Transcriptional and Posttranscriptional Activation of Urokinase Plasminogen Activator Gene Expression in Metastatic Tumor Cells


Medical Oncology Unit, Department of Medicine, University of Sydney, Westmead Hospital, Westmead, New South Wales 2145 [B. R. H., W. P. T., R. F. K.] and Division of Cell Biology, John Curtin School of Medical Research, Australian National University, P. O. Box 334, Canberra, A.C.T. 2601 [S. M. P., I. A. R.], Australia

ABSTRACT

Urokinase plasminogen activator (uPA) is a serine protease which has frequently been implicated in the process of tumor cell invasion and metastasis. The degree of expression and mode(s) of regulation of the uPA gene in metastatic compared with nonmetastatic tumor cells have not yet been addressed. We have cloned and sequenced a full-length rat uPA complementary DNA and utilized Northern blot analysis to report that the uPA gene is expressed at levels 3.5- to 70-fold higher in metastatic cell lines than in nonmetastatic cell lines derived from two independent rat mammary adenocarcinomas. Nuclear run-on assays and RNA half-life estimations indicated that metastatic MAT 13762 rat mammary adenocarcinoma cells expressed 3.5-fold higher levels of uPA RNA than a nonmetastatic derivative (J-clone), due to a combined increase in uPA gene transcription and cytoplasmic RNA stability. By contrast, uPA RNA (and enzyme) levels were elevated by up to 70-fold in metastatic clones of dimethylbenz(a)anthracene-induced rat mammary adenocarcinoma (DMBA-8) due to predominantly posttranscriptional mechanisms. Moreover, treatment of nonmetastatic DMBA-8 cell lines with protein synthesis inhibitors led to an increase in nuclear and cytoplasmic uPA RNA levels, without altering the rate of uPA gene transcription. These results suggest that in addition to gene transcription, posttranscriptional events localized in the nucleus and cytoplasm are key determinants of uPA gene activation in rat mammary adenocarcinomas.

INTRODUCTION

The ability of certain primary tumor cells to invade normal tissue and metastasize to distant sites continues to present a major barrier to the treatment of human cancer. Metastasis is a multilevel process comprising sequential steps at which the tumor cell must interact with host tissue or cells (1). These discrete steps may be accompanied by transient shifts in the expression of specific genes (2, 3). Several genes differentially expressed in metastatic or nonmetastatic cells have been identified (4); however, in most cases the significance of their expression or function is unclear. Recently, a great deal of research has focused on what may be one of the rate-limiting steps of the metastatic cascade: tumor cell invasion and exit from the vasculature (1). Tumor cell invasion involves degradation of the extracellular matrix by tumor or host-secreted proteases (5). Biochemical support has been found for uPA as a fundamental initiator of the invasive process (6–8). uPA is a serine protease, which through catalytic activation of the general protease plasmin has been documented to play numerous indirect roles in normal physiological processes requiring cell migration and in metastasis (6). Although certain studies have found no correlation between uPA enzyme production (9) or uPA RNA levels (10) and the malignant state, it is proposed that anchorage of uPA molecules to specific cell-surface receptors may provide a platform for initiating localized proteolysis and consequently at least one mechanism facilitating tumor cell invasion (11–13).

uPA is overexpressed by many malignant tumors in vivo, at the levels of both protein (6) and gene (14) expression. Unfortunately, any study of uPA gene regulation in primary malignant tumors is complicated by the inherent mixture of host stromal cells, infiltrating macrophages, and subpopulations of tumor cells which might vary in their metastatic capacity and ability to synthesize uPA (4, 6). Rodent models based on clonal variants derived via a minimal-step in vivo selection from chemically induced RMAs have recently been characterized, in which a correlation between uPA synthesis and metastatic potential was described (15, 16). These tumor cell models have provided an experimental system highly suited to the direct investigation of the activation of uPA gene expression in metastatic tumor cells. In this paper we describe experiments which demonstrate that the uPA gene is down-regulated in nonmetastatic RMA cell lines due to a decrease in the rate of transcription and to posttranscriptional regulatory events, localized in the cytoplasmic and/or nuclear compartments.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

All tumor cell lines were adapted to culture either directly from the metastatic MAT 13762 and nonmetastatic DMBA-8 rat mammary tumors or indirectly as clonal variants of differing metastatic potential (15, 16). These include a nonmetastatic 6-thioguanine-resistant variant of the MAT 13762 parent cell line, called J-clone (originally termed TG9), and, from the DMBA-8 parent line, the derivatives NM4, MA, and MC2 and MC4. All cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum as described (15, 16) and were Mycoplasma-negative.

