Transcription factor PEA3 participates in the induction of urokinase plasminogen activator transcription in murine keratinocytes stimulated with epidermal growth factor or phorbol-ester

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ABSTRACT

Keratinocytes in culture represent cells which exhibit continued and controlled growth in the organism. We have investigated the synthesis of urokinase plasminogen activator mRNA in exponentially growing cultures of primary murine keratinocytes and the keratinocyte cell line BALB/MK. The tumor promotor 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and epidermal growth factor (EGF) induced urokinase mRNA synthesis. We made a series of progressive 5′ deletions as well as internal deletions in the region upstream of the murine uPA gene. These were joined to the *cat* reporter gene, and used to map the TPA and EGF responsive regions of the promoter. We found both responsive sequences within a 90 base pair Hae III fragment, located 2.4 kb. upstream of the mRNA cap site. This DNA fragment conferred TPA inducibility on reporter gene expression independent of its distance and orientation to the transcription initiation site. Footprinting and gel retardation studies identified the responsible sequence to be a binding site for PEA3 juxtaposed to an octameric TRE-element. Transfections with point mutants showed that these target sequences were necessary for TPA and EGF induction of transcription.

INTRODUCTION

Urokinase plasminogen activator (uPA) is one of two plasminogen activators which can convert plasminogen to plasmin. Plasminogen activation releases general extracellular proteolytic activity, since plasmin can activate still other proteolytic pro-enzymes as well as being an active protease itself. The presence of uPA protein in mice is restricted to few cells and tissues(1), and uPA expression correlates with tissue remodelling and cell migration(2).

Normal skin contains very little uPA protein, but healing skin contains appreciable amounts, located to the migrating and hyperproliferating keratinocytes that participate in wound-healing processes(3). The amount of uPA in primary cultures of human keratinocytes can be increased by wounding the cultured cells with a surgical blade, and elevated amounts of uPA is associated with the migrating keratinocytes that appear in the wounded area within 10 hours(4). Plasminogen, the substrate for uPA, is present in the skin(5).

In vitro cell culture experiments have shown that the level of uPA mRNA is controlled by several effectors in many different cell types. In general, the uPA gene is only transcribed in normal cells when they are growing and dividing(6). In contrast, lines of transformed cells often contain uPA mRNA even when starved for serum(7).

In quiescent normal BALB/MK keratinocytes, epidermal growth factor (EGF) stimulates synthesis of uPA mRNA(6). In this paper we show that TPA addition to exponentially growing cultures of primary mouse keratinocytes and BALB/MK cells leads to an increase in uPA mRNA level in the cultures.

We have mapped and sequenced the region responsible for EGF and TPA stimulation in a 90 bp region which showed strong homologies to the published regulatory sequences of the human uPA gene(8). Transcriptional stimulation was mediated through the sequence AGGAAATGAGGTCA, closely related to the combined PEA3/AP-1 site in the a-domain of the polyoma virus enhancer(9). PEA3 is involved in the regulation of the collagenase promotor (10); our data show that it participates in the regulation of uPA expression as well.

MATERIALS AND METHODS

Cells, Cell Growth

Primary keratinocytes were isolated from newborn BALB mice as described (11) and maintained in Eagle's MEM with 1% antibiotic/antimycotic mixture (Gibco) and 8% chelex-treated fetal calf serum (FCS) from Reheis.

BALB/MK cells are non-tumorigenic epidermal keratinocytes which are dependent on EGF for growth, and undergo terminal

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differentiation when presented with Ca^{++}-ions in concentrations larger than 1.0mM(12); we obtained these cells were from P. Di Fiore, Laboratory of Cellular and Molecular Biology, NCI, NIH, Bethesda.

BALB/MK cells were grown in S-MEM, pH 7.6 at 37 C, 5.5% CO2 (Gibco cat. # 042−01650) with 0.05mM Ca++, 100U/ml Penicillin-100μg/ml Streptomycin (Gibco), 10% dialysed FCS (Gibco) and 4ng/ml EGF(Collaborative Research inc.).

For EGF induction, cells were plated in the absence of EGF (but in the presence of serum) and received fresh medium with or without 4ng/ml EGF 48 hours later. For TPA induction, exponentially growing cells received fresh medium with 100ng/ml TPA (Sigma) or solvent alone (DMSO).

Transfections

BALB/MK cells were transfected by electroporation, using an apparatus built as described (13). The optimal conditions were: 1000μF capacity, transfection voltage 150V, volume and cell density: 0.25ml. and 8×10^6 cells/ml, respectively. Cells were stimulated with TPA 48 hours after electroporation.

In the experiments shown in figures 4 and 7, BALB/MK cells were transfected by a modified calcium-phosphate technique as described(14).

Each of the transfection experiments were done with a mixture of 10μg of a uPA-promotor:*cat* plasmid DNA and 2μg DNA of pCH110, serving as an internal control. The pCH110 plasmid has the *lacZ* gene under control of the SV40 early promoter(15).

Plasmid constructions

All genomic murine uPA DNA used in this work was derived from a clone of a 12kb. Sal I fragment, most kindly donated to us by Rene Bernard from the laboratory of Robert Weinberg. Unidirectional deletions for sequencing and further cloning were generated with exonuclease III as described by Henikoff(16) or using the restriction enzyme sites indicated on figure 2A.

The Xba I site at uPA position +395 defines the 3′ end of uPA DNA in all reporter constructs. pUC18-uPA DNA fragments from the pUC Aat II site through the Hind III site in the pUC polylinker were cloned into the Aat II-Hind III sites in pCH110. From these *lacZ* reporters, *cat* reporter constructions (uPA-*cat*) were isolated by cloning the Bam HI−Hind III fragment that contains the uPA promoter into the Bam HI−Hind III sites in pSV2*cat*. (17) The Δ-series constructs were obtained by combining uPA sequences upstream of the Bgl II site, position −2175, with the Bam HI site in the polylinker region of the pUC-derivative that contains uPA sequences −114 through +395. In pPRR97−102 the EcoR I (in the pUC polylinker)-Hae III, Hae III-Hae III or Hae III-Mae I fragment (see fig. 3) was cloned in Sma I in the same pUC-uPA (−114 to +395), and inserted in front of the *cat* gene The identity and orientation of these inserts was checked by nucleotide sequencing. The −3150ΔHae III plasmid was constructed from the −3150 uPA−*cat* reporter plasmid by deleting Hae III −2446 to Hae III −2356. Full details of the various cloning steps are given in(18).

