Quantification of tissue-type plasminogen activator (t-PA) mRNA in human endothelial-cell cultures by hybridization with a t-PA cDNA probe

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We describe the construction of a recombinant DNA plasmid, consisting of the vector pBR322 and full-length tissue-type plasminogen-activator (t-PA) cDNA, by using polyadenylated RNA from cultured Bowes melanoma cells as substrate. A 1280-base-pair *PstI* restriction fragment, covering the 3′ untranslated region and part of the coding region for the t-PA L-chain, was used as a radiolabelled probe to determine the size and the number of t-PA mRNA molecules in cultured endothelial cells of different origin from the same individual. Northern blotting showed that in all these cells a t-PA mRNA is synthesized of about 2500 nucleotides, indicating that transcriptional initiation, splicing and polyadenylation is similar. The number of t-PA mRNA molecules per cell measured, by using a dot-blotting technique and t-PA mRNA made *in vitro*, with a plasmid DNA preparation harbouring a specific promotor of the *Salmonella typhimurium* bacteriophage SP6, t-PA cDNA and SP6 RNA polymerase as standard, is approx. 10000 in all cultured endothelial cells from adult vessels. However, the amount of t-PA antigen synthesized and/or secreted differs by a factor of 6–20. Relatively large amounts of t-PA antigen secreted were detected in conditioned medium from vena-cava-derived cells, whereas low amounts were found in conditioned medium from arteria-iliaca-derived cells.

INTRODUCTION

The serine-proteinase tissue-type plasminogen activator (t-PA) is believed to play a key role in fibrinolysis, the dissolution of a blood clot. t-PA promotes the conversion of the zymogen plasminogen to plasmin, a serine proteinase of broad specificity which is the major enzyme involved in degradation of fibrin. t-PA in the blood-stream is presumed to be derived from the vascular wall, where it is localized in the endothelial cells (Rijken *et al.*, 1980). Therefore, the endothelium may play an important role in the regulation of the blood-fibrinolytic activity. However, little is known at the molecular level about the mechanism(s) by which t-PA synthesis or secretion is regulated.

t-PA is known to be secreted by the human Bowes melanoma cell line (Rijken & Collen, 1981). The construction of a t-PA-cDNA-containing plasmid from a Bowes-melanoma cDNA library made it possible to develop a sensitive t-PA mRNA assay. Together with the development of tissue- and cell-culture techniques and specific assays for t-PA antigen (Rijken *et al.*, 1984), this assay will enable us to study the regulation of t-PA biosynthesis *in vitro*, which may provide insight into the causes of thrombotic diseases.

In this investigation we have measured the t-PA mRNA in, and t-PA antigen secreted by, cultured endothelial cells from different vascular origin, derived from one adult individual.

MATERIALS AND METHODS

Cell culture

Endothelial cells from the aorta, vena cava superior and arteria iliaca communis of an adult female human donor were isolated by the method of Jaffe *et al.* (1973) and grown on fibronectin-coated T25 flasks (Costar) in M-199 medium supplemented with 20% (v/v) human serum, 200 μg of bovine hypothalamus extract ('endothelial cell growth factor'; Maciag *et al.*, 1979)/ml, 100 units of penicillin/ml and 100 μg of streptomycin/ml under air/CO_2 (19:1).

At confluency, the cells were released with trypsin/EDTA and passaged with a 1:5 split ratio to obtain subcultures. No visible contamination with smooth-muscle cells was observed by phase-contrast microscopy. The endothelial cells showed a typical 'cobblestone' morphology at confluency, contained factor-VIII-related antigen and bound *Ulex europeus* lectin I.

Endothelial cells from human umbilical veins and arteries were isolated as described above and cultured as previously reported (Van Hinsbergh *et al.*, 1983). The conditions for culturing these cells were similar to those

Abbreviations used: t-PA, tissue-type plasminogen activator; poly(A)$^+$ RNA, polyadenylated RNA; SSC, 0.15 M-NaCl/0.015 M-sodium citrate; bp, base-pairs.

