MOLECULAR AND CELLULAR BIOLOGY, Mar. 1997, p. 1075–1083 Vol. 17, No. 3
0270-7306/97/$04.00+0
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Posttranscriptional Regulation of Urokinase Receptor mRNA: Identification of a Novel Urokinase Receptor mRNA Binding Protein in Human Mesothelioma Cells

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Received 19 June 1996/Returned for modification 19 July 1996/Accepted 26 November 1996

Treatment of human pleural mesothelioma (MS-1) cells with phorbol myristate acetate (PMA) and cycloheximide results in 17- and 10-fold, respectively, increases in steady-state expression of urokinase-type plasminogen activator receptor (uPAR) mRNA. Studies of transcriptional inhibition by actinomycin D showed four- and sixfold extensions of uPAR mRNA half-life in MS-1 cells treated with PMA and cycloheximide, respectively, suggesting that uPAR gene expression involves a posttranscriptional regulatory mechanism. Using gel mobility shift and UV cross-linking assays, we identified a 50-kDa uPAR mRNA binding protein (uPAR mRNABp) that selectively bound to a 51-nucleotide (nt) fragment of mRNA corresponding to the uPAR coding region. We investigated the possibility that this 51-nt protein binding fragment of uPAR mRNA contains regulatory information for message stability. Chimeric β-globin/uPAR/β-globin mRNA containing the 51-nt protein binding fragment was able to destabilize otherwise stable β-globin mRNA. Conversely, a control chimeric β-globin/uPAR/β-globin mRNA containing a 51-nt fragment of the uPAR coding region that does not bind uPAR mRNABp was stable under identical conditions. Binding of uPAR mRNABp to uPAR mRNA was abolished after treatment with cycloheximide and rapidly down-regulated by PMA. These data suggest that the 51-nt protein binding fragment of uPAR mRNA may be involved in mRNA turnover as well as in cycloheximide-induced uPAR message stabilization. Our results indicate a novel mechanism of uPAR gene regulation in which *cis* elements within a 51-nt coding region interact with a uPAR mRNABp to regulate uPAR message stability.

Normal and neoplastic cells contain surface receptors for urokinase-type plasminogen activator (uPA). The receptor (uPAR) binds both uPA and its proenzyme (8). Because many biological activities of uPA depend on association with its receptor, uPAR plays a central role in localized uPA-mediated plasminogen activation. Regulation of this receptor could thus influence the range and extent of uPA activity.

In human mesothelial (MeT5A) or mesothelioma (MS-1) cells, we found that phorbol myristate acetate (PMA) increased both uPAR mRNA and uPAR expression on the cell surface (26). Others reported similar findings for other cell lines (19, 22, 32), and earlier reports showed that PMA and cycloheximide increased uPAR mRNA in various cells (16, 22, 27). These results suggest that short-lived proteins may influence mRNA stability. It has previously been reported that uPAR mRNA contains a single-copy AUUUA sequence in the 3′ untranslated region (25) and that mRNA species containing multiple copies of AUUUA sequences, such as granulocyte-macrophage colony-stimulating factor mRNA, are selectively degraded (27).

The steady-state level of any mRNA reflects its rate of decay as well as synthesis. Moreover, the half-lives of many mRNAs seem to be the major determinants of their abundance; that is, mRNA levels correlate directly with persistence of the mRNA in the cytoplasm rather than with the rapidity of synthesis. Among the various mechanisms by which different cell types and tissues influence mRNA stability, regulation of mRNA decay is a potentially important process for determining the level of gene expression. For instance, high-level lability of an mRNA allows rapid down-regulation of protein synthesis after arrest of transcription and thus provides an efficient mechanism for transient expression. This type of regulation is a key feature in normal physiological processes (3, 25), and down-regulation of this type of control mechanism can contribute to neoplastic transformation (13, 24).

Several determinants of rapid c-*fos* or c-*myc* mRNA turnover were found in both coding and untranslated regions (29, 33). AU-rich sequences in heterologous mRNA, as well as at least two other regions that confer instability independently, appear to influence the growth and metastasis of tumors. We report here the influence of various proinflammatory agonists on the stability of uPAR mRNA in cultured human mesothelioma (MS-1) cells. In addition, we demonstrate that a specific sequence of uPAR mRNA interacts with a novel binding protein (uPAR mRNA binding protein [uPAR mRNABp]) and that this interaction appears to destabilize uPAR mRNA. Overexpression of a chimeric β-globin/uPAR/β-globin gene containing a 51-nucleotide (nt) uPAR mRNABp binding sequence inserted into the β-globin cDNA coding region produced a destabilized chimeric mRNA transcript.

MATERIALS AND METHODS

Materials. Culture media, penicillin, streptomycin, fetal calf serum (FCS), proteinase K, and RNase T_1 were purchased from Gibco BRL Laboratories (Grand Island, N.Y.). Tissue culture plastics were from Becton Dickinson Labware (Lincoln Park, N.J.). Tris base, dithiothreitol, RNase A, heparin, phenylmethylsulfonyl fluoride, ammonium persulfate, cycloheximide, PMA, tRNA, and homoribonucleotides poly(A), poly(C), poly(G), and poly(U) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Acrylamide, bisacrylamide, and related chemicals were from Bio-Rad Laboratories (Richmond, Calif.). In vitro transcription and RNase protection assay kits were purchased from Ambion (Austin, Tex.). HEPES and other reagents were from Fisher Scientific (Pitts-

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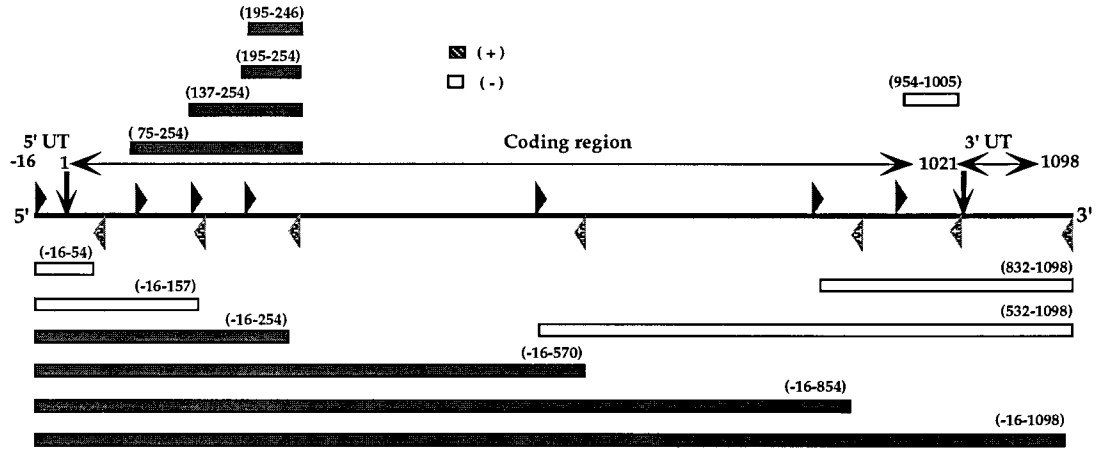