To obtain point mutations in the pPR99 background, all uPA sequence from pPR99 was cloned into M13 mp19. Mutagenesis was by the Eckstein method(19), using the oligos shown in figure 5 as primers. Oligo-numbers correspond to the plasmid-numbers of the mutated vectors. Isolated mutants were re-cloned in the pPR99 vector and sequenced.

RNA isolation and Northern Blots

RNA was isolated by guanidinium-isothiocyanate solubilization of the cells and centrifugation through 5.7M CsCl(20). Total cellular RNA was separated on a 1.2% agarose formaldehyde-gel (20μg per lane) and blotted onto GeneScreen membranes (DuPont) or Schleicher & Schuell BA85 nitrocellulose. ^{32}P labeled random primed probes were used for the Northern blots: uPA: a 1.1kb. Eco RI-Bgl II fragment of murine uPA cDNA(21); keratin K14: 200 bp. Xmn I fragment of 3′ non-coding, transcribed DNA(22); β_2-microglobulin: 0.62 kb. Sac I-Kpn I fragment including exon 2(23). Hybridization was in 50% formamide, 5×SSC, 1% SDS, 2×Denhardts solution and 0.2mg/ml denatured salmon sperm DNA or 0.2mg/ml yeast RNA at 42°C for 16−20 hours. The high stringency washes were in 0.1×SSC, 0.5% SDS 65°C.

Transcriptional run-on

Preparation of nuclei and elongation were performed as described by Greenberg and Ziff(24), except 100μCi ^{32}P-α-UTP (160Ci/mmole) and 2×10^7 BALB/MK nuclei were used. After elongation and proteinase K treatment, the sample was twice extracted with H_2O-saturated phenol, and ethanol precipitated. The nucleic acids were digested with RQ1 DNase (Promega Biotech) at 37°C, extracted and ethanol precipitated. Filters (GeneScreen™) were prepared with 10μg HCl-depurinated and NaOH-denatured plasmid DNA per slot. The DNAs applied were p1519 (containing 2kb uPA cDNA in pUC19), pG3B (2kb beta-actin cDNA(25). in pGEM3) and pUC19. Labeled RNA was exposed to 0.2M NaOH on ice for 5 minutes before addition of 10^7cpm to each prehybridized filter. Hybridization was in 50% formamide, 5×SSC, 2×Denhardts solution, 0.25%SDS, 50mM Na-phosphate pH6.5, 0.5 mg/ml yeast tRNA and 0.5 mg/ml denatured salmon sperm DNA, at 40°C for 48 hours. Filters were then washed at 65°C in 2×SSC, 0.5%SDS for 1 hour and in 0.1×SSC, 0.1%SDS for 4×30 minutes.

Enzyme assays

Harvested cells were divided for chloramphenicol acetyl-transferase (CAT) and beta-galactosidase assay. The two-phase CAT assay was preformed essentially as described(26). Normally, the ^3H counts in the scintillator phase was determined after 0, 3 and 10 hours at 37°C. CAT enzyme activity was calculated as nmoles of Acetyl-Coenzyme A reacted per minute of incubation.

For β-galactosidase assays, cells were resuspended in 0.5ml 0.03% deoxycholate in PM2 buffer (39mM NaH_2PO_4, 60mM Na_2HPO_4, 3mM $MgSO_4$, 2mM EDTA, 0.2mM $MnCl_2$) and 0.1ml. 2mg/ml ortho-nitro phenyl β-D-galacto-pyranoside (ONPG), 100mM beta-mercaptoethanol in PM2 was added at 37°C. The reaction was stopped with 0.25 ml 1M Na_2CO_3 and the increase in OD_{420} per minute used as measure of enzyme activity.

Final ratios of reporter enzyme levels were calculated after normalization to protein concentration and subtraction of mock-transfected cellular background.

Preparation of nuclear extracts

5×10^8 BALB/MK cells were used for each extract preparation; when preparing extracts from TPA stimulated cells, TPA was added to the medium 1 hour before harvest. The extracts were prepared essentially according to Dignam et al.(27). Extracts to

be used in footprinting analysis were subjected to a 47% ammonium sulfate precipitation by the addition of 300mg ammonium sulfate (Schwarz-Mann, enzyme grade) per ml extract while still in buffer C. After stirring 30 min at 2°C the precipitate was spun down, resuspended in buffer D in 0.25 of the original volume and dialyzed against buffer D. For bandshift experiments with labeled PEA3 oligo, extracts were dialysed without precipitation.

DNase I footprint analysis

5′ end labeling of DNA fragments was done with γ-^{32}P-ATP (0.8μM, New England Nuclear; >6000 Ci/mmole) and polynucleotide kinase. When labeling 3′ ends the digested plasmid was filled in with Klenow enzyme (New England Biolabs) in the presence of dTTP and α-^{32}P-dATP (1.7μM, >3000 Ci/mmole). The specific activity of the gel purified fragment DNA was greater than 2000 cpm/femtomole.

Binding was in 16 μl of a buffer containing 20mM HEPES (pH 7.9), 50mM KCl, 2mM $MgCl_2$,17% glycerol, 0.1mM EDTA, 0.25mM DTT and 0.25mM PMSF. 5−20 000cpm of DNA fragment and 1μg poly d(I-C) (Boehringer Mannheim), and the indicated amount of nuclear extract. After 20 minutes incubation at 20°C, 4μl of a 12.5mM $CaCl_2$ solution was added followed by DNase I (Boehringer Mannheim grade I) freshly diluted in 50% glycerol; 3−5ng for samples without protein addition 20−1000ng for reactions with extract. After 2 minutes at 20°C the reactions were stopped with 50μl of stop solution (40mM EGTA, 0.1% SDS), phenol/chloroform extracted, and ethanol precipitated. Finally, the DNA was separated on 8% denaturing polyacrylamide gels, using standard Maxam-Gilbert G+A sequencing reactions of the fragments as markers.

Oligonucleotide synthesis, bandshift assays

Oligonucleotides were synthesized on an Applied Biosystems Model 381 DNA synthesizer and purified on gels. After annealing 5′ end-labeled (^{32}P) complementary oligos, double stranded oligos were gel-purified. A specific activity of at least 2−5000cpm/fmole was obtained.

