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[73] Proprietor 專利所有人
GENENTECH, INC., 460 Point San Bruno Boulevard South, San Francisco 94080, United States of America 美利堅合眾國

[72] Inventor 發明人
ANDERSON, STEPHEN
BENNETT, WILLIAM F.
BOTSTEIN, DAVID
HIGGINS, DEBORAH L.
PAONI, NICHOLAS F.
ZOLLER, MARK

[74] Agent and / or address for service 代理人及/或送達地址
Deacons Graham & James, 3/Fl., Alexandra House, Central, Hong Kong
的近律師行，香港中環歷山大廈 3 樓

[54] TISSUE PLASMINOGEN ACTIVATOR HAVING ZYMOGENIC OR FIBRIN SPECIFIC PROPERTIES 具發酵性或纖維朊特性的纖維蛋白溶酶原活化質

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Nature, vol. 339, 29 June 1989, E.L. MADISON et al.: "Serpin-resistant mutants of human tissue-type plasminogen activator", pages 721-724

(73) Proprietor: **GENENTECH, INC.**
460 Point San Bruno Boulevard
South San Francisco
California 94080 (US)

(72) Inventor: **ANDERSON, Stephen**
158 Springdale Road
Princeton, NJ 08540 (US)
Inventor: **BENNETT, William, F.**
3437 Monterey Street
San Mateo, CA 94403 (US)
Inventor: **BOTSTEIN, David**
2535 Somerset Drive
Belmont, CA 94002 (US)
Inventor: **HIGGINS, Deborah, L.**
3984 Pasadena Drive
San Mateo, CA 94403 (US)
Inventor: **PAONI, Nicholas, F.**
51 Shuey Drive
Moraga, CA 94556 (US)

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Inventor: **ZOLLER, Mark**
2410 Pacific Avenue, Apartment 9
San Francisco, CA 94115 (US)

(74) Representative: **Armitage, Ian Michael et al**
MEWBURN ELLIS
York House
23 Kingsway
London WC2B 6HP (GB)

Description

The present invention is directed to particular tissue plasminogen activator (t-PA) zymogens, to methods for preparing such zymogens, and to methods and compositions utilizing such zymogens in pharmaceutical applications. In addition, the invention relates to variants having a modified structure that includes substituted amino acids within the protease domain of t-PA, which modification renders the variant zymogenic, i.e., relatively inactive in its one-chain form but active when converted to its two-chain form in the presence of fibrin, and/or more fibrin (or plasma clot) specific than wild-type (wt) t-PA.

Plasminogen activators are enzymes that activate the zymogen plasminogen to generate the serine proteinase plasmin (by cleavage at Arg561-Val562) that degrades various proteins, including fibrin. Among the plasminogen activators studied are streptokinase, a bacterial protein, urokinase, an enzyme synthesized in the kidney and elsewhere and originally extracted from urine, and human tissue plasminogen activator (t-PA), an enzyme produced by the cells lining blood vessel walls.

The mechanism of action of each of these plasminogen activators differs: Streptokinase forms a complex with plasminogen or plasmin, generating plasminogen-activating activity, urokinase cleaves plasminogen directly, and t-PA, fibrin, and plasminogen all interact to yield maximum activity.

t-PA has been identified and described as a particularly important and potent new biological pharmaceutical agent that has shown extraordinary results in the treatment of vascular diseases, such as myocardial infarction, due in part to its high fibrin specificity and potent ability to dissolve blood clots *in vivo*.

Although the existence of t-PA prompted numerous investigations by several scientific groups, it was first identified as a substantially pure isolate from a natural source, and tested for requisite plasminogen activator activity *in vivo*, by Collen et al., U.S. Pat. No. 4,752,603 issued June 21, 1988. See also Rijken et al., J. Biol. Chem., 256: 7035 (1981).

Subsequently, t-PA was fully identified and characterized by underlying DNA sequence and deduced amino acid sequence based on successful work employing recombinant DNA technology resulting in large quantities of t-PA in a distinct milieu. This work was reported by Pennica et al., Nature, 301: 214 (1983)) and in U.S. Patent No. 4,766,075, issued 23 August 1988.

Based on these disclosures, it seems now clear that the t-PA molecule contains five domains that have been defined with reference to homologous or otherwise similar structures identified in various other proteins such as trypsin, chymotrypsin, plasminogen, prothrombin, fibronectin, and epidermal growth factor (EGF). These domains have been designated, starting at the N-terminus of the amino acid sequence of t-PA, as 1) the finger region (F) that has variously been defined as including amino acids 1 to about 44, 2) the growth factor region (G) that has been variously defined as stretching from about amino acids 45 to 91 (based upon its homology with EGF), 3) kringle one (K1) that has been defined as stretching from about amino acid 92 to about amino acid 173, 4) kringle two (K2) that has been defined as stretching from about amino acid 180 to about amino acid 261, and 5) the so-called serine protease domain (P) that generally has been defined as stretching from about amino acid 264 to the C-terminal end of the molecule. These domains, which are situated generally adjacent to one another, or are separated by short "linker" regions, account for the entire amino acid sequence of from 1 to 527 amino acids of the putative mature form of t-PA.