Fragment	Foward primer (5'-3')	Name	Reverse Primer (5'-3')	Name
(-16-54)	GAGCTGCCCTCGCGACAT	F -16-21	TGGGACGCAGGTGTGG	R 39-54
(-16-157)	GAGCTGCCCTCGCGACAT	F -16-21	AGCGCACGATCGTGGT	R 142-157
(-16-254)	GAGCTGCCCTCGCGACAT	F -16-21	ATCTTCAAGCCAGTCCGATA	R 235-254
(-16-570)	GAGCTGCCCTCGCGACAT	F -16-21	CAGGAAGTGGAAGGTGTCGT	R 551-570
(-16-854)	GAGCTGCCCTCGCGACAT	F -16-21	ACATCAATGTGGTTCATGCT	R 835-854
(-16-1098)	GAGCTGCCCTCGCGACAT	F -16-21	GGTCACACAGCAAGTCTGTA	R1079-1098
(532-1098)	TCCAATGGTTTCCACAACGA	F 532-551	GGTCACACAGCAAGTCTGTA	R1079-1098
(532-1098)	TTCAGCATGAACCACATTGA	F 832-851	GGTCACACAGCAAGTCTGTA	R1079-1098
(75-254)	CATGCAGTGTAAGACCAACG	F 75-94	ATCTTCAAGCCAGTCCGATA	R 235-254
(137-254)	GCAGGACCACGATCGTGC	F 137-154	ATCTTCAAGCCAGTCCGATA	R 235-254
(195-254)	AAGCTGTACCCACTCAGA	F 195-212	ATCTTCAAGCCAGTCCGATA	R 235-254
(195-246)	AAGCTGTACCCACTCAGA	F 195-212	GCCAGTCCGATAGCTCAG	R 229-246
(954-1005)	TCACCCTGCTAATGACTGCC	F 954-973	GGTCCAGAGGAGAGTGCCTG	R 996-1005

FIG. 1. Deletion map of uPAR to identify the protein binding site on uPAR mRNA. Different-size uPAR cDNA fragments were made by PCR-based deletion. These fragments were cloned into TA cloning vector PCR II and transcribed in vitro, using [^{32}P]UTP. Deletion transcripts were subsequently used as probes for mobility shift studies to localize the protein binding sequence. Numbers indicate the distance (in nucleotides) downstream from the translation initiation codon of uPAR mRNA. The lengths of various protein coding regions are denoted by the bars of various lengths; + indicates detection of a band shift complex; − indicates that no band shift complex was detected with the RNA probes. Results are based on gel mobility shift assay data for various deletion transcripts. UT, untranslated region.

burgh, Pa.). Restriction enzymes were from New England Biolabs (Beverly, Mass.). [^{32}P]UTP was from Dupont (Wilmington, Del.).

Cell culture. Human mesothelioma (MS-1) or human mesothelial (MeT5A) cells were cultured and maintained in RPMI 1640 medium containing 10% FCS as described previously (10, 11). Confluent monolayers of cells in T75 flasks were switched to serum-free medium containing 1% glutamine and 0.5% bovine serum albumin for 12 h. The cells were treated in serum-free medium with PMA, lipopolysaccharide (LPS), transforming growth factor β (TGF-β), tumor necrosis factor alpha (TNF-α), or cycloheximide, using concentrations and conditions that we previously found to be effective in inducing uPAR mRNA (28). Total RNA was isolated by a standard procedure (7), and RNA stability was measured by a transcription chase method. In this system, cells stimulated with selected agonists are treated with actinomycin D for various lengths of time to inhibit ongoing transcription, after which total RNA is isolated at selected time points.

Plasmid construction. Plasmid uPAR/pBluescript was obtained from the American Type Culture Collection. The human uPAR mRNA template containing a complete coding sequence of uPAR cDNA (nt −16 to 1144) from uPAR/pBluescript was subcloned to pRC/CMV and pcDNA3 (InVitrogen, San Diego, Calif.). Various deletion products of uPAR cDNA were created by PCR, using forward and reverse primers as indicated in Fig. 1. These PCR products were cloned directly to the TA cloning vector PCR II (InVitrogen). The orientations and sequences of the clones were confirmed by sequencing. Plasmids containing full-length (1.1-kb) uPAR cDNA or its deletion products were linearized by either *Hin*dIII or *Xba*I and used as templates for in vitro transcription.

In vitro transcription. Linearized plasmids containing the human uPAR mRNA transcriptional template of complete or deletion products of uPAR cDNA were transcribed in vitro with T7 or Sp6 polymerase (Ambion). The uPAR mRNA or deletion transcripts were synthesized according to the supplier's protocol except that 50 μCi of [^{32}P]UTP (800 Ci/mmol) was substituted for unlabeled UTP in the reaction mixture. Passage through a Sephadex G-25 column removed unincorporated radioactivity. The specific activities of the products were 4.9×10^8 to 5.2×10^8 cpm/μg. The sizes of labeled mRNA transcripts were confirmed by electrophoresis on 5% urea gels.

Steady-state mRNA assessment. An RNase protection assay was used to assess the steady-state level of uPAR mRNA; this assay was done with only minor modifications to the supplier's protocol. Total RNA (20 μg) prepared as described above was incubated with ^{32}P-labeled cRNA (approximately 8×10^4 cpm) in 20 μl of hybridization buffer, heated for 3 min at 95°C, and hybridized overnight at 42°C. The hybridization mixtures were then digested with a mixture of RNase A and 200 U of RNase T_1 per ml for 30 min at 37°C. The reaction was stopped, and the protected fragments were precipitated by adding 300 μl of RNase inactivation-precipitation buffer and ethanol at −70°C. They were washed with 75% ethanol, separated on Tris borate-EDTA–5% polyacrylamide-urea gels, dried, and autoradiographed at −70°C. Individual bands were scanned with a scanning densitometer and normalized by comparison with corresponding β-actin controls.

Preparation of cytosolic extracts. MS-1 cells treated with medium alone or with proinflammatory agents were detached from culture flasks with trypsin solution, collected by centrifugation, and washed three times in phosphate-buffered saline (PBS). Cytosolic extracts were prepared by suspending the cell pellet in a buffer containing 25 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. The cells were lysed by four cycles of freezing and thawing as described previously (24) and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was collected, and a cytosolic extract was obtained. Intact nuclei from MS-1 cells were isolated by the protocol of Antalis and Godbolt (2), and a nuclear extract was prepared. The protein contents of the cytosolic and nuclear extracts were measured with a Bio-Rad protein assay kit, with serum albumin as a standard.

Gel mobility shift assay. Twenty micrograms of cytoplasmic or nuclear protein was incubated with 2×10^4 cpm of ^{32}P-labeled transcript in a mixture containing 15 mM KCl, 5 mM $MgCl_2$, 0.25 mM EDTA, 0.25 mM dithiothreitol, 12 mM HEPES (pH 7.9), 10% glycerol, and *Escherichia coli* tRNA (200 ng/μl) in a total volume of 20 μl at 30°C for 30 min. The reaction mixtures were treated with 50 U of RNase T_1 or RNase A and incubated at 37°C for 30 min. To avoid nonspecific protein binding, 5 mg of heparin per ml was added, and the mixture was incubated at room temperature for an additional 10 min. Samples were then separated by electrophoresis on a 5% native polyacrylamide gel with 0.25× Tris borate-EDTA running buffer. The gels were dried and developed by autoradiography at −70°C.

