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The urokinase-type plasminogen activator converts the zymogen plasminogen into plasmin, a serine protease with broad substrate specificity (1, 2). Several studies have shown that urokinase can bind to a Mr 55,000–60,000 heavily glycosylated, disulfide-linked, cell surface receptor specifically and with high affinity (Ko, −0.5 nM) (3, 4). This receptor is comprised of three similar repeats approximately 90 residues each (5), the first of which is required for the binding of urokinase and the last of which is anchored to the cell membrane via a glycosyl-phosphatidylinositol chain (6).

The urokinase-type plasminogen activator receptor (u-PAR) has been implicated in tissue remodeling in a number of physiological and pathological processes, such as re-epithelialization in skin wounds and tumor cell invasion and metastasis (7, 8). Romer et al. (9) demonstrated using in situ hybridization that mRNA encoding this receptor was present in keratinocytes at the leading edge of regenerative epithelial outgrowths of mouse skin wounds. The signal was strongest in the keratinocytes just beginning to move 12 h after wounding. Likewise, wounding of an endothelial cell monolayer triggers a rapid and sustained increase in u-PAR expression at the surface of the migrating cells, and this increase is mediated by endogenous fibroblast growth factor (10). In a number of cancers, the expression of the u-PAR is required for the invasive and metastatic phenotype (8, 11, 12). Thus, overexpression of a human u-PAR cDNA increased the ability of human osteosarcoma cells to penetrate a barrier of reconstituted basement membrane (11). Conversely, Crowley et al. (7) reported that blockade of the u-PAR by the expression of a catalytically inactive enzyme

**Requirement of an Upstream AP-1 Motif for the Constitutive and Phorbol Ester-inducible Expression of the Urokinase-type Plasminogen Activator Receptor Gene**

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1 The abbreviations used are: u-PAR, urokinase-type plasminogen activator receptor; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol; wt, wild type; mt, mutated type; PMA, phorbol 12-myristate 13-acetate; bp, base pair(s).
decreased, over 10-fold, the metastases of PC3 prostate cancer cells to the regional lymph nodes, lung, and brain tissue. Similarly, a study by Kook et al. (8) demonstrated that the expression of an antisense u-PAR cDNA in HEp3 squamous cell carcinoma cells decreased their invasiveness into a modified chorioallantoic membrane. The u-PAR promotes tissue remodeling in at least two ways. 1) It accelerates extracellular matrix degradation by increasing the rate of plasmin formation at the cell surface (13); this is associated with a 40-fold reduction in the \( K_m \) of urokinase for its zymogen plasminogen (13, 14). 2) The binding site can internalize urokinase that is covalently linked to its physiological inhibitor PAI-1, with the complex being subsequently degraded in the lysosomal compartment (15).

The seven-exon human u-PAR gene has been located to chromosome 19q13 (16, 17). Transcription of the gene gives rise to a 1.4-kilobase mRNA or, in some instances, an alternatively spliced variant that lacks the carboxyl-terminal membrane attachment peptide sequence (18). The 5' flanking sequence of the u-PAR gene contains putative binding sites for AP-1, AP-2, Sp1, and NF-kB but no potential TATA or CAAT boxes, and there is evidence that suggests the involvement of a proximal Sp1 motif in the basal transcription of the gene (19, 20).

Several investigators have demonstrated that synthesis of this receptor protein is increased by a diverse set of agents including epidermal growth factor (21), vascular endothelial growth factor (22), hepatocyte growth factor/scatter factor (23), fibroblast growth factor (10), transforming growth factor \( \beta \) (24), okadaic acid (25), and phorbol ester (26); this is ascribed largely, but not entirely (21), to increased gene transcription. Although the aforementioned studies have shown that u-PAR expression can be regulated by a diverse set of exogenous stimuli, there is relatively little information on the transcriptional regulation of this gene. In this regard, Soravia et al. (20) demonstrated the presence of multiple Sp1-binding regions in the proximal 122 bp of the u-PAR promoter and concluded that these were necessary for driving the basal transcription of this gene (20). Since that study addressed the requirements for basal transcription of the gene, we undertook a study to identify elements in the u-PAR promoter required for elevated expression of this binding site in cell lines either constitutively overexpressing this gene or transcriptionally activated in response to phorbol ester treatment.