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for adult human endothelial cells, except that the media were supplemented with 10% (v/v) human serum and 10% (v/v) newborn-calf serum (not heat-inactivated). All endothelial cells used in the experiments were from the third passage of the cultures.

Bowes melanoma cells were kindly provided by Dr. D. B. Rifkin (Department of Cell Biology, New York University School of Medicine, New York, U.S.A.). The cells were routinely cultured in 25 cm^2 flasks in Dulbecco's modification of Eagle's medium, supplemented with heat-inactivated newborn-calf serum (10%, v/v), glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and $NaHCO_3$ (1.75 mg/ml) at 37 °C under air/CO_2 (19:1). Cells were subcultured by trypsin/EDTA treatment.

Isolation of RNA

After collecting the medium from the cells, the cell monolayers were washed twice with ice-cold phosphate-buffered saline (10 mM-sodium phosphate/0.14 M-NaCl, pH 7.4). Ice-cold phosphate-buffered saline (2 ml) was added to each of the dishes, and cells were scraped off from the dishes with a rubber policeman. Cell suspensions were collected by centrifugation (1000 *g* for 10 min) and stored at −90 °C.

To isolate RNA, cells were suspended in 50 mM-sodium acetate, pH 5.2, containing 10 mM-vanadyl–ribonucleoside complex to prevent RNA degradation during the extraction. Cells were lysed by adding SDS to a final concentration of 1%. Total RNA was extracted by a hot-phenol method (Warner *et al.*, 1966). The efficiency of the isolation procedure was over 90% as determined by the addition of radiolabelled SP6 RNA synthesized *in vitro*. Poly(A)$^+$ RNA was selected by two cycles of oligo(dT)–cellulose chromatography (Aviv & Leder, 1972).

cDNA cloning

t-PA-producing human Bowes melanoma cells were cultured on micro carriers (Kluft *et al.*, 1983). Poly(A)$^+$ RNA from 2×10^9 cells was size-fractionated on a 5–30% (w/v) sucrose gradient containing 0.1 M-Tris/HCl (pH 7.5), 1 mM-EDTA, 0.2% SDS and 50% (v/v) formamide. After ethanol precipitation of the fractions, small amounts were micro-injected into *Xenopus laevis* oocytes. The oocytes were subsequently cultivated in fibrin plates containing Barth's medium (Gurdon *et al.*, 1971). Oocytes that were secreting biologically active t-PA could be identified by the appearance of lysis zones, whereas in fibrin plates with anti-t-PA IgG (2 mg/ml) no lysis zones could be detected (A.-J. van Zonneveld, unpublished work). The 20–22 S fractions contained biologically active t-PA mRNA; 3 μg of 20–22 S poly(A)$^+$ RNA was used as a substrate for oligo(dT)-primed cDNA synthesis. The RNA was denatured in 10 mM-methylmercury hydroxide, and pancreatic ribonuclease inhibitor (RNasin; Biotec, Madison, WI, U.S.A.) (1 unit/μl) was added as a ribonuclease inhibitor. First-strand synthesis, RNA hydrolysis and second-strand synthesis were done by standard procedures (Maniatis *et al.*, 1982). Cleavage of the hairpin structure of the double-stranded cDNA was performed by incubation with mung-bean nuclease. The resulting double-stranded cDNA was size-fractionated on a Sepharose CL-4B column, and cDNA larger than 600 bp was C-tailed and annealed to a G-tailed pBR322 vector.

Transformation was carried out with *Escherichia coli* DH1 cells (Hanahan, 1983). Colonies were screened for t-PA cDNA clones by colony hybridization using a synthetic oligonucleotide probe (20-mer) complementary to t-PA mRNA (nucleotides 1872–1891; Pennica *et al.*, 1983).

