Transgenic Mice Demonstrate Novel Promoter Regions for Tissue-Specific Expression of the Urokinase Receptor Gene

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The urokinase-type plasminogen activator receptor (u-PAR) contributes to cell migration and proteolysis in normal and cancerous tissues. Currently, there are no reports on the regulatory regions directing tissue-specific expression. Consequently, we undertook a study to identify novel promoter regions required for expression of this gene in transgenic mice bearing a LacZ reporter regulated by varying amounts (0.4, 1.5, and 8.5 kb) of upstream sequence. The 0.4-kb u-PAR upstream sequence directed weak and strong LacZ expression in the placenta and epididymis, respectively, both of which are tissues that express endogenous u-PAR. Conversely, transgene expression in the apical cells of the colon positive for endogenous u-PAR protein required 1.5 kb of upstream sequence for optimal expression. Furthermore, chromatin accessibility assays coupled with real-time polymerase chain reaction suggested a putative regulatory region spanning -1295/-1192 driving u-PAR expression in colonic cells. Interestingly, placental transgene expression was augmented with the 8.5-kb upstream fragment compared with the shorter 1.5-kb fragment indicating contributing element(s) between -1.5 and -8.5 kb. Thus, while 0.4 kb of upstream sequence directs u-PAR expression in the epididymis, sequences located between -0.4 and -1.5 kb and between -1.5 and -8.5 kb are required for optimal tissue-specific expression in the colon and the placenta, respectively. (*Am J Pathol* 2003; 163:453-464)

The urokinase-type plasminogen activator receptor (u-PAR), a 45- to 60-kd glycosylated cell surface receptor (u-PAR)¹ linked to the cell surface via a glycolipid chain^{2,3} plays a critical role in cell migration, adhesion and extracellular matrix degradation in both physiology and pathology. In cancer, these cellular functions are

critical requirements in tumor cell invasion⁴⁻⁷ and indeed, several previous observations indicate a prominent role for u-PAR in tumor cell invasion and metastasis. Thus, a high u-PAR protein level is predictive of shortened survival for patients with colon cancer^{8,9} and gene expression profiling has revealed up-regulated expression of this binding site in various malignancies.¹⁰ Furthermore, studies with pharmacological and peptide antagonists or antisense strategies directed against the u-PAR have demonstrated their efficacy against growth, angiogenesis, and invasiveness of divergent malignancies.^{6,11-14}

The u-PAR contributes to the aforementioned cellular functions via different mechanisms. First, the serine protease urokinase bound to this receptor activates plasminogen at a much faster rate than fluid-phase plasminogen activator, thereby augmenting extracellular matrix degradation.¹⁵ Second, the binding site clears urokinase-inhibitor complexes from the extracellular space^{16,17} via an $\alpha 2$ macroglobulin receptor-dependent mechanism. Third, the u-PAR interacts with the extracellular domain of integrins thereby mediating cell adhesion and migration.^{18,19} Recently, it has been shown that the seven-transmembrane receptor FPR-like receptor-1/lipoxin A4 receptor, a G-protein-coupled receptor directly interacts with a soluble cleaved form of u-PAR to induce chemotaxis.²⁰

The amount of u-PAR protein is controlled mainly at the transcriptional level, although altered message stability and receptor recycling also contributes to the quantity of this gene product.²¹ Our laboratory and others have previously reported several upstream transcriptional elements regulating u-PAR expression in tissue culture. In the first study, Soravia et al²² demonstrated that the basal expression of the gene was regulated via Sp1 motifs located about 100 bp upstream of the transcriptional start site. Subsequently, our laboratory showed that both constitutive and phorbol 12-myristate 13-acetate (PMA)-inducible expression of the gene required a footprinted region (-190/-171) of the promoter containing an AP-1

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motif²³ as well as a second footprinted region (–148/–124) bound with an AP-2 α -related factor and Sp1/Sp3.²⁴ Hapke and co-workers²⁵ implicated a PEA3/Ets silencing motif located at –248 while Wang et al²⁶ demonstrated a novel NF- κ B element (located at –45) required for expression of this gene in cultured cells.

While these studies have been informative, they have two limitations. First, they provide no information on tissue-specific regulation of gene expression. Second, since the aforementioned studies all used transiently transfected, non-integrated reporter plasmids, the influence of the chromatin structure (DNA wrapped around core histone proteins in the nucleus) on gene expression is not addressed. To overcome these limitations, we have used a transgenic approach to identify previously undescribed regions of the u-PAR promoter required for its expression in its chromatinized environment in u-PAR-expressing tissues in the mouse.

