Tissue plasminogen activator mRNA in murine tissues

Richard J. Rickles[+][*] and Sidney Strickland[*]

[+]Department of Microbiology and [*]Department of Molecular and Cellular Pharmacology, State University of New York at Stony Brook, Stony Brook, NY 11794, USA

The urokinase-type and tissue-type plasminogen activators are the two enzymes found in mammals, which specifically convert the zymogen plasminogen to plasmin. Using cDNA probes, we have assayed for the presence of the two types of plasminogen activator mRNAs in murine tissues. We demonstrate that tissue-type plasminogen activator mRNA can be detected in a wide variety of tissues. In contrast, the accumulation of urokinase-type plasminogen activator mRNA is observed in only a few of the tissues analyzed. Using an S_1 nuclease assay, we demonstrate that the tPA mRNA detected contains the complete sequences encoding the non-protease finger, growth-factor and kringle domains.

Tissue-type plasminogen activator; Urokinase-type plasminogen activator; RNA analysis; (Murine tissue)

1. INTRODUCTION

The activation of the zymogen plasminogen to plasmin by plasminogen activators is associated with processes such as ovulation [1,2], embryo implantation [3], mammary gland involution [4], spermatogenesis [5], fibrinolysis [6] and in some instances metastasis [7,8]. Since plasminogen is a ubiquitous serum protein that permeates vertebrate tissues, much interest has centered on the role of plasminogen activators in these diverse physiologic events. There are two types of mammalian plasminogen activators: tPA and uPA. These two proteins, the products of different genes, are antigenically distinct and their mRNAs can be distinguished from each other using nucleic acid probes [8]. It has now become apparent that the type of plasminogen activator produced and the cell type expressing the enzyme are both important determinants of function.

In humans and rodents, plasminogen activators have been observed in almost all tissues though at variable levels [9–14]. Three methods have generally been used to assay for the presence of plasminogen activators: enzyme assays of tissue extracts in conjunction with tPA and uPA antibodies [8], immunohistochemical analysis of tissue sections [12,13] and polyacrylamide gel electrophoresis of tissue extracts followed by zymography [14,15]. All three methods allow distinction to be made between tPA and uPA.

Interestingly, some investigators have observed tPA size heterogeneity [16–20]. One interpretation of these observations is that the lower molecular mass tPA molecules are the result of partial proteolysis, perhaps by plasmin, during extraction. In support of this proposal, plasmin has been used to remove domains from tPA in attempts to study the properties of the altered tPA enzyme [21]. Another possibility is that the tPA size heterogeneity could be the result of carbohydrate heterogeneity between individual tPA molecules. With Bowes melanoma cells, for instance, two tPA proteins are observed that differ in molecular mass by

Correspondence address: S. Strickland, Department of Molecular and Cellular Pharmacology, State University of New York at Stony Brook, Stony Brook, NY 11794, USA

Abbreviations: tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator

2-3 kDa, due to carbohydrate differences [22]. The results of Kagitani and collaborators [23], however, suggest another cause for the lower molecular mass tPA molecules. This group isolated a human tPA cDNA from Detroit 562 cells that lacks the sequences which code for the finger domain. Their results are consistent with alternate splicing of the tPA mRNA for the generation of additional forms of the enzyme. In addition, Nagamine and co-workers [24] have demonstrated the presence of two mRNAs for uPA which would generate proteins that differ in size by nine amino acids. Clearly, tissue-specific splicing of plasminogen activator transcripts would be of great significance in expanding the biological diversity of the plasminogen activator molecule.

We were interested in whether there were other examples of altered forms of plasminogen activator, generated as a result of tissue-specific splicing. Using the mouse tPA cDNA as a probe, we have addressed this question by Northern blot and S_1 analysis of mouse tPA mRNA. We also have determined the relative steady-state levels of uPA mRNA in mouse tissues.

2. MATERIALS AND METHODS

2.1. Animals
Adult CD-1 mice were supplied by Charles River (Wilmington, MA). For tissue dissection, animals were killed by CO_2 asphyxiation.