Experimental Lung Colonization Assay

All tumor cell lines described in this study were reassessed for experimental metastatic potential following tail vein injection into 3- to 4-week-old syngeneic host female Fischer 344 rats. About 16 to 20 days following tail vein injection of tumor cell suspensions (10^6 cells in 0.1 ml), animals were sacrificed, and the number of lung colonies was counted after inflation with India ink. For assessment of tumor-forming ability, cells were injected s.c., and tumors were detected by palpation after 2 months.

PA Gel Zymography

Secreted uPA activity in serum-free tumor cell culture supernatants was assessed by gel zymography as described previously (16). Parallel polyacrylamide gels lacking plasminogen provided a negative control.

cDNA Cloning and Sequence Analysis

In order to isolate a rat-specific uPA gene probe, a total cDNA library was constructed in pGemI (Promega, WI) from DMBA-8 MA
polyadenylated RNA and screened using a 32P-labeled porcine uPA cDNA probe, pPK79 (17), as previously described (18). The positive clone pRAT-UK1 contained the largest insert, 2.1 kilobases, and was selected for sequence analysis. To facilitate this analysis, the cDNA insert was excised from pGEM1 with Sall and BamHI, treated with T4 DNA polymerase, and subcloned (in both orientations) into the Smal site of pGEM 7zf+ (Promega). Unidirectional deletions from both the 5' and 3' ends of the cDNA insert were prepared using the Erase-a-Base system (Promega) as directed by the manufacturer, and sequencing was carried out by the chain termination method of Sanger et al. (19). Full sequence information was obtained for both strands of the cDNA insert.

While the rat uPA cDNA appeared complete at the 3' end (as judged by the presence of a polyadenylation signal and polyadenylated tail), primer extension analysis (data not shown) revealed that the cDNA was lacking approximately 370 base pairs at the 5' end. The additional 5' uPA sequence was cloned using the RACE method of Frohman et al. (20) as described. Briefly, 1 µg of total cytoplasmic BC1 tumor cell RNA (21) was reverse transcribed with AMV reverse transcriptase using the synthetic oligonucleotide primer 5'-AGGGTCGCCCTCTAGGGTTGACACTATAGT-3' (complementary to nucleotide positions 479-501 in Fig. 2B). The single-stranded cDNA was then tailed with DATP and terminal deoxynucleotidyl transferase and purified through a Centricron-100 microconcentrator (Amicon, MA). The 5' end of the rat uPA cDNA was amplified using the polymerase chain reaction and three oligonucleotide primers:

5'-CAGGCTAGGCTAGGCTAAGAGCAGCAT-3' (complementary to positions 436-455)
5'-TTTAGGTGACACTATAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
The uPA cDNA contains a single large open reading frame encoding a 432-amino acid protein with a predicted molecular weight of 48,000, in keeping with PA gel zymography data (Fig. 1). Amino acid sequence homology with the other uPA proteins is high (88, 76, 70, and 69%, as above) and considerably higher if conservative amino acid substitutions are ignored (98, 93, 93, and 84%). Unlike the human and pig uPA proteins (27, 29), rat uPA has no potential sites for glycosylation but retains the conservation of sequence in the epidermal growth factor, Kringle, and serine protease domains exhibited by all the uPAs. As previously reported, A + T-rich sequence motifs previously shown to confer RNA instability (30) were well conserved in the 3' untranslated region of the rat uPA gene when compared to other uPA genes (31).