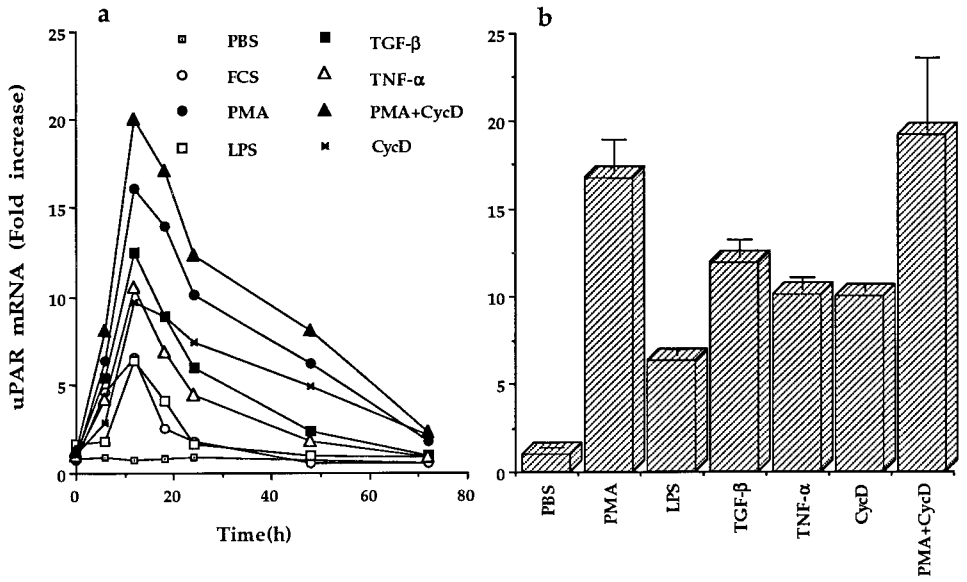

FIG. 2. Expression of uPAR mRNA in human pleural mesothelioma cells. Confluent mesothelioma cells (10^7) were treated with PBS, PMA (150 ng/ml), LPS (10 μg/ml), TGF-β (2 ng/ml), TNF-α (10 ng/ml), cycloheximide (CycD; 10 μg/ml), or a combination of PMA and cycloheximide. Total RNA (20 μg) was hybridized with a ^{32}P-labeled uPAR antisense probe and analyzed by RNase protection assay. RNase-resistant fragments were separated on 5% urea-polyacrylamide gels, dried, and autoradiographed. uPAR mRNA was measured by spectrophotometric scanning of the autoradiograms and normalized against the corresponding amount of β-actin mRNA in the sample. (a) Time-dependent expression of uPAR mRNA induction as detected by RNase protection assay. Total RNA (20 μg) from cells treated with various agents as indicated above or FCS (10%) was hybridized with the ^{32}P-labeled uPAR antisense probe at 42°C for 12 h. RNase-resistant fragments were separated on 5% urea-polyacrylamide gels, dried, and autoradiographed. uPAR mRNA at each time point was estimated by scanning densitometry after normalization to corresponding amounts of β-actin mRNA. (b) Induction of uPAR mRNA after 12 h of treatment with selected stimuli.

UV cross-linking. RNA-protein binding reactions were done as described above. Following the addition of heparin, reaction mixtures were transferred to 96-well microtiter plates and irradiated at 4°C at 2,500 μJ for 10 min with a UV-Stratalinker chamber apparatus (Strategene, La Jolla, Calif.). The samples were then boiled for 5 min and separated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel under reducing and nonreducing conditions. The gel was dried, and ^{32}P-labeled proteins were visualized by autoradiography.

Competitive inhibition by sense and antisense mRNA or polyribonucleotides. Cytosolic extracts were incubated with various amounts (0- to 500-fold excess) of unlabeled uPAR sense or antisense mRNA at 30°C for 30 min and then with ^{32}P-labeled uPAR sense mRNA for an additional 30 min at 30°C. The extracts were treated with RNase T_1 and heparin as described above, and the reaction mixture was run on 5% native gels, dried, and autoradiographed. In separate experiments to determine the specificity of the RNA-protein complex, cytosolic extracts were pretreated with a molar excess of ribonucleotide poly(A), poly(C), poly(G), or poly(U) for 30 min at 30°C prior to the ^{32}P-labeled uPAR mRNA and RNase T_1 steps.

Effects of SDS and proteinase K. Cytosolic extracts were treated with SDS (0.1%) or proteinase K (2.5 mg/ml) for 30 min at 30°C prior to addition of ^{32}P-labeled uPAR mRNA. The reaction mixtures were subjected to RNase T_1 and heparin digestion as described above, and then the complexes were resolved on 5% native gels, dried, and exposed to X-ray film at −70°C.

Effect of proinflammatory agents on uPAR mRNABp. Confluent monolayer of MS-1 cells were treated with PBS, PMA, LPS, TGF-β, and cycloheximide for 24 h, using concentrations and conditions as described earlier (28). Cells were also treated with PMA and cycloheximide for various time periods (0 to 24 h). Cytosolic extracts were prepared as described above and used for gel mobility shift assay.

Construction of β-globin/uPAR/β-globin chimeric message. The plasmid (pSP6βc) containing the complete human β-globin cDNA was kindly provided by Richard A. Spritz (University of Wisconsin). The complete human β-globin cDNA was excised from pSP6βc and inserted into the *Hin*dIII-*Xba*I site of plasmid pBluescript. Two 51-bp DNA fragments, one corresponding to the 5′ (uPAR 195-246) and other corresponding to the 3′ (uPAR 954-1005) coding sequences, were prepared from uPAR cDNA by PCR. The forward and reverse primers used for PCR amplification of uPAR 195-246 were 5′-agaattcacagctgta cccactcagag and 5′-gtgaattctgccagtccgatagctcag, respectively; those used for uPAR 954-1005 were 5′-agaattcacctgctaatgactgccag and 5′-gaattcggtccagaggag agtgcctcc, respectively. Each of these primers contained an *Eco*RI site, which permitted insertion of these fragments into the unique *Eco*RI site of human β-globin cDNA subcloned to pBluescript. The orientations and sequences of β-globin/uPAR/β-globin chimeric clones were verified by sequencing. These clones were then inserted into a eukaryotic expression vector containing the cytomegalovirus promoter, pcDNA3 (InVitrogen). MS-1 cells were transfected with the prepared chimeric plasmid constructs by lipofection using Lipofectamine (Gibco BRL), and transient transfectants were grown in culture flasks in the presence or absence of PMA (150 ng/ml) for 12 h. Total RNA was isolated at various time points after the inhibition of transcription by actinomycin D. Chimeric β-globin/uPAR/β-globin mRNA was then measured by RNase protection assay using ^{32}P-labeled antisense mRNA.