Materials and Methods

Construction of u-PAR Promoter-LacZ Reporter Constructs

The –0.4 u-PAR LacZ transgene was constructed as follows. The nucleotide fragment (–392/+52 relative to the transcription start site) derived from –398 u-PAR²³ was digested with *Xba*I and the fragment cloned into the *Sma*I site of pBSSK-AUG- β -Gal plasmid upstream of the β -Galactosidase (LacZ) coding sequence. To generate the –1.5 u-PAR LacZ transgene, the nucleotide sequence (–1469/+52 relative to the transcription start site) was generated by PCR from normal human genomic DNA and subcloned into pBSSK-AUG- β -Gal as described for the shorter fragment. To generate the –8.5 u-PAR LacZ transgene, a nucleotide fragment spanning –8500/+52 (relative to the transcription start site) of the u-PAR gene was derived by PCR from cosmid R23816 (kindly provided by Lawrence Livermore National Laboratory, Livermore, CA). This cosmid includes the nucleotide sequence spanning chromosome 19q 13.2 from AKT 2 to D19S178 of which approximately 15 kb corresponds to the u-PAR flanking sequence. The PCR-amplified insert was subcloned into the *Not*I/*Xba*I site of the pJ251 reporter plasmid upstream of the β -Galactosidase coding sequence. DNA sequencing, restriction digestions and PCR were used to confirm orientation, nucleotide sequence and integrity of the 3 constructs.

Generation of Transgenic Mice

Founder mice were generated by the MD Anderson Cancer Transgenic Core Facility using B6D2F1J mice (a cross of the C57BL6 and DBA2 mouse strains) essentially as described previously.²⁷ Briefly, purified, linear DNA comprised of the fused u-PAR promoter fragment-LacZ reporter (1 ng/ μ l) dissolved in a TE buffer (10 mmol/L Tris (pH 7.4), 0.1 mmol/L EDTA) was microinjected into the pronuclei of fertilized mouse eggs when the eggs were at their maximum size but before the nuclear membrane

disappeared before first cleavage. Eggs that survived the injections were transferred into the oviduct of 0.5-day post-coitus pseudopregnant female mice. Mice born from microinjected eggs (founders) were subsequently screened by Southern blotting for transgene integration (see below). Positive founder lines were maintained by crossing with C57 Black breeders.

Analysis of LacZ Expression

Tissues were fixed for 1 hour at 4°C with a 0.1 mol/L phosphate-buffered solution (pH 7.3) containing 0.2% glutaraldehyde, 2% formalin, 5 mmol/L EGTA, 2 mmol/L MgCl₂. Tissues were subsequently rinsed three times in a 0.1 mol/L phosphate-buffered solution (pH 7.3) containing 0.1% sodium deoxycholate, 0.2% NP40, 2 mmol/L MgCl₂. After this, tissues were stained for LacZ expression overnight with a solution containing 1 mg/ml X-gal, 5 mmol/L potassium ferricyanide, and 5 mmol/L potassium ferrocyanide. The following day, tissues were rinsed and paraffin-embedded. Counterstaining with Nuclear Fast Red was accomplished after de-paraffinization. X-gal staining intensity was quantified with Optima (version 6.5) software (Media Cybernetics, Silver Spring, MD) using a minimum of five independent measurements (3 μ m) per slide. For each construct, transgene expression was determined in mice derived from at least three independent founder lines. Results were only regarded as valid if the observations were made in at least two of the three founder lines.

Western Blotting for u-PAR

Western blotting for u-PAR was performed as described previously with modifications.²⁸ Tissues were homogenized in a buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1.0% Triton-X 100, 0.5% NP-40, 1 mmol/L EDTA (pH 8), 1 mmol/L EGTA (pH 8), 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μ g/ml Aprotinin. After centrifugation, proteins in the supernatant (10 μ g) were resolved in a 10% polyacrylamide gel under non-reducing conditions. Following protein transfer to a polyvinylidene difluoride membrane, the membrane was blocked in a 3% bovine serum albumin solution overnight. Subsequently, the blot was probed with 2 μ g/ml of a rabbit polyclonal anti-mouse u-PAR antibody²⁹ for 2 hours. After multiple rinses, the blot was incubated with an anti-rabbit secondary antibody horseradish peroxidase-conjugate (1:5000) and immunoreactive products visualized by enhanced chemiluminescence. Loading equality was checked with an antibody to GAPDH (# 374-Chemicon; Temecula, CA).

Immunohistochemical Detection of Mouse u-PAR

This method was carried out essentially as described previously²⁹ with minor modifications in that blocking was accomplished with 5% normal horse serum/1% normal

goat serum and 3,3' diaminobenzidine tetrahydrochloride (DAB) was used as substrate for visualization of immunoreactive products. As a control, the primary anti-u-PAR antibody was replaced with an equivalent amount of non-immune rabbit IgG. Counterstaining was achieved with hematoxylin.