Nuclear extract was mixed with poly d(I-C) and double stranded competitor oligo on ice in 18 μl 10mM HEPES (pH 7.9), 50mM KCl, 10% glycerol, 0.1mM EDTA, 0.25mM DTT and 0.25mM PMSF. After 15 minutes at 0°C, 10.000cpm double stranded oligo was added and the incubation continued for 15 minutes at 25°C. Alternatively, the competitor oligo was added along with the labeled oligo and the incubation was at RT. The samples were loaded onto a native polyacrylamide gel and electrophoresis was in 0.5 or 0.25×TBE (1×TBE: 89mM Tris, 89mM Boric acid, 1mM EDTA) at 300V.

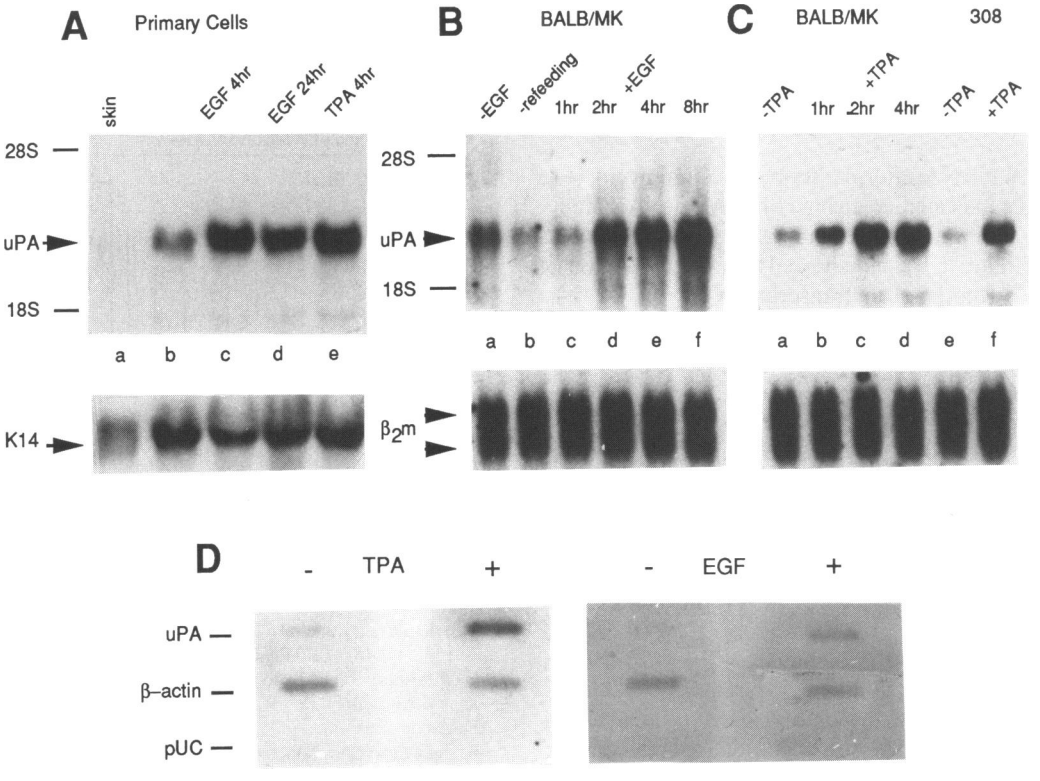

Figure 1. Regulation of the endogenous uPA expression in primary keratinocytes and keratinocyte cell lines. A, B and C: Northern blots, the 28S and 18S rRNAs were localized by Ethidium Bromide staining of a marker lane. **Panel A:** Primary keratinocytes and skin Lane a: RNA isolated from skin of newborn BALB mice, lanes b,c,d and e: RNA from primary keratinocytes harvested 4 hours after feeding with: lane b: medium without EGF, lane c: medium+EGF d: medium+EGF (cells harvested after 24 hours), lane e: medium+TPA. Keratin gene K14 as standard. **Panel B:** Effect of EGF addition in BALB/MK cells. BALB/MK cells were starved for EGF 48 hrs., then recieved fresh medium and were harvested at the indicated times. lane a: −EGF 4 hours, lane b: no medium change, lanes c,d,e and f: +EGF: 1, 2, 4 and 8 hours after medium change. β2-microglobulin mRNA served as standard to correct for differences in the amount of RNA loaded in individual lanes. **Panel C:** Effect of TPA addition in BALB/MK and 308 cells. Exponentially growing cells recieved full medium with or without TPA and were harvested at the indicated times. Lanes a, b, c and d: BALB/MK, lane a: −TPA 4 hours., lanes b, c and d : +TPA: 1, 2 and 4 hours after medium change. Lanes e and f: 308 cells, lane e: −TPA 4 hours, lane f: +TPA 4 hours. β2-microglobulin mRNA as standard. **Panel D:** Run-on transcriptional analysis in BALB/MK cells. TPA experiment: Exponentially growing cells were harvested 1 hour after addition of TPA in DMSO (+TPA) or DMSO (−TPA) to the medium. EGF experiment: After 48 hours EGF starvation, cells recieved medium with (+EGF) or without (−EGF) EGF 2 hours before harvest.

RESULTS

Induction of uPA mRNA level by treatment with EGF and TPA in primary keratinocytes and keratinocyte cell lines

We isolated RNA from cultures of primary keratinocytes and BALB/MK cells, both normally growing and exposed to either TPA or EGF. TPA was added to exponentially growing cells. When analyzing EGF mediated effects in the EGF-dependent BALB/MK cells, these were initially starved for EGF for 48 hours, thus were quiescent at the start of the experiment. The isolated RNA was analyzed for uPA mRNA content by Northern blotting, and the autoradiograms are shown in figure 1. The observed changes in uPA mRNA levels were reflected in measurements of extracellular uPA enzymatic activity (data not shown).

The level of uPA mRNA was high in primary keratinocytes when compared to mRNA isolated directly from newborn mouse skin, and could be elevated by addition of EGF or TPA to the growth medium (panel A; lanes c,d and e). In BALB/MK cells, readdition of EGF after 48hours of EGF-starvation induced an increase in the uPA mRNA level (panel B) as has already been shown(6). Refeeding with 10% serum without EGF gave a smaller, but significant increase (lane a). In all later experiments with EGF-addition, serum refeeding was used as control to correct for this effect.

TPA stimulation of cells growing in full serum resulted in a maximal accumulation of uPA mRNA 4hours after stimulation (panel C), thus the stimulation by TPA was faster than that by EGF. We found a similar increase of uPA mRNA after TPA addition in a papilloma-forming keratinocyte line, namely 308(28).

