GENE 1198

# Isolation of a human tissue-type plasminogen-activator genomic DNA clone and its expression in mouse L cells

(Recombinant DNA; cosmid vector; bacteriophage $\lambda$; fibrinolysis; Bowes melanoma)

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SUMMARY

We have isolated a cDNA clone corresponding to a substantial portion of the human tissue-type plasminogen activator (t-PA) protein. It encodes almost all of the protein B chain and part of the 3' untranslated region. We have used this clone to screen bacteriophage $\lambda$ and cosmid libraries of human genomic DNA. Several related genomic clones were isolated. One of these, a cosmid clone, carried approx. 40 kb of human DNA. Mapping experiments indicate that the region containing the protein-coding exons is approx. 20 kb in length. The cosmid, containing the t-PA gene and the aminoglycosyl-3'-phosphotransferase dominant-selection marker, was introduced into mouse L cells. Approximately half of the transformants were shown to produce human t-PA. We demonstrated that the fibrinolytic t-PA activity could be specifically quenched by anti-t-PA antibody and that the recombinant t-PA was of similar size (by SDS-polyacrylamide gel electrophoresis) to the t-PA produced by the human Bowes melanoma cell line. Our results suggest that the cosmid clone carries the whole t-PA coding region together with the regulatory elements necessary for its expression.

INTRODUCTION

Plasminogen activators appear to play an important role in the fibrinolytic system. These enzymes are capable of converting inactive plasminogen to its catalytically active form, plasmin, which degrades the fibrin network associated with blood clots. There are two major types of human plasminogen activator, namely urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). These proteins are immunologically distinct, differ in $M_r$ and are encoded by different genes (Heyneker et al., 1982; Pennica et al., 1983). Particular attention has been drawn to the clinical use of t-PA since recent studies indicate that administration of t-PA may provide therapeutic benefit in iliofemoral thrombosis (Weimar et al., 1981) and in thrombolysis of myocardial infarctions (Van de Werf et al., 1984).

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Abbreviations: bp, base pair(s); EtBr, ethidium bromide; HPLC, high-performance liquid chromatography; kb, 1000 bp; SDS, sodium dodecyl sulphate; SSC, 0.15 M NaCl, 0.015 M $Na_3 \cdot$ citrate, pH 7-8; t-PA, human tissue-type plasminogen activator; u-PA, human urokinase-type plasminogen activator.

The current method of production of t-PA from the human Bowes melanoma cell line is, however, unlikely to provide sufficient t-PA for future clinical needs (Rijken and Collen, 1981; Van de Werf et al., 1984). As a consequence, a number of laboratories are attempting to produce t-PA by means of recombinant DNA technology. Thus cDNA clones have been obtained (Pennica et al., 1983; Edlund et al., 1983) and the t-PA cDNA expressed in *Escherichia coli* (Pennica et al., 1983). We decided to isolate the genomic DNA for t-PA in order to provide a basis for a better understanding of the t-PA molecule. We were also interested in the feasibility of expressing the t-PA gene in mammalian cell culture since t-PA synthesised in *E. coli* is not glycosylated (Pennica et al., 1983). We took advantage of recent advances in cosmid cloning technology which, combined with new dominant selection markers, facilitate the isolation of genomic DNA clones spanning even relatively large genes and the direct expression of such genes in eukaryotic systems (Grosveld et al., 1982).

MATERIALS AND METHODS

## (a) Enzymes and special materials

Restriction endonucleases, T4 DNA ligase and polynucleotide kinase were obtained from Bethesda Laboratories (U.K.) Ltd. Nitrocellulose filters (BA 85) were from Schleicher & Schüll GmbH, Dassel, W. Germany, and GeneScreen™ was from New England Nuclear, Boston, MA, U.S.A. Urokinase was from Leo Laboratories Ltd., U.K. Human fibrinogen was from Flow Laboratories, Irvine, U.K. Radioisotopically labelled compounds and nick-translation kits were from Amersham International plc, U.K. Fuji-RX film was from Fujimex Ltd., Dorcan, U.K. Antibiotic G418 sulphate was from Gibco Europe Ltd., Paisley, U.K.