In order to isolate a full-length t-PA cDNA clone, a second Bowes-melanoma cell cDNA bank was made by using cDNA prepared by the ribonuclease-H method (Gubler & Hoffman, 1983). A 5′ restriction fragment of a partial t-PA cDNA plasmid, obtained from the first Bowes-melanoma cDNA bank, was employed as probe in the identification and isolation of a full-length t-PA cDNA plasmid.

Synthesis of SP6-t-PA mRNA *in vitro*

The 3′ 1280-bp *Pst*I restriction fragment of full-length t-PA cDNA was cloned into the *Pst*I site of plasmid pSP64 (Melton *et al.*, 1984) in parallel to the SP6 promotor (pSP1280). *Bam*HI-linearized pSP1280 DNA was used as a DNA template for the synthesis of SP6-t-PA mRNA *in vitro*, by using SP6 RNA polymerase. Reaction conditions were essentially as described by Melton *et al.* (1984). Routinely, about 5–10 μg of t-PA mRNA was made by using 1 μg of linear SP6-t-PA DNA.

To purify the SP6-t-PA mRNA, SDS was added to a final concentration of 0.1% and the reaction mixture was layered on a 1.5% (w/v) low-melting-agarose gel. After electrophoresis, gel slices containing SP6-t-PA mRNA were cut out, melted and purified by repeated extractions with phenol and precipitated with ethanol. The RNA concentration was determined spectrophotometrically.

Dot-blot hybridization

Nitrocellulose filters (BA 85; Schleicher and Schüll, Dassel, W. Germany) were wetted thoroughly with sterile distilled water and equilibrated with 10 × SSC. RNA was collected by centrifugation for 30 min at 12000 *g* at 4 °C, and to the pellet 15% (v/v) formaldehyde (100 μl) was added for denaturation, which led to a better RNA retention on the filter. An equal volume (100 μl) of 20 × SSC was added, and the RNA was applied to the filter by using a manifold filtration apparatus (Bio-Rad, Richmond, CA, U.S.A.). Tobacco-mosaic-virus RNA and poly(A)$^+$-depleted RNA from human liver were used as controls to determine non-specific hybridization. After application of the samples, the filter was baked for 2 h at 80 °C under vacuum, and pre-hybridized overnight at 60 °C in 100 ml of 3 × SSC/5 × Denhardt's [Ficoll (1 μg/ml), polyvinylpyrrolidone (1 μg/ml), bovine serum albumin (1 μg/ml)] 0.1% sodium pyrophosphate/5 mM-EDTA (pH 8.0)/0.1% SDS containing 50 μg of sonicated, heat-denatured herring sperm DNA/ml.

Hybridization was performed overnight at 60 °C in the same solution (5 ml) containing 10^6 c.p.m. of ^{32}P-labelled heat denatured cDNA probe/ml. Radiolabelling of a 1280 bp *Pst*I restriction fragment of t-PA cDNA was done by 'nick' translation (Maniatis *et al.*, 1982). The specific radioactivity of the ^{32}P-labelled probe was approx. 10^8 c.p.m./μg of DNA.

Northern-blot analysis

Electrophoresis of RNA samples was performed in a 1.5% agarose gel in E-buffer (0.018 M-Na_2HPO_4, 0.002 M-NaH_2PO_4) containing 6% (v/v) formaldehyde. After centrifugation for 30 min at 12000 *g*, RNA was

dissolved in 10 μl of E-buffer containing 50% (v/v) formamide and 6% formaldehyde. After incubation for 5 min at 60 °C, samples were quickly chilled in ice and 5 μl of E-buffer containing 50% formamide, 20% (v/v) glycerol and 0.12% Bromophenol Blue was added. After application of the samples to the gel, electrophoresis was performed overnight at 25V. The gel was washed twice with 10 mM-sodium phosphate (pH 7.0) for 10 min each at room temperature. RNA was transferred to a nitrocellulose filter in 20 × SSC overnight at room temperature (Maniatis *et al.*, 1982). After the transfer the filter was dried and hybridization was performed as described above.