Southern Blotting

Southern blotting was carried out as described by our lab.³⁰ Tail tissues were digested with proteinase K in a buffer containing 0.5% SDS, 25 mmol/L EDTA, 10 mmol/L NaCl, and 10 mmol/L Tris-Cl (pH 8.0). Genomic DNA purified by multiple extractions with phenol/chloroform: isoamyl-alcohol (24:1) and RNA-ase treatment (37°C, 1 hour) was digested at 37°C, overnight with restriction endonucleases (*EcoRV*/Asp718- for the -0.4 and -1.5 and *EcoRV*/*EcoRI* for the -8.5 u-PAR LacZ constructs, respectively). Cleavage products were resolved by electrophoresis and transferred to a nylon filter after acid/base treatment. The transgene was detected using a radioactive *Clal*/*EcoRV*-generated 0.3 kb cDNA complementary to the LacZ coding sequence.

Chromatin Accessibility Assays

These were performed essentially as described elsewhere.^{31,32} Briefly, 5×10^6 cells were resuspended in hypotonic buffer (10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl₂, 0.15 mmol/L spermine, and 0.5 mmol/L spermidine), and incubated on ice for 5 minutes. NP-40 was then added (final concentration, 0.5%) and the cells vortexed and incubated on ice for another 5 minutes. Nuclei were harvested by centrifugation, washed twice with RE buffer (10 mmol/L Tris-HCl, pH 7.4, 50 mmol/L NaCl, 10 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.2 mmol/L EGTA, 1 mmol/L DTT, 0.15 mmol/L spermine, and 0.5 mmol/L spermidine), and then resuspended in 90 μ l buffer H (Roche Applied Science, Indianapolis, IN). Restriction digestions were performed at 37°C with 100 U of *PstI* for the indicated times. The reactions were terminated with 2X proteinase K buffer (100 mmol/L Tris-HCl, pH 7.5, 200 mmol/L NaCl, 2 mmol/L EDTA, 1% SDS). Reaction mixtures were then supplemented with 50 μ l 2X proteinase K buffer, 50 μ l RE buffer, and 50 μ g RNase A and incubated for 30 minutes. Subsequently, 80 μ g proteinase K was added and the reaction continued at 37°C overnight. Genomic DNA was harvested by phenol/chloroform extractions and ethanol precipitation, and then dissolved in TE buffer. Real-time PCR was then used to determine the amount of uncut DNA.

Real-Time PCR

This method was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. DNA samples (100 ng) were mixed with 1X SYBR Green PCR master mix (Applied Biosystems) and 1 μ mol/L of each primer (-1295/-1275- 5'GCAGTGGTGCATCAT-

AGCTC3' and -1192/-1213- 5'AGTGGCCCGTACTTG-TAGTCCT 3) and loaded into the ABI Detection System. After incubating at 95°C for 10 minutes to activate the AmpliTaq Gold enzyme, the mixes were subjected to 40 amplification cycles (15 seconds at 95°C for denaturation and 1 minute for annealing and extension at 60°C).

After PCR, a Ct value was obtained using the software provided by the manufacturer. A Δ Ct value reflecting the difference in Ct values between the digestion samples at different time points (Sn) and undigested sample (S0) was calculated³² by subtracting the Ct value for the former from the latter ie, Δ Ct = Ct(S0) - Ct(Sn). The *PstI* uncut DNA amount in the digested samples was then calculated by raising 2 to the Δ Ct power followed by multiplying by 100 (100 ng is the uncut DNA amount for the undigested sample), ie, uncut DNA amount for the samples = $100 \times 2^{\Delta Ct}$. Therefore, the cut DNA amount equals to $100 - 100 \times 2^{\Delta Ct}$, and the cut % was calculated as follows: cut % = $100 \times (\text{cut amount}/\text{total amount}) = 100 \times ((100 - 100 \times 2^{\Delta Ct})/100) = 100 \times (1 - 2^{\Delta Ct}) = 100 \times (1 - 2^{(Ct(S0) - Ct(Sn))})$.

Results

Generation of Transgenic Mice Harboring a LacZ Reporter Regulated by Varying Length 5' u-PAR Sequences

Transgenic mice bearing a LacZ reporter regulated by varying amounts of the 5' u-PAR sequence (Figure 1, A to D) were generated. Southern blotting (Figure 1, B to D) using a *Clal*/*EcoRV*-generated cDNA corresponding to the LacZ coding sequence (Figure 1A, probe) verified the presence of the integrated constructs bearing 0.4, 1.5, and 8.5 kb of upstream sequence (Figure 1, B to D, respectively) in the transgenic mice. Subsequent analysis of transgene expression was performed on mice showing a similar band intensity in Southern blots indicative of comparable copy number.