Analysis of uPA mRNA after addition of Actinomycin D to BALB/MK cultures indicated no change of uPA mRNA stability by EGF or TPA addition. Transcriptional run-on experiments were performed (figure 1 D) and showed that the regulation of uPA mRNA was at the level of transcription.

Nucleotide sequencing, determination of mRNA 5′ end

The nucleotide sequence of the murine uPA gene up to and including the Bgl II site at position −2175 in figure 3 has previously been published(29). Since our initial experiments indicated that sequences upstream of this site were responsible for the observed induction by TPA, we sequenced additional upstream DNA. Our new sequence data has been deposited with the EMBL data library (accession number X52971), and can be obtained from us on request. We found very few differences from the published uPA sequence(29) in the region −2175 to +466, and assume that the differences (which include a few restriction site polymorphisms) are due to allelic variations.

By nuclease S1 mapping and primer extension analysis of polyA-RNA purified from BALB/MK cells, we found the same mRNA cap site of the endogenous uPA mRNA as published by Degen et al.(29). This was true whether or not the cells had been stimulated with TPA. The same mRNA 5′ end was also found upon transfection of a reporter plasmid tested in this way, containing 4.2kb of uPA upstream DNA fused to the *lacZ* gene (data not shown).

Localization of the responsive sequences

To map the regulatory sequences, we constructed a series of unidirectional deletions, all with the same 3′ endpoint of uPA DNA (position +395), but varying in uPA 5′ endpoint, as indicated in figure 2A. These uPA sequences were placed 5′ to the *cat* reporter gene.

The time-course of *cat* gene induction by TPA from a transfected full-length uPA-cat construct showed an almost linear increase in CAT enzyme activity from 4 to 8 hours after TPA

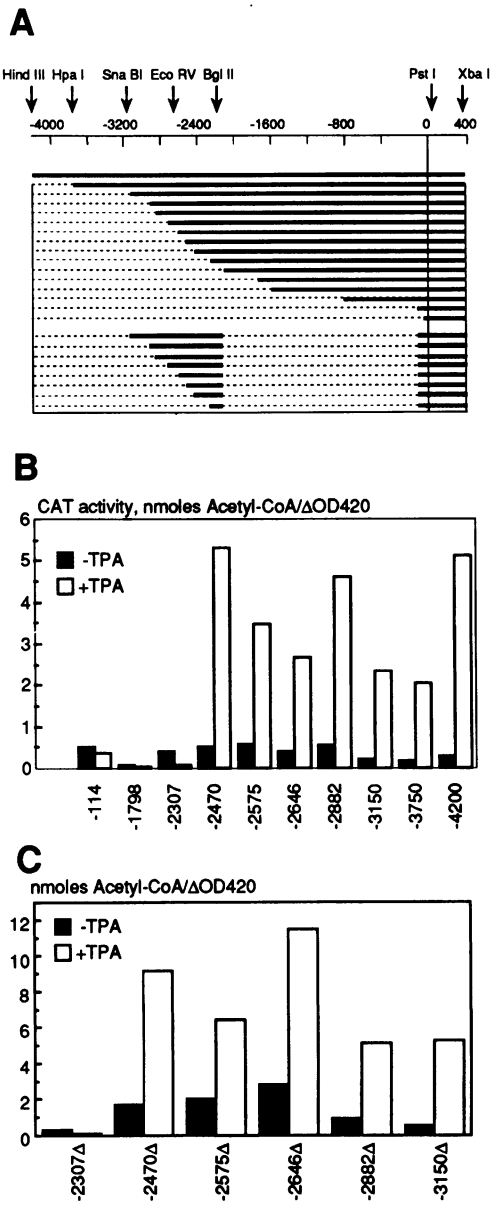

Figure 2. Mapping the TPA responsive region with unidirectional deletions. Panel A: Deletions in the uPA 5′ region that have been used in uPA-cat constructs for short-term transfection experiments. The XbaI site at position +395 was used as 3′ end of all uPA promoter fragments and positioned immediately 5′ of the Hind III site of pSV2cat. The reporter constructions have the SV40 early polyadenylation site, and pUC18 origin and *bla* gene. For constructions −4200, −3150, −2646, −2175 and corresponding Δ-constructions, the shown restriction enzyme sites were used to generate 5′ ends of the cloned uPA sequence. In all other constructions the 5′ end was generated using Exo3 and S1 nuclease. In the Δ-constructions a deletion was made from Bgl II (−2175) to BamHI in a pUC polylinker positioned immediately 5′ of the uPA promoter (−114 to +395). **Panel B:** 10μg of uPA-cat test-plasmid (see panel A) +2μg internal control plasmid (pCH110) transfected into BALB/MK. Enzyme assays performed as described in materials and methods 8 hours after addition of TPA or of solvent alone. **Panel C:** As in **B**, using Δ-constructions of uPA-cat. All constructs were tested at least twice with two different plasmid preparations, −2307 and −2470 constructs 4−5 times.

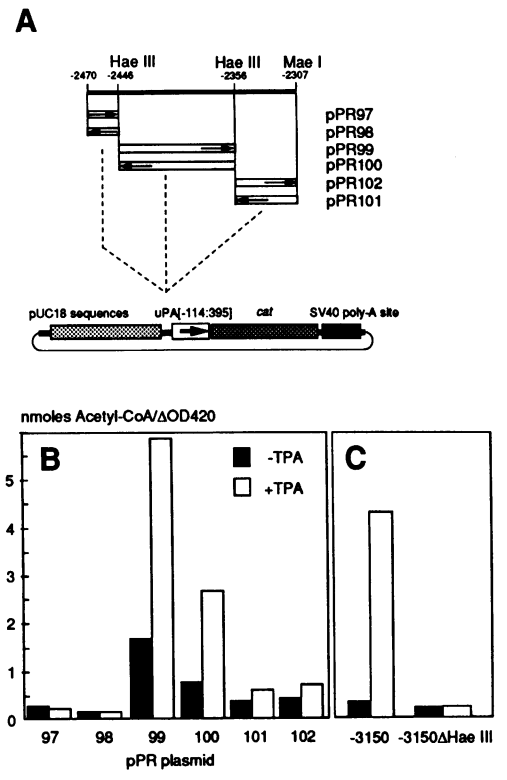

Figure 3. A 90 bp. fragment is sufficient to confer TPA inducibility. **Panel A:** Subcloning of the region −2470 to-2307 upstream of the uPA promoter. The vector is the −114 uPA:cat construct with pUC polylinker inserted at the −114 position, and the fragments were cloned into the linker's Sma I site. **Panel B:** CAT activity from the constructs transiently transfected into BALB/MK cells, 8 hours after addition of TPA or solvent alone. Panel C: CAT activity from the −3150 construction and a corresponding construct −3150ΔHae with the 90 base pair Hae III fragment deleted (−2446 to −2356). Experiment performed as in panel B.