Determination of t-PA antigen secreted

Cultured endothelial cells derived from the umbilical-cord vein and artery secrete a molar excess of the fast-acting plasminogen-activator inhibitor versus t-PA (Emeis *et al.*, 1983). The same observation was made with cultured endothelial cells derived from adult vessels (T. Kooistra, J. H. Verheijen & C. Kluft, unpublished work). Consequently, t-PA synthesized and secreted is fully complexed with the inhibitor. Determination of t-PA antigen was performed with an enzyme-linked immunosorbent assay ('ELISA') as described by Rijken *et al.* (1984). In this assay the concentration of t-PA antigen is measured as follows: t-PA in conditioned medium is absorbed on to microtitre plates coated with rabbit anti-human t-PA antibodies and then quantified by successive incubation with goat anti-human t-PA antibodies and rabbit anti-goat IgG conjugated with alkaline phosphatase. The detection limit of the assay is about 1 ng of t-PA antigen/ml, and experiments have shown that quenching by complex-formation of t-PA with the fast-acting inhibitor produced by endothelial cells is approx. 50%. Thus there are no differences in the percentage of quenching in the ELISA, since, with both the adult cells and the umbilical-cord-derived cells, the molar excess of the inhibitor is at least 10-fold. Finally, the assay specifically detects t-PA antigen and not urokinase-like plasminogen activators.

RESULTS AND DISCUSSION

Construction of full-length t-PA DNA

We intend to study the regulation of fibrinolysis. The biosynthesis of t-PA is a crucial factor in this process. We have applied recombinant-DNA techniques to construct both partial and full-length t-PA cDNA. These cDNA probes have been used to monitor the quality and the amount of t-PA mRNA in endothelial cells from different vessels.

Two approaches were used to construct t-PA cDNA and to create human cDNA libraries in *E. coli* DH1 cells. For both methods poly(A)$^+$ RNA from large-scale cultures of Bowes melanoma cells served as substrate for cDNA synthesis. These RNA preparations were shown to contain biologically active t-PA mRNA (as outlined in the Materials and methods section). The first cDNA synthesis was done with a conventional protocol in which mung-bean nuclease is used to degrade the 'hairpin' structure that arises after first-strand synthesis with reverse transcriptase. A cDNA library, with G-tailed pBR322 as vector, of 42000 independent colonies was screened by colony hybridization with a synthetic radiolabelled 20-mer, derived from the established sequence of the 3′ untranslated region (Pennica *et al.*, 1983). Two t-PA cDNA plasmids were found which contained overlapping inserts of 1050 and 1700 bp, spanning the 3′ untranslated region and the L-chain of t-PA (Fig. 1). The 5′ 435 bp *Pst*I fragment of the longest t-PA cDNA plasmid was radiolabelled by 'nick translation' and used as probe for another cDNA library. The second cDNA synthesis was performed essentially by the ribonuclease-H method: 60 ng of size-fractionated (larger than 600 bp) C-tailed cDNA was annealed to G-tailed pBR322 DNA to generate a cDNA library in *E. coli* DH1 of 120000 colonies. We found eight positive colonies, of which one colony appeared to harbour a plasmid with a full-length t-PA cDNA insert of about 2540 bp (ptPA8FL), including a 90 bp 5′ untranslated region, the entire coding sequence and a 773 bp 3′ untranslated region. A comparison of restriction-enzyme-