Expression of a LacZ Reporter Driven by 0.4 kb of u-PAR Upstream Sequence

Because our studies using transiently transfected plasmids in tissue culture had revealed that 0.4 kb of upstream sequence was sufficient for "driving" expression of this gene, we first asked whether this amount of upstream sequence could also regulate expression of a LacZ reporter in transgenic mice in a manner coincident with expression of the endogenous u-PAR gene. In normal mice, the placenta, characterized by its extensive tissue remodeling reminiscent of invasive cancer, has the highest constitutive expression of the u-PAR gene of all organs examined.³³ Thus, we first determined whether the -0.4 u-PAR LacZ transgene was expressed in this organ. F1 mice shown to be positive for this transgene (Figure 1B) by Southern blotting were mated, 14-day placentas harvested, fixed, and either stained with X-gal or subjected to immunohistochemistry for the endoge-

Figure 1. Verification of transgene integration into the mouse genome by Southern blotting. **A:** Schematic showing the various u-PAR LacZ transgenes used. Relevant restriction cleavage sites and predicted fragment sizes are indicated. The open rectangle indicates the LacZ cDNA probe used in Southern blotting. **B** to **D:** DNA (10 μ g for the shorter two constructs and 20 μ g for the longest transgene) from mice bearing the -0.4, -1.5, and -8.5 u-PAR LacZ constructs (**B** to **D**, respectively) was digested with *EcoRV*Asp718 (**B** and **C**) or *EcoRV*/*EcoRI* (**D**). Digested DNA was resolved by gel electrophoresis and, after transfer to a nylon filter, probed with a *Clal*/*EcoRV*-generated cDNA complementary to the LacZ coding sequence.

nous u-PAR protein. Expectedly, strong u-PAR protein immunoreactivity was evident in this tissue (Figure 2A, arrows) and staining was specific because it was abolished with non-immune rabbit IgG. More importantly, transgene expression as indicated by X-gal staining (Figure 2B, right, arrows) was evident in the placentas although the X-gal staining intensity was weak. LacZ ex-

pression was evident in placentas derived from at least four separate founder lines (Table 1). No X-gal staining was observed in placentas derived from breeder mice (Figure 2B, left). Thus, the 0.4-kb upstream sequence directs weak u-PAR expression in this tissue.

Although it has not been reported previously, we made the serendipitous observation that the epididymis strongly

Figure 2. Expression of a LacZ reporter driven by 0.4 kb of u-PAR 5' flanking sequence in the placenta and epididymis. **A** and **B:** Placentas (approximately 14 days) derived from mice positive for the -0.4 u-PAR LacZ transgene, or from breeder mice, were either subjected to immunohistochemistry (**A**) using a specific rabbit anti-mouse u-PAR antibody or an equivalent amount of non-immune rabbit IgG or stained with 1 mg/ml X-gal (**B**). Counterstaining was achieved with hematoxylin and Nuclear Fast Red for **A** and **B**, respectively. **B:** Arrows indicate the LacZ expression and the magnification was ×40. **C** and **D:** Epididymal tissue derived from transgenic or breeder mice from approximately 10-week-old male mice was fixed and either subjected to immunohistochemistry (**C**) for endogenous u-PAR protein or stained with X-gal (**D**) as described above. For the LacZ staining, magnifications were ×40.

expresses endogenous u-PAR protein as evident by immunohistochemistry (Figure 2C, arrows). We therefore determined if 0.4 kb of upstream sequence was sufficient for regulating LacZ expression in this tissue (Figure 2D). Although no X-gal staining was observed in breeder mice (Figure 2D, left), widespread strong LacZ expression was evident (Figure 2D, right, arrows) in the pseudostratified columnar epithelial cells of the epididymis derived from mice positive for the construct. Moreover, LacZ expression was coincident with the strong immunoreactivity evident in

this tissue. On the other hand, spermatozoa apparent in the luminal space were negative for expression of the endogenous u-PAR and the transgene. Thus for two organs that constitutively express the endogenous mouse u-PAR gene, 0.4 kb of 5' flanking sequence weakly activates the LacZ reporter in the placenta whereas this reporter is strongly activated in the epididymis.