Each domain has been described variously as contributing certain specific biologically significant properties. The finger domain has been characterized as containing a sequence of at least major importance for high binding affinity to fibrin. (This activity is thought important for the high specificity that t-PA displays with respect to clot lysis at the locus of a fibrin-rich thrombus.) The growth factor-like region likewise has been associated with cell surface binding activity. The kringle 2 region also has been strongly associated with fibrin binding and with the ability of fibrin to stimulate the activity of t-PA. The serine protease domain is responsible for the enzymatic cleavage of plasminogen to produce plasmin.

Despite the profound advantages associated with natural t-PA as a clot-dissolving agent, it is not believed that the natural protein necessarily represents the optimal t-PA agent under all circumstances. Therefore, several variants have been proposed or devised to enhance specific properties of t-PA. Certain of those variants address disadvantages associated with the use of natural t-PA in situations where an agent with a longer half-life or slower clearance rate would be preferred, e.g., in the treatment of deep-vein thrombosis and following reperfusion of an infarct victim, or where a single-chain agent is preferred.

For example, removal of a substantial portion or all of the finger domain results in a molecule with substantially diminished fibrin binding characteristics, albeit in return there is a decrease in the overall rate of clearance of the resultant entity - See WO 89/00197 published 12 January 1989.

Variants are described in EPO Pat. Publ. No. 199,574 that have amino acid substitutions at the proteolytic cleavage sites at positions 275, 276, and 277. These variants, characterized preferentially as t-PA variants having an amino acid other than arginine at position 275, are referred to as protease-resistant one-chain t-PA variants in that, unlike natural t-PA, which can exist in either a one-chain or two-chain form, they are resistant to protease cleavage at position 275 and are therefore not converted metabolically *in vivo* into a two-chain form. This form is thought to have certain advantages biologically and commercially, in that it is more stable while the fibrin binding and fibrin stimulation are increased relative to two-chain t-PA. Furthermore, plasminogen activators are described that comprise one domain capable of interacting with fibrin and the protease domain of urokinase, with one embodiment being urokinase altered to make it less susceptible to forming two-chain urokinase. See WO 88/05081 published 14 July 1988.

For further patent literature regarding modification of the protease cleavage site of t-PA, see, for example, EPO Pat. Nos. 241,209; EP 201,153 published November 12, 1986; EP 233,013 published August 19, 1987; EP 292,009 published November 23, 1988; EP 293,936 published December 7, 1988; and EP 293,934 published December 7, 1988; and WO 88/10119.

EP-A-0 351 246, which is prior art under Article 54(3) EPC, discloses t-PA analogues modified in the region from what is called therein position 414 to position 433. The numbering used in that application differs from that used in the present application. The amino acid positions are given in EP-A-0 351 246 a number with value three greater than the number used in the present application to identify a particular amino acid.

Glycosylation mutants at 117-119, 184-186, and 448-450 exhibited higher specific activity as the mole percent carbohydrate was reduced. See EPO Pub. No. 227,462 published July 1, 1987. This patent application additionally discloses using an assay of fibrin/fibrin degradation products and teaches that one may also modify the t-PA molecule at positions 272-280 or delete up to 25 amino acids from the C-terminus. Further, the t-PA mutants with Asn119, Ala186 and Asn450, which have the N-glycosylation sites selectively removed by DNA modification but contain residual O-linked carbohydrate, were found to be about two-fold as potent as melanoma t-PA in an *in vitro* lysis assay. See EPO Publ. No. 225,286 published June 10, 1987.

Replacement of the amino acid at 449 of t-PA with any amino acid except arginine to modify the glycosylation site, as well as modification of Arg275 or deletion of the - 3 to 91 region, is also taught. See WO 87/04722 published Aug. 13, 1987. An amino acid substitution at position 448 of t-PA is disclosed as desirable to remove glycosylation. See EPO Pub. No. 297,066 published December 28, 1988. The combination of modifications at positions 448-450 and deletion of the N-terminal 1-82 amino acids is disclosed by WO 89/00191 published January 12, 1989. Additionally, urokinase has been modified in the region of Asp302-Ser303-Thr304 to prevent glycosylation. See EPO Pub. No. 299,706 published 18 January 1989. However, alteration of the glycosylation sites, and in particular that at amino acid 117, seems invariably to result in a molecule having affected solubility characteristics that may result additionally in an altered circulating half-life pattern and/or fibrin binding characteristics. See EPO Pat. Publ. No. 238,304, published 23 September 1987.