## (b) Purification of messenger RNA

Total RNA was isolated from Bowes melanoma cells (Rijken and Collen, 1981) by phenol extraction (Scott et al., 1983) and mRNA was purified by single cycles of poly(U)-Sepharose or oligo(dT)-cellulose chromatography (Legon et al., 1982).

## (c) Identification of a t-PA cDNA clone

10 $\mu$g of total mRNA was used to prepare a cDNA library of approx. 50 000 recombinants. The cDNA was inserted into the pCD5 plasmid vector by dC:dG tailing and transformed into *E. coli* DH1 cells as described by Scott et al. (1983). The library was propagated on nitrocellulose filters and replicas were prepared for screening (Hanahan and Meselson, 1980). Hybridising colonies were detected by autoradiography.

## (d) Oligonucleotide probes

Oligonucleotide probes were synthesised from dimer building blocks by the solid phase phosphotriester method (Gait et al., 1982) and purified by preparative ion-exchange HPLC. Probes were end-labelled to a specific activity of $5 \times 10^7$ dpm/$\mu$g with $[\gamma\text{-}^{32}P]$ ATP as described by Maxam and Gilbert (1980). Hybridisations were performed, at 37°C for 16 h, essentially as described by Dalbadie-Mac-Farland et al. (1982), except that 0.1% SDS was substituted for NP40 in the hybridisation buffer. Filters were then washed for $4 \times 15$ min in $6 \times$ SSC containing 0.1% SDS at 45°C.

## (e) Identification of genomic t-PA clones

An amplified $\lambda$ Charon 4A bacteriophage human DNA library, kindly provided by T. Maniatis (Lawn et al., 1978), was propagated in *E. coli* LE392, and nitrocellulose copies were prepared for screening (Benton and Davis, 1977). A further human DNA library of $5 \times 10^5$ independent recombinants was prepared in the pTCF cosmid vector and nitrocellulose replicas were prepared for screening as described previously (Grosveld et al., 1982). Genomic libraries were screened using the appropriate nick-translated probe as follows. Nitrocellulose filters were prehybridised in a solution of $3 \times$ SSC, 0.1% SDS, 50 $\mu$g denatured sonicated salmon sperm DNA, 0.2% Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone for 2 h at 65°C. Hybridisation was for 16 h at 65°C in the same solution. Filters were washed for $6 \times 30$ min at 65°C in 0.1 $\times$ SSC containing 0.1% SDS, followed by $2 \times 30$-min washes at room temperature in 3 mM Tris base.

## (f) Northern and Southern blotting of nucleic acids

mRNA was fractionated by electrophoresis in a 1% agarose gel containing formaldehyde and then transferred to GeneScreen (Thomas, 1980). Human 18 and 28 S ribosomal RNAs, stained with EtBr, were used as size markers. DNA was fractionated on agarose gels and blotted onto nitrocellulose as described by Southern (1975).

## (g) DNA sequencing

The sequences of the oligonucleotide probes were verified by the method of Maxam and Gilbert (1980). Sequencing of cloned DNA was performed using the chain-termination method (Smith, 1980; Messing et al., 1981).

## (h) Transformation of mouse L cells

Transformation of mouse L cells using cosmid DNA was carried out essentially as described previously (Grosveld et al., 1982). 10 $\mu$g of cosmid DNA (without carrier) was precipitated onto approx. $10^6$ cells per 90-mm dish. Transformants were selected using 400 $\mu$g/ml G418 sulphate. Colonies of 1 to 2 mm diameter were clearly visible 12 to 14 days after transformation and were individually harvested for propagation and assay of t-PA production.