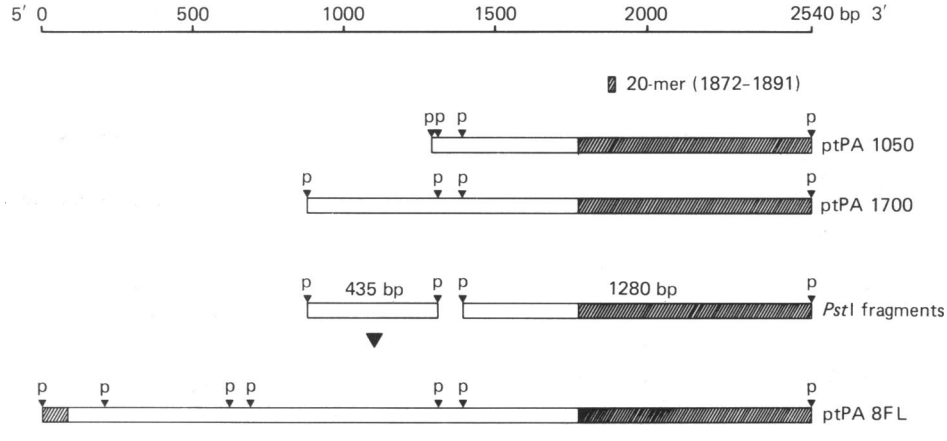

Fig. 1. Schematic representation of the isolated partial (pt-PA1050 pt-PA1700) and full-length (ptPA8FL) cDNA inserts

A synthetic 20-nucleotide probe (nucleotides 1872–1891) was used to isolate ptPA1050 and ptPA1700. The 5′ 435-bp *Pst*I fragment of ptPA1700 was used as a probe to isolate ptPA8FL. Numbers are according to Pennica *et al.* (1983). Key: p, *Pst*I restriction sites; 5′ and 3′ untranslated sequences are presented by shaded boxes.

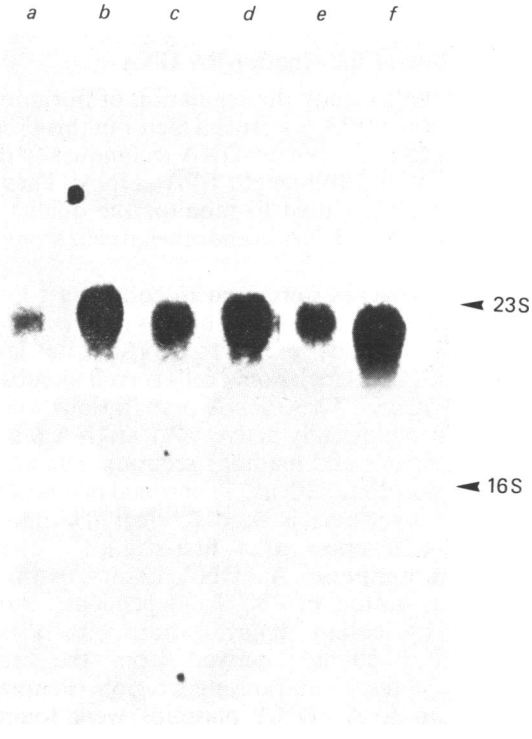

Fig. 2. Northern-blot analysis of total RNA preparations of the endothelial-cell cultures and the Bowes melanoma cell culture

Total RNA preparations were treated with formamide and formaldehyde, subjected to electrophoresis on a 1.5%-agarose/formaldehyde gel and transferred to nitrocellulose. The RNA blot was hybridized with ^{32}P-labelled 1280 bp *Pst*I fragment of ptPA8FL. Total RNA from endothelial cells derived from the aorta (*a*), vena cava superior (*b*), arteria iliaca communis (*c*), umbilical-cord vein (*d*) and umbilical-cord artery (*e*). Total RNA from Bowes melanoma cells (*f*).

digestion patterns and DNA sequence analysis with that reported by Pennica *et al.* (1983) confirmed the authenticity of the t-PA-cDNA-containing plasmids (results not shown).

