The urokinase receptor overexpressed in invasive cancers promotes laminin degradation. The current study was undertaken to identify cis elements and trans-acting factors activating urokinase receptor expression through a footprinted (−148/−124) region of the promoter containing putative activator protein-2- and Sp1-binding motifs. Mobility shifting experiments using nuclear extract from a high urokinase receptor-expressing cell line (RKO) indicated that Sp1, Sp3, and a factor similar to, but distinct from, activator protein-2α bound to this region. Mutations preventing the binding of the activator protein 2α-related factor diminished urokinase receptor promoter activity. In RKO cells, the expression of a negative regulator of activator protein-2 function diminished urokinase receptor promoter activity, protein, and laminin degradation. Conversely, urokinase receptor promoter activity in low urokinase receptor-expressing GEO cells was increased by activator protein-2α expression. Although using GEO nuclear extract, little activator protein-2α-related factor bound to the footprinted region, phorbol 12-myristate 13-acetate treatment, which induces urokinase receptor expression, increased complex formation. Mutations preventing the activator protein-2α-related factor and Sp1/Sp3 binding reduced urokinase receptor promoter stimulation by this agent. Thus, the constitutive and phorbol 12-myristate 13-acetate-inducible expression of the urokinase receptor is mediated partly through trans-activation of the promoter via a sequence (−152/−135) bound with an activator protein-2α-related factor.

The urokinase-type plasminogen activator (urokinase) is a serine protease that converts the inert zymogen plasminogen into plasmin, a protease with broad substrate specificity leading to extracellular matrix degradation and tumor invasion (1–3). Urokinase can bind specifically and with high affinity to the urokinase receptor (u-PAR) 1 (4, 5) composed of three similar repeats (6, 7). The amino-terminal domain binds the plasminogen activator with the carboxyl terminus domain serving to anchor the binding protein to the cell surface via a glycosyl-phosphatidylinositol chain (6, 7).

The u-PAR has multiple functions. First, urokinase bound to the u-PAR activates plasminogen at a much faster rate than fluid phase plasminogen activator (8, 9), and this contributes to type IV collagenase activation (10). Second, the binding site clears urokinase-inhibitor complexes from the extracellular space (11) via a α2-macroglobulin receptor-dependent mechanism (12). Third, the u-PAR interacts with the extracellular domain of integrins to connect to the cytoskeleton, thereby mediating cell adhesion and migration (13–15). Fourth, the u-PAR is chemotactic for human monocytes and mast cells, and this may require the cleavage of the binding site between domains 1 and 2 (16, 17).

The u-PAR gene is 7 exons long and is located on chromosome 19q13 (18, 19). Transcription of the u-PAR gene yields a 1.4-kilobase mRNA or an alternatively spliced variant lacking the membrane attachment peptide sequence (20, 21). The amounts of u-PAR are controlled mainly at the transcriptional level, but altered message stability and receptor recycling may represent other means of controlling the amount of this gene product at the cell surface (22–25).

The transcriptional regulation of the u-PAR gene is still poorly understood. Soravia et al. (26) reported that the basal expression of the gene was regulated via Sp1 motifs proximal and upstream of the transcriptional start site. Our laboratory showed that both the constitutive and PMA-inducible expression of the gene required a footprinted region (−190/−171) of the promoter containing an AP-1 motif (22). We also observed a second footprinted region of the promoter (−148/−124), and deletion of this region caused a dramatic reduction in the constitutive u-PAR promoter activity in a colon cancer cell line characterized by its high expression of this gene. Interestingly, this region of the promoter contained noncanonical AP-2 (−142/−134) and Sp1 (−147/−138) motifs overlapping with each other as well as nonconsensus polyomavirus activator 3 (−133/−127) motifs.

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Transactivation of the Urokinase-type Plasminogen Activator Receptor Gene through a Novel Promoter Motif Bound with an Activator Protein-2α-related Factor*

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The urokinase receptor overexpressed in invasive cancers promotes laminin degradation. The current study was undertaken to identify cis elements and trans-acting factors activating urokinase receptor expression through a footprinted (−148/−124) region of the promoter containing putative activator protein-2- and Sp1-binding motifs. Mobility shifting experiments using nuclear extract from a high urokinase receptor-expressing cell line (RKO) indicated that Sp1, Sp3, and a factor similar to, but distinct from, activator protein-2α bound to this region. Mutations preventing the binding of the activator protein 2α-related factor diminished urokinase receptor promoter activity. In RKO cells, the expression of a negative regulator of activator protein-2 function diminished urokinase receptor promoter activity, protein, and laminin degradation. Conversely, urokinase receptor promoter activity in low urokinase receptor-expressing GEO cells was increased by activator protein-2α expression. Although using GEO nuclear extract, little activator protein-2α-related factor bound to the footprinted region, phorbol 12-myristate 13-acetate treatment, which induces urokinase receptor expression, increased complex formation. Mutations preventing the activator protein-2α-related factor and Sp1/Sp3 binding reduced urokinase receptor promoter stimulation by this agent. Thus, the constitutive and phorbol 12-myristate 13-acetate-inducible expression of the urokinase receptor is mediated partly through trans-activation of the promoter via a sequence (−152/−135) bound with an activator protein-2α-related factor.