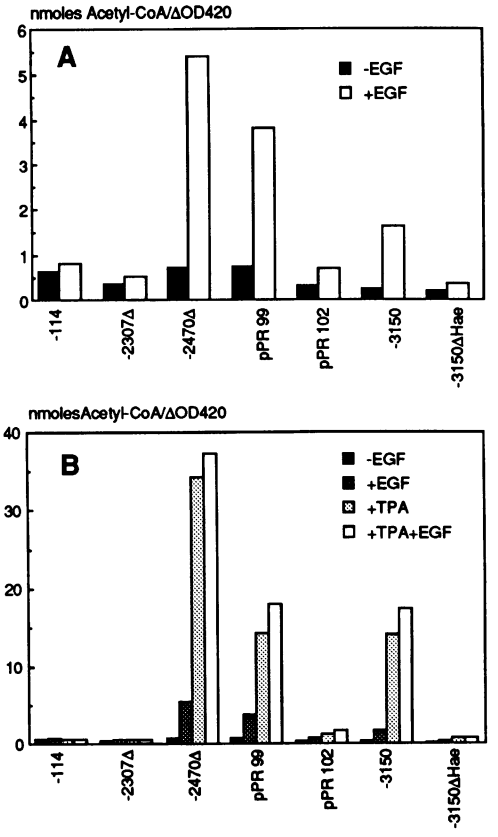

Figure 4. Induction by EGF in transient transfection of BALB/MK cells. **Panel A:** Each 100mm dish (4×10^6 cells) recieved 20μg of the indicated test plasmid and 4μg pCH110 as transfection control. 6 hours after transfection, cells were replated without EGF in four 50mm dishes. After 48 hours of EGF starvation, the cells recieved fresh medium with or without EGF for 8 hours before harvest for enzyme assays. **Panel B:** Experiment as in panel A, but the cells recieved medium −EGF, −TPA; +EGF, −TPA; −EGF, +TPA or +EGF, +TPA for 8 hours before harvest.

addition, and slower increase after 8 hours (not shown). Thus, in all later experiments reporter enzyme activity was assayed 8 hours after TPA addition.

Figure 2B shows reporter gene activity from the 5′ deletion plasmids in BALB/MK cells in the presence and absence of TPA. An additional series of transfection experiments (figure 2C) was performed with the Δ-series of plasmids, constructed by deletion of base pairs −2174 through −115 in several of the unidirectional deletions (see figure 2A). Transfections with these plasmids showed that the region −2307 to −2470 was absolutely necessary for TPA induction. Deletion of sequences between nucleotides −2175 and −114 did not influence TPA-inducibility, but resulted in higher overall activity. All values have been normalized to the activity of the co-transfected, SV40-*lacZ* reporter plasmid. In these and subsequent mapping experiments we found no significant difference in the activity of the SV40 early promoter whether TPA was present or not (data not shown, note that these experiments are done with cells growing in full serum). Similar transfection experiments with uPA-*lacZ* plasmids mapped the same region as necessary for TPA induction in 308 cells (data not shown).

To further analyze the sequences −2470 through −2307, this region was divided in three DNA fragments, that were cloned in both orientations directly upstream of a minimal uPA promoter (−114, containing mRNA cap site, TATA-box and GC-boxes), fused to the *cat* gene (figure 3A). These plasmids, pPR97-pPR102, were transfected into BALB/MK cells and assayed for TPA inducibility (figure 3B). Sequences −2470 through −2446 shows no activity. The 90 bp. fragment −2446 through −2356 is sufficient to confer higher level of basal expression as well as TPA inducibility to the minimal promoter. The fragment is functional in both orientations, with the native orientation being twice as active as the opposite. In comparison, the effect of the 49 bp. fragment −2356 through −2307 is negligible. The activity of the −2470Δ construct in figure 4 is therefore accounted for almost exclusively by the 90 bp Hae III fragment. We deleted the 90 bp. fragment in the full-length −3150 uPA-*cat* construct (giving the −3150ΔHae III construct), and transfected this plasmid into BALB/MK. As shown in figure 3C, TPA induction is practically abolished.

A subset of the uPA-*cat* constructs described above were used to define sequences responsible for the EGF induction of uPA transcription in BALB/MK cells. Transient transfection experiments showed that the same 90bp. Hae III fragment contains information for the induction by EGF (figure 4A).

EGF and TPA induction were not cooperative. Cells were transfected with the same plasmids as above, starved for EGF, and analyzed after addition of i) EGF, ii) TPA and iii) EGF+TPA. The simultaneous addition of EGF and TPA resulted only in a slightly larger stimulation of reporter gene transcription

than did addition of TPA alone (figure 4B). This might indicate that the two effectors act through the same DNA sequences.

Footprint analysis of the responsive region

The sequence of the identified regulatory region in the murine uPA gene, positions −2470 to −2307, is shown in figure 5A, aligned with the homologous region in the human uPA upstream sequences. DNase I footprint experiments were carried out to characterize interactions between this region of the murine uPA gene and nuclear proteins.

We prepared nuclear extracts from BALB/MK cells, either treated with TPA or not; in none of the experiments described below did we find any differences between extracts from stimulated versus non-stimulated cells. We subjected both strands of the uPA DNA to footprint analysis. Within the crucial 90 bp. sequence, we found clear protection of bases −2441 through −2421 (indicated by an open box in figure 6A) and a protection spanning −2398 to −2381. The latter protection was not studied further.

The −2441/−2421 protected region has significant homology to the part of the Polyoma virus enhancer which interacts with transcription factors PEA3 and AP-1(9), with the notable difference that the 3′ sequence of the site in the uPA enhancer is more comparable to an ATF/CRE site than to an AP-1 site (see figure 5B). Thus, we tentatively named this region the PEA3/URTF site (URTF for Urokinase Transcription Factor).