**Table 1** Summary of metastatic potential and uPA expression of RMA cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Experimental metastasisa</th>
<th>Experimental tumorsb</th>
<th>uPA enzyme activityc</th>
<th>uPA RNA levelsd</th>
<th>uPA RNA half-lifef</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT 1376z</td>
<td>&gt;200</td>
<td>+</td>
<td>++++</td>
<td>73 ± 7</td>
<td>&gt;16 h</td>
</tr>
<tr>
<td>J-CLONE</td>
<td>0</td>
<td>±</td>
<td>±</td>
<td>21 ± 10</td>
<td>19 h</td>
</tr>
<tr>
<td>DMBA-8fe</td>
<td>0</td>
<td>+</td>
<td>–</td>
<td>1</td>
<td>12 h</td>
</tr>
<tr>
<td>NM4fe</td>
<td>0</td>
<td>+</td>
<td>–</td>
<td>0.1</td>
<td>NDf</td>
</tr>
<tr>
<td>MA</td>
<td>&gt;200</td>
<td>+</td>
<td>++++</td>
<td>70 ± 10</td>
<td>&gt;16 h</td>
</tr>
<tr>
<td>MC2</td>
<td>&gt;200</td>
<td>+</td>
<td>++++</td>
<td>25 ± 9</td>
<td>ND</td>
</tr>
<tr>
<td>MC4</td>
<td>&gt;200</td>
<td>+</td>
<td>++++</td>
<td>25 ± 5</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Experimental metastasis data correlated well with the previously determined spontaneous metastatic abilities of the RMA cell-lines studied (24; data not shown). Data shown represent the average number of pulmonary nodules counted 16 days after tail vein injection of 10⁶ cells into host Fischer 344 rats. There was a minimum of 4 animals/group. Data for MC2 and MC4 were taken from Ramshaw et al. (16).

* Ability to form primary tumors following s.c. injection of 10⁶ cells into Fischer 344 rats. Data taken from Ramshaw and Badenoch-Jones (15), except for J-clone, where 2 out of 5 animals receiving injections formed primary tumors within 60 days.

* Relative uPA enzyme activity. Data are arbitrary units and taken from Fig. 1, Ramshaw et al. (16) and B. R. Henderson, Ph.D. thesis, University of Sydney, 1991.

* Relative levels of uPA cytoplasmic RNA, where the DMBA-8 cell uPA RNA level is set at a value of 1%. Average uPA values (range) were determined by a densitometric analysis of autoradiographs from at least two independent Northern blots (Fig. 5; data not shown).

* uPA RNA half-lives were estimated by actinomycin D chase (see Fig. 6) and confirmed by [³H]uridine pulse-chase experiments for the cell lines MAT 13762 and DMBA-8 (data not shown).

* Parental cell line initially adapted to culture.

* Cell lines which grow as monolayers in culture.

* ND, not determined.

Fig. 1. Gel zymogram of PA derived from DMBA-8 metastatic MA (A) and nonmetastatic NM4 (B) cell lines. Supernatants were concentrated 10-fold using a Millipore CX-10 concentrator. Ten-μl aliquots were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and PA was localized by fibrin-overlay zymography as previously described (16). Ordinate, molecular weight kilodaltons.

Nuclear “Run-on” Analysis of uPA Gene Transcription. To investigate the possibility that the elevated uPA RNA levels observed in metastatic RMA cell lines result from increased transcription of the uPA gene template, nuclear run-on assays were performed (see Fig. 4). Specificity of the uPA gene signal was judged by comparison to reference genes (α-tubulin, γ-actin, and GAPDH) and effective interference by the RNA polymerase II inhibitor α-amanitin (data not shown). Furthermore, a single-stranded uPA antisense template (but not the uPA sense strand) produced a pattern of signals similar to that of the double-stranded pRAT-UK1 uPA plasmid, confirming binding of [³²P]uPA nuclear transcripts to the noncoding strand of the uPA gene. The results shown (Fig. 4) are representative of at least two independent experiments. Quantitative densitometry (Fig. 5) revealed a 2-fold difference in uPA gene transcription, which largely accounts for the average 3.5-fold
higher level of uPA RNA in MAT 13762 cells than in J-clone cells (Fig. 5).