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This paper is in tribute to Barbara Young for her dedicated work.

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1 The abbreviations used are: u-PAR, urokinase-type plasminogen activator receptor; AP-1 and -2, activator protein-1 and -2, respectively; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; RSV, Rous sarcoma virus.
though it is not critical for mouse development (27). In cancer, several experimental and clinical findings support the view that the u-PAR plays a prominent role in tumor cell invasion and metastasis. For example, the u-PAR mRNA is expressed in the tumor cells of invasive colon cancers (28, 29), and a high u-PAR protein level is predictive of short survival times for patients with this disease (30). Further, earlier studies have shown that the overexpression of a human u-PAR cDNA increased the ability of human osteosarcoma cells to invade into an extracellular matrix-coated porous filter (31). Conversely, down-regulating u-PAR levels using antisense expression constructs, oligonucleotides, or synthetic compounds reduced the ability of divergent invasive cancers to invade in vitro and in vivo (32–36). Since the u-PAR is a key factor in promoting tumor-associated proteolysis, down-regulation of its expression could be a promising strategy for inhibiting cancer invasion and metastasis. We therefore undertook a study with two objectives: (a) to identify cis-elements and trans-acting factors regulating constitutive and PMA-inducible u-PAR gene expression via the footprinted region spanning nucleotides −148/−124 and (b) to determine the effect of interfering with transcription factors binding to this region on u-PAR-directed laminin degradation.

**EXPERIMENTAL PROCEDURES**

**Vectors and Antibodies**—The u-PAR CAT reporter consisted of 449 base pairs of sequence (37) stretching from −398 to +51 (relative to the transcription start site) cloned into the XbaI site of the pCAT-Basic vector (Promega, Madison, WI). Reportor constructs were regulated by truncated u-PAR promoter fragments as were described previously (22). The urokinase CAT reporter consisted of 2345 base pairs of a 5′-flanking region fused directly to the reporter (38). Antibodies to Sp1, Sp2, Sp3, and AP-2 isoforms were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Jun-D expression construct was described elsewhere (22). Oligonucleotides were purchased from Genosys Biotechnologies (The Woodlands, TX). Recombinant AP-2α and Sp1 (full-length human proteins) were obtained from Promega (Madison, WI). Expression vectors for AP-2αA, AP-2αB, and AP-2 α antisense (39) consisted of the cloned sequences inserted into the EcoRI site of pBSG (Stratagene, La Jolla, CA) and were kindly provided by Dr. Michael Tainsky. The AP-2α, AP-2β, and AP-2γ reporter constructs were also regulated by the footprinted region II, since preliminary EMSA utilizing a Sp1-specific consensus oligonucleotide revealed that 5′ of the pBLCAT2 reporter (40). For the generation of the R2 CAT reporter construct, an oligonucleotide spanning nucleotides −148/−128 was cloned into the XbaI site of pCATBasic (Promega).

**Preparation of Nuclear Extracts and EMSA**—Nuclear extracts and EMSA were carried out as described elsewhere (22). EMSA was carried out using nuclear extract (8 μg), 0.6 μg of poly(dI/dC), and 2 × 10^6 cpm of a T4 polynucleotide kinase-labeled [32P]ATP oligonucleotide. The sequences of the AP-2 and Sp1 consensus oligonucleotides were 5′-GAT CGA ACT GCC CCG CCC GT-3′ (Santa Cruz Biotechnology catalog no. sc-2513) and 5′-ATT CGA TCG GGG CCC GAC C-3′ (Santa Cruz Biotechnology catalog no. sc-2502), respectively. The sequence of the mutated (underlined nucleotides) AP-2 consensus-containing oligonucleotide was 5′-GAT CGA ACT GCC CCG CCC GT-3′ (Santa Cruz Biotechnology catalog no. sc-2516).

**Site-directed Mutagenesis**—This was performed according to the protocol of the Site-Directed Mutagenesis Kit (52701) of CLONTECH (Palo Alto, CA). For generation of the Sp1/Sp3mt u-PAR CAT, pCATBasic (Promega) regulated by 398 base pairs of the u-PAR promoter (37) served as a template. A mutation primer substituting A for C at positions −148, −147, −144, and −142 and its corresponding selection primer (5′-CTTATGTCGTCTGGATCCCCCGGAATTC-3′) changing the 5′-end (−142) of the footprinted region I was annealed to the denatured template plasmid, and the plasmid was amplified according to the protocol. Remaining wild-type plasmid was eliminated by two BanHI digestions for 4 h, each of them followed by transformation of nondigested DNA into BMH 71–18 S cells (CLONTECH, Palo Alto, CA). DNA of selected clones was isolated and sequenced using the Amersham Pharmacia Biotech T7-Sequnese 2.0 Kit.

For generation of the AP-2α/Sp1mt u-PAR CAT construct, the Sp1/Sp3mt u-PAR CAT plasmid served as a template. The second mutation primer substituted A for C at positions −146, −145, −142, and −141 of the u-PAR promoter, and the selection primer (5′-CTTATGTCGTCTGGATCCCCCGGAATTC-3′) changed the 5′-end back to BamHI. Selection for AP-2α/Sp1mt-mutated plasmids was done by KpnI digestion. The procedure was continued as described above.