Northern-blot analysis of endothelial RNA

Total RNA was isolated from cultured endothelial cells of different vessels of the same adult individual. Moreover, RNA preparations were made from cultured endothelial cells of both the vein and an artery of an umbilical cord. These preparations were subjected to electrophoresis under denaturing conditions, blotted on to nitrocellulose membrane filters and hybridized with a ^{32}P-labelled 1280 bp *Pst*I t-PA cDNA fragment (Fig. 2).

The results show that in all these cells a single t-PA mRNA species is synthesized, with a length of approx. 2500 nucleotides. Apparently, transcriptional initiation, polyadenylation and splicing proceed with the same specificity. A control experiment with Bowes melanoma RNA shows that also in this cell line a single species of about 2500 nucleotides is found. We conclude that this analysis specifically detects t-PA mRNA.

Quantification of t-PA mRNA synthesis by dot-blotting

To avoid discrepancies in transfer of RNA during Northern blotting, we have chosen dot-blotting to estimate the number of t-PA mRNA molecules synthesized in cultured endothelial cells from different origins. The reliability of the RNA–DNA hybridization assay was demonstrated in a dose–response experiment (Fig. 3). Various amounts of a Bowes melanoma total RNA preparation were dot-blotted and hybridized with an apparent excess of ^{32}P-labelled t-PA cDNA probe. Hybridized material was either counted for radioactivity or scanned after autoradiography. The linear relationship between dose of RNA and hybridization shows that under these conditions the amount of spotted RNA is limiting.

As a standard for quantitative determinations we used synthesized t-PA mRNA *in vitro*. For that purpose we cloned the 1280 bp *Pst*I restriction fragment of full-length t-PA cDNA on vector pSP64. This pUC12-derived plasmid harbours a promotor which is exclusively recognized by RNA polymerase of the *Salmonella typhimurium* phage SP6. In this construction the orientation of t-PA cDNA is in parallel with the SP6 promotor. Linearization with *Bam*HI, which cleaves the

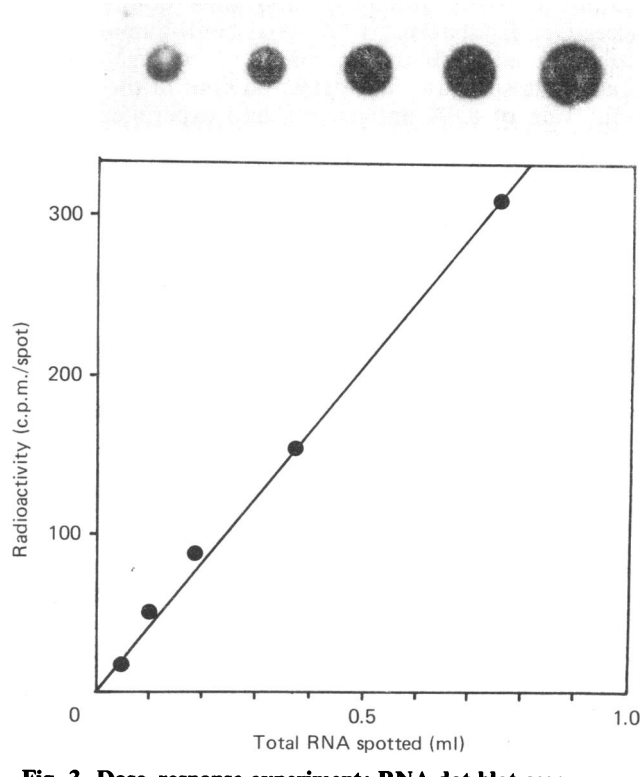

Fig. 3. Dose–response experiment: RNA dot-blot assay

A serial dilution of a Bowes melanoma total RNA preparation was dot-blotted on nitrocellulose and hybridized with ^{32}P-labelled 1280 bp *Pst*I fragment of ptPA8FL. The amount of probe that hybridized to the RNA on the nitrocellulose filter (upper panel) was determined by transferring the nitrocellulose filters to scintillation vials and counted for radioactivity in a liquid-scintillation counter. The indicated values represent averages of duplicate determinations (S.D. approx. 10%).