2.2. RNA preparation and analysis
The preparation of tissue RNA [25], Northern gel analysis [26] and dot blot analysis [27] were performed as described. To determine the relative levels of plasminogen activator RNA in tissues, dot blot autoradiograms were scanned using an LKB Ultrascan XL laser densitometer. The values obtained for the plasminogen activator samples were normalized using those obtained for 18 S rRNA to account for any variability in the amount of RNA present on the filters.

2.3. Probe synthesis and hybridization conditions
^{32}P-labeled tPA [28], uPA [29] and 18 S ribosomal [30] DNA probes were prepared using a random priming procedure [31]. Probe specific activities were routinely $0.8-2 \times 10^9$ cpm/μg. Hybridization and washing conditions were as in [28]. The nitrocellulose filters were washed for 30 min with 50 mM NaOH at room temperature after autoradiography to remove radiolabeled DNA and allow successive filter hybridizations with tPA, uPA and 18 S ribosomal probes.

2.4. Single-stranded DNA probe preparation and S_1 protection assay
Bacteriophage DNA was uniformly labeled with [^{32}P]ortho-phosphate in vivo as described [32]. The hybridizations and nuclease reactions were carried out as in [33] at a hybridization temperature of 52°C and using 500 U S_1 nuclease (Sigma) for each protection assay.

3. RESULTS

Previously, we isolated a tPA cDNA from F9 cells after cells were induced to differentiate using retinoic acid and dibutyryl cAMP. Characterization of the mouse cDNA revealed homology with the human tPA coding sequences with a finger, growth factor and two kringle domains [28]. As a preliminary step before analyzing tPA mRNA for tissue-specific splicing events, we used the mouse cDNA to assay murine tissues for the presence of tPA mRNA.

To assay for tPA mRNA, 5-40 μg total cellular RNA from tissues was electrophoretically fractionated on denaturing formaldehyde/agarose gels, transferred to nitrocellulose and hybridized to a ^{32}P-labeled tPA cDNA probe. The results from one experiment are shown in fig.1. When the tPA cDNA was used as a probe, a transcript approx. 2800 nucleotides in size was detected in most of the tissues. In contrast, detectable accumulation of uPA mRNA was observed only in the kidney and ovary.

Overall, RNA was extracted from tissues on two or three separate occasions and analyzed for plasminogen activator RNA content by Northern gel and dot blot analysis. While tPA mRNA was detected in 20 out of 22 tissues analyzed, uPA mRNA accumulation was more restricted and observed, by this technique, only in the kidney, vas deferens and ovary. A summary of these results is shown in table 1.