**CAT Assays**—Cells were transfected at 60% confluency using poly-L-ornithine as described previously (41). All transient transfections were performed in the presence of a luciferase expression vector (4 μg), and transfection efficiencies were determined by assaying for luciferase activity. CAT activity was measured as described previously (22). The amount of acetylated [14C]chloramphenicol was determined using a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software. Student’s t test analysis was performed via the SPSS software (SPSS Inc., Chicago, IL). Statistical significance was defined as p ≤ 0.05.

**Magnetic Separation of Transfected and Nontransfected Cells**—Transfected cells were enriched by the MACS-Select method of Miltenyi Biotech (Auburn, CA). RKO cells were co-transfected with the AP-2αB expression construct and a plasmid encoding a mutated CD4 molecule (pMACS 4) in a 3:1 ratio. Cells were harvested after 42 h in 320 μl of PBE buffer (phosphate-buffered saline, 0.5% bovine serum albumin, 5 mM EDTA) and incubated for 15 min with 80 μl of a magnetic bead-conjugated antibody directed against the mutated CD4 molecule. The cell suspension was then run through V8+ separation columns using the VarioMACS magnet according to the manufacturer’s protocol.

**Enzyme-Linked Immunosorbent Assay for u-PAR Protein**—RKO cells were harvested into a buffer (10 mM Tris, pH 7.4, 0.15 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonfluoride, 1 mM EDTA) and incubated for 10 min at 4 °C. Insoluble material was removed by centrifugation, and 750 μg protein of cell extract was immunoprecipitated at 4 °C for 16 h with 0.25 μg of a polyclonal anti-u-PAR antibody and protein A-agarose beads. The polyclonal antibody (kindly provided by Dr. Andrew Mazar, Angstrom Pharmaceuticals, San Diego, CA) was raised in rabbits against amino acids 1–281 of the human u-PAR and purified on a Sepharose-immobilized u-PAR column. The immunoprecipitated material was subjected to standard Western blotting (42), and the blot was probed with 5 μg/ml of an anti-u-PAR monoclonal antibody (catalog no. M010, Transduction Laboratories, Lexington, KY). After varying times at 37 °C, tissues were homogenized and probed with 10 μg/ml of anti-u-PAR antibodies (catalog no. 148/128, American Diagnostica, Greenwich, CT) and a horseradish peroxidase-conjugated goat anti-mouse IgG. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

For the determination of u-PAR by enzyme-linked immunosorbent assay, resected tissue was prepared as described by the manufacturer (American Diagnostica).

**Laminin Degradation Assays**—These were carried out as described previously (43). RKO cells were harvested with 3 mM EDTA/phosphate-buffered saline, washed twice, and seeded (500,000 cells) on radioactive laminin-coated (2 μg/dish) dishes. The cells were allowed to attach overnight. Subsequently, cell surface u-PARS were saturated by incubating the cells at 37 °C for 30 min with 5 nM urokinase, and unbound plasminogen activator was removed by washing. The cells were then replenished with serum-free medium with or without 10 μg/ml plasminogen activator. After varying times at 37 °C, aliquots of the culture medium were withdrawn and counted for radioactivity. Solubilized laminin represents the degraded glycoprotein.

**RESULTS**

**Region II of the u-PAR Promoter Footprinted by Nuclear Extract from a High u-PAR-expressing Cell Line Is Bound with Sp1, Sp3, and an AP-2α-related Factor**—We previously reported (22) that nuclear extract from a high (3 × 10^6 binding sites/cell) u-PAR-expressing colon cancer cell line (RKO) footprinted a region (referred to as region II) of the u-PAR promoter (nucleotides −146/−124). As a first step to identifying transcription factor(s) bound to this region, EMSA was carried out using an oligonucleotide spanning nucleotides −154/−128 (Fig. 1A). The oligonucleotide was not extended to the 3′-end (−124) of the footprinted region II, since preliminary EMSA utilizing a probe that included sequences 3′ of −128 has not revealed any specific binding complex. EMSA data (Fig. 1A) showed three slower migrating bands (indicated by a brace, arrow, and asterisk) were apparent (Fig. 1B, lanes 2 and 8) with a 100-fold excess of the nonradioactive oligonucleotide eliminating (lane 3) all of these bands. Computer analysis of this region of the u-PAR promoter revealed the presence of putative AP-2 (−142/−134) and Sp1/Sp3 (−147/−138) bind-
ing motifs all bearing one mismatch with the corresponding canonical (AP-2, GCCNNNGGC; Sp1, RYYCCGCCCM) sequences. The addition of a 100-fold excess of a consensus AP-2-containing oligonucleotide (Fig. 1B, lane 7 and 11) from the human metallothionein IIa promoter sequence (44) eliminated one of the shifted bands (*). In contrast, substitution of this oligonucleotide at the AP-2 motif (CC to TT) prevented it from competing for the shifted bands (Fig. 1B, lane 10). Increasing