RESULTS

Effects of PMA, LPS, TGF-β, TNF-α, and cycloheximide on uPAR mRNA. Confluent monolayers of MS-1 cells treated with proinflammatory agents demonstrated increased amounts of uPAR mRNA compared to control cells treated with medium alone. Total RNA was isolated from control or treated cells at different times and analyzed for uPAR mRNA by the RNase protection method, using a uPAR antisense cRNA probe. Densitometric scanning of autoradiographs indicated a hybridization signal in serum-starved cells; however, uPAR mRNA increased severalfold in response to treatment of the cells with selected inflammatory mediators (Fig. 2a). Under the conditions of our experiments, uPAR mRNA starts accumulating at 6 h and increases with time. It is maximal within 12 h of treatment with proinflammatory reagents and then starts declining. uPAR mRNA increased 6- to 19-fold when the cells were stimulated by either PMA, LPS, TGF-β, TNF-α, or cycloheximide. As shown in Fig. 2b, PMA increased the uPAR mRNA level 17-fold as measured by densitometry; LPS increased it by 6.3-fold, and TGF-β and TNF-α increased it approximately 10- to 12-fold. Treatment with cycloheximide alone increased uPAR mRNA 10-fold, but in combination with PMA the effect was additive, with a 19-fold increase over basal levels. These results suggest that the uPAR gene is regulated at the level of transcription or by posttranscriptional destabilization of the mRNA.

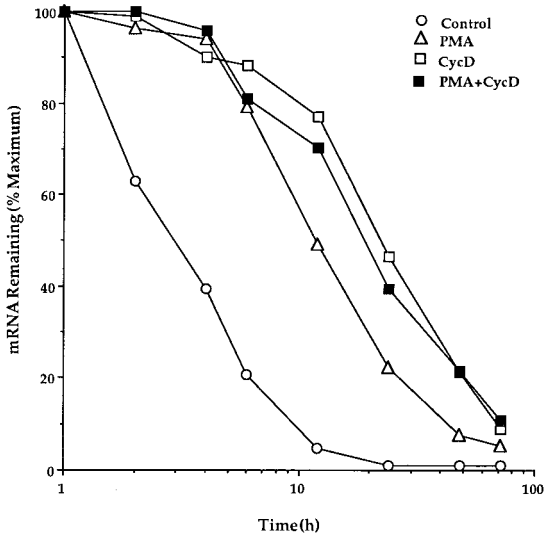

FIG. 3. Effects of PMA and cycloheximide on uPAR mRNA stability in MS-1 cells. Cells were treated with PBS, PMA, cycloheximide (CycD), or a combination of PMA and cycloheximide for 12 h, after which the medium was removed and fresh medium containing actinomycin D (10 μg/ml) was added for various periods of time. Total RNA (20 μg) was then hybridized with the ^{32}P-labeled uPAR antisense probe at 42°C for 12 h, and RNase-resistant fragments were separated on 5% urea-polyacrylamide gels, dried, and autoradiographed. uPAR mRNA from each treatment was estimated by scanning densitometry after normalization to corresponding amounts of β-actin mRNA.

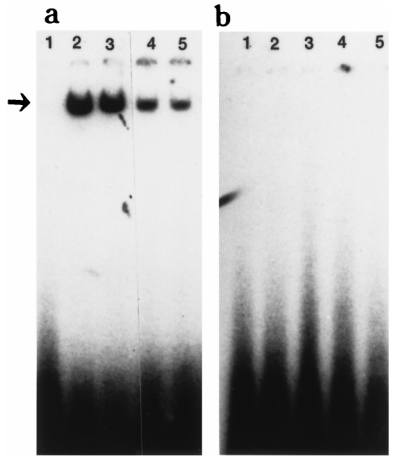

FIG. 4. Identification of uPAR mRNABp by gel mobility shift assay. Crude cytosolic and nuclear extracts (10 μg of protein) were incubated with the ^{32}P-labeled uPAR sense transcript at 30°C for 30 min and digested with RNase A or T_1 at 37°C for 30 min; heparin (final concentration, 5 mg/ml) was then added at room temperature for 10 min. RNase-resistant complexes were resolved on 5% polyacrylamide gels, dried, and autoradiographed. (a) Cytosolic extract of nonmalignant human MeT5A mesothelial cells and MS-1 human malignant mesothelioma cells. Lane 1, probe alone; lane 2, cytosolic extract of MeT5A cells treated with RNase A; lane 3, cytosolic extract treated with RNase T_1; lanes 4 and 5, corresponding extracts of MS-1 cells. (b) Nuclear extracts of MeT5A and MS-1 cells. Lanes 1 to 5 are the same as in panel a. The arrow indicates the RNA-protein complex.

Decay of uPAR mRNA. To determine the rate of degradation of uPAR mRNA, MS-1 cells were stimulated with PMA and cycloheximide separately or in combination and were treated with actinomycin D for various lengths of time. The total RNA was analyzed by the RNase protection assay, as shown in Fig. 3. The uPAR mRNA half-life was 3 h in untreated cells. However, treatment with PMA or cycloheximide alone or in combination increased the uPAR mRNA half-life to 13, 22, or 23 h, respectively. The cells remained viable (93%) after 24 h of exposure to actinomycin D, as judged by trypan blue exclusion. The data suggest the possibility that a *trans*-acting factor(s) contributes to uPAR mRNA stability.

Identification of uPAR mRNABp by gel mobility shift assay. To investigate the regulatory mechanism involved in uPAR mRNA stability, we first examined whether the uPAR mRNA transcript binds to a *trans*-acting factor(s). We used gel mobility shift assays to detect *trans*-acting factors that conferred lability on uPAR mRNA. Uniformly labeled RNA containing a full-length (1.1-kb) uPAR sequence was incubated with cytoplasmic or nuclear extracts from unstimulated MS-1 or MeT 5A cells. The binding mixtures were then digested with RNase T_1 or RNase A and treated with heparin to eliminate nonspecific RNA-protein interactions. The RNase-resistant, stable RNA-protein complex was analyzed by using 5% nondenaturing polyacrylamide gels. Figure 4a shows that a single RNase-resistant complex was detected in the gel mobility shift assay of unstimulated cells. This complex was not observed when nuclear extracts were used (Fig. 4b) or when cytosolic extracts were mixed with either tissue factor or β-globin mRNA transcripts (data not shown).