It should be noted that the Northern blot analysis performed with the tissue RNAs is not adequate for detecting small differences in composition between the various tPA transcripts. For example, if a particular transcript lacked the growth factor or finger domain (111 and 138 nucleotides in length, respectively) these differences in molecular mass might not be detected. In addition, as discussed, there is one example where plasminogen activator mRNAs (uPA) differed by 27 nucleotides [24]. Therefore, we used an S_1 protection assay to screen the mouse tissue RNAs for the presence of differentially spliced
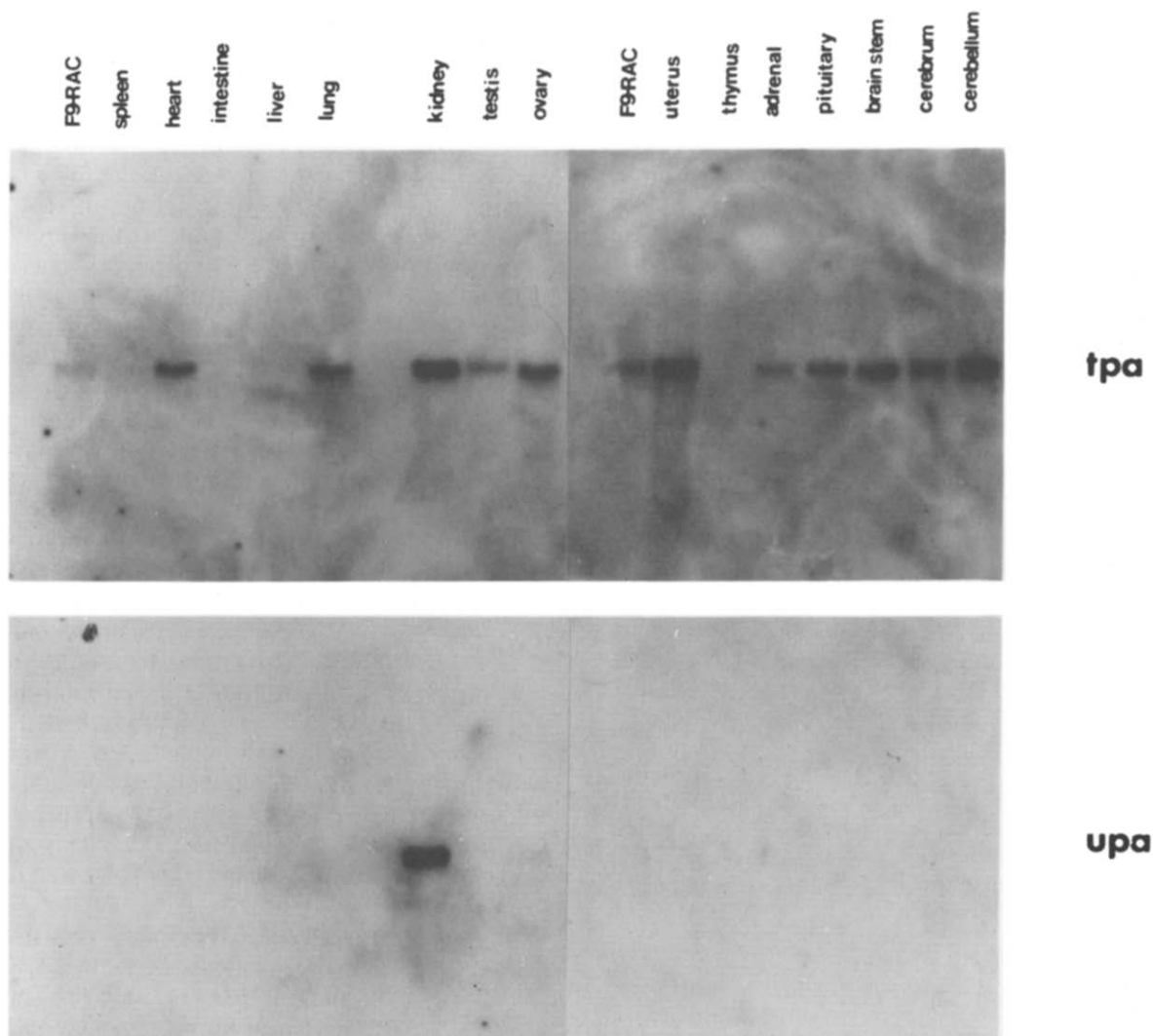
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Fig.1. Northern blot analysis of plasminogen activator mRNA in murine tissues. Total cellular RNA from various tissues was electrophoretically fractionated on a denaturing 1% agarose gel, blotted and hybridized sequentially with ^{32}P-labeled tPA and uPA probes. The amount of RNA analyzed was as follows: F9-RA/dbcAMP cells (F9 cells treated with retinoic acid and dibutyryl cAMP), 2.5 μg; thymus, 10 μg; uterus, adrenals, pituitary and brain stem, 15 μg; cerebrum, cerebellum, spleen, heart, small intestine, liver, lung, kidney, testis and ovary, 40 μg. Approximate sizes for the transcripts observed: tPA, 2.8 kb; uPA, 2.4 kb.