**FIG. 1.** Binding of Sp1, Sp3, and an AP-2-related factor to footprinted region II of the u-PAR promoter. A, schematic representation of the footprinted region II of the u-PAR promoter (22) and the oligonucleotide used in EMSA in this figure. B, RKO nuclear extract (8 μg) was incubated at 21 °C for 20 min with an end-labeled oligonucleotide (−154/−128 u-PAR) in the presence or absence of a 100-fold excess of competitor sequences. After this time, 2 μg of the indicated antibodies were added, and complexes were subsequently analyzed by gel electrophoresis. C, EMSA was carried out as described for B with the exception that the amount of the Sp1 consensus sequence was varied. D and E, RKO nuclear extract was incubated where indicated with an anti-AP-2α, an anti-AP-2β, or an anti-AP-2γ antibody (2 μg) or an equivalent amount of an unspecific IgG for 50 min at 4 °C followed by protein A-agarose beads. Beads were subsequently removed by centrifugation, and treated and untreated nuclear extract was analyzed by EMSA as described for B. The data are typical of duplicate experiments.
Regulation of u-PAR Expression by an AP-2α-related Factor

amounts of a nonradioactive oligonucleotide bearing a consensus Sp1 motif caused a dose-dependent decrease in the intensity of two of the shifted bands (indicated with a brace and arrow) (Fig. 1C) while having little effect on the band (*) competed with the AP-2 motif-containing oligonucleotide.

The ability of the consensus Sp1 motif to compete for the binding of nuclear-extracted proteins to the u-PAR promoter footprinted region II oligonucleotide suggested that transcription factors recognized by these motifs were bound to the u-PAR promoter. To examine this possibility, “supershifting” experiments were carried out. The addition of an anti-Sp1-specific antibody to the RKO nuclear extract resulted (Fig. 1B, lane 4) in a slower migrating band (indicated with a line) with a concomitant decreased intensity of the complex (indicated by a brace) competed with the Sp1 consensus sequence. On the other hand, the addition of an antibody against Sp2 had no effect (lane 5) on the migration pattern, while an antibody directed at the DNA-binding domain of Sp3 completely abolished (Fig. 1B, lane 6) the shifted band with the intermediate mobility (arrow). These data suggested that the region of the u-PAR promoter footprinted with nuclear extract from a high u-PAR-expressing cell line (22) is bound with Sp1 and Sp3.

Since the fastest migrating complex evident in the EMSA (indicated by an asterisk) was competed with an AP-2 consensus motif (Fig. 1B), we hypothesized that the bound protein was an AP-2 isoform. To test this hypothesis, two sets of experiments were carried out. First, RKO nuclear extract was mixed with 2 μg of an anti-AP-2α antibody or an equal amount of IgG and subsequently with protein A-agarose beads. The supernatant (depleted of AP-2 and subsequently with protein A-agarose beads. The supernatant of the u-PAR promoter footprinted region II oligonucleotide suggested that transcription factors recognized by these motifs were bound to the u-PAR promoter.

To delineate the minimal sequence of footprinted region II required for the binding of nuclear-extracted proteins to the u-PAR promoter, while the binding of Sp1/Sp3 and the AP-2α antibody-reactive factor demonstrated different 5’ requirements, the binding of these transcription factors showed identical requirements for 3’ sequences. Thus, the removal of up to 5 base pairs from the 3’ terminus of oligonucleotide −154/−130 (generating oligonucleotide −154/−135) had little effect on transcription factor binding. However, the deletion of an additional 3 base pairs from the 3’-end (oligonucleotide −154/−138) completely abolished the binding of these three transcription factors. Thus, sequences in the u-PAR promoter extending 3’ to −135 are required for the optimal binding of Sp1, Sp3, and the factor recognized by the anti-AP-2α antibody.

While the anti-AP-2α antibody “supershifted” (arrow) authentic AP-2α bound to the u-PAR promoter oligonucleotide −154/−128 (Fig. 2), in contrast, we were unable to detect a “supershift” of the fastest migrating band (using RKO nuclear extract) with this antibody (data not shown). However, this could be due to the presence of multiple complexes evident in the EMSA masking a “supershifted” band. Thus, while the addition of RKO nuclear extract to this probe yielded a slower migrating band (indicated with an asterisk), which was abolished with an excess of unlabeled AP-2 consensus oligonucleotide. However, the addition of the anti-AP-2α antibody, effective in supershifting authentic AP-2α bound to the −147/−128 probe (arrow), failed to produce a “supershifted” complex. Likewise, antibodies to other AP-2α isoforms did not yield a “supershift” of the RKO-derived nuclear factor. Another possible explanation for the lack of a
“supershift” using RKO nuclear extract is that there may be an inhibitor present in the extract. However, this is unlikely, since the addition of nuclear extract from this cell line to authentic AP-2\(\alpha\) did not prevent a supershift of the latter in EMSA employing the \(2154/2128\) probe (data not shown). These data suggest that a DNA-binding factor related to AP-2\(\alpha\) (hereafter referred to as AP-2\(\alpha\)-related factor), derived from RKO nuclear extract, is bound to region II of the u-PAR promoter sequence.