MATERIALS AND METHODS

Vectors and Antibodies—A 449-nucleotide u-PAR promoter fragment (19) stretching from −398 to +51 (relative to the transcription start site) was cloned into the XhoI site of the pCAT-Basic vector (Promega Corp., Madison, WI). The dominant-negative Jun-D expression construct (RSV-Jun-D162) consists of the coding sequence of Jun-D, lacking the first 162 amino acid residues, inserted into a RSV long terminal repeat-driven expression vector. A Jun-D expression construct was generated by cloning of the \( \alpha \) SV promoter fragments in the EcoRI cloning site of the pCMV5 vector. The 3X AP1 pBLCAT construct consists of three AP1 tandem repeats upstream of a thymidine kinase minimal promoter-CAT reporter (pBLCAT) (28). The following antibodies used in mobility shift assays were purchased from following antibodies used in mobility shift assays were purchased from DakoCytomation: 1) SC 60X, which is specific for Fra-1; 2) SC 46X, which is specific for Fra-2; 3) SC 604X, which specifically reacts with Fra-2; 4) SC 82X, directed against c-Jun; 5) SC 46X, which specifically recognizes Jun-B; and 6) SC 241X, which reacts with ATF-1.

Transfections—RKO cells were transfected by a calcium phosphate method (29) as described previously but with minor modifications. All transient transfections were performed in the presence of 5 μg of a luciferase expression vector to correct for differences in transfection efficiencies. Briefly, DNA precipitate formed in the presence of 124 μM calcium chloride in a buffer containing 15 mM HEPES, pH 7.1, 280 mM NaCl, and 1.5 mM Na₂HPO₄, was added to 50% confluent cells. After 5 h, the cells were shocked with 25% glycerol, changed to fresh 10% fetal bovine serum-containing medium and cultured for an additional 36 h. The cells were harvested and lysed by repeated freeze-thaw cycles in a buffer containing 0.25 M Tris-HCl, pH 7.8. Transfections were determined by assaying for luciferase activity. CAT activity was measured by incubating cell lysates (normalized for transfection efficiency) at 37 °C for 6 h with 4 μM \([^{14}C]\)chloramphenicol and 1 mg/ml acetyl CoA. After 3 h, the acetyl CoA was replenished. The mixture was extracted with ethyl acetate, and acetylated products were subjected to thin layer chromatography using chloroform:methanol (95:5) as a mobile phase. The amount of acetylated \([^{14}C]\)chloramphenicol was determined by using a 603 Betascope.

Northern Blotting—The level of steady-state u-PAR transcript expressed by RKO and GEO cells was determined by Northern analysis (30). Total cellular RNA was extracted from 90% confluent cultures using 5.0 M guanidine isothiocyanate and purified on a cesium chloride cushion (5.7 M) by centrifugation at 150,000 × g for 20 h. Purified RNA was electrophoresed in a 1.5% agarose-formaldehyde gel and transferred to Nytran-modified nylon by capillary action using 10 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.4). The Northern blot was probed at 42 °C with a random primed, radiolabeled 0.65-kilobase cDNA specific for u-PAR mRNA (31). The blots were washed at 65 °C using 0.25 × SSC in the presence of 0.75% SDS. Loading efficiencies were checked by probing with a radioactive cDNA, which hybridizes with the GPDH mRNA.

Nuclear Run-on Experiments—Approximately 6 × 10⁷ cells were scraped into cold PBS and resuspended in a hypotonic buffer containing 10 mM HepES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5% Nonidet P-40. The cells were lysed with a Dounce homogenizer, and following centrifugation, the nuclear pellets were resuspended in transcription buffer (150 mM KCl, 5 mM MgCl₂, 1 mM MnCl₂, 20 mM HepES, pH 7.9, 10% glycerol, and 5 mM DTT). Nuclei were incubated at 20 °C for 30 min in the presence of 1 μM nucleotide triphosphate and 250 μM \( [^{32}P]UTP \), after which they were collected by centrifugation. Nuclei were treated with DNase I and proteinase K and subsequently with phenol/chloroform, and the RNA was precipitated. Treatment with DNase I/proteinase K followed by phenol/chloroform extraction was repeated. Newly synthesized RNA (6.5 × 10⁵ cpm/ml per reaction) was incubated for 48 h at 42 °C with Nylon-immobilized cDNAs (5 μg/mm²) corresponding to the u-PAR mRNA sequence (31), a housekeeping mRNA (GAPDH), or the linearized vector. After hybridization, the filter was washed at 65 °C with 0.1 × SSC in the presence of 1% SDS and then exposed to x-ray film.