## (i) Characterisation of t-PA

Cell cultures were washed twice with serum-free medium and were incubated in serum-free medium for 48 h to harvest secreted t-PA. The medium was collected, Tween-80 added to 0.01% (w/v) and activity measured on fibrin plates (Granelli-Piperno and Reich, 1978). The $M_r$ of plasminogen activators was determined by electrophoresis on 8% SDS-polyacrylamide gels using a 4% stacking gel (Laemmli, 1970) and the fibrinolytically active species identified by fibrin zymography (Granelli-Piperno and Reich, 1978). Where appropriate t-PA was purified by continuous chromatography using zinc chelate (Rijken and Collen, 1981) and lysine Sepharose.

Antibodies to t-PA (from Bowes melanoma cells) or u-PA were raised in rabbits and the IgG fraction purified according to Hjelm et al. (1972).

## RESULTS AND DISCUSSION

## (a) Identification and isolation of a t-PA cDNA clone

We constructed a nonadecameric oligonucleotide (probe 1) of sequence 5'-d(CCTGGTCACGGTC-GCATGT)-3' which we reasoned would provide a unique probe for t-PA mRNA and its corresponding cDNA. This oligonucleotide sequence spans the boundary between the t-PA B chain coding region and the 3' untranslated region of the mRNA (Pennica et al., 1983). The selectivity of the oligonucleotide was assessed by probing a Northern blot of Bowes melanoma cell mRNA. Under appropriate conditions we observed a single hybridising band which migrated between the 18 S and 28 S ribosomal RNA markers. This result is consistent with previous estimates for the size of the t-PA mRNA (Pennica et al., 1983; Edlund et al., 1983). A cDNA library of approx. 50000 recombinants was prepared from Bowes melanoma cell mRNA and was probed under the same conditions as the Northern blot. A single clone (pTR5) was isolated and sequence analysis confirmed that it encoded part of the t-PA protein. The clone extends from nucleotide 1084 to nucleotide 2109 of the full cDNA molecule (Pennica et al., 1983) and corresponds to the majority of the t-PA B chain (lacking only 69 bp at the 5' end) plus a large segment of the 3' untranslated region.

## (b) Identification and isolation of t-PA genomic DNA clones

The plasmid pTR5 was digested with ClaI and SalI to release the t-PA cDNA (Legon et al., 1982) which was then separated from vector DNA by agarose gel electrophoresis (Maniatis et al., 1982) and nick-translated to a specific activity of 1 to $3 \times 10^8$ dpm/$\mu$g (Rigby et al., 1977).

$1.3 \times 10^6$ recombinants from the bacteriophage library were screened with the t-PA cDNA probe as described in MATERIALS AND METHODS, section e, and seven recombinants were isolated. Restriction enzyme analysis of these cloned genes revealed that they were all very similar (not shown).

$8 \times 10^5$ colonies from the cosmid library, which originally contained $5 \times 10^5$ colonies, were also screened with the t-PA cDNA probe and two colonies were isolated. Restriction enzyme analysis

showed both of the cosmids to be identical (presumably originating from the same initial recombinant) and that they carried approximately 40 kb of human DNA.

### (c) Characterisation of t-PA genomic DNA

The bacteriophage and cosmid clones were compared to the uncloned t-PA gene (in a total placental DNA preparation) by restriction enzyme analysis and Southern blotting using the nick-translated t-PA cDNA probe. $Bgl$II digestion revealed the presence of a common single hybridising fragment of approx. 5.8 kb in length. Thus most, if not all, of the t-PA B chain is coded on this $Bgl$II fragment. We have mapped the cosmid with $Bam$HI, $Cla$I, $Kpn$I, $Pvu$I and $Sal$I (Fig. 1) and located the 5.8-kb $Bgl$II fragment. Further mapping experiments (not shown) enabled us to assign the orientation of the $Bgl$II fragment with respect to the cDNA clone and (using probe 1) to locate the 3' end of the coding region (Fig. 1).