The specific interaction of the observed RNA-protein complex was confirmed by several subsequent experiments. Addition of a molar excess of homologous unlabeled RNA to the binding reaction mixture diminished complex formation in a concentration-dependent manner (Fig. 5a). However, incubation with the same amount of antisense transcript did not inhibit complex formation (Fig. 5b). Preincubation of cell lysates with ribonucleotides poly(A), poly(C), poly(G), or poly(U) did not affect complex formation (Fig. 6). Predigestion of cell lysates with proteinase K abolished formation of the complex, and addition of SDS prior to the binding reaction inhibited the RNA-protein interaction (Fig. 6). These results

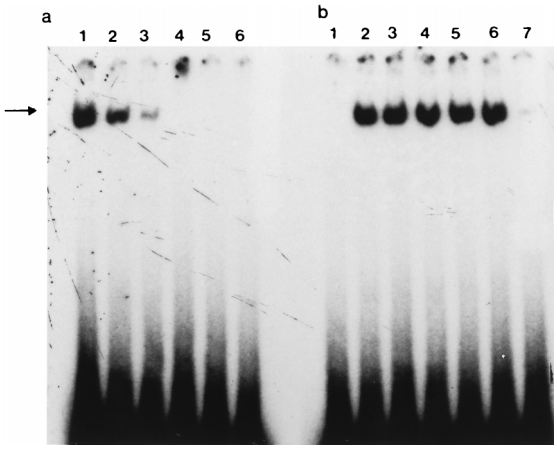

FIG. 5. Demonstration of a specific interaction between uPAR mRNA and MS-1 cytoplasmic extracts by gel mobility shift assay. ^{32}P-labeled uPAR mRNA was incubated with crude MS-1 cytoplasmic extract (10 μg of protein) for 30 min at 30°C prior to RNase T_1 digestion. The binding mixtures were analyzed by electrophoresis on a nondenaturing, low-ionic-strength 5% polyacrylamide gel. (a) ^{32}P-labeled probe mRNA was incubated with cytoplasmic lysate alone (lane 1) or with a 10-, 25-, 50-, 100-, or 400-fold molar excess of unlabeled sense mRNA transcript (lanes 2 to 6). (b) Cytoplasmic lysate was subjected to competitive inhibition with a 0- to 400-fold molar excess of unlabeled antisense mRNA transcript (lanes 2 to 6). Lane 1, free probe; lane 7, reaction mixture preincubated with a 200-fold molar excess of unlabeled uPAR sense transcript. Arrows indicate the RNA-protein complex.

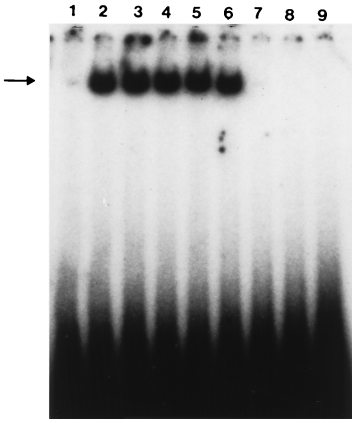

FIG. 6. Effects of polyribonucleotides, proteinase K, and SDS on uPAR mRNA interaction with the binding protein. Cell lysates were treated with a 400-fold-excess of poly(A), poly(C), poly(G), and poly(U), proteinase K (2.5 mg/ml), and 0.1% SDS for 30 min at 30°C. The ^{32}P-labeled uPAR mRNA probe was added; the mixture was digested with RNase T_1 and analyzed by gel mobility shift assay. Lane 1, probe alone; lane 2, cytosolic extract; lanes 3 to 6, cytosolic extracts pretreated with poly(A), poly(C), poly(G), and poly(U); lanes 7 to 9, extracts pretreated with SDS (0.1%), proteinase K (2.5 mg/ml), and a 200-fold molar excess of unlabeled probe. The arrow indicates the RNA-protein complex.

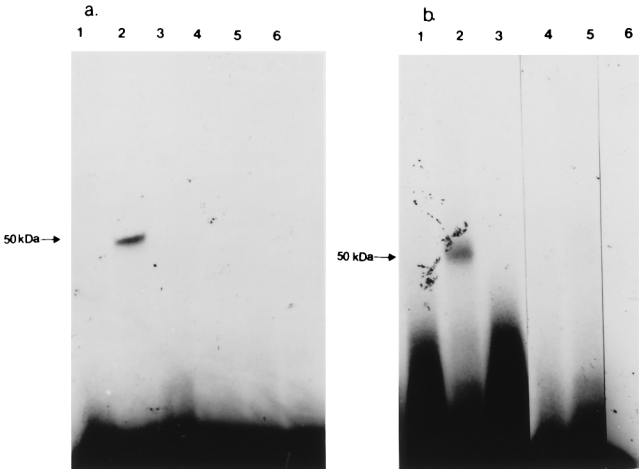

FIG. 8. Characterization of uPAR mRNABp by UV cross-linking and SDS-PAGE. Cytoplasmic extracts of cells treated with the agents used for Fig. 7 were hybridized with RNase T_1 and UV irradiated on ice at 2,500 μJ for 10 min. The samples were resolved by SDS-PAGE (10% polyacrylamide gel) under nonreduced (a) or reduced (b) conditions. Lane 1, free probe; lanes 2 to 6, extracts of cells treated with PBS, PMA, LPS, TGF-β, and cycloheximide, respectively, for 24 h. Concentrations of these agents are given in the legend to Fig. 2.

suggest that a cellular protein(s) forms a specific complex with uPAR mRNA.

Effects of proinflammatory agents on uPAR mRNABp. In cells treated with proinflammatory agents (PMA, LPS, TGF-β, and TNF-α), the RNA-protein complex was down-regulated, while in those treated with cycloheximide, it disappeared entirely (Fig. 7a). In a separate experiment, we treated MS-1 cells with PMA or cycloheximide for various lengths of time (Fig. 7b and c); PMA decreased complex formation by at least 50 to 60%, and the effect occurred as early as 3 h after treatment. However, prolonged exposure to PMA, for up to 24 h, caused no further change, and cycloheximide totally abolished the complex in a time-dependent manner. These data suggest that decreased RNA-proteins binding in the presence of proinflammatory agents may contribute to enhanced uPAR mRNA stability.

Characterization of uPARBp by UV cross-linking. We next used UV-induced cross-linking to establish the apparent molecular weight of uPAR mRNABp and to characterize the complex. The binding mixtures were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% polyacrylamide gel following UV irradiation. Figure 8a shows a 50-kDa