Site-directed Mutagenesis—This was performed using the Transformizer kit from Clontech (Palo Alto, CA) following the manufacturer's procedure. A selection primer was used (5′-TTTATCATGTCCTGTTACCCCGGAAATTC)-3′, which changed a BamHI site in the vector to an Asp 718 site. After annealing of the selection and mutagenesis primers, the strand DNA was filled in with the mod DNA polymerase and T4 DNA ligase. The plasmid was cut with BamH I and transformed in repair-deficient bacteria (BMH 71–18 Rts). Miniprep DNA was subsequently cut with BamHI to enhance the mutation efficiency, and DH5 43 bacteria was transformed. Colonies were expanded, and DNA was harvested and sequenced. 5′ deletion constructs were generated as follows. The −197 and −8 uPAR CAT constructs were generated by digestion of the XhoI fragment containing 449 bp of the 5′ flanking sequence upstream of the translation start site of the u-PAR gene with Sau3AI or Ddel, respectively, and the fragments were resolved in a 5% polyacrylamide gel. The overhangs were filled in with Klenow, and the promoter fragments were ligated into the CAT reporter.
tion. The purified DNA fragments were mixed with 2 μg poly(dI-dC) and 8–50 μg of a 52-bp DNA fragment extracted and buffered with 7.5% glycerol, 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl₂, and 1.5 mM DTT in a total volume of 20 μl. Binding was allowed to proceed for 15 min at room temperature, after which DNase I (final concentration, 10 μg/ml) was added for 30 s. A stop solution containing 10 mM EDTA, 0.1% SDS, and 50 μg/ml of proteinase K was added, and the reaction mixture was incubated at 37 °C for 30 min. The mixture was extracted with phenol/chloroform, precipitated, washed with ethanol, and redissolved in water. The material was dissolved in denaturing solution containing formamide and, and electrophoresed in a DNA sequencing gel. The G and A ladder was made by the Maxam-Gilbert chemical degradation method (33).

Methylation Interference Assays—Methylation interference assays were carried out as described previously but with modifications (34). A 52-bp DNA fragment stretching from −291 to −150 relative to the transcription start site was generated by digestion of the XhoI u-PAR promoter fragment with AluI and HpaII, and the fragment was 32P-end-labeled with Klenow enzyme. The radioactive 52-bp fragment was purified by gel electrophoresis and methylated for 17.5 min in the presence of a 1:200 dilution of a 99% w/v dimethylsulfate solution. The partially methylated, radioactive probe was incubated at room temperature for 30 min with nuclear extract and subsequently subjected to preparative band shifting. The shifted bands, along with unbound probe, were extracted with 0.3 M NaCl and purified by ethanol/salt precipitation. The extracted probe was cleaved with piperidine, and the reaction products were resolved in a sequencing gel as described above. The position of the G and A nucleotides was determined by dimethylsulfate (G) and formic acid (G) products were resolved in a sequencing gel as described above. The activity of a CAT reporter driven by 398 nucleotides of the upstream sequence. Deletion of 201 bp from the 5′ end caused a modest augmentation in promoter activity (−197 uPAR construct), suggesting that this region might contain a silencer element. However, deletion of an additional 189 bp (−8 u-PAR) of 5′ flanking sequence resulted in a substantial loss of promoter activity (−11%). These data indicated that a region of the sequence residing between −197 and −8 was required for optimal activation of the u-PAR promoter in RKO cells.

As a first step toward identifying the DNA binding factor(s) regulating the u-PAR promoter in RKO cells, fragments of the 5′ flanking sequence of the u-PAR gene generated either by AluI/Ddel (Fig. 1D) or RsaI (Fig. 1E) digestion, were incubated with nuclear extract and subsequently subjected to DNase I digestion and electrophoresis. At least three protected regions were identified in these footprinting experiments. The first region (I) spanning nucleotides −190 to −171 (relative to the major transcription start site) with a second region (II) residing between −148 and −124 (Fig. 1, D and F). A third region (III) stretching from −99 to −70 was also evident (Fig. 1, D-F) and may be identical to that reported by Soravia et al. (20) as an Sp-1-binding region. Interestingly, region I contained a consensus AP-1 binding site (TGAGTCA) located at −184. To determine the importance of these regions for u-PAR expression, site-directed mutations or deletion constructs were generated and assayed for CAT activity (Fig. 2). Deletion of a large part of region II (del 144–123 construct) reduced CAT activity by approximately 70%, consistent with the idea of a regulatory role for this region. Similarly, deletion of most of region III (del 98–78 construct) also impaired u-PAR promoter activity. In contrast, deletion of a region (from −17 to −8) of the promoter, which lies outside of the footprinted regions (construct del 17–8), had only a modest effect on the u-PAR promoter activation. Since the expression of several inducible genes is regulated through AP-1 binding sites (39, 40), we determined the effect of mutating the AP-1 motif in region I of the footprint. Mutation of this consensus sequence, which is located 184 bp upstream of the transcription start site (AP-1 184 nt), reduced the activation of the u-PAR promoter by over 80% (Fig. 2). On the other hand, the mutation of a nuclear factor-xB-like site (NF-xB-like 43 nt), which lay outside of the footprinted regions, did not impair the activity of the u-PAR promoter in RKO cells.