**Binding of Authentic Sp1 and AP-2\(\alpha\) to the u-PAR Promoter Region II Is Mutually Exclusive**—There is substantial overlap between the Sp1/Sp3 (\(2147/2138\)) and AP-2 motifs (\(2142/2134\)) in the footprinted region II of the u-PAR promoter. Thus, we considered it unlikely that Sp1 and the AP-2\(\alpha\)-related factor were simultaneously binding to the u-PAR oligonucleotide probe. Nevertheless, to examine this possibility, the u-PAR oligonucleotide spanning \(2154/2128\) was incubated with authentic Sp1 and authentic AP-2\(\alpha\) proteins alone or in combination. Binding of the oligonucleotide with the individual purified proteins resulted in slower migrating complexes (Fig. 4). However, the simultaneous addition of both purified proteins to oligonucleotide \(2154/2128\) did not yield a ternary complex and in fact resulted in a decrease in binding of either protein. The topmost band represents material remaining in the well and failed to resolve as a third band even with continuous electrophoresis. These data would suggest that the binding of Sp1 and AP-2\(\alpha\) to the u-PAR promoter sequence spanning \(2154/2128\) is mutually exclusive. However, it should be emphasized that this conclusion is based on the use of authentic AP-2\(\alpha\) rather than the AP-2\(\alpha\)-related factor present in RKO nuclear extract.

**Effect of Inhibiting Sp1/Sp3 Binding to Region II on u-PAR Promoter Activation**—To determine whether u-PAR promoter activation was dependent on the binding of Sp1/Sp3 to region II, G nucleotides at positions \(2148, 2147, 2144, \) and \(2142\) (within the Sp1-like motif) were substituted for T (Fig. 5A). EMSA using this substituted oligonucleotide indicated that the slower migrating bands identified as Sp1 (brace) and Sp3 (arrow) by their reactivity with specific antibodies (see Fig. 1B) were markedly diminished in their intensity when compared with an oligonucleotide corresponding to the wild type u-PAR promoter sequence (Fig. 5B). In contrast, the intensity of the complex (*) recognized with the anti-AP-2\(\alpha\) antibody (see Fig. 1D) was not decreased by this change. RKO cells were then transfected with a CAT reporter driven by either the wild type u-PAR promoter (u-PAR CAT) or the promoter harboring the mutations that reduced Sp1 and Sp3 binding to region II. In duplicate experiments, the activity of the mutated u-PAR pro-
promoter was similar to that achieved with the wild type u-PAR promoter (Fig. 5C).

Elimination of Sp1/Sp3 and AP-2α-related Factor Binding to u-PAR Region II Abolishes Constitutive Promoter Activation—We were unable to interfere with the binding of the AP-2α-related factor to region II of the u-PAR promoter without having a deleterious effect on Sp1/Sp3 binding, and this was consistent with the large overlap of these motifs and band shifting experiments (see Fig. 3A), which revealed identical 3' nucleotide requirements. We therefore determined the effect of eliminating the binding of these three transcription factors on constitutive u-PAR promoter activity. Nucleotide substitutions (Fig. 6A) of the Sp1-like and AP-2-like motifs, which prevented (Fig. 6B) these transcription factors binding to oligonucleotide −154/−128, were engineered into the u-PAR promoter CAT reporter construct. The reporter construct was then compared with the wild type promoter for activation in the u-PAR-over-expressing RKO cells. We found in duplicate experiments that the activity of the mutated u-PAR promoter was reduced by over 90% in comparison with the wild type promoter construct (Fig. 6C). These data, combined with the observation that the mutation of region II, which prevented the binding of Sp1/Sp3 (but not the AP-2α-related factor), had minimal effect on the activity of the u-PAR promoter (see Fig. 5C), implies that binding of the AP-2α-related factor to this region is critical for the constitutive activity of this promoter in RKO cells.

Down-regulation of u-PAR Promoter Activity by the Co-expression of either a Dominant Negative or an Antisense AP-2α—As indicated above, any conclusions drawn as to the role of the AP-2α-related factor in the regulation of u-PAR promoter activity were confounded by the fact that nucleotide substitutions of region II affecting the binding of this factor also disrupted the binding of Sp1 and Sp3. To circumvent this problem, we determined the effect of interfering with AP-2α on u-PAR promoter activity. RKO cells were transiently transfected with an AP-2α expression construct. AP-2αB is an alternatively spliced product of AP-2α and acts as a negative regulator of AP-2 transcriptional activity (39). In three separate experiments, increasing amounts of the AP-2αB expression construct caused a dose-dependent repression of u-PAR promoter activity (Fig. 7). An input of 0.5 μg of the dominant negative expression construct brought about an 81 ± 4% inhibition of u-PAR promoter activity compared with the empty expression vector (pSG5). Increasing the amount of AP-2αB DNA to 5 μg was marginally more effective, resulting in a 93 ± 5% inhibition of u-PAR promoter activity.

As an alternative to using the dominant negative expression construct, parallel experiments were also carried out using an antisense AP-2α expression vector. Similar to the results achieved with the AP-2αB, the antisense expression construct caused an inhibition of u-PAR promoter activity (data not shown), with 10 μg of DNA yielding a 90 ± 14% repression of u-PAR promoter activity when compared with the vector backbone (pSG5). To rule out the possibility that interfering with AP-2α activity was having a general suppressive effect on transcription, RKO cells were transiently co-transfected with the AP-2αB expression construct and a CAT reporter driven by the urokinase promoter. The AP-2αB expression construct failed to repress the urokinase reporter construct using 1 μg of the effect on an amount that inhibited u-PAR promoter activity by over 90 ± 7% (data not shown). Thus, it is unlikely that the effect of AP-2αB on u-PAR promoter activity is due to a general shut down of transcription.