Kristensen and co-workers³⁴ previously reported that the luminal surface epithelial cells of the gastrointestinal tract were positive for endogenous u-PAR expression,

Table 1. Summary of LacZ Expression Data

Construct	Transgene Expression							
	Placenta		Colon		Skin (wounding)		Epididymis	
	Expression	Intensity	Expression	Intensity	Expression	Intensity	Expression	Intensity
0.4 u-PAR	4/4	+	0/5	—	0/4	—	2/3	+++
1.5 u-PAR	3/3	+	5/6	++	0/4	—	7/7	+++
8.5 u-PAR	5/6	++	4/5	+	0/4	—	3/3	+++

Figure 3. Expression of a LacZ reporter driven by 1.5 kb of u-PAR 5' flanking sequence in the luminal epithelial cells of the colon. **A:** Transverse sections of the large intestine were subjected to immunohistochemistry for endogenous u-PAR protein using an antibody specific for the mouse protein or, as a control, an equivalent amount of non-immune IgG. **B:** Breeder mice (**left**) or mice positive for the -1.5 u-PAR LacZ reporter construct (**right**) were stained with X-gal as described in Figure 2. Magnifications: **B**, $\times 40$; (**left**) and $\times 100$ (**right**).

possibly related to their ability to shed from the crypt in the normal progression from the basal to the apical region. Thus, we examined LacZ expression in the colon derived from multiple founders. However, in no case was LacZ expression evident in this tissue (Table 1). Thus, it is likely that regulatory region(s) outside of the 0.4-kb upstream fragment are required for expression in the crypt cells at the luminal surface.

1.5 kb of u-PAR Upstream Sequence Directs LacZ Expression in the Luminal Surface Colonic Epithelial Cells As Well As in the Placenta

We therefore investigated the expression of a LacZ reporter regulated by a longer fragment (1.5 kb) of the

u-PAR upstream sequence. F1 offspring positive for the transgene were sacrificed and the large intestine was harvested and analyzed either for endogenous u-PAR protein or for LacZ expression. Expectedly, the luminal surface epithelial cells were strongly positive for u-PAR protein (Figure 3A) and abolition of this immunoreactivity by omission of the anti-u-PAR antibody confirmed the specificity. Parallel studies using X-gal to stain for LacZ expression confirmed strong expression of the transgene in the luminal epithelial cells of the colon (Figure 3B, right, arrows) coincidental with the expression of the endogenous u-PAR gene. In contrast, colon from non-transgenic breeder mice proved negative for LacZ expression (Figure 3B, left panel). LacZ expression in the transgenic mice was evident in mice derived from multiple founders

Figure 4. Induction of u-PAR expression in the skin requires elements outside of the 1.5-kb upstream sequence. **A** and **B**: Incisional, superficial wounds were made in mice positive for the transgene. After varying times, mice were sacrificed and the skin analyzed for endogenous u-PAR and GAPDH (as a loading control) proteins by Western blotting (**A**) or for transgene expression (**B**) as described in the legend to Figure 2. Counterstaining was achieved using Nuclear Fast Red. **C** and **D**: Mice positive for transgene integration, were treated with 100 μ g PMA for 24 hours, sacrificed, tissues fixed and either examined for endogenous u-PAR protein by immunohistochemistry (**C**) or for LacZ expression (**D**) as described in the legend to Figure 2. **E**: Total RNA extracted from the wounded tissue was analyzed by Northern blotting for LacZ mRNA. mRNA integrity was determined by re-probing of the blot with a GAPDH cDNA. The positive control corresponds to total RNA from LacZ-positive HT-1080 cells.

(Table 1). Thus, these findings suggest that a region residing between -0.4 and -1.5 kb is required for expression of the endogenous u-PAR gene in the luminal epithelial cells of the colon.

Similar to the shorter construct, the -1.5 -kb regulatory sequence also directed LacZ expression in the epididymis and in the placenta (data not shown). However, we saw no greater LacZ staining (either in intensity or the amount of tissue) in the placenta (Table 1), suggesting that this longer fragment provided no additional regulatory elements for controlling u-PAR expression in this tissue.

During re-epithelialization, u-PAR mRNA expression is strongly expressed by the keratinocytes at the leading edge of the mouse skin wound.⁴ Thus, we also determined whether 1.5 kb of u-PAR 5' flanking sequence was sufficient for transgene expression in repair of this tissue. Mice positive for the transgene were wounded superficially and, after varying times, sacrificed and analyzed either for endogenous u-PAR expression by Western blotting (Figure 4A) or for LacZ expression (Figure 4B). As expected, endogenous u-PAR expression was strongly induced within 24 hours of wounding as apparent from Western blotting (Figure 4A). Surprisingly, however, there was absolutely no LacZ expression in the wounded area at any of the time points examined (24 to 72 hours after wounding). The absence of LacZ expression was not restricted to mice from a particular founder because transgene expression was undetectable in the offspring from at least three other founder lines (Table 1). The

absence of LacZ expression could also not be attributed to a silencer element residing between -0.4 and -1.5 kb; studies with mice generated using the shorter construct also indicated undetectable LacZ expression in response to skin wounding (Table 1).