In cell lines derived from the DMBA-8 tumor, uPA gene transcription was also decreased 2-fold in nonmetastatic DMBA-8 (parent) and NM4 (clone) cell lines relative to the metastatic clones (Figs. 4 and 5). However, this modest transcriptional difference is unlikely to account for the 25- to 70-fold difference in uPA cytoplasmic mRNA levels (Fig. 5).

Transcription of the metalloprotease gene transcri, previously correlated with malignancy (33), was virtually undetectable in nonmetastatic RMA cells. uPA mRNA transcripts demonstrated a higher degree of stability in metastatic MAT 13762 cells than in J-clone cells (Fig. 5).

Stability of Cytoplasmic uPA mRNA. The stability of cytoplasmic uPA RNA transcripts was determined in various cell lines by actinomycin D chase (Fig. 6) and [3H]uridine pulse-chase experiments (data not shown). uPA mRNA transcripts demonstrated a higher degree of stability in metastatic MAT 13762 and MA cells (t1/2 > 16 h) than in the nonmetastatic cell lines J-clone and DMBA-8, in which uPA mRNA decayed with half-lives of 19 and 12 h, respectively (see Table 1). Stability of uGAPDH RNA was less variable between cell lines (Fig. 6). In the MAT 13762/J-clone model, the combination of increased uPA gene transcription (average, 2-fold; Figs. 4 and 5) and mRNA stability accounts for the 3.5-fold greater amount of uPA RNA in MAT 13762 cells compared with J-clone. However, the 12-h half-life of cytoplasmic uPA RNA in DMBA-8 cells appears too long to explain the almost undetectable levels of uPA RNA in these nonmetastatic cells.

Comparison of Steady-state uPA Nuclear RNA Levels. Relative steady-state levels of uPA nuclear RNA in cell lines derived from both MAT 13762 and DMBA-8 tumors were assessed by Northern analysis (Fig. 7). Particular care was taken to purify nuclei and minimize cytoplasmic RNA contamination (see "Materials and Methods"). The experiment shown (Fig. 7) is typical of at least three independent determinations. Densitometric quantitation revealed little difference between the level of mature-sized (2.6 kilobases) uPA transcripts in the nuclei of MAT 13762 and nonmetastatic J-clone cells. A smaller (1.8 kilobases) uPA transcript (Fig. 3) was present in metastatic MA cells synthesized (on
Fig. 3. Northern blot analysis. Total cytoplasmic RNA was isolated from nonmetastatic (DMBA-8, NM4, J-clone) and metastatic (MA, MC2, MAT 13762) RMA cell lines. Fifteen μg of RNA were loaded per lane, and equivalent loading was confirmed by ethidium bromide staining of the 1% formaldehyde agarose gel. Filters were probed with 32P-labeled pRAT-UK1 (uPA) cDNA and reprobed with pHcGAP (GAPDH) cDNA.

Fig. 4. Nuclear run-on transcription analysis. Assays were performed using 32P-labeled nuclear RNAs derived from the indicated RMA cell lines as described in "Materials and Methods." Labeled RNA was hybridized to the following filter-immobilized cDNAs: pRAT-UK1 (uPA) sense single-strand [uPA ss (-)] and antisense single-strand [uPA ss (+)], pRAT-UK1 plasmid DNA (uPA DS), pG7TRI (TRANSIN), pGem7zf+ (G7z+), pHFaT (TUBULIN), pHcGAPDH (GAPDH), and pHFA-1 (ACTIN).

average) 10- and 18-fold more mature uPA nuclear RNA than nonmetastatic DMBA-8 and NM4 cell lines, respectively. A specific, 7-kilobase nucleotide uPA pre-mRNA was also overexpressed in MA cell nuclei (and was weakly detectable in MAT 13762 cells; data not shown) at levels proportional to the expression of the fully processed transcript. The discrepancy between differences in uPA gene transcription (2-fold) and steady-state uPA nuclear RNA levels (>10-fold) may suggest