tPA transcripts. We used as a probe M13.9l9. This M13 bacteriophage recombinant contains the nucleotide sequences encoding 16 amino acids of leader sequence, the finger domain, growth factor homology region, kringle no. 1 and the sequences encoding 25 amino acids of kringle no. 2. M13.9l9 bacteriophage DNA was uniformly labeled in vivo using [^{32}P]orthophosphate to a specific activity of 4.37×10^6/μg. The single-stranded bacteriophage

DNA was hybridized to tissue RNAs for 14 h to allow duplex formation. Samples were then digested with S_1 nuclease to degrade unprotected (non-duplexed) radiolabeled DNA. The nucleic acids, post-S_1 treatment, were analyzed on 6% acrylamide/8 M urea gels. tPA transcripts present in murine tissues that have identity with F9 cell tPA generate a 675 bp fragment after S_1 digestion (see F9-RA/dbcAMP, fig.2). RNA from F9 stem

Table 1

Relative levels of plasminogen activator RNA in murine tissues[a,b]

Tissue	tPA[c]
Uterus	100
Oviduct	100
Pituitary	69
Cerebellum	49
Heart	38
Ovary	35
Cerebrum	34
Brain stem	31
Kidney	30
Lung	28
Adrenal	15
Testis	14
Epididymis	14
Trachea[d]	9
Vas deferens	7
Muscle[e]	3
Intestine	3
Thymus	2
Stomach	2
Spleen	0.5
Pancreas	N.D.
Liver	N.D.

[a] Relative levels of plasminogen activator RNA were determined by dot blot analysis (see Section 2)

[b] uPA RNA was observed only in kidney, vas deferens and ovary. While kidney and vas deferens contain similar amounts of uPA RNA, ovary contains 2% of the amount found in these tissues

[c] Results reflect analysis of 2-3 separate RNA preparations. Amount of tPA RNA observed for uterine tissue given an arbitrary value of 100. N.D., none detected

[d] Tracheal tissue samples contain, in addition, RNA from the thyroid and parathyroid

[e] Dissected from hind limb

cells and liver, which do not contain detectable amounts of tPA mRNA (as determined by Northern blot analysis) served as negative controls. For all tissues containing tPA mRNA, only the 675 bp fragment was observed (fig.2).

4. DISCUSSION

We have performed Northern gel and dot blot analysis with total cellular RNA to define mouse tissues that accumulate detectable amounts of tPA mRNA. After obtaining this information, we used a portion of the mouse tPA cDNA to perform S_1 protection assays to determine whether tPA mRNAs were alternately spliced. Our results

demonstrate that the tPA mRNAs in these tissues encode a protein with the same non-protease structural domains found for the F9 cell protein. tPA proteins translated from these RNAs should contain signal sequences, a growth factor homology region, finger domain and two kringles. The data suggest that the apparent molecular mass differences observed with tPA may be the result of proteolysis or carbohydrate differences.

The results of this study should not be interpreted as conclusive evidence that tissue specific tPA RNA splicing products do not exist. First, our study analyzed RNA from untreated animals. In a number of cell types, plasminogen activator production is subject to specific regulatory controls [34]. With rat granulosa cells, for instance, tPA mRNA accumulates only after exposure to gonadotropins [35]. It may be that additional forms of tPA RNA exist in mouse tissues, but they are not present at the time of killing. Alternatively, these RNAs may have been a minor component of the total tPA RNA population and therefore were not detected and/or the cells producing these RNAs may constitute only a small percentage of the total tissue mass. With this in mind, it would be useful to determine for Detroit 552 cells [23] the percentage of 'finger-less' tPA RNAs present in the total tPA mRNA population. Similar studies for the two uPA mRNA transcripts [24] could also be performed.

The two mammalian plasminogen activators, tPA and uPA, have the same substrate specificity, converting the zymogen plasminogen to plasmin. However, the plasminogen activator gene products are not similarly expressed in the mouse. While tPA mRNA is observed in the majority of the tissues analyzed, uPA mRNA accumulation is seen with only three of the tissues examined. The significance of this observtion is not clear, but the data are consistent with the production of the protease tPA by a wide variety of tissues.