RESULTS

Requirement of an AP-1-containing Region of the u-PAR Promoter for Transactivation of the Gene in RKO Cells—The RKO colon cancer cell line is a highly tumorigenic cell line with a high clonogenicity in soft agarose (35). These cells demonstrate high plating efficiencies and have elevated cell saturation densities (35). In contrast, the GEO colon cancer cell line demonstrates enterocyte differentiation in low glucose and forms polarized cysts with tight apical junctions toward the lumen of the cysts (36). RKO cells incubated with urokinase are highly invasive in an in vitro invasion assay, whereas GEO cells are weakly active in this regard (37). We reported previously that RKO cells were characterized by their elevated display of u-PAR (3 × 10⁵ receptors/cell), whereas the GEO cells were equipped with approximately 10⁴ binding sites/cell (38). This difference was largely due to differences in the steady-state mRNA levels encoding the urokinase receptor (Fig. 1A). Nuclear run on experiments indicated that the larger amount of u-PAR mRNA in the RKO cells could be ascribed to a higher rate of transcription at this gene (Fig. 1B).

To drive a CAT reporter construct by 398 nucleotides of the sequence upstream of the major transcriptional start site (19, 20) was nearly as high (Fig. 1C) as that achieved with a RSV-driven reporter, indicating activation of the u-PAR pro-
and GEO cells, which are high and low-u-PAR-displaying cell lines, respectively. The wt end-labeled oligonucleotide was incubated with nuclear extract from RKO or GEO cells (Fig. 3B). AP-1-binding proteins were detected (Fig. 3B, arrow) in both RKO and GEO nuclear extracts by gel-shifts using the wt AP-1-containing oligonucleotide. However, the intensity of the retarded band was greater for RKO than for GEO cells. The Jun-D- and c-Jun-specific antibodies produced strong supershifts, suggesting that these proteins are major components of the DNA-binding complex. On an equal protein basis, RKO cells had higher levels of Jun-D and c-Jun than did GEO cells, which only weakly expressed the u-PAR gene. In contrast, antibodies to either Jun-B or ATF-1 did not result in a discernible supershift.

To identify Fos family members interacting with the u-PAR promoter fragment, similar experiments were carried out using
antibodies that specifically recognize Fos and Fos-related antigen (Fra-1 and Fra-2) proteins (Fig. 3C). A Fra-1-specific antibody supershifted the oligonucleotide complex formed with RKO nuclear extracted proteins. Although GEO nuclear extract also gave rise to a supershifted band with the anti-Fra-1 antibody, the intensity of the band was lower than that achieved with RKO nuclear extract. The amount of supershifted complex was not further increased by the addition of a greater amount of antibody. A c-Fos-specific antibody was found capable of inducing a supershift using nuclear extracts from GEO cells, but no c-Fos was detected in DNA-binding complexes with RKO extracts.

FIG. 2. Effect of site-specific mutations and deletions of the u-PAR promoter on its function. RKO cells were transfected and assayed for CAT activity as described in the legend to Fig. 1. The CAT reporter constructs used were as follows: wt-u-PAR, includes 449 nucleotides sequence upstream of the translation start site; AP-1 184 mt, the AP-1 motif at −184 was mutated to TactTCA; nuclear factor-xB-like 43 mt (NF-xB-like 43mt); this motif was mutated to GGAGTTTTGGG; del 17–8 lacks the nucleotide sequence spanning −17 to −8; del 144–123 lacks the nucleotide sequence spanning 144–123; del 98–78 lacks the sequence including nucleotides −98 to −78. All modified promoter sequences were in context of 449 nucleotides of 5’ flanking sequence. All nucleotide positions are indicated relative to the transcription start site. The data are representative of four different experiments, the range of which did not exceed 20%.

An Expression Vector Encoding a wt Jun-D cDNA Induces u-PAR Promoter Activity in GEO Cells—Since mobility shift assays indicated Jun-D to be the predominant Jun family member bound to the AP-1 motif at −184, we determined the function of this transcription factor in regulating u-PAR expression. We first asked whether the expression of an exogenous cDNA encoding Jun-D was inductive for u-PAR promoter activity. Toward this end, the receptor-deficient GEO cells were transfected with an expression vector encoding the wt Jun-D and a CAT reporter driven by either the wt (wt-u-PAR CAT) or the AP-1 (−184) mutated (AP-1 184 mt) u-PAR promoter (Fig. 4). Expression of the wt Jun-D caused a dose-dependent increase in u-PAR promoter activity with 15 μg of plasmid, resulting in a 8-fold stimulation over the empty vector. On the other hand, the activation of the u-PAR promoter was greatly reduced (3-fold stimulation) when the promoter was mutated at the AP-1 motif at −184.