Phorbol ester is a well-established potent inducer of u-PAR transcription.^{23,35} Consequently, parallel experiments were undertaken to determine whether LacZ expression in the skin could be induced by this agent. Mice positive for the transgene were treated with 100 μ g PMA³⁶ or carrier (ethanol) for 24 to 96 hours. Immunohistochemical staining for endogenous u-PAR protein (Figure 4C) in the PMA-treated skin showed intense u-PAR immunoreactivity (see boxed area) which was maintained throughout the time points examined (24 to 72 hours). No staining was apparent in skin receiving the carrier alone. Similar to the previous results of the skin wounding experiment, we observed no LacZ expression (Figure 4D) in response to the phorbol ester, which induces a parallel increase in the endogenous gene expression. LacZ expression was absent over the three time points examined (24, 48, and 72 hours) and from mice generated from at least two other founder lines.

To rule out the possibility that the absence of LacZ expression was due to the lack of X-gal penetration into the excised skin tissue, we performed Northern blotting for LacZ mRNA. Mice were wounded and sacrificed at the indicated time points, and wounded tissue was analyzed for LacZ mRNA (Figure 4E). No LacZ mRNA was detected by Northern blotting in the wounded skin (Fig-

Figure 5. A 8.5-kb u-PAR promoter fragment directs optimal expression of a LacZ reporter in the placenta but represses its expression in colonic cells. F1 mice positive for the -8.5 u-PAR LacZ transgene were mated. After 14 days, the females were sacrificed, placental tissue fixed and analyzed for LacZ expression (**A**) as described in the legend to Figure 2. **Arrows** indicate pronounced LacZ expression. Magnification, $\times 20$. **B:** Mice positive for transgene integration were sacrificed, the colon fixed, stained with X-gal, and counterstained with Nuclear Fast Red, respectively, as described in the legend to Figure 2. Magnification, $\times 40$. **C:** Male mice were sacrificed at approximately 10 weeks of age, and epididymal tissue was fixed and analyzed for LacZ expression using X-gal and as described in the legend to Figure 2. Magnification, $\times 20$.

ure 4E and data not shown), although this transcript was readily detectable in LacZ-expressing HT-1080 cells (Figure 4E, positive control). We therefore conclude that, while 1.5 kb of u-PAR 5' flanking sequence regulates expression of the endogenous u-PAR gene in the epididymis and luminal epithelial cells of the colon, it does not allow for inducible expression in response to skin wounding or phorbol ester treatment.

The -8.5 u-PAR LacZ Transgene Shows Greater Activity in the Placenta but Is Weaker than the -1.5 u-PAR LacZ Construct in the Colon

Based on precedents with other genes including the tissue-type plasminogen activator³⁷ and the 92-kd type IV collagenase (MMP-9),³⁸ we entertained the notion that a fragment longer than -1.5 kb contained additional elements regulating u-PAR expression. To address this issue, we used transgenic mice harboring a LacZ reporter flanked by 8.5 kb of upstream sequence. F1 offspring positive for the transgene were bred, and 14-day placentas were harvested and stained with X-gal (Figure 5A). Similar to the two shorter constructs, X-gal staining was easily apparent in this tissue. However, a fourfold greater intensity (determined with Optima quantitation software) of the X-gal staining compared with placentas derived from mice harboring either of the two shorter constructs (see Figure 2B) indicated stronger transgene expression with the longer promoter fragment. These findings were reproducible using mice from at least five independent founder lines in which densitometric analysis (Optima quantitation software) indicated between a twofold and 15-fold increase in transgene activity for the -8.5 u-PAR LacZ construct (Table 1). Since the observations were reproducible with multiple founder lines, it is unlikely that the more prominent LacZ expression evident with the longer fragment is an artifact of varying transgene insertion sites and/or a different gene copy number. These data would suggest the existence of additional

regulatory elements residing between -1.5 and -8.5 kb, directing u-PAR expression in the placenta.

We also examined LacZ expression in the luminal epithelial cells of the colon from mice positive for the -8.5 u-PAR LacZ construct (Figure 5B; Table 1). Similar to the results generated with the -1.5 u-PAR LacZ construct, X-gal staining was evident in the apical cells of the colonic crypts (Figure 5B). However, we found that staining intensity was reduced 50% (determined using Optima quantitation software) and a smaller amount of tissue stained positive for the LacZ reporter when compared with mice harboring the two shorter u-PAR promoter fragment transgenes. These findings were consistent among mice derived from four separate founders (Table 1) with densitometric analysis indicating a reduction in staining intensity ranging from 40% to 85%. These data would implicate an element residing between -1.5 and -8.5 kb that represses u-PAR expression in the colonic crypt cells (at the luminal surface).