Table 1. Number of t-PA mRNA molecules per cell, the number of t-PA molecules per cell present in the culture medium after 24 h and the ratio of these parameters for the endothelial-cell cultures and the Bowes melanoma cell lines

The number of t-PA molecules/cell was calculated as follows: for each total RNA preparation, serial dilutions were dot-blotted and the amount of radioactivity (c.p.m.) hybridized per cell (*a*) was determined. The number of cells used for each total RNA preparation was counted with a haemocytometer. With a series of standard SP6-t-PA mRNA dilutions (with a known number of t-PA molecules/ml) we determined the number of t-PA mRNA molecules per c.p.m. hybridized (*b*). $a \times b$ gives the number of t-PA mRNA molecules/cell. The number of t-PA molecules present in the medium of the cells per 24 h was determined as described in the Materials and methods section.

Cell type	10^{-4} × No. of t-PA mRNA molecules/cell	10^{-4} × No. of t-PA molecules (antigen) secreted in medium/cell	t-PA molecules secreted/t-PA mRNA molecules
Aorta	2.1	90	43
Vena cava superior	1.3	320	246
Arteria iliaca communis	1.2	14	11
Umbilical-cord vein	0.18	7.5	41
Umbilical-cord artery	0.22	6.4	29
Melanoma cell line (Bowes)	0.78	800	1026

SP6-t-PA plasmid 3′ of the t-PA cDNA insertion, provides a template for efficient 'run-off' transcription *in vitro* with SP6 RNA polymerase.

Serial dilutions of total RNA preparations of cultured adult endothelial cells, derived from the aorta, vena cava superior and arteria iliaca communis, and from the vein and artery of an umbilical cord, were dot-hybridized with an excess of the radiolabelled probe. The results are presented in Table 1.

Using a standard series of SP6-t-PA mRNA dilutions we can estimate the number of t-PA mRNA molecules per cell. We find that the endothelial cells derived from the adult vessels (aorta, vena cava superior and arteria iliaca communis) have a comparable t-PA mRNA content; 5–10-fold lower amounts were detected in umbilical-cord-derived cells. For the Bowes melanoma cells we can estimate that the amount of t-PA mRNA is about 0.1% of the poly(A)$^+$ RNA in the cells, assuming that poly(A)$^+$ RNA amounts to 2–5% of total RNA. This value is substantially higher than was expected from the number of t-PA-cDNA-containing clones in a Bowes melanoma cDNA library (Pennica *et al.*, 1983; the present work). Apparently, in this case the number of cDNA clones is not a true reflection of the abundancy of the mRNA. This observation is in contrast with a previous statement by McKnight & McConnaughy (1983) that for various *Saccharomyces cerevisiae* genes the number of cDNA clones in a library correlated with the abundancy of the mRNA.

Ratio of t-PA mRNA synthesis and t-PA antigen secreted in cultured endothelial cells