To confirm that the repressive effect of AP-2αB on u-PAR promoter activity was mediated via the footprinted region II, two experiments were carried out. First, RKO cells were transiently co-transfected with an AP-2αB expression construct and a CAT reporter regulated by either 148 or 105 base pairs of 5'-flanking sequence of the u-PAR gene. Based on band shifting experiments (see Fig. 3A) the −148 u-PAR promoter fragment can, via region II, bind the AP-2α-related factor, whereas the u-PAR promoter regulated by only 105 base pairs of upstream...
sequence cannot. It should be noted that Sp1/Sp3 binding is preserved in the 2148 u-PAR construct due to the contribution of plasmid sequences. The AP-2 αβ expression construct caused a marked reduction (80%) in promoter activity using the reporter construct flanked by 148 base pairs of upstream sequence while bringing about only a modest repression (20%) of the reporter construct regulated by 105 base pairs of the u-PAR regulatory sequence (data not shown). Second, RKO cells were co-transfected with the AP-2 αβ expression construct and a CAT reporter regulated by either an oligonucleotide (2154/2128) spanning region II (R2 CAT) of the u-PAR promoter or 398 base pairs of 5′-flanking sequence (u-PAR CAT). The CAT reporter regulated by region II (R2 CAT) was activated (Fig. 8) in RKO cells, albeit to a lesser extent than that achieved with 398 base pairs of flanking sequence (u-PAR CAT). In two separate experiments, the activity of the R2 CAT reporter construct was inhibited by over 75% by the co-expression of the AP-2α-related factor when compared with an equivalent amount of the empty expression construct (pSG5). This inhibition was quantitatively similar to that achieved with the u-PAR CAT reporter. Taken together, these data suggest that the u-PAR promoter activity is indeed regulated by an AP-2α-related factor and possibly Sp1/Sp3 in RKO cells and that this occurs via region II of the promoter.

Nuclear Extract from u-PAR-rich Cells Demonstrate Increased Binding of the AP-2α-related Factor to the u-PAR Promoter Region II Compared with Nuclear Extract from Low u-PAR-expressing Cells—Considering the evidence implicating the AP-2α-related factor in the regulation of u-PAR expression in RKO cells, we speculated that nuclear extract derived from cells rich in u-PAR protein would contain more of this transcription factor bound to region II of the u-PAR promoter compared with nuclear extract derived from cells characterized by their low u-PAR protein. Toward this end, we made use of another colon cancer cell line (GEO), which displays 10-fold fewer u-PARs compared with RKO as a consequence of reduced transcription of the gene (22). Nuclear extracts were generated from each cell line, and equal protein amounts were incubated with the radioactive oligonucleotide (2154/2128) in the presence or absence of excess oligonucleotide competitors (Fig. 9). Binding complexes were then analyzed by electrophoresis. Nuclear extract from GEO cells gave rise to a retarded band with a mobility similar to that identified as the AP-2α-related factor (*) using RKO nuclear extract. An excess of the AP-2 consensus sequence reduced the intensity of this band, consistent with the notion that it represents an AP-2α-related factor-bound complex. Interestingly, the intensity of the complex (*) was reduced in nuclear extract from the low u-PAR-expressing GEO cells when compared with RKO cells, the latter of which display over 10-fold more u-PAR. Equally important, treatment of GEO cells with PMA, which increases u-PAR gene transcription in this cell line (22, 45), brought about a dramatic increase in the amount of this complex (*). In contrast, the intensity of the retarded band, which was indistinguishable from Sp3 (arrow),
was decreased by PMA treatment of GEO cells.

To determine if the altered amounts of the region II-bound factors were required for the stimulation of u-PAR promoter activity by PMA, GEO cells were co-transfected with a CAT reporter flanked by the wild type or mutated u-PAR promoter and a vector bearing AP-2α and subsequently treated with the phorbol ester. Treatment of the cells with PMA caused a strong (10–20-fold) induction of the wild type (u-PAR CAT) u-PAR promoter, which was prevented in cells made to co-express AP-2α (Fig. 10). Mutation of the u-PAR promoter to prevent Sp1/Sp3 binding (Sp1/Sp3mt u-PAR CAT) reduced the stimulation by PMA. This stimulation was further reduced when nucleotide substitutions of the u-PAR promoter were undertaken to abolish the binding of the AP-2α-related factor as well as Sp1/Sp3 (AP-2/Sp1/Sp3mt u-PAR CAT). Interestingly, the ability of the AP-2αB expression construct to repress u-PAR promoter activity was diminished by mutations that prevented the binding of Sp1/Sp3 (Sp1/Sp3mt u-PAR CAT).

Taken together, these data would suggest that PMA stimulation of u-PAR gene expression in GEO cells requires, at least in part, the binding of the AP-2α-related factor as well as Sp1/Sp3 to region II of the promoter.