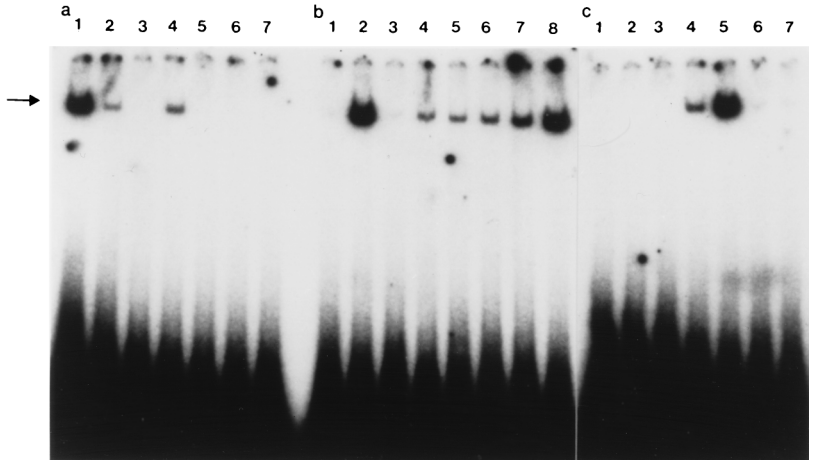

FIG. 7. (a) Effects of PMA, LPS, TGF-β, TNF-α, and cycloheximide on the uPAR mRNA-uPARBp interaction. Cells were treated with agents for 24 h at 37°C, and the cytosolic extracts were incubated with labeled uPAR mRNA. Specific complexes were identified by the gel mobility shift following RNase T_1 digestion. Extracts of cells treated with PBS (lane 1), PMA (lane 2), LPS (lane 3), TGF-β (lane 4), TNF-α (lane 5), or cycloheximide (lane 6) are shown. The concentrations of these agents are given in the legend to Fig. 2. Lane 7, free probe. (b) Time-dependent down-regulation by PMA of uPAR mRNABp and uPAR mRNA interaction. Confluent cells were treated with PMA (150 ng/ml) for 0 to 24 h. Cytosolic extracts were hybridized with the labeled mRNA probe, digested with RNase T_1, and analyzed by gel mobility shift. Lane 1, ^{32}P-labeled uPAR mRNA probe; lane 2, cytosolic extract from PBS-treated cells; lane 3, cytosolic extract incubated with a 200-fold molar excess of unlabeled sense uPAR mRNA; lane 4, cytosolic extract from cells treated with PMA (24 h); lanes 5 to 8, extract from cells treated with PMA for 12, 6, 3, and 0 h, respectively. (c) Time-dependent inhibition of uPAR mRNABp and uPAR mRNA interaction by cycloheximide. Confluent cells were treated with cycloheximide (10 μg/ml) for 0 to 24 h. Cytosolic extracts were hybridized with ^{32}P-labeled mRNA probe, digested with RNase T_1, and analyzed by gel mobility shift assay. Lanes 1 to 5, cytosolic extract of cells treated with cycloheximide for 24, 12, 6, 3, and 0 h, respectively; lane 6, cytosolic extract incubated with a 200-fold excess of unlabeled sense transcript; lane 7, ^{32}P-labeled uPAR mRNA probe. The arrow indicates the RNA-protein complex.

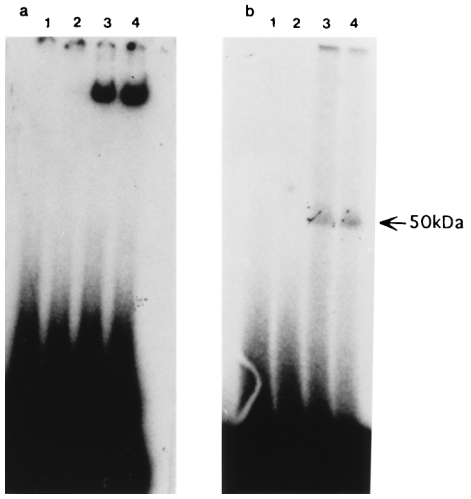

FIG. 9. Interaction between uPAR 195-246 mRNA and uPAR mRNABp by gel mobility shift, UV cross-linking, and SDS-PAGE. ^{32}P-labeled full-length uPAR or uPAR 195-246 mRNA transcripts were incubated with crude MS-1 cytosolic extracts and then subjected to gel mobility shift assays as described in the legend to Fig. 4 (a) or UV cross-linking and SDS-PAGE as described in the legend to Fig. 8 (b). Lane 1, ^{32}P-labeled uPAR mRNA probe alone; lane 2, cytosolic extract incubated with the radiolabeled uPAR 954-1005 transcript; lane 3, cytosolic extract incubated with the radiolabeled uPAR 195-246 mRNA transcript; lane 4, cytosolic extract incubated with the radiolabeled full-length uPAR mRNA transcript. The arrow indicates the RNA-protein complex.

radioactive protein band detected by this method in resting cells. Treatment of UV-cross-linked samples with β-mercaptoethanol also yielded a single protein band of the same molecular mass (Fig. 8b). The band was not visible in samples treated with PMA, LPS, TGF-β, TNF-α, or cycloheximide, nor did it appear in samples pretreated with a molar excess of either unlabeled uPAR mRNA, SDS (0.1%), or proteinase K (data not shown). These data confirm the effects of these treatments as depicted in Fig. 5 and 6.

Determination of the protein binding site on uPAR mRNA. To identify the minimal binding region on uPAR mRNA, we made transcripts of uPAR cDNA as shown in Fig. 1. The deletion products from uPAR cDNA were made by PCR amplification and cloned directly to the TA cloning vector, PCR II. Initially, we made four transcripts of 270, 586, 870, and 1,114 bases from the 5′ end. Surprisingly, the protein bound to all of the transcripts, indicating that it bound to translated sequences. We made two more deletion products to confirm this. There was no binding to deletion product 532-1098, covering 473 bp of the coding region and 93 bp of the 3′ untranslated region, or to a second one, 832-1098, containing 266 bp that covered the entire 93-bp 3′ untranslated region. These data indicated that the protein binding sequence was located somewhere within the region from bp 0 to 532. We made two more deletions covering bp −16 to 254 and 254 to 532 from the 5′ end and found specific binding only in the region from bp −16 to 254. We next constructed six more deletion fragments within the binding site between bp −16 and 254. Fragments 75-254, 137-254, 195-254, and 195-246 underwent a gel shift, whereas two fragments, −16-54 and −16-157, did not. The mRNA corresponding to fragment 195-246 was the smallest protein binding region that we identified within the uPAR mRNA transcript. This 51-bp uPAR sequence was found to be the consensus sequence among all protein binding transcripts of uPAR cDNA. We confirmed that this sequence bound the uPAR mRNABp in gel shift experiments (Fig. 9). These results demonstrate that bp 195 to 246 in the coding region of uPAR mRNA specifically interacts with the cytosolic protein.

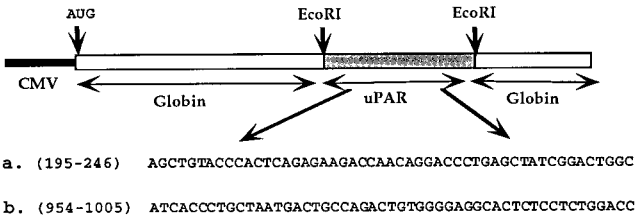

FIG. 10. Structure of β-globin/uPAR/β-globin chimeric cDNA. The uPAR mRNABp binding region of 51 nt corresponding to nt 195 to 246 (a) and a control sequence of similar size corresponding to the non-binding region from nt 954 to 1005 (b) of uPAR cDNA were inserted into the *Eco*RI site of the β-globin coding region. The chimeric β-globin/uPAR/β-globin cDNA was constructed without altering the reading frame of the β-globin gene.