An Expression Vector Encoding a Dominant-Negative Jun-D Represses u-PAR Promoter Activity in RKO Cells—We then determined whether the expression of a dominant-negative Jun-D could repress the u-PAR promoter in RKO cells that overexpress this binding site. RKO cells were transiently transfected with the u-PAR promoter-driven CAT reporter and an expression vector encoding a mutant Jun-D protein, which lacks amino acids 1–162 corresponding to the transactivation domain (RSV Δjun D). Transfection of the expression vector encoding the mutant Jun-D into RKO cells reduced the activity of the u-PAR promoter-driven CAT reporter in a dose-dependent manner (Fig. 5). The highest input amount of the RSV Δjun D repressed the promoter by 80% relative to the empty expression vector (RSV vector). All together, these results suggest that the expression of the u-PAR requires trans-
Stimulation of u-PAR Expression by Phorbol Ester Requires the AP-1 Binding Site at −184—We then asked whether the presence of these transcription factors. However, GEO cells transfected with the AP-1-mutated (AP-1 184 mt) u-PAR promoter. After 5 h, the cells were shocked with 25% glycerol and cultured for an additional 18 h. The cells were then treated with (+) or without (−) 100 nM PMA for 24 h and extracted. Promoter activation is shown as fold induction of [14C]chloramphenicol acetylation, where 1-fold indicates no change. mock, no DNA added. The data are typical of two experiments, with the fold induction, between experiments, not exceeding 20% of that shown.

In contrast, mutation of the AP-1 site located at −184 abolished this PMA-dependent induction.

The binding of nuclear factors from PMA-treated and -untreated GEO cells to the AP-1 motif at −184 was characterized by both electrophoretic mobility shift assay and methylation interference assays. Nuclear extracts were prepared and incubated with an end-labeled 30-bp oligonucleotide spanning the AP-1 site at −184 (see Fig. 3 for sequence). A retarded complex (Fig. 7A, arrow) was apparent with nuclear extract from both untreated and PMA-treated cells. Complex formation could be substantially reduced with an excess of unlabeled oligonucleotide. Antibodies specific for c-Jun and Jun-D supershifted the complex formed between the oligonucleotide and nuclear factors from both untreated and PMA-treated GEO cells, indicating the presence of these transcription factors. However, GEO cells treated with 100 nM PMA contained a greater amount of c-Jun and Jun-D (Fig. 7A), as judged by the intensity of the supershifted bands. Both of these proteins are also present in the nuclear extract from the receptor-overexpressing RKO cells. Interestingly, we saw little Fra-1 in PMA-exposed GEO cells, an observation divergent with the finding of this transcription factor in the supershifted complex formed between the u-PAR promoter oligonucleotide and nuclear factors extracted from RKO cells (Fig. 3E). Instead, nuclear extract from PMA-stimulated GEO cells contained a large amount of c-Fos, as judged from the strong supershift achieved with the anti-Fos antibody.

We then characterized the interaction of the AP-1-binding proteins by methylation interference assays. Nuclear extracts from GEO cells treated with or without PMA and RKO cells were incubated with a u-PAR fragment corresponding to nucleotides −201 to −150 (relative to the major transcription start site) of the promoter and spanning the AP-1 binding site at −184. Complex formation was assessed by gel electrophoresis. Expectedly, the promoter fragment bound nuclear factor(s)

**Fig. 4.** An expression vector encoding a wt Jun-D induces u-PAR promoter activity in GEO cells. Transfections and CAT assays were performed as described in the legend to Fig. 1. GEO cells were co-transfected with a CAT reporter driven by the wt (wt u-PAR CAT) or AP-1-mutated (AP-1 184 mt) u-PAR promoter and varying amounts of wt jun-D cDNA (junD) or the empty vector (CMV Vector). Transfections were in pairs (bracketed), with each amount of junD having a corresponding equimolar amount of the empty vector. [14C]Chloramphenicol conversions were determined with a 603 Betascope and are shown as mean values of duplicate experiments. The variation between experiments did not exceed 15% of the value shown.

**Fig. 5.** An expression vector encoding a mutated Jun-D represses u-PAR promoter activity in RKO cells. Transfections and CAT assays were performed as described in the legend to Fig. 1. RKO cells were co-transfected with a CAT reporter driven by the wt u-PAR promoter (wt u-PAR CAT) and the indicated amount of an expression vector encoding a transactivation domain-lacking jun-D (RSV-junDΔ162) or the empty expression vector (RSV Vector). [14C]Chloramphenicol conversions were determined with a 603 Betascope and are shown as mean values of duplicate experiments the range not exceeding 15% of the values shown.