Since the analysis of plasminogen activator RNA content was carried out in animals not treated with exogenous substances prior to killing, the factor(s) that could influence tPA gene expression is (are) not known. However, it may be that the high levels of tPA mRNA in many tissues reflect, in part, that RNA production is constitutive. The accumulation of tPA mRNA does not necessarily ensure that proportionate levels of
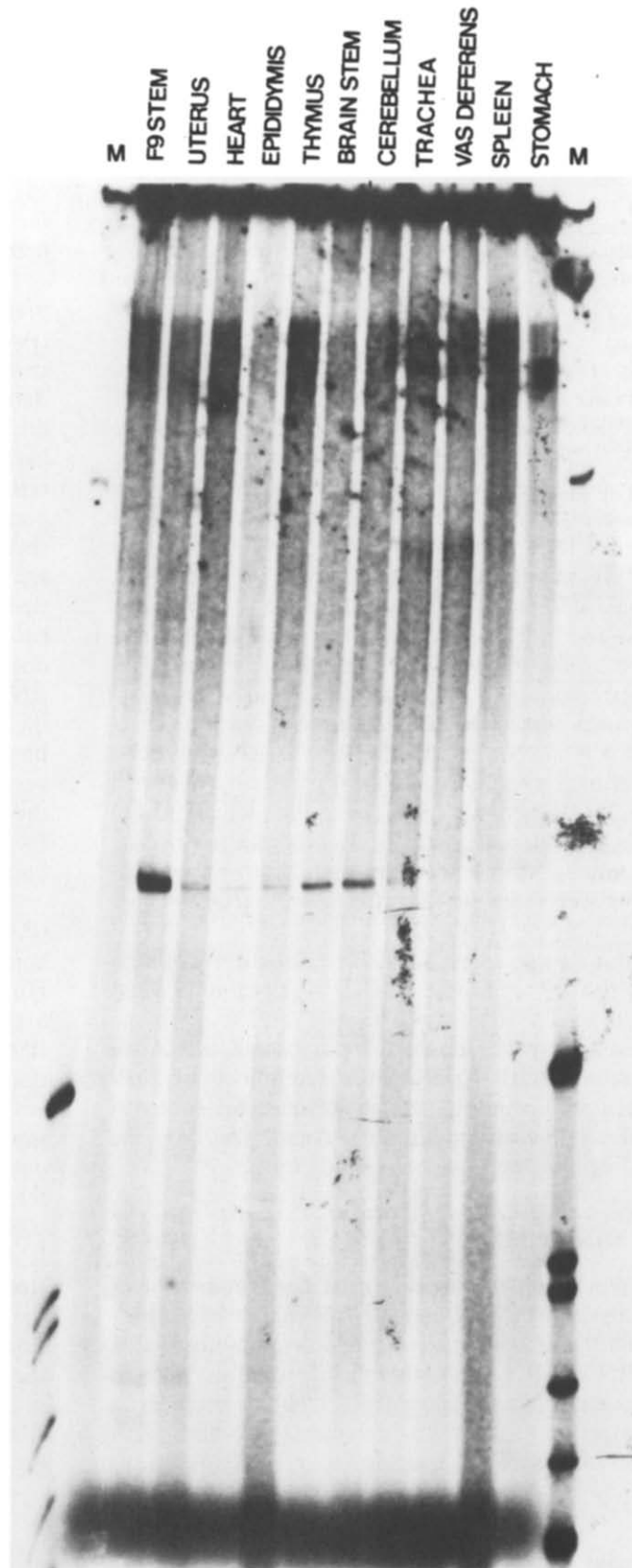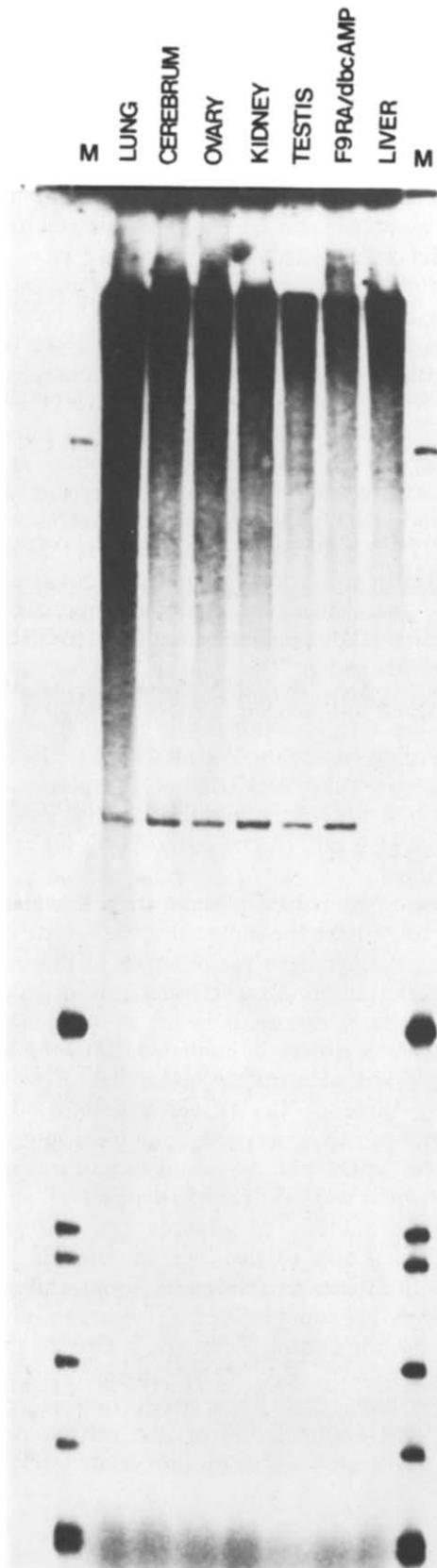
Fig.2. S_1 protection analysis of tissue tPA mRNAs using M13.9L9. M13.9L9 single-stranded bacteriophage DNA (120 ng, 524 000 cmp) was hybridized with the following amount of total cellular RNA: (A) liver, 100 μg; F9-RA dbcAMP (F9 cells treated with retinoic acid and dibutyryl cAMP), 30 μg; testis, 50 μg; kidney, 50 μg; ovary, 50 μg; cerebrum, 50 μg; lung, 35 μg. (B) F9-stem, 100 μg; F9-RAC, 7.5 μg; uterus, 7.5 μg; oviduct, 7.5 μg; brain stem, 25 μg; heart, 25 μg; trachea, 15 μg; adrenal, 7.5 μg; epididymis, 25 μg; cerebellum, 25 μg; vas deferens, 7.5 μg; spleen, 100 μg; stomach, 100 μg. Radiolabeled pBR322 HinfI restriction fragments were included as molecular mass markers (lane M).

extracellular active enzyme will be produced; certain cells may exhibit translational control [36], or the cells producing tPA may be able to regulate the extent to which the protein is secreted. Finally, plasminogen activator inhibitors may be present to regulate the levels of enzyme activity.

The present data document the accumulation of tPA mRNA in a wide variety of murine tissues. However, the question of which cell types produce the observed tPA mRNA is not addressed. One candidate is vascular endothelial cells which are known producers of tPA enzyme [37] and which would be present in all of our tissue preparations. However, the fact that there were low or undetectable levels of tPA mRNA in liver, intestine, and spleen – organs with considerable vascularization – suggests that in addition, other cell types produce the tPA mRNA observed in our experiments. For example, tPA can be synthesized by neurons [38,39], neuronal accessory cells [20], pituitary cells [13,40], pancreatic β-cells [16], granulosa cells [2] and oocytes [36]. It is probable that many additional cell types produce tPA, but these cell types have not been defined.

Our tissue survey, while not allowing us to identify the cell types that produce tPA, serves as good background information for defining tissue localization. The mouse tPA cDNA should prove to be a useful probe for identifying in situ the cell types that produce tPA, which should lead to a better understanding of tPA gene expression.

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