Destabilization of β-globin mRNA by a 51-nt region of uPAR mRNA. To test the hypothesis that the 51-nt protein binding region of the uPAR coding region destabilizes uPAR mRNA by specific interaction of this mRNA with a cytosolic protein, we inserted this 51-nt fragment into a full-length β-globin cDNA. The 51-nt uPAR mRNA binding protein sequence (195 to 246) and a control uPAR coding sequence (954 to 1005) were cloned at the unique *Eco*RI site of full-length β-globin cDNA in such a way as to maintain the reading frame of β-globin transcript (Fig. 10). The chimeric construct was cloned to a eukaryotic expression vector, pcDNA3, and was transfected into MS-1 cells by lipofection. Because MS-1 cells express the uPAR mRNABp and a low steady-state level of endogenous β-globin mRNA, we used these cells to overexpress β-globin and chimeric β-globin/uPAR/β-globin genes. The transiently transfected cells were treated with actinomycin D for various lengths of time, and the effects on stability of chimeric and wild-type β-globin mRNAs were compared. Figure 11A depicts the decay of mRNA transcripts corresponding to full-length uPAR, β-globin, and chimeric β-globin constructs containing either the 51-bp uPAR mRNA binding protein sequence (bp 195 to 246) or the 51-bp control uPAR coding sequence (bp 954 to 1005) that were overexpressed in MS-1 cells. The half-life of the β-globin transcript in MS-1 cells was >24 h. By densitometry, insertion of the protein binding sequence of uPAR mRNA into β-globin mRNA shortened the half-life of the chimeric transcript to 5 h, which approximated that of the overexpressed uPAR mRNA, 7 h, in MS-1 cells. However, insertion of the control non-protein binding sequence into β-globin did not alter the half-life of the corresponding β-globin chimeric transcript. Transcripts from chimeric β-globin cDNA incorporating the binding sequence bound uPAR mRNABp by gel shift assay, whereas those from chimeric β-globin incorporating a control nonbinding sequence of identical size did not (data not shown). Parallel experiments done with 171 nt of uPAR cDNA containing the 51-nt binding or nonbinding sequence yielded identical results (data not shown). As shown in Fig. 11B, PMA treatment stabilized transcripts of the chimeric cells transfected with uPAR 195-246, while the mRNA of cells containing the control uPAR coding sequence (bp 954 to 1005) was unchanged. The half-lives of the uPAR 195-246 mRNA and that of the uPAR mRNA, as determined by densitometry, approximated each other at 16 and 18 h, respectively, while that of the control uPAR 954-1005 mRNA and that of the β-globin mRNA were both >24 h. These results indicate that a specific 51-nt uPAR mRNA protein binding sequence is involved in the regulation of message stability. The results further suggest that an inter-

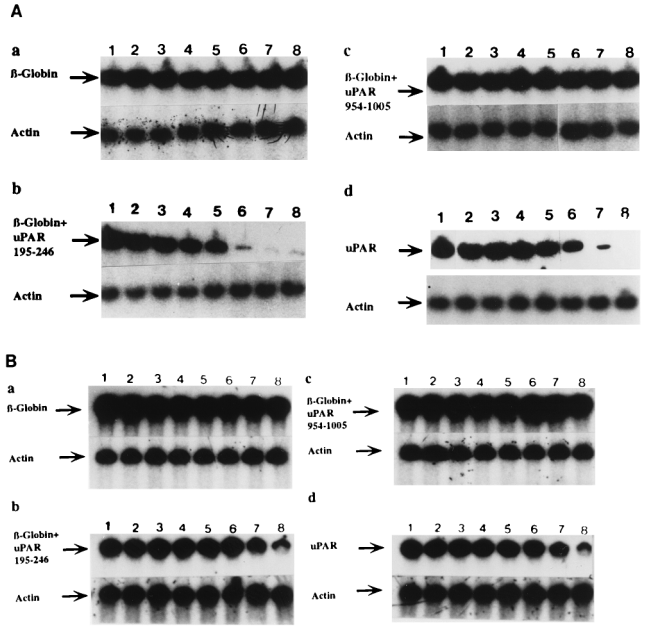

FIG. 11. Decay of β-globin/uPAR/β-globin chimeric mRNA. (A) MS-1 cells were transfected with β-globin (a), the chimeric β-globin/uPAR/β-globin gene containing the 51-nt (nt 195 to 246) nucleotide uPAR mRNABp binding sequence (b), non-protein binding control sequence of the uPAR coding region (nt 954 to 1005) (c), and the uPAR gene (d) in pcDNA3. Total RNA was isolated at different time intervals after treatment with actinomycin D in serum-free medium and analyzed by RNase protection assay using a ^{32}P-labeled antisense transcript as described above. Lanes contain RNA samples after treatment with actinomycin D (10 μg/ml) for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 6 h (lane 5), 12 h (lane 6), 18 h (lane 7), and 24 h (lane 8). Actin mRNA loading controls are shown. (B) MS-1 cells transiently transfected as described above were then treated with PMA (150 ng/ml) for 12 h, and the overexpressed mRNAs were compared as described above.

action between this 51-nt region of uPAR mRNA and its specific uPAR mRNABp is involved in the regulation of uPAR mRNA turnover in MS-1 cells.

DISCUSSION

Synthesis of uPA and its cell surface receptor (uPAR) is regulated by various agents, including cytokines and growth factors which appear to act at both transcriptional and posttranscriptional levels (9, 16, 22, 31). The uPAR localizes plasmin generation at the cell surface and thereby regulates percellular proteolysis.

In previous studies, we observed that PMA increased uPAR mRNA in MS-1 cells approximately 17-fold and that uPAR mRNA was increased by LPS, TGF-β, and TNF-α in MS-1 cells (28). PMA treatment increased uPAR mRNA 40- to 50-fold in U937 cells (16). Recently Lund et al. (17) reported that PMA and TGF-β increased the stability of uPAR mRNA in A549 cells besides increasing uPAR transcription. Elevated levels of uPAR mRNA in PMA-stimulated cells could be due to induction of growth factors (18), the activation of protein kinase C (21), increased transcription factor AP1 (1), increased uPAR mRNA half-life, or all of these mechanisms.

We found that cycloheximide, a protein synthesis inhibitor, induced uPAR mRNA 10-fold in MS-1 cells and had an additive effect with PMA. Cycloheximide in combination with PMA exerts an additive effect on MS-1 cell uPAR mRNA. The observed increase in specific mRNA could be due to an increased rate of transcription and/or decreased posttranscriptional mRNA turnover. However, in U937 cells, cycloheximide reduced the PMA-induced increase in uPAR mRNA (16). This finding underscores the diversity and complexity of the regulation of uPAR gene expression in different types of cells.

We tested the hypothesis that the increase in uPAR mRNA levels in PMA- and cycloheximide-treated MS-1 cells involves posttranscriptional regulation. In cycloheximide-treated cells, elevated levels of uPAR mRNA are maintained at least for 18 h. These experiments indicate that regulation of uPAR mRNA involves a posttranscriptional mechanism. To prevent new RNA synthesis during this time, the cells were treated with actinomycin D, which inhibits nearly all RNA synthesis by intercalation into DNA. We examined the rate of decay of uPAR mRNA in actinomycin D-treated cells and found that uPAR mRNA has a half-life of 3 h. However, in MS-1 cells pretreated with PMA, the half-life of uPAR mRNA was maintained for up to 13 h after treatment with actinomycin D. These observations are consistent with earlier findings of studies using A549 cells (17). A potential link between mRNA degradation and translation emerged from in vitro studies showing that cycloheximide superinduced uPAR mRNA. These observations suggested the participation of a labile *trans*-acting protein in uPAR mRNA degradation.