As shown in figure 5B, we made point mutants in the region homologous to the PEA3 binding sequence (mutant 119) and in the ATF/CRE homology (mutant 121). These mutants, as well as the double mutant 123, which contains both the 119 and the 121 mutations, were characterized by footprint-analysis (figure 6B). The PEA3 site mutation leads to a slightly altered foot-print. A single band (marked with an arrow on figure 6B), disappeared in mutants 119 and 123 when compared to wild-type 99 and mutant 121, respectively. The URTF site mutation in 121 and 123 DNAs substantially weakened the protection of several bands between bases −2441 and −2421 (indicated with a box in figure 6B).

Bandshift analysis

To demonstrate presence of PEA3 activity in crude BALB/MK nuclear extracts, we used the same PEA3 specific oligo as Wasylyk et al.(30) (figure 6C). Protein binding to the PEA3 oligo could be competed with the wild-type o-99 oligo, but not with the o-119 mutant, supporting the conclusion from the foot-print experiments that binding of PEA3 to the PEA3/URTF site is severely hampered by the 119 mutation.

Transfections with point mutations

In addition to the already described mutants 119 and 121, we constructed the 127 mutation (5′-AGGAGGAAATGAGGagA TCTTGCTC), which destroys the dyad symmetry of the URTF site. The point mutations were introduced into the pPR99 uPA-*cat* plasmid background, and transfected into BALB/MK cells. Results of TPA-stimulation analysis are shown in figure 7A. Mutation 119, in the PEA3 binding site, reduced both the basal level of expression and the inducibility of the reporter gene. Mutation 121, which alters the central bases in the URTF binding site, showed a less dramatic phenotype. The 123 double mutation (119+121), and the 127 mutation, which destroys the symmetry in the URTF site, both abolish TPA-inducibility and reduces transcription level to that of pPR126 (containing only the minimal uPA promoter).

Analysis after EGF-starvation and readdition showed the PEA3/URTF site to be crucial also for EGF stimulation of transcription (figure 7B). As observed also after TPA stimulation, mutant 119 (no PEA3 binding) has residual activity, whereas mutant 127 (abolished URTF binding) is devoid of any activity. Therefore, the URTF site is absolutely necessary and the PEA3 site is important for the activity of the PEA3/URTF site.

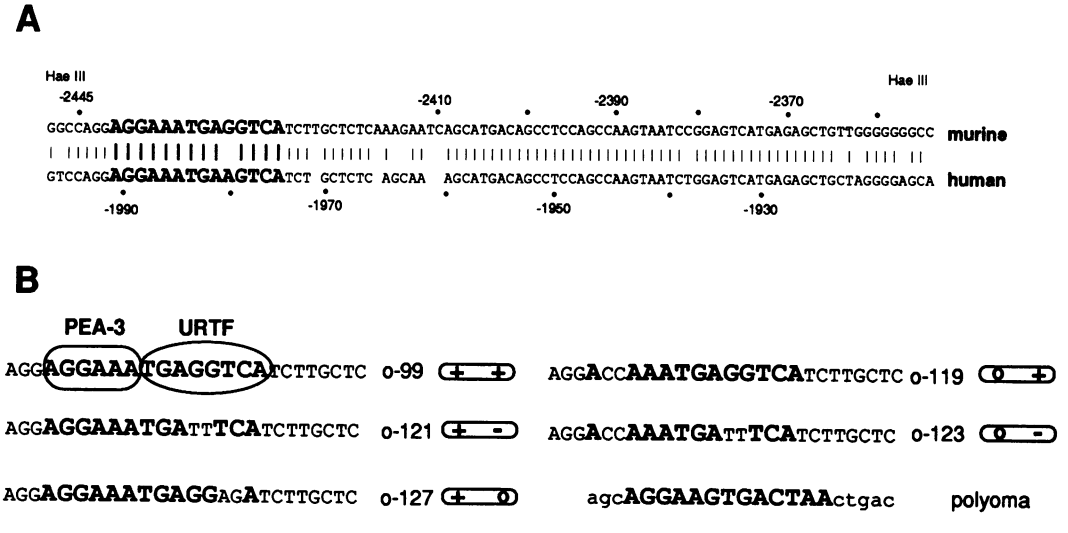

Figure 5. Panel A: Homology between the murine and the human uPA regulatory sequences. The sequence of the murine 90 bp Hae III fragment (−2446 to −2356) is shown together with the homologous human DNA (numbering of the human sequence is taken from (8). The combined PEA-3/UTF site is shown enhanced and the open bar corresponds to the protection shown in the footprints in figure 6.. **Panel B:** Oligos. The oligos used for mutagenesis of the uPA PEA3/URTF site in the pPR99 construction are listed, along with the wildtype sequence. These oligos were also used (in double-stranded form) for the band shift analysis shown in figures 7 and 8. The numbering of the mutant plasmids corresponds to the numbering of the oligos. A symbolic representation of the oligos and their protein binding sites is shown to the right of the sequence (+ indicating wildtype, − indicating a weaker binding and 0 indicating no binding). For comparison the sequence of the polyoma enhancer PEA3/AP-1 site is shown below the uPA oligos.

DISCUSSION

In this paper we have shown that EGF and the phorbol ester TPA induce uPA mRNA synthesis in exponentially growing murine keratinocytes. This was due to the presence of a sequence, largely contained in a 90 bp. Hae III fragment spanning nucleotides −2446 to −2356 relative to the mRNA 5′ end. The fragment had characteristics of an inducible enhancer, in that it was able to confer TPA induction on the uPA promoter, irrespective of its orientation and distance relative to the mRNA cap site (Figures 2 and 3).

We sequenced 5′ flanking region of the murine uPA gene and found that the TPA/EGF responsive region is in an area with extensive homologies to human uPA gene upstream sequences (figure 5A). This part of the human promoter is responsible for high uPA expression in several human cell lines, and functions