Fig. 5. Comparison of relative uPA mRNA levels and uPA gene transcription rate in RMA cell lines. Relative intensities of uPA RNA autoradiograph signals from Northern blot (Fig. 3) and nuclear run-on (Fig. 4) experiments were quantitated by scanning densitometry. Columns, mean from at least two independent experiments; bars, SE. Densitometry was performed on two different autoradiograph exposures from each experiment, and values were graphed in arbitrary units where DMBA-8 was set at values of 1 (RNA levels) and 10 (transcription rate).

Fig. 6. Estimation of cytoplasmic uPA RNA stability. Cytoplasmic RNA was isolated from the indicated RMA cell lines treated with 5 μg/ml actinomycin D for different times (h). Ten μg RNA of each sample were assessed by Northern blot analysis as described in Fig. 3. Autoradiograph signals were quantitated by densitometry and graphed, setting untreated samples (0 h) at 100%. RNA half-life estimates (see Table 1) were determined from decay curves as shown for uPA (●) and GAPDH (■) genes.
that additional posttranscriptional events down-regulate nuclear uPA RNA levels in nonmetastatic DMBA-8 cells.

**Translation Inhibitors Increase the Level of uPA Cytoplasmic and Nuclear Transcripts in Nonmetastatic DMBA-8 Cells.** The expression of several posttranscriptionally down-regulated genes can be induced by protein synthesis inhibitors, which presumably act by causing the depletion of short-lived negatively regulating proteins (34). We observed that 10 μg/ml CHX induced uPA RNA levels from within 2 h of treatment of nonmetastatic DMBA-8 cells (data not shown), with the effect peaking at about 8-fold by 8 to 16 h, suggesting a short-lived protein(s) may at least partly account for decreased uPA RNA accumulation in these cells. CHX did not influence uPA RNA levels in DMBA-8 metastatic cell lines (data not shown). We next tested two distinct protein synthesis inhibitors, CHX and anisomycin, which block translation through different mechanisms (35), for their effects on uPA gene expression in nonmetastatic DMBA-8 and NM4 cell lines. The inhibitors were used at concentrations which blocked 25% methionine incorporation into protein (>95%) (data not shown). Cells were treated for 4 h, and modulation of both cytoplasmic and nuclear uPA RNA levels was analyzed by Northern blotting (see Fig. 8A). Quantitation of the autoradiographs revealed a gene-specific 3- to 8-fold increase in cytoplasmic uPA RNA levels in nonmetastatic lines from both the MAT 13762 and DMBA-8 tumors (Fig. 8B; data not shown). However, a strong induction of nuclear uPA RNA levels was observed only in the DMBA-8 tumorderived cell lines, DMBA-8 and NM4. In contrast to a previous report (36), nuclear run-on assays revealed that the stimulation by CHX of nuclear uPA RNA levels in NM4 and DMBA-8 cell lines did not accompany an increase in uPA gene transcription, despite a 6-fold induction of α-tubulin gene transcription in NM4 cells (Fig. 8C). These results are consistent with the relatively high constitutive rate of uPA gene transcription observed in these cell lines (Fig. 4).

**DISCUSSION**

The regulatory mechanism(s) responsible for the observed activation of uPA gene expression in malignant tumor cells (14) has not yet been investigated. We have cloned and sequenced a rat uPA cDNA, which demonstrated 91% coding sequence similarity with the mouse uPA gene (26), and shown that down-regulation of the uPA gene in nonmetastatic RNA cells was mediated in part by a decreased transcription rate and by posttranscriptional mechanism(s).