Since the above studies indicated the existence of elements residing between -1.5 and -8.5 kb regulating u-PAR expression in the placenta and the colon, we considered the possibility that epididymal expression of the transgene was also subject to regulatory sequences residing in this region. Thus, epididymis derived from F1 mice positive for the -8.5 u-PAR LacZ construct was analyzed for transgene expression (Figure 5C; Table 1). However, both the intensity of the X-gal staining and the amount of the epididymal tissue positive for staining was unchanged relative to tissues derived from mice harboring either of the shorter constructs. We conclude that the upstream sequence residing between -0.4 and -8.5 kb provides no additional regulatory elements governing expression of the endogenous gene in the epididymis.

Since no LacZ expression was evident in skin re-epithelialization using the -0.4 and -1.5 u-PAR LacZ constructs, it was possible that sequences residing between -1.5 and -8.5 kb were necessary for expression in this tissue. However, like the other two reporter constructs, LacZ expression could not be detected in re-epithelializing skin (from multiple founders) subsequent to wound-

Figure 6. Increased chromatin accessibility in the u-PAR promoter spanning $-1295/-1192$ in high u-PAR-expressing colon cancer cells. **A:** Schematic indicating the u-PAR promoter region amplified by real-time PCR after *PstI* digestion of chromatin. **B to D:** Nuclei isolated from RKO or PMA (100 nmol/L/1 hour)-treated, and untreated GEO cells were incubated with 100 U *PstI* for the indicated times. DNA was purified and either subjected to agarose gel electrophoresis followed by ethidium bromide staining (**B**) or the amount of *PstI*-cut genomic DNA quantified by real-time PCR (**C** and **D**). The positive control in **B** represents amplification of the u-PAR promoter fragment derived from the -8.5 u-PAR LacZ transgene. The data are shown as average values $\pm$ SD of three separate determinations. Statistical analysis was performed using Student's *t*-test.

ing, despite clear evidence of endogenous u-PAR expression (Table 1). Thus, regulatory sequences residing outside of the -8.5 -kb fragment must be required for skin-specific expression.

Chromatin Accessibility Assays of Genomic DNA Suggest a Putative Regulatory Region in the u-PAR Promoter Spanning $-1295/-1192$

Our transgenic data indicated that a region residing between -1.5 and -0.4 kb was required for optimal u-PAR expression in colonic cells in the transgenic mice. To further delineate the region required, we performed chromatin accessibility assays on intact nuclei derived from colon cancer cell lines that express a large ($\sim 3 \times 10^5$ per cell-RKO) or small ($\sim 10^4$ per cell-GEO) number of u-PAR.^{23,39} Nuclei were prepared from RKO and GEO colon cancer cells and incubated with *PstI*, which cleaves the u-PAR promoter sequence at -1269 (Figure 6A). Subsequently, DNA was purified and assayed by real-time PCR for the amount of *PstI*-cut genomic DNA using

primers that span the restriction site (Figure 6A). Gel electrophoresis confirmed that the PCR reaction specifically amplified the genomic u-PAR promoter DNA sequence (Figure 6B, line). Chromatin spanning $-1295/-1192$ from u-PAR-rich colon cancer cells (RKO) showed increased accessibility to the endonuclease when compared with that derived from the u-PAR-deficient GEO cells (Figure 6C). These differences were statistically significant ($P < 0.01$) at all time points. To further corroborate these data, similar experiments were conducted for GEO cells treated with PMA, a known transcriptional inducer of the u-PAR gene as previously shown by our group and others.^{23,35} Chromatin from GEO cells, induced for u-PAR expression by the phorbol ester, showed enhanced hypersensitivity (Figure 6D) in the $-1295/-1192$ region when compared with chromatin derived from untreated cells in which u-PAR expression is low.

A search ([http://www.cbil.upenn.edu/cgi-bin/tess/tess33?RQ = WELCOME](http://www.cbil.upenn.edu/cgi-bin/tess/tess33?RQ=WELCOME)) for putative consensus transcription factor binding sites revealed several matches, including c-Ets 1 (-1267), Sp1 (-1245 , -1243 , -1232),

C/EBP α (–1291), and C/EBP β (–1258). Ets and Sp1 binding sites have previously been implicated in the regulation of constitutive and inducible gene expression of other genes by diverse stimuli including phorbol ester.^{24,40–43} Thus, taken together, these assays suggest a potential regulatory region spanning the –1295/–1192 sequence required for driving u-PAR expression in colonic cells.