Previous work has shown that cultured endothelial cells do not store t-PA antigen, but display a linear release over many passages (Rijken *et al.*, 1984). Furthermore, all cultured endothelial cells secrete a molar excess of the fast acting plasminogen-activator inhibitor over t-PA antigen. Therefore, an ELISA was developed which detects t-PA antigen in the presence of excess inhibitor (Rijken *et al.*, 1984). It was reported that complex-formation between these components causes a 2-fold decrease in the amount of t-PA antigen detected. Hence, all t-PA antigen values found have been corrected, except for the control (Bowes melanoma) cells, which produce 'free' t-PA antigen. The data on the amount of t-PA antigen secreted by various cultured endothelial cells are given in Table 1. It is evident that substantially different amounts of t-PA antigen are secreted among these cultured adult endothelial cells from different vascular origin. Vena-cava-derived cells secrete about 6-fold more than aorta-derived cells and about 20-fold more than the endothelial cells from the arteria iliaca communis. Cultured endothelial cells derived from a newborn secrete t-PA antigen in amounts similar to adult arteria-iliaca-communis-derived cells. Thus, in spite of a comparable number of t-PA mRNA molecules synthesized in these different adult endothelial cells, the number of t-PA molecules per mRNA molecule greatly differs. The Bowes melanoma cell line displays exceptionally high secretion of this protein and, for that reason, is considered to be a convenient source for the production of t-PA. Opdenakker *et al.* (1985) have demonstrated that the t-PA mRNA content in Bowes melanoma cells increases 15–20-fold after exposure to the tumour promotor 12-*O*-tetradecanoylphorbol 13-acetate. Exposure to the letter of the Bowes melanoma cells used in the present work, under the same conditions as Opdenakker *et al.* (1985), does not affect the t-PA mRNA content, although we obtained the cells from the same source (Dr. R. B. Rifkin) (T. Kooistra, J. H. Verheijen & C. Kluft, unpublished work). Apparently, continuous propagation of the Bowes melanoma cells and clone secretion may cause this striking difference on regulation of expression. Further experiments are required to determine whether the amount of t-PA mRNA determined in our experiments corresponds to the induced amount as reported by Opdenakker *et al.* (1985).

Furthermore, it should be noted that identical results on secretion of t-PA antigen were found with different donors (both males and females) for adult vascular endothelial cells and with endothelial cells which had not been cultured, but were assayed immediately on collection of cells (results not shown). This implies that the observations that we have presented in this paper are

not caused by an artifact of culturing these cells. We conclude that a sensitive and reliable assay for the determination of t-PA mRNA is a valuable tool to study regulation of fibrinolysis.

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REFERENCES

Aviv, H. & Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. **69**, 1408–1412

Emeis, J. J., van Hinsbergh, V. W. M., Verheijen, J. H. & Wijngaards, G. (1983) Biochem. Biophys. Res. Commun. **110**, 392–398

Gubler, U. & Hoffman, B. J. (1983) Gene **25**, 263–269

Gurdon, J. B., Lane, C. D., Woodland, H. R. & Marbaix, G. (1971) Nature (London) **233**, 177–181

Hanahan, D. (1983) J. Mol. Biol. **166**, 557–580

Jaffe, E. A., Nachman, R. L., Becker, C. G. & Minick, C. R. (1973) J. Clin. Invest. **52**, 2745–2756

Kluft, C., van Wezel, A. L., van der Velden, C. A. M., Emeis, J. F., Verheijen, J. H. & Wijngaards, G. (1983) Adv. Biotechnol. Processes **2**, 97–110

Maciag, T., Cerlindolo, J., Iesley, S., Kelley, P. R. & Forand, R. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 5674–5678

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor

McKnight, G. L. & McConnaughy, B. L. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 4412–4416

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) Nucleic Acids Res. **12**, 7035–7056

Opdenakker, G., Billiau, A., Volckaert, G. & De Somer, P. (1985) Biochem. J. **231**, 309–313

Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Venar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L. & Goeddel, D. V. (1983) *Nature* (London) **301**, 214–221

Rijken, D. C. & Collen, D. (1981) J. Biol. Chem. **256**, 7035–7041

Rijken, D. C., Wijngaards, G. & Welbergen, J. (1980) Thromb. Res. **18**, 815–830

Rijken, D. C., Van Hansbergh, V. W. M. & Sels, E. H. C. (1984) Thromb. Res. **33**, 145–153

Van Hinsbergh, V. W. M., Havekes, L., Emeis, J. J., van Corven, E. & Scheffler, M. (1983) Arteriosclerosis **3**, 547–559

Warner, J. R., Soeiro, R. & Birnboim, H. C. (1966) J. Mol. Biol. **19**, 349–361

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