**Fig. 6.** PMA induces the wt but not the AP-1-mutated u-PAR promoter in GEO cells. In A, GEO cells were at 80% confluency treated with 100 nM PMA for a 24-h period. Total RNA was extracted, and 20 μg were electrophoresed in a 1.5% agarose-formaldehyde gel. The separated RNA was transferred to a Nytran-modified nylon filter, and the filter was probed with cDNAs corresponding to the u-PAR or GAPDH mRNAs. Stringency washes were performed at 65°C using 0.5 × SSC. The positions of the 28 S and 18 S ribosomal RNA are shown. In B, GEO cells were transfected as described in the legend to Fig. 1 with a CAT reporter (pSV0 CAT) driven by the wt (wt u-PAR CAT) or the AP-1-mutated (AP-1 184 mt) u-PAR promoter. After 5 h, the cells were treated with (+) or without (−) 100 nM PMA for 24 h and extracted. Promoter activation is shown as fold induction of [14C]chloramphenicol acetylation, where 1-fold indicates no change. mock, no DNA added. The data are typical of two experiments, with the fold induction, between experiments, not exceeding 20% of that shown.

acting factor(s) that bind to the AP-1 site located at −184.
from RKO cells (Fig. 7B, arrow), and this was specific for the AP-1 site since it could be competed with an excess of unlabeled wt oligonucleotide but not the oligonucleotide that had been mutated in the AP-1 motif. In contrast, nuclear extract from GEO cells gave rise to two complexes; one had the same mobility as that observed with RKO nuclear extract (Fig. 7B, bottom band), whereas the other (Fig. 7B, top band) displayed a slower mobility. The slower moving band evident with GEO cells could very well represent transcription factor(s), which are absent from RKO cells (e.g., c-Fos) or altered in GEO cells, bound to the DNA fragment. The amount and/or activity of the binding factor(s) in untreated GEO cells was reduced, as judged from the combined intensities of the top and bottom bands when compared with RKO cells. Stimulation of the GEO cells with phorbol ester, which augments u-PAR expression, dramatically increased the intensity of the two bands, indicating an increase in the amount and/or binding activity of both these AP-1-binding complexes. The u-PAR promoter fragment was then methylated, reacted with nuclear extracts, and subjected to preparative gel electrophoresis. The retarded bands were eluted with 0.3 M NaCl, chemically cleaved with piperidine and then resolved in a sequencing gel (Fig. 7C). Methylation of the two G nucleotides at −183 and −181 (Fig. 7C, arrow) within the AP-1 consensus sequence (TGAGTCA) of the DNA frag-
ment strongly interfered with binding of nuclear factors from GEO (PMA treated or untreated) and RKO cells. In contrast, methylation of three consecutive G nucleotides located immediately downstream of the AP-1 motif (indicated by an *) did not affect binding of proteins extracted from GEO (PMA treated or untreated) or RKO cells. These data indicate that the contact points are identical for nuclear factors from RKO and PMA-treated and -untreated GEO cells and localized to the two G nucleotides within the AP-1 consensus site.

DISCUSSION

The u-PAR accelerates plasmin formation at the cell surface and has been implicated in tissue remodeling in a number of physiological and pathological processes. However, the mechanism(s) by which the expression of this gene is regulated is poorly understood. We report herein that a previously uncharacterized AP-1 motif located 184 nucleotides upstream of the major transcriptional start site is required both for the elevated expression of the gene in a cell line (RKO), which overexpresses the u-PAR (3 × 10^5/cell), as well as the PMA-inducible expression in a receptor-deficient cell line (GEO).

The AP-1 consensus sequence at −184 was bound with multiple transcription factors including Jun-D (GEO and RKO), c-Jun (GEO and RKO), Fra-1 (RKO), and c-Fos (GEO). Although caution should be exercised in drawing any conclusions as to the identity of the transcription factors regulating u-PAR expression through this motif, several of our observations are consistent with a role for Jun-D and/or c-Jun. (a) Mobility shift assays indicated that the amount of these transcription factors bound to the oligonucleotide spanning the AP-1 motif at −184 of the promoter was greater for RKO cells when compared with untreated GEO cells, which display 10-fold fewer receptors. (b) Stimulation of GEO cells with phorbol ester, which is inductive for u-PAR expression, caused a marked increase in the amount of Jun-D and c-Jun bound to the AP-1 motif (−184) in the u-PAR promoter. (c) An expression vector encoding a Jun-D cDNA was a potent stimulus for u-PAR promoter activity in the receptor-deficient GEO cells.

Since we could readily detect Jun-D bound to the AP-1-containing oligonucleotide, it is conceivable that the regulation of u-PAR expression in RKO cells is mediated in part through a homodimer of Jun-D binding to this motif. Indeed, Welter et al. (43) reported that a distal and a proximal AP-1 site, which bound Jun-D, was essential for the high transcriptional activity of the human involucrin promoter achieved by phorbol ester stimulation. Similarly, Yoon and Lau (44) found that in PC12 cells, the orphan receptor gene nur77 was transcriptionally activated by Jun-D through an AP-1-binding element that was bound only with this transcription factor. Alternatively, it is equally possible that u-PAR expression in these cells is a consequence of heterodimerization of Jun-D with Fra-1 since this transcription factor was also bound to the AP-1 motif of the u-PAR promoter. Indeed, previous studies by other investigators would indicate that a Jun-D/Fra-1 heterodimer is a more efficient activator of AP-1-dependent gene expression than Jun-D alone. For example, the half-life of the AP-1-bound Jun-D/Fra-1 heterodimer is substantially greater than that for the Jun-D homodimer (45, 46). Additionally, Suzuki et al. (47) observed that the coexpression of Jun-D and Fra-1 led to a much more efficient activation of an AP-1-driven minimal promoter compared with the expression of Jun-D by itself.