Until recently, studies of uPA gene control have focused largely on transcriptional regulation, as is evident from the number of reports observing the stimulation of uPA gene transcription by cAMP (37), phorbol esters and growth factors (32, 38), serum (39), or oncogene transformation (40). Recent studies on LLC-PK, pig kidney cells, however, revealed an enhancement of uPA mRNA levels by Ca2+ (41) and CHX (42), mediated through an increase in the uPA mRNA half-life from 70 to 160 min and >20 h, respectively. Thus posttranscriptional regulation may also be an important determinant of uPA gene expression. We recently noted the conservation of A + T-rich sequences in the untranslated region of the uPA genes (31), which in oncogenes and cytokine genes mediate mRNA degradation (30, 43), suggesting indirectly a mechanism by which uPA mRNA stability may be regulated. Despite differences observed in the level of cytoplasmic uPA RNA stability in the cell lines reported here, all uPA RNA half-lives were estimated...
to extend beyond 12 h. Therefore we tentatively conclude that uPA gene expression is repressed, at least in part, at the additional level of nuclear RNA stability and/or processing in nonmetastatic DMBA-8 cells.

We interpret the induction of uPA nuclear RNA levels by protein synthesis inhibitors to indicate the presence of short-lived proteins which decrease uPA nuclear RNA levels in nonmetastatic DMBA-8 tumor cell lines. This interpretation is supported indirectly by CHX wash-out experiments (data not shown), which showed that CHX induction of DMBA-8 uPA RNA levels was not immediately reversible over an 8-h period, and suggest that CHX does not act by simply stabilizing cytoplasmic uPA RNA. Since CHX did not alter uPA RNA levels in metastatic DMBA-8 cells (data not shown), these putative labile proteins may be inactive or absent in metastatic DMBA-8 cells, thereby acting specifically in nonmetastatic DMBA-8 cells to decrease nuclear uPA RNA stability. Processing. Recently, CHX was similarly observed to elicit a preferential induction of granulocyte-macrophage colony-stimulating factor mRNA in nonmetastatic tumor cells (44), although the mechanism of gene induction was not addressed.

It is unlikely that such putative unstable proteins would down-regulate uPA expression solely by blocking the efficacy of uPA RNA processing in nonmetastatic DMBA-8 and NM4 cells, inasmuch as an overaccumulation of uPA precursor transcripts was not observed. In addition, the fact that uPA mature nuclear transcripts do not normally accumulate to high levels in these cells excludes nuclear RNA export as a rate-limiting factor. Enrichment of nuclear RNA was deemed sufficient, however, as is evident from our ability to detect high-molecular-weight pre-rRNA (data not shown) and a 7-kilobase uPA pre-mRNA as previously described for the ribosomal genes. As is evident from our ability to detect high-molecular-weight pre-rRNA (data not shown) and a 7-kilobase uPA pre-mRNA as previously described for the ribosomal protein L1 gene (45).

Previous studies have suggested that posttranscriptional mechanisms control uPA mRNA levels (at least in part) in LLC-PK1 porcine kidney cells (41, 42) and mouse fibroblasts (39). Moreover, we have recently observed that primary rat embryo fibroblasts and Rat-1 fibroblast cells efficiently transcribe the uPA gene at a basal rate comparable to that observed in Rat-1 fibroblasts (data not shown). The putative unstable protein(s) may act by directly degrading uPA nuclear precursor or mature mRNA or perhaps by causing a block to splicing or polyadenylation, which in turn might signal the rapid degradation of uPA pre-mRNA as previously described for the ribosomal protein L1 gene (45).

Previous studies have suggested that posttranscriptional mechanisms control uPA mRNA levels (at least in part) in LLC-PK1 porcine kidney cells (41, 42) and mouse fibroblasts (39). Moreover, we have recently observed that primary rat embryo fibroblasts and Rat-1 fibroblast cells efficiently transcribe the uPA gene at a basal rate comparable to that observed in Rat-1 fibroblasts (data not shown). The putative unstable protein(s) may act by directly degrading uPA nuclear precursor or mature mRNA or perhaps by causing a block to splicing or polyadenylation, which in turn might signal the rapid degradation of uPA pre-mRNA as previously described for the ribosomal protein L1 gene (45).