It was also of interest to determine whether any of the clones encoded the whole amino acid sequence of the t-PA protein. A second probe, with the sequence 5'-d(CCCTCTCTTCATTGCATCC)-3', corresponding to the N-terminal region of the t-PA hydrophobic signal peptide (Pennica et al., 1983), was used to examine the genomic clones. None of the bacteriophage $\lambda$ clones hybridised to probe 2 indicating that they probably lacked the 5' end of the gene. Probe 2 hybridised to a 3.9-kb $Bam$HI-$Kpn$I fragment on the cosmid clone (Fig. 1). The fidelity of

hybridisation was confirmed by subcloning and sequencing part of the 3.9-kb $Bam$HI-$Kpn$I fragment (not shown). Location of probe 2 on this fragment indicates that the region containing the amino acid coding exons is between 17 and 21 kb long.

### (d) Expression of the t-PA cosmid clone

The isolation of human t-PA bacteriophage (Pennica et al., 1983) and cosmid (Ny et al., 1984) genomic clones has been described previously. In neither case was it demonstrated whether the whole t-PA genetic locus had been cloned. While it is possible to define the protein-coding areas of a gene by mapping and DNA sequencing, there are no definitive means of detecting regulatory elements (such as promoters) solely by structural analysis of cloned DNA. To demonstrate that the whole gene was present on our cosmid we examined its ability to direct the synthesis of human t-PA, using the dominant aminoglycosyl-3'-phosphotransferase selection marker present in the pTCF vector (Grosveld et al., 1982).

Mouse L cells were chosen as the host for the t-PA cosmid since we had found that these cells do not normally secrete plasminogen activator(s). Additionally, in the unlikely situation that manipulation of the L cells might induce the expression of an endogenous murine plasminogen activator, we would be able to distinguish this from human t-PA on the basis of $M_r$. Thus it is known that there are two major types of human plasminogen activator: u-PA ($M_r$ 53 000) and t-PA ($M_r$ 63 000–65 000) (Heyneker
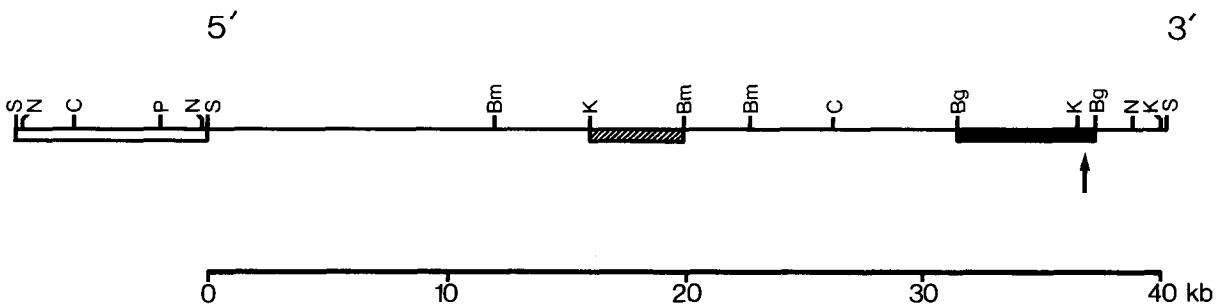


Fig. 1. Map of the t-PA cosmid clone. All $Cla$I, $Kpn$I, $Pvu$I and $Sal$I sites are shown; only relevant $Bam$HI and $Bgl$II sites are shown. Black box represents the sole $Bgl$II (5.8-kb) fragment hybridising to the t-PA cDNA probe; hatched box represents the $Bam$HI-$Kpn$I fragment hybridising to probe 2 (corresponding to the N-terminus of hydrophobic signal peptide); the arrow indicates the $Bgl$II-$Kpn$I fragment hybridising to probe 1 (3' end of protein-coding region); open box represents pTCF vector. The total length of inserted human DNA is 40 kb. Bg = $Bgl$II; Bm = $Bam$HI; C = $Cla$I; K = $Kpn$I; P = $Pvu$I; S = $Sal$I.

et al., 1982; Pennica et al., 1983). There is also a minor human t-PA component of approx. $M_r$ 56000 which is believed to be the product of limited proteolysis of the major species (Banyai et al., 1983). There are two analogous murine activators, one ($M_r$ 48000) immunologically related to u-PA and one ($M_r$ 79000) immunologically related to t-PA (Marotti et al., 1982).