Stimulation of u-PAR Promoter Activity in the Low u-PAR-expressing GEO Cell Line by the Expression of Exogenous AP-2α—While the above experiments suggested a requirement for the AP-2α-related factor in the stimulation of u-PAR gene expression by phorbol ester, it was not clear as to whether this transcription factor alone was sufficient to augment u-PAR promoter activity. To address this issue, GEO cells, which have low u-PAR protein, were co-transfected on three separate occasions with a u-PAR promoter-regulated CAT reporter and an expression vector bearing the full-length form of AP-2α (AP-2α). The activity of the promoter alone was below the detection limit of the assay (Fig. 11), consistent with the low expression of the u-PAR gene in this cell line. However, the co-transfection of AP-2α into these cells caused a dose-dependent increase in u-PAR promoter activity with up to 0.05 μg of the effector plasmid. This amount of the expression con-
struct brought about over a 13-fold stimulation of promoter activity. Higher amounts of the AP-2αA diminished u-PAR promoter stimulation, presumably as a consequence of squelching (46). The AP-2αA-dependent stimulation of the u-PAR promoter was greater than that achieved with 2 μg of a JunD expression vector, which we had shown previously (22) to stimulate u-PAR expression via a separate footprinted region (2190/2171) of the promoter containing a classical AP-1 motif. The transfection of GEO cells with JunD and AP-2αA together stimulated u-PAR promoter activity to an extent greater than the sum of the individual expression constructs, indicative of synergism. Thus, it is likely that these transcription factors cooperate with each other to regulate u-PAR expression.

Notwithstanding these observations, our data indicate that the expression of AP-2αA is sufficient to up-regulate u-PAR gene expression in GEO cells.

Expression of AP-2αB Decreases Endogenous u-PAR Protein Amount in RKO Cells—We then determined if the expression of AP-2αB, which acts as a negative regulator of AP-2 function, reduces the expression of the endogenous u-PAR gene in RKO cells. Cells were co-transfected with an expression vector encoding a mutated CD4 and varying amounts of an expression construct bearing AP-2αB. Cells were harvested 48 h later, and transfected cells were enriched with magnetic beads coated with an anti-mutated CD4 antibody (which is non-cross-reactive with wild type CD4) and assayed for u-PAR protein by Western blotting (Fig. 12A). A band whose molecular mass was indistinguishable from that of u-PAR (55 kDa) (47) was detected in the immunoblot. Increasing amounts of the AP-2αB expression vector caused a dose-dependent decrease in the amount of this protein. The reduced amount of u-PAR protein was associated with the attenuated activity of a CAT reporter regulated by three tandem AP-2 motifs upstream of a thymidine kinase minimal promoter (Fig. 12B). Thus, the expression of a negative regulator of AP-2 reduces the expression of the endogenous u-PAR gene in RKO cells.

Inhibition of u-PAR-directed Laminin Degradation in RKO Cells Made to Express AP-2αB—One of the functions of the u-PAR is to accelerate plasminogen-dependent proteolysis (8), and this is a requirement for the invasive potential of a divergent set of cancers (31, 32, 48). We were therefore interested in determining whether interfering with the transcriptional activation of the gene leading to reduced u-PAR synthesis would diminish extracellular matrix degradation. RKO cells in serum-free medium (RKO-SF) demonstrated minimal solubilization of laminin (Fig. 12C). However, the addition of plasminogen to the RKO cells transfected with the vector backbone (RKO-pSG5-Pl-SF) resulted in a strong time-dependent increase in laminin degradation, indicating plasmin-dependent proteolysis. After a 2-h incubation in the presence of zymogen,
nearly 500,000 cpm of solubilized laminin was evident in the culture supernatant. In contrast, RKO cells transfected with the AP-2αB expression construct (RKO-AP-2B-Pl-SF) showed markedly reduced plasminogen-dependent degradation (80% reduction) of this glycoprotein after a 2-h incubation. These data suggest that reduced u-PAR synthesis brought about by interfering with the AP-2α-dependent transcriptional activation of the u-PAR gene in RKO cells attenuates plasminogen-dependent proteolysis.

DISCUSSION

The u-PAR plays a critical role in extracellular matrix degradation and tumor invasion (34, 49). We have identified an important regulatory region in the u-PAR promoter (−152/−135) that is trans-activated by an AP-2α-related factor. Further, our finding that the expression of AP-2αB, which interferes with the transcriptional activation of the u-PAR gene by the AP-2α-related factor and Sp1/Sp3, diminishes u-PAR protein amounts and laminin degradation serves to illustrate that transcriptional studies of a target gene can be utilized to bring about a more indolent phenotype of cancer at least in an experimental setting.

Interestingly, in a previous report, Soravia et al. (26), using HeLa nuclear extracts, failed to detect, by EMSA, the binding of AP-2 to the urokinase receptor promoter. The reason for this difference is unclear at the present time. One possibility is that urokinase receptor expression is regulated differently in the separate cell lines used in the two studies.

