNA and 100 μg/ml salmon sperm DNA 37°C for 4 days. Washing of blots was carried out twice at room temperature in 2 × SSC, 0.1% SDS, followed by four washes at 48°C.

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