Previous studies have suggested that posttranscriptional mechanisms control uPA mRNA levels (at least in part) in LLC-PK1 porcine kidney cells (41, 42) and mouse fibroblasts (39). Moreover, we have recently observed that primary rat embryo fibroblasts and Rat-1 fibroblast cells efficiently transcribe the uPA gene at a basal rate comparable to that observed in Rat-1 fibroblasts (data not shown). The putative unstable protein(s) may act by directly degrading uPA nuclear precursor or mature mRNA or perhaps by causing a block to splicing or polyadenylation, which in turn might signal the rapid degradation of uPA pre-mRNA as previously described for the ribosomal protein L1 gene (45).

Previous studies have suggested that posttranscriptional mechanisms control uPA mRNA levels (at least in part) in LLC-PK1 porcine kidney cells (41, 42) and mouse fibroblasts (39). Moreover, we have recently observed that primary rat embryo fibroblasts and Rat-1 fibroblast cells efficiently transcribe the uPA gene at a basal rate comparable to that observed in Rat-1 fibroblasts (data not shown). The putative unstable protein(s) may act by directly degrading uPA nuclear precursor or mature mRNA or perhaps by causing a block to splicing or polyadenylation, which in turn might signal the rapid degradation of uPA pre-mRNA as previously described for the ribosomal protein L1 gene (45).

Previous studies have suggested that posttranscriptional mechanisms control uPA mRNA levels (at least in part) in LLC-PK1 porcine kidney cells (41, 42) and mouse fibroblasts (39). Moreover, we have recently observed that primary rat embryo fibroblasts and Rat-1 fibroblast cells efficiently transcribe the uPA gene at a basal rate comparable to that observed in Rat-1 fibroblasts (data not shown). The putative unstable protein(s) may act by directly degrading uPA nuclear precursor or mature mRNA or perhaps by causing a block to splicing or polyadenylation, which in turn might signal the rapid degradation of uPA pre-mRNA as previously described for the ribosomal protein L1 gene (45).

Previous studies have suggested that posttranscriptional mechanisms control uPA mRNA levels (at least in part) in LLC-PK1 porcine kidney cells (41, 42) and mouse fibroblasts (39). Moreover, we have recently observed that primary rat embryo fibroblasts and Rat-1 fibroblast cells efficiently transcribe the uPA gene at a basal rate comparable to that observed in Rat-1 fibroblasts (data not shown). The putative unstable protein(s) may act by directly degrading uPA nuclear precursor or mature mRNA or perhaps by causing a block to splicing or polyadenylation, which in turn might signal the rapid degradation of uPA pre-mRNA as previously described for the ribosomal protein L1 gene (45).

Previous studies have suggested that posttranscriptional mechanisms control uPA mRNA levels (at least in part) in LLC-PK1 porcine kidney cells (41, 42) and mouse fibroblasts (39). Moreover, we have recently observed that primary rat embryo fibroblasts and Rat-1 fibroblast cells efficiently transcribe the uPA gene at a basal rate comparable to that observed in Rat-1 fibroblasts (data not shown). The putative unstable protein(s) may act by directly degrading uPA nuclear precursor or mature mRNA or perhaps by causing a block to splicing or polyadenylation, which in turn might signal the rapid degradation of uPA pre-mRNA as previously described for the ribosomal protein L1 gene (45).

Previous studies have suggested that posttranscriptional mechanisms control uPA mRNA levels (at least in part) in LLC-PK1 porcine kidney cells (41, 42) and mouse fibroblasts (39). Moreover, we have recently observed that primary rat embryo fibroblasts and Rat-1 fibroblast cells efficiently transcribe the uPA gene at a basal rate comparable to that observed in Rat-1 fibroblasts (data not shown). The putative unstable protein(s) may act by directly degrading uPA nuclear precursor or mature mRNA or perhaps by causing a block to splicing or polyadenylation, which in turn might signal the rapid degradation of uPA pre-mRNA as previously described for the ribosomal protein L1 gene (45).


Transcriptional and Posttranscriptional Activation of Urokinase Plasminogen Activator Gene Expression in Metastatic Tumor Cells


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/52/9/2489

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.