Discussion

The study herein reports on the use of transgenic mice to identify novel u-PAR regulatory regions required for tissue-specific expression. Our investigations revealed that while 0.4 kb of upstream sequence was sufficient for regulating reporter activity in the epididymis, a region residing between –0.4 and –1.5 kb was critical for expression in the luminal epithelial cells of the colonic crypt. Moreover, an additional region between –1.5 and –8.5 kb yielded optimal transgene expression in the placenta while repressing expression in the apical colonic crypt cells.

The identification of novel regions directing u-PAR expression in a tissue-specific manner is reminiscent of previous transgenic studies with other genes^{37,44,45} and highlights the utility of this approach. Yu and co-workers³⁷ determined that 9.5 kb of upstream sequence of the tissue-type plasminogen activator (t-PA) gene was required for constitutive and inducible expression in specific regions of the brain including the dentate gyrus, hippocampus, and the thalamus. For the gonadotropin-releasing hormone receptor gene, 9.1 kb of 5' flanking sequence, which is devoid of transcriptional activity in tissue culture of gonadotrope-derived cell lines, directs tissue-specific expression in transgenic mice and is highly responsive to both gonadotropin-releasing hormone and estradiol.⁴⁶ Transgenic approaches also identified previously unknown elements (eg, –2722/–7745) critical for MMP-9 expression in osteoclasts and migrating keratinocytes and in development.^{38,47} Further, an element spanning –655/+52 required for expression of the Rp365 gene in a tissue-specific manner⁴⁵ was discovered using transgenic mice.

The present study advances our previous investigations of u-PAR expression conducted in tissue culture using transiently transfected plasmids. In those *in vitro* studies, we demonstrated that 0.4 kb of upstream sequence was sufficient for both the constitutive and inducible (c-Src) expression of the u-PAR gene.^{23,28} Thus, we were surprised by the finding that 1.5 kb of 5' flanking sequence was required for expression in apical colonic crypt cells of the transgenic mice. Three potential explanations might reconcile these observations. First, the requirements for u-PAR expression in colon cancer may differ from those required for expression of this gene in the normal tissue (ie, the luminal cells of the colonic crypt). Second, it may be that analyses performed with transiently transfected non-integrated constructs, as carried out in our previous studies, do not accurately reflect transcriptional requirements for the endogenous gene as

reported for the fatty-acid synthase gene.⁴⁸ Indeed, while integrated transgenes are appropriately chromatinized, transiently transfected plasmids are known to be poorly chromatinized and there is now abundant evidence for a prominent role of the chromatin environment in regulating gene expression.^{49–51} Third, it is possible that the differing transcriptional requirements reflect the contribution of the stromal compartment (absent in our tissue culture experiments) to u-PAR expression in the transgenic mice.

The optimal LacZ expression in the epididymis with the shortest u-PAR promoter construct (–0.4 u-PAR LacZ) deserves comment. We previously reported, albeit using tissue-cultured cells, that u-PAR expression required a GC-rich region of the u-PAR promoter –152/–135 bound with Sp1/Sp3.²⁸ It is interesting to note that the columnar epithelial cells of the epididymis strongly express Sp1⁵² bringing up the possibility that optimal u-PAR expression in this tissue reflects trans-activation by this transcription factor.

The absence of transgene expression in the skin, in response to either wounding or phorbol ester, was surprising and deserves comment. Several explanations are possible. First, it may be that the high level of u-PAR mRNA evident in re-epithelializing skin⁴ is largely a consequence of message stabilization.²¹ Alternatively, it may be that sequences outside of the –8.5-kb fragment examined are necessary for inducible expression in the skin akin to that reported for the MMP-9 gene where macrophage-specific expression required sequences outside of –522/+12.⁵³ A third possibility is that the human 5' flanking sequence used in our studies lacks a mouse-specific regulatory region required for skin expression or that the *cis*-elements that we previously determined to contribute to PMA responsiveness at least in cultured human cell lines are not conserved in the murine sequence. However, we found that the murine regulatory sequence does indeed include *cis*-elements that we previously demonstrated to be critical for induction of expression of the human u-PAR gene. Thus, like the corresponding human sequence, the murine sequence includes AP-1- (–72) and AP-2-like sequences (–42) (1-bp mismatch) as well as a consensus Sp1 motif all located within the 5' flanking 200-bp sequence. We previously showed that these transcription factor binding sites were required for PMA-inducible u-PAR gene expression in tissue-cultured human cells.^{23,24}

In conclusion, using transgenic approaches, we have identified novel regulatory regions in the u-PAR flanking sequence required for tissue-specific expression in the placenta and colon. These transgenic mice may be of utility in screening candidate agents for their ability to repress u-PAR transcription *in vivo* and could provide a tool for identifying regulatory regions directing u-PAR expression in invasive cancer.

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