Another possibility is that u-PAR expression in RKO cells is mediated, in part, through a c-Jun homo- or heterodimer binding to the AP-1 motif at −184. Indeed, c-Jun alone is an efficient activator of AP-1-regulated gene expression in a variety of cell types, including F9 teratocarcinoma cells (47). On the other hand, it is unlikely that u-PAR expression in RKO cells is regulated by a c-Jun/Fra-1 heterodimer since studies by Suzuki et al. (47) indicated this complex to be an extremely poor activator of AP-1-regulated gene expression.

We detected strong supershifts with antibodies to c-Jun, Jun-D, and c-Fos in nuclear extracts from PMA-stimulated GEO cells, indicating the presence of these transcription factors. Therefore, in these cells it is possible that u-PAR expression is activated by c-Jun or Jun-D homodimers or heterodimers of these proteins with c-Fos (45, 47, 48). In fact, the co-expression of c-Fos and Jun-D strongly transactivates other AP-1-responsive genes, including collagenase I and tissue inhibitor of metalloproteinase-1 (47, 49). Likewise, the ability of c-Fos and c-Jun co-expression to efficiently activate AP-1-dependent gene expression is well established (50) and exemplified by the stimulation of a CAT reporter driven by five copies of the human papillomavirus type 18 AP-1 binding sequence (48). On the other hand, since, unlike RKO cells, we could only detect a small amount of Fra-1 in nuclear extract from PMA-treated GEO cells it is less likely that the induction of u-PAR promoter activity in these cells following PMA stimulation is achieved via a Jun-D/Fra-1 heterodimer.

The absence of a PE3A3-binding site juxtaposed with the AP-1 motif at −184 was of interest since the inducible expression of several, but not all (44, 51), AP-1-regulated genes, including collagenase (40) and the glutathione transferase P gene (52), is mediated through such juxtaposed motifs. It could be that either the AP-1 motif acts independently of PE3A3-binding (44, 51) or that it interacts with transcription factors bound further upstream or downstream to regulate u-PAR expression.

DNase I footprinting revealed two other protected regions (referred to as II and III) in addition to the AP-1-containing region (region I). Deletion of a large part of either protected region II or III reduced promoter activity in RKO cells by over 50%. This finding may be explained if transcription factor(s) binding to these regions are required for optimal u-PAR promoter activity. Computer analysis of the protected region II revealed the presence of two Ets-binding (GGA) (53) motifs at −136 and −131. However, we found that mutation of either of these motifs had little effect on promoter activity in RKO cells (data not shown). Thus, if region II is required for u-PAR promoter activation in RKO cells, it is unlikely that this is by way of Ets factors binding to this region of the regulatory sequence. On the other hand, a previous report by Soravia et al. (20) suggested that the footprinted region I may indeed have a functional role regarding the regulation of u-PAR expression. In that study, the authors had found Sp1-class proteins bound to this region of the promoter. Furthermore, an Sp1 expression vector activated the u-PAR promoter in Drosophila Schneider cells (20), a finding consistent with the idea that u-PAR expression is regulated by this transcription factor(s). Indeed, Soravia et al. (20) concluded that the Sp1 motif (located in our footprinted region III of the promoter) was required for the basal expression of the u-PAR gene similar to that reported for other genes, such as glutathione S-transferase (54). If this is the case, then it is conceivable that elevated u-PAR expression is a function of two different classes of transcription factors: one responsible for basal expression of the gene, and the other regulating its overexpression. Transcription factors binding to the AP-1 motif at −184 would fall into the second category, as evidenced by the observation that the mutation of this motif abrogates the induction of the u-PAR promoter by phorbol ester as well as repressing the strong constitutive activation of the promoter in a cell line (RKO) characterized by its elevated display of the binding site.

In conclusion, using cell lines which either constitutively overexpress u-PAR or are stimulated to do so with phorbol
ester treatment, we have demonstrated that the activation of this promoter requires a previously undescribed AP-1 motif located 184 nucleotides upstream of the transcription start site. The AP-1 binding site is bound with multiple transcription factors, including Jun-D and c-Jun. Since there is now ample evidence indicating that both activated protein tyrosine kinase transmembrane receptors and PMA use a common signaling cascade (involving the sequential activation of c-Raf-1, mitogen-activated protein kinase kinase, and the extracellular signal-regulated kinase 55–60), it is tempting to speculate that the ability of epidermal growth factor, fibroblast growth factor, and hepatocyte growth factor/scatter factor to elevate u-PAR synthesis (21, 61, 62) also requires the AP-1 motif at -184. If this is the case, these findings could lead to the development of novel antiinvasive/antimetastatic agents that suppress the autocrine and/or paracrine-driven synthesis of the u-PAR in invasive cancers (8, 11, 12).