The identity of the AP-2α-related factor bound to the u-PAR promoter region II is unknown at the present time. Certainly, it is strongly related to AP-2α, since depletion of nuclear extract with an anti-AP-2α antibody and protein A-agarose beads severely diminished the intensity of the retarded band in EMSA and since the u-PAR promoter region II can clearly bind authentic AP-2α. On the other hand, in EMSA it had a distinct mobility and the anti-AP-2α antibody was unable to supershift the RKO nuclear factor bound to a region II oligonucleotide while effecting a supershift with authentic AP-2α bound to this oligonucleotide. We were also unable to supershift or immunodeplete the AP-2α-related factor from RKO nuclear extract using recently available antibodies (Santa Cruz Biotechnology) to AP-2α, making it unlikely that the RKO-derived nuclear factor was one of these isoforms. Lee et al. (50) also noted an AP-2α-related factor in THP-1 monocytes that regulated the expression of the B2 subunit of the V-ATPase. Similar to our study, the THP-1-derived AP-2α-related factor was not supershifted in EMSA and had a
different size from authentic AP-2α based on its mobility in gel retardation studies. It remains to be determined whether the AP-2α-related factor found in the current study and in the study by Lee et al. (50) are the same.

In addition to the AP-2α-related factor recognized by footprinted region II of the u-PAR promoter, both Sp1 and Sp3 were also bound, as evident in EMSA using nuclear extract from the high u-PAR-expressing cell line RKO. However, we found that mutations of the u-PAR promoter that prevented the binding of either of these transcription factors had a negligible effect on promoter activation, arguing against a role for these factors in regulating at least the constitutive u-PAR expression in RKO cells.

The requirement of the u-PAR promoter region II for the PMA-dependent elevation of u-PAR gene expression (53) merits discussion. We had shown previously (22) that the induction by this phorbol ester required an intact AP-1 motif (at −184) in a separately footprinted region (−190/−171) of the u-PAR promoter. However, several observations in the current study would indicate that the induction of the u-PAR promoter by PMA requires, in addition, other transcription factor binding sites including the motif bound with the AP-2α-related factor. Thus, nucleotide substitutions of the u-PAR promoter that prevented the binding of the AP-2α-related factor substantially reduced the stimulation of the promoter by the phorbol ester. Further, the co-expression of AP-2αB, which is a negative regulator of AP-2 function, completely ablated the stimulation of the u-PAR promoter by PMA. Finally, an increased amount of the AP-2α-related factor bound to the u-PAR promoter region II was evident using nuclear extract from PMA-treated GEO cells (compared with nuclear extract from untreated GEO cells). Our results are reminiscent of other studies in which the stimulation of the PAC-1 (phosphatase of activated cells) phosphatase and neuropeptide tyrosine genes by PMA was shown to be mediated partly through an AP-2-related site and coincided with induced DNA binding of AP-2 (54, 55). It was also clear that interfering with the binding of Sp1/Sp3 (but not affecting the binding of the AP-2α-related factor) to region II of the
u-PAR promoter had a deleterious effect on the activation of the u-PAR promoter by PMA. A similar requirement for Sp1 has been reported for the stimulation of thromboxane receptor gene expression by PMA (56), although, in contrast to that study, we did not detect increased binding of this transcription factor. Taken together, it is likely that the stimulation of the u-PAR gene expression by phorbol ester is complex, requiring the interactions of multiple transcription factors with their cognate binding sites in the upstream sequence of this gene.

The mechanism by which the AP-2αB alters u-PAR promoter activity deserves comment. Initially, we assumed that this was through a direct antagonism of the AP-2α-like factor bound to the u-PAR promoter region II, presumably as a consequence of heterodimerization of the two proteins (39). However, one experiment suggested that this proposal might not be entirely accurate. Thus, the ability of the AP-2αB to counter the stimulation of u-PAR promoter activity by PMA was diminished by mutations in the u-PAR promoter that prevented the binding of Sp1/Sp3. These data suggested that the expressed AP-2αB was mediating its effect, at least in part, through the Sp1/Sp3-binding motif.

Our data based on mobility shift assays using purified proteins strongly suggested that the binding of at least AP-2αA and Sp1 to the u-PAR promoter region II is mutually exclusive,
and this is consistent with the considerable (about 50%) overlap of the Sp1/Sp3 and AP-2 motifs. Thus, it is unlikely that the AP-2-related factor and Sp1 are physically interacting on the u-PAR promoter region II sequence. The biological significance of having two overlapping transcription factor binding motifs in the u-PAR promoter region II, which can be bound with only one transcription factor at any one time, can only be speculated on. Certainly, for the regulation of keratin 3 gene expression in corneal epithelial cells, overlapping AP-2 and Sp1 motifs act as a switch to up-regulate expression of this gene in response to the latter transcription factor (57). For the u-PAR promoter, since this motif can be bound with Sp3, which for some genes is a trans-repressor (58), it may be that this represents a mechanism for either up- or down-regulating u-PAR expression.

In conclusion, we have shown that the constitutive and PMA-inducible expression of the u-PAR gene requires, at least in part, the trans-activation of a region of the promoter spanning -152/-135 by an AP-2-related factor. A high u-PAR protein level in colon cancer, which promotes the invasive phenotype (31, 32) and portends a poor patient outcome (30), is probably partly a consequence of trans-activation of this gene by a greater amount and/or binding activity of the AP-2-related factor. Further, interfering with the trans-activation of the promoter by the AP-2-related factor reduced endogenous u-PAR expression and diminished laminin degradation. These findings raise the exciting possibility that interfering with the AP-2-related factor-dependent trans-activation of the u-PAR gene may represent a novel means of diminishing extracellular matrix degradation and consequently reducing colon cancer invasion and metastasis.

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