We assumed that some protein factor(s) interacts with uPAR mRNA to alter its stability and confirmed this assumption by identifying a cytosolic protein that specifically binds to uPAR mRNA transcripts. Nuclear extracts from the same cells failed to form a specific complex. The cytosolic protein-RNA complex is resistant to both RNase A and RNase T_1 digestion. The specificity of the uPAR mRNABp was assessed by competition experiments in which an unlabeled uPAR sense probe was effectively competed by its labeled analog. An antisense transcript had no effect. Further, a molar excess of a homoribonucleotide polymer [poly(A), poly(U), poly(C), or poly(G)] did not compete for specific probe binding, indicating that the binding of the uPAR mRNABp requires a specific sequence. The involvement of a specific protein factor is indicated by the finding that pretreatment with either SDS or proteinase K completely destroyed the complex.

PMA causes the down-regulation of the uPAR mRNA-uPAR mRNABp complex. A similar phenomenon occurs in T lymphocytes, where costimulation with PMA correlates inversely with the binding of lymphokine mRNA by an AU binding protein (5). Treatment of 3T3 cells with tumor promoter (tetradecanoyl phorbol acetate) down-regulates a ribonucleotide reductase R1 mRNA binding protein (6). In this instance, PMA induces an AU binding protein.

Cross-linking experiments with UV light indicate that the binding protein has an approximate molecular mass of 50 kDa. Other authors reported several 3′ untranslated region AUUUA multimer sequence binding proteins (5). We identified a cytoplasmic RNA binding factor that binds to uPAR coding sequences. Binding activity of uPAR mRNABp is significantly affected by serum induction or pretreatment of cells with agents known to stabilize uPAR mRNA.

From our experiments and those of others, it appears that PMA induces uPAR mRNA by increasing the longevity (16) as well as by increasing transcription of the uPAR gene (17). The precise mechanism of PMA-induced stabilization of uPAR mRNA remains elusive. The observed increase of uPAR mRNA by PMA probably results, at least in part, from down-regulation of uPAR mRNABp.

Treatment with cycloheximide increased uPAR mRNA severalfold in various cell lines (16, 28). Cycloheximide has an additive effect with other agonists (16). We find that treatment of cells with cycloheximide abolishes the uPAR mRNABp-

uPAR mRNA complex. We conclude that cycloheximide induces uPAR mRNA by inhibiting synthesis of the binding protein that selectively binds and degrades the uPAR transcript.

The half-lives of most mRNAs are influenced by the 3′ untranslated region. Most studies dealing with mRNA stability determinants have identified an mRNA decay signal in the 3′ untranslated region. The full-length uPAR mRNA transcript used in this study contains a small (93-nt) 3′ untranslated region. The results of deletion experiments showed no protein binding sequence in this 3′ untranslated region or elsewhere in the coding sequence other than the 51-nt region corresponding to the uPAR cDNA sequence from bp 195 to 246. This 51-nt protein binding fragment corresponds to exon 3 of the uPAR gene and codes for amino acids 66 to 85 of the uPAR protein. Prediction of RNA secondary structure did not show any significant stem-loop structure-forming sequence within this 51-bp protein binding region.

To test the ability of the 51-nt protein binding fragment of uPAR mRNA to influence mRNA stability, we used a strategy that exploits the comparison of chimeric mRNAs of genes with stable and unstable mRNAs linked in such a way as to maintain the reading frame. In this study, we identified a region on uPAR mRNA that is relevant for uPAR mRNA destabilization, using a β-globin/uPAR/β-globin hybrid gene construct transfected into MS-1 cells as a model system. Our data indicate that a 51-bp sequence of the uPAR coding region is involved in uPAR mRNA destabilization. Insertion of a non-protein binding control sequence of the uPAR mRNA coding region did not significantly alter the turnover rates of the hybrid message following actinomycin D treatment. As expected, PMA treatment extended the half-life of the mRNA of cells transiently transfected with the 51-bp uPAR binding sequence, while there was no detectable change in the half-life of the mRNA of cells transfected with the control coding sequence (bp 954 to 1005). There was no detectable decrease in the baseline (0 h of actinomycin D treatment) steady-state levels of the overexpressed β-globin–uPAR 195-246 compared with β-globin mRNA, suggesting that such differences may be small. Coding-region stability determinants have also been described for c-*myc*, c-*fos*, tubulin, and interleukin-2α receptor mRNAs (4, 21, 29, 30, 34). Our observations suggest that PMA- or cycloheximide-induced uPAR mRNA stabilization is controlled by information within the 51-nt sequence. Although the 51-nt region is involved in message destabilization, our experiments do not rule out the possibility that other sequences within uPAR mRNA (not included in the 51-nt fragment) also play a role in message stability.

Several factors may account for message destabilization conferred by the 51-nt region, including the possibility that the message is rendered susceptible to endonuclease attack due to insertion-specific conformational change. However, this is highly unlikely as the insertion of non-protein binding sequences did not alter the half-lives of the β-globin/uPAR/β-globin chimeric transcript. We believe that the most likely explanation is that this region of the message contains a *cis* element that interacts with a cytoplasmic protein involved in message degradation. Our results are also consistent with experiments describing *trans*-acting factors that influence the half-lives of specific mRNAs in intact cells (5, 15, 19). A similar mechanism was shown in other studies with an iron-responsive element and the AUUUA binding proteins (5, 14) or ribonucleotide reductase R1 mRNA binding protein (6). Our results with an in vitro transcription system and RNA band shift, combined with UV-cross-linking assays, support this view. The findings that the cytoplasmic binding protein binds to the 51-nt sequence and that the uPAR mRNABp-uPAR mRNA binding activity was rapidly down-regulated by PMA and cycloheximide add further support to this interpretation.

We find that the uPAR coding region is primarily involved in destabilization of uPAR mRNA; this region contains neither a long AU-rich segment nor the pentanucleotide AUUUA found in all known AU-rich elements. Our data suggest a model for regulation of uPAR mRNA stability by a protein-mRNA complex, but the nature of the complex, its relationship to translation, whether the protein actually binds within the 51-nt coding region, and how degradation of the mRNA is accomplished remain unclear. Purification and characterization of the protein are currently in progress and will likely define its role in the regulatory mechanism.

We conclude that the uPAR mRNA-uPAR mRNABp interaction likely represents a unique pathway for regulation of the uPAR with only limited similarity to previously described mechanisms. While the physiologic role of this regulatory pathway remains to be clarified, there are potential links to the pathogenesis of pleural diseases. This degradation pathway, which ensures rapid turnover of uPAR mRNA, may have evolved to serve uPAR-mediated functions in neoplasia, such as remodeling of tumor stroma and tumor extension. Because overexpression of the uPAR has been linked to neoplastic invasiveness, it is possible that this pathway influences the growth of mesothelioma.

ACKNOWLEDGMENTS

The work was supported by NIH grant R0145018-06, the RGK Foundation, a Gina Sabatasse Research grant award, and a grant from the American Heart Association, Texas affiliate (S.S.).

We are grateful to Alice Johnson and G. C. Das for reading the manuscript and to Kathy Koenig for technical assistance.

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