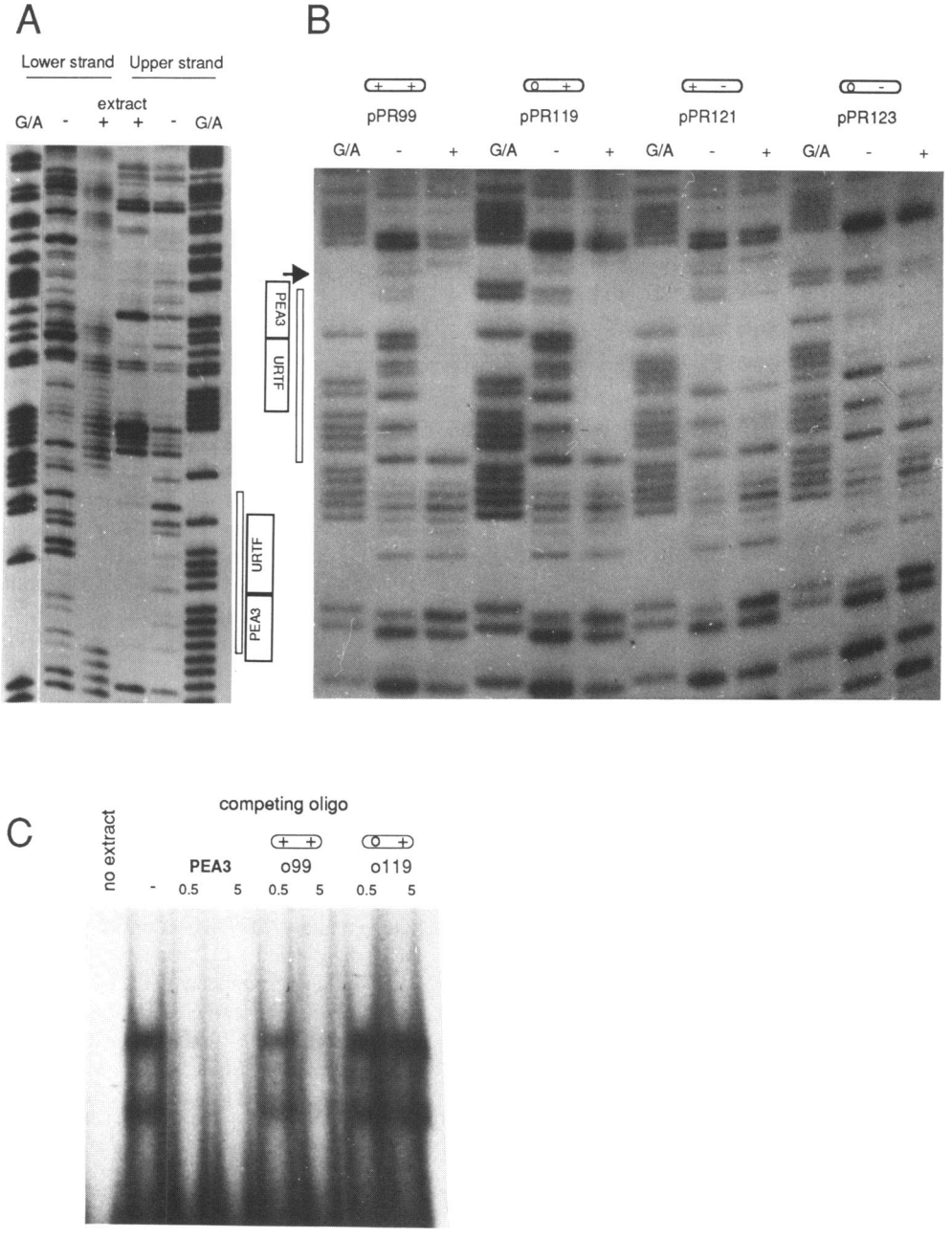

Figure 6. Footprint analysis of the 90bp sequence. **Panel A**. DNaseI protection in the −2446bp to −2356bp region of uPA 5′ sequence by BALB/MK nuclear extract. 37μg $(NH_4)_2SO_4$ precipitated extract used per lane. Both lower (α-^{32}P-dATP labeled) and upper (γ-^{32}P-ATP labeled) strands were labeled in the pUC EcoRI site,immediately adjacent to position −2470, and thereafter cut with BglII (−2175). **Panel B**. DNaseI footprints on wildtype and mutated 90bp sequences of pPR99, pPR119, pPR121 and pPR123. Plasmids were γ-^{32}P-ATP labeled at the BamHI site in the linker region 3′ to uPA position −2356 . −and + above the lanes indicates without or with BALB/MK nuclear extract (as in A). The PEA3 and URTF recognition sequences are indicated next to the autoradiograms. **Panel C**. Band shift analysis with a PEA3 binding oligo. The PEA3 oligo sequence is identical to the one used by Wasylyk et al (Wasylyk, C. et al 1989): 5′-TCGAGCAGGAAGTTCGA-3′. The wildtype (o-99) and PEA3 mutant (o-119) uPA competitor oligos are shown in figure 5. Each reaction contained: 35μg BALB/MK nuclear extract (+TPA), 5fmoles ^{32}P-labeled PEA3 oligo, 2μg poly d(I-C) and competitor as indicated (in pmoles). Extract and competitor oligo were preincubated on ice before addition of labeled oligo. Complexes were separated on a 6% polyacrylamide gel in 0.25×TBE.

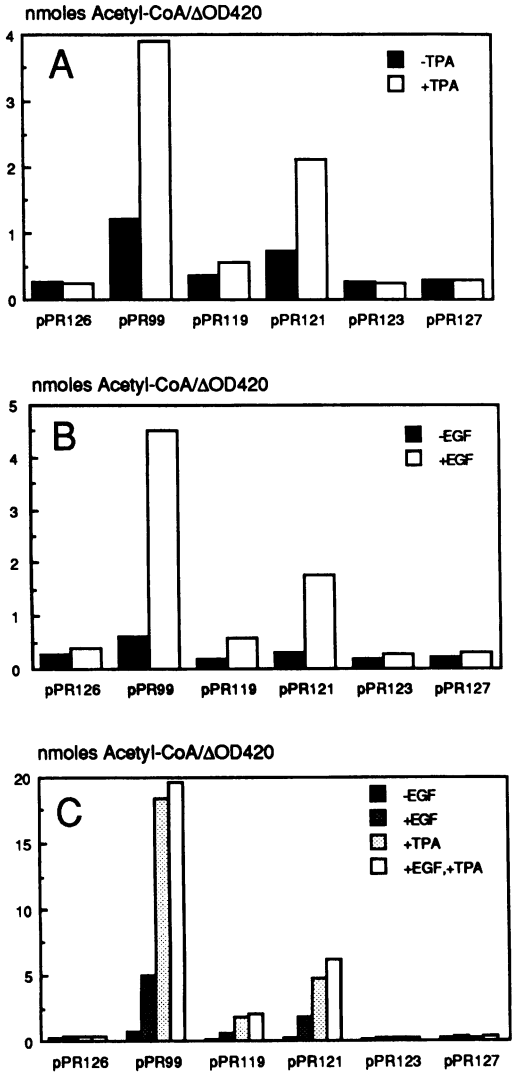

Figure 7. TPA and EGF effects on mutant uPA-*cat* vectors transfected into BALB/MK. Site-directed mutagenesis was performed on the pPR99 construction shown in figure 3, the specific mutations are shown in figure 5. pPR126 contains the uPA promoter and the *cat* reporter gene, but not the 90bp insert. **A.** TPA stimulation experiments were done essentially as described in legend to figure 2; **B** and **C.** EGF stimulation experiments as described in legend to figure 4.

as a transcriptional enhancer(8). Sequences between these identified regulatory regions and the transcription start site are much less conserved between the two species, which suggests important in vivo functions of the conserved nucleotides. Human uPA sequences −2350 through +30(8), cloned in front of the *lacZ* reporter gene were active and regulated by both TPA and EGF in BALB/MK cells (PR, unpublished).