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REFERENCES
1. Nielsen, L., Hansen, J., Skriver, L., Wilson, E., Keltoff, K., Zenthen, J., and Dano, K. (1982) Biochemistry 21, 6410–6415
2. Robbins, K. C., Summaria, L., Hsieh, B., and Shah, R. J. (1967) J. Biol. Chem. 242, 2353–2362
3. Stopelli, M. P., Tacchetti, C., Cubelli, M., Corti, A., Hearing, V., Cassani, G., Appella, E., and Blasi, F. (1986) Cell 45, 675–684
4. Vassalli, J., Baccino, D., and Belin, D. (1985) J. Cell Biol. 100, 86–92
5. Behrendt, N., Ploug, M., Pathy, L., Houen, G., Blasi, F., and Dano, K. (1991) J. Biol. Chem. 266, 7842–7847
6. Ploug, M., Ronne, E., Behrendt, N., Jensen, A., Blasi, F., and Dano, K. (1991) J. Biol. Chem. 266, 1926–1933
7. Crowley, C. W., Cohen, R., Lucas, B. K., Liu, G., Shuman, M. A., and Levinson, J. (1994) J. Biol. Chem. 269, 1151–1156
8. Romer, J., Lund, L. R., Erikson, J., Pyke, C., Kristensen, P., and Dano, K. (1994) J. Invest. Dermatol. 103, 519–522
9. Pepper, M. S., Matsumoto, K., Nakamura, T., Orci, L., and Montesano, R. (1985) Cancer Res. 45, 701–709
10. Pepper, M. S., Sappino, A. P., Stocklin, R., Montesano, R., Dano, K., and Brattain, M. G. (1988) Cancer Res. 48, 4534–4542
11. Higazi, A. A.-R., Cohen, R. L., Henkin, J., Kniss, D., Schwartz, B. S., and Cines, B. D. (1990) J. Biol. Chem. 265, 6494–6498
12. Schlezewski, S., Puig, M., Wollheim, C. A., and Orci, L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4939–4943
13. Pyke, C., Erikson, J., Solberg, H., Schnack, B., Nielsen, S., Kristensen, P., Lund, L. R., and Dano, K. (1995) FEBS Lett. 366, 69–74
14. Wang, Y., Dang, J., Johnson, L. K., Selhamer, J. J., and Doe, W. F. (1995) Eur. J. Biochem. 230, 71–82
15. Soravia, E., Grebe, A., De Luca, P., Helin, K., Suh, T. T., Degen, J. L., and Blasi, F. (1995) Blood 86, 624–635
16. Lund, L. R., Ellis, V., Ronne, E., Pyke, C., and Dano, K. (1995) Biochem. J. 310, 345–352
17. Mandriota, S. J., Seghezzi, G., Vassalli, J.-D., Ferrara, N., Wasi, S., Mazzieri, R., Mignatti, P., and Pepper, M. S. (1995) J. Biol. Chem. 270, 9709–9716
18. Pepper, M. S., Matsuomoto, K., Nakamura, T., Orei, L., and Montesano, R. (1992) J. Biol. Chem. 267, 20493–20496
19. Lund, L. R., Romer, J., Ellis, V., Blasi, F., and Dano, K. (1992) EMBO J. 10, 3389–3403
20. Lund, L. R., and Dano, K. (1992) FEBS Lett. 298, 177–181
21. Kato, T., Murakami, J., and Yamada, K. M. (1987) Cancer Res. 47, 467–474
22. Kato, T., Hattori, K., Sasaki, G., Blasi, F., and Asooian, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4929–4933
23. Pfarr, C. M., Mecha, F., Spyrou, G., Lallemand, D., Carrillo, S., and Yaniv, M. (1994) Cell 76, 747–760
24. Angel, P., Hattori, K., Sasaki, G., Blasi, F., and Asooian, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4929–4933
25. Angel, P., Hattori, K., Sasaki, G., Blasi, F., and Asooian, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4929–4933
26. Stoppelli, M. P., Tacchetti, C., Cubelli, M., Corti, A., Hearing, V., Cassani, G., Appella, E., and Blasi, F. (1986) Cell 45, 675–684
27. Kato, T., Hattori, K., Sasaki, G., Blasi, F., and Asooian, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4929–4933
28. Angel, P., Hattori, K., Sasaki, G., Blasi, F., and Asooian, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4929–4933
29. Stoppelli, M. P., Tacchetti, C., Cubelli, M., Corti, A., Hearing, V., Cassani, G., Appella, E., and Blasi, F. (1986) Cell 45, 675–684