Cosmid DNA was introduced into L cells as described in MATERIALS AND METHODS, section h. 27 L-cell clones were isolated and assayed for t-PA production: 55% of the clones secreted t-PA. The quantity produced by individual clones varied considerably; the highest yielding clone, TRL25, pro-
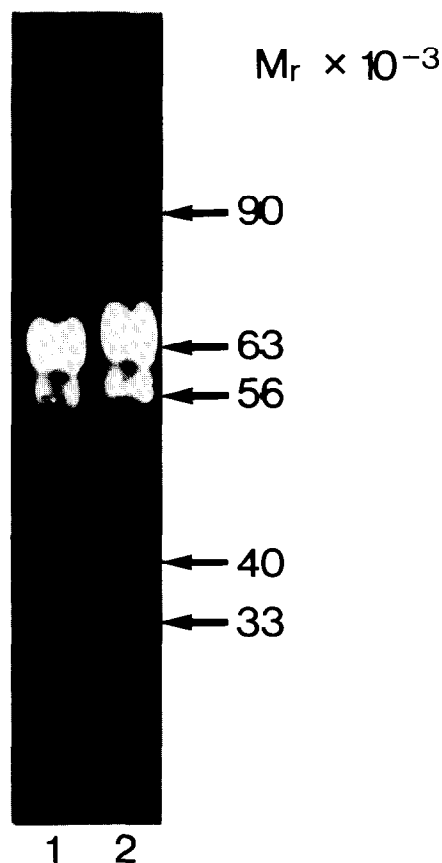


$$M_r \times 10^{-3}$$

Fig. 2. SDS-polyacrylamide gel electrophoresis of t-PA. 20 μl aliquots of Bowes melanoma cell-derived t-PA (lane 1) or recombinant mouse L cell (TRL25)-derived t-PA (lane 2) were electrophoresed in SDS-containing gels until the marker dye reached the bottom of the gel. Plasminogen activator activity, visualised as clear lysis areas against the Amido Black-stained background, was detected by fibrin zymography as described in MATERIALS AND METHODS, section i. The $M_r$'s of the plasminogen activators were determined by comparing their mobility to known standards.

duced approx. 70 ng/ml t-PA. Analysis of the purified protein by SDS-polyacrylamide gel electrophoresis and fibrin zymography showed that the fibrinolytically active species were of similar size to those produced by the human Bowes melanoma cell line (Fig. 2).

The identity of the recombinant protein was further confirmed as its fibrinolytic activity could be specifically quenched with anti-t-PA antibody whereas anti-u-PA antibody was without effect (not shown). Human t-PA and u-PA may also be distinguished on the basis of their dose–response curves in the fibrin plate assay (Rijken et al., 1979); we observed that the recombinant t-PA dose–response curve was similar to that of Bowes melanoma cell t-PA (not shown).

Expression of the human t-PA protein in mouse L cells suggests that our cosmid encodes all of the t-PA protein molecule and in addition carries all the relevant structures (such as the promoter and poly(A) addition site) necessary for expression. The cosmid clone is thus likely to encompass the whole of the t-PA genetic locus.

The present study has also demonstrated the feasibility of expression of a cloned human t-PA gene in mammalian cell culture. While the yield of t-PA, even from our highest producing clone, is not at present optimal, there are now many methods of manipulating genes to increase expression (Rigby, 1982).

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