The 90bp. murine enhancer fragment contained an essential sequence, AGGAAATGAGGTCA, which we have called a PEA3/URTF site, as we found it bound the transcription factor PEA3 and a second regulatory protein. We found that transcription factor PEA3 activity is present in cultured keratinocytes, and that its target sequence, AGGAAA, was necessary for full transcriptional regulation of the uPA gene by TPA and EGF in these cells. It has recently become clear that PEA3 activity is encoded by the c-*ets* oncogene family(31), which includes the *Spi-1* oncogene, coding for the transcription factor PU.1(32, 33).

The presently best characterized PEA3 binding site is within the Polyoma virus enhancer, where it is situated immediately 5′ to an AP-1 site(9) Analysis of the polyoma PEA3 site mutant pA425 shows an impaired TPA response in MPC11 murine melanoma cells growing in full serum(34). The wild type sequence of this binding site is AGGAAG, but the sequence of revertant B122−5134 (9), shows that the uPA enhancer site, AGGAAA, is functional in the Py enhancer. Induction of the human collagenase promoter by TPA also requires PEA3, acting in synergy with AP-1(10).

The urokinase enhancer mutation 119, which destroyed PEA3 binding, retained slight enhancing activity as well as some TPA inducibility. Mutant 127, which abolished URTF binding, resulted in total loss of activity of the 90 bp. fragment. Thus, URTF binding alone could stimulate transcription, but an isolated PEA3 site showed no enhancing ability. Mutant 121, in which URTF binding is only somewhat weakened, had significantly higher activity than both the mutant pPR127 (no URTF binding) and the double mutant pPR123 (no PEA3 binding and weakened URTF binding). We conclude that a weak URTF site is active only in the presence of PEA3, implying functional cooperation of the two proteins.

The proximity of the PEA3 and URTF binding sites suggests interactions between the two proteins, but our experiments have not directly adressed this question.

Stimulation of gene transcription after treatment with phorbol esters has been associated with transcription factor AP-1 (35, 36). This factor binds the TRE consensus sequence (TGACTCA) and consists of dimers of related proteins, encoded by several genes of the *jun-* and *fos* families. The protein complexes have been shown to have varying DNA binding affinities (reviewed by Kouzarides and Ziff(37)).

The URTF recognition sequence, TGAGGTCA, is closely related to the Activation Transcription Factor (ATF) site and cyclic AMP response element, TGACGTCA(38, 39). Three murine *jun*-gene products have been found to bind ATF/CRE sites as well as the TRE sequence(40). ATF, CREB and AP-1 proteins show immunological cross-reactivity(41), and have closely related amino acid sequences in their DNA-binding regions(42). The *c-jun* encoded protein can form a complex with a member of the CREB family, this complex binds the ATF/CRE site, but not the TRE sequence(43, 44, 45), providing a link between the ATF/CRE-site and a TPA-inducible transcription factor. We tried to characterize the URTF protein further by band-shift experiments with the o-99 oligo, including competitions with known AP-1 and CREB targets, but the results did not allow us to further identify URTF, which may be an already known activator protein (data not shown).

Nucleotides −2446 to −2356 not only conferred TPA and EGF inducibility, but raised the basal level of expression from pPR99 and pPR100 as well. This may reflect that during exponential growth, the TPA/EGF-responding target sequence is sufficiently loaded with activating proteins to stimulate a nearby promoter. This hypothesis is supported by the observation that all point mutations which diminished the induction after stimulation affected the basal level of transcription to a similar extent.

Analysis of other enhancers has shown that in some cells, an activated *ras* gene stimulates PEA3 and AP-1 activity(10, 30). We made a transformed derivative of the BALB/MK line which expressed high levels of the v-Ha-*ras* oncogene. Transfection experiments in these cells with various uPA-*cat* constructs gave

no indications of heightened enhancer activity of the 90 bp. Hae III fragment as a result of *ras* transformation. Neither did we find an altered TPA response of the reporter constructs (data not shown). These differences between our results and published work may reflect differences in the responses of URTF and AP-1 to cellular transformation. Alternatively, *ras* transformation does not alter the relevant transcription factor activity in exponentially growing keratinocytes. Finally, previously published analyses were done by co-transfecting the *ras* gene with a reporter construct, whereas our analysis was done in a cell line where the activated *ras* gene was already established. These situations are not equivalent at least in terms of *jun* expression(46).

It seems highly plausible that uPA transcription in vivo is regulated through the PEA3/URTF sequence. The significant amount of uPA mRNA (and uPA activity) seen in uninduced primary keratinocytes may reflect the general hyperproliferation induced by culture conditions, indicated by analysis of other relevant markers(47). Increased level of uPA protein is associated with cultured human keratinocytes that are actively migrating(4). EGF and TGF-α stimulates human and murine keratinocytes to migrate(48, 49) (H. Hennings, personal communication). These findings suggest that EGF and TGF-α could be the active agents for induction of uPA expression in migrating keratinocytes. We found that regulation by TPA and EGF shared responsible uPA DNA sequences. Whether we looked at EGF or TPA mediated induction of transcription, we found identical phenotypes of all mutants we tested. Moscat et al.(50) report an activation of phosphoinositide turnover and protein kinase C by EGF in BALB/MK cells. Thus, the effects of EGF and TPA on uPA mRNA level are likely both to be mediated through this signal transduction pathway.

Expression of uPA is abnormally high in invasive areas of some tumors and in many transformed cells, and uPA activity plays an important part in the invasive capacity of several tumor cell lines (reviewed in (2, 51)). Our findings that high uPA expression is controlled by proto-oncogene encoded transcription factors may thus be relevant for the metastatic potential of malignant cells.

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