When the growth factor domain of t-PA is deleted, the resultant variant is still active and binds to fibrin, as reported by A. J. van Zonneveld et al., Thrombos. Haemostas., 54 (1) 4 (1985). Various deletions in the growth factor domain have also been reported in the patent literature. See EPO Publ. No. 241,209 (des-51-87), EPO Publ. No. 241,208 (des-51-87 and des-51-173), PCT 87/04722 (deletion of all or part of the N-terminal 1-91), EPO Publ. No. 231,624 (all of growth factor domain deleted), and EPO Publ. No. 242,836 and Jap. Pat. Appl. Kokai No. 62-269688 (some or all of the growth factor domain deleted).

It has further been shown that t-PA can be modified both in the region of the first kringle domain and in the growth factor domain, resulting in increased circulatory half-life. See EPO Pat. Publ. No. 241,208 published October 14, 1987. The region between amino acids 51 and 87, inclusive, can be deleted from t-PA to result in a variant having slower clearance from plasma. Browne et al., J. Biol. Chem., 263: 1599-1602 (1988). Also, t-PA can be modified, without adverse biological effects, in the region of amino acids 67 to 69 of the mature, native t-PA, by deletion of certain amino acid residues or replacement of one or more amino acids with different amino acids. See EPO Pat. Publ. No. 240,334 published October 7, 1987.

A hybrid of t-PA/urokinase using the region of t-PA encompassing amino acids 273-527 is also disclosed. See EPO 290,118 published November 9, 1988.

Serpin-resistant mutants of human t-PA with alterations in the protease domain, including d296-302 t-PA, R304S t-PA, and R304E t-PA, are disclosed in Madison et al., Nature, 339: 721-724 (1989); see also the accompanying article by Dagmar Ringe in the same issue.

WO90/10649, published on 20 September 1990, contains disclosure of d296-302tPA, R304E t-PA and R304S t-PA, which is prior art according to Article 54(3) EPC.

A general review of plasminogen activators and second-generation derivatives thereof can be found in Harris, Protein Engineering, 1: 449-458 (1987). Other reviews of t-PA variants include Pannekoek et al., Fibrinolysis, 2: 123-132 (1988) and Ross et al., in Annual Reports in Medicinal Chemistry, Vol. 23, Chapter 12 (1988).

While the foregoing disclosures provide evidence that newer and, in various respects, better t-PA agents are at hand, there are currently no t-PA molecules described that only become activated when they reach the site of the clot to be dissolved. Currently, the t-PA molecules are active in the presence of fibrin and/or plasma proteins or whole blood, whether they are in the one-chain or two-chain form. It would be desirable to have a zymogenic t-PA that in the presence of fibrin requires clipping of its one-chain form to its two-chain form to become fully active. Such variant molecules would likely exhibit fewer side effects, such as less bleeding, and have fibrinogen sparing properties, thereby providing medical science important new alternatives in the treatment of cardiovascular disease and numerous other medical conditions that arise from thromboembolic occlusion of blood vessels, as well as in the prevention of the formation of adhesions.

It would also be desirable to provide a t-PA molecule that, relative to wild-type t-PA, has a higher fibrin-stimulated (or a plasma clot-stimulated) activity than fibrinogen-stimulated (or plasma-stimulated) activity, i.e., is fibrin (or plasma clot) specific, so that it will act only at the site of the clot and not systemically.

Accordingly, it is an object of this invention to provide zymogenic and/or fibrin-specific t-PA molecules that exhibit improved therapeutic and pharmaceutical characteristics.

It is another object to provide for the treatment of conditions that admit the use of clot-dissolving agents that are active only at the site of the clot and are useful at higher levels than other such agents.

These and other objects will be apparent to one of ordinary skill in the art.

These objects are achieved by the provision of a tissue plasminogen activator (t-PA) zymogen capable of converting to the enzymatically active form of t-PA upon cleavage by plasmin. In another aspect, the invention provides a t-PA variant having an amino acid alteration at a site or sites within the protease domain of t-PA as compared with the corresponding wild-type t-PA, which alteration renders the variant zymogenic as compared to the corresponding wild-type t-PA.

In one particularly preferred embodiment, the t-PA is human t-PA and the alteration is in the region of 305: an amino acid other than phenylalanine being substituted at amino acid position 305, or an amino acid being inserted after one or both of amino acid positions 304 and 305, or one or more amino acids being deleted at amino acid positions 297 to 305 inclusive; the numbering of amino acid positions corresponding with that of figure 1.

In other embodiments, this invention relates to a DNA sequence encoding the zymogen and variant described above, replicable expression vectors capable of expressing the DNA sequence in a transformant host cell, and microorganisms and cell cultures transformed with the vector.

In still another embodiment, the invention provides a method for identifying tissue plasminogen activator (t-PA) variants which have a larger differential activity between the one-chain form and the two-chain form than wild-type recombinant t-PA, comprising:

(a) substituting an amino acid other than phenylalanine at amino acid position 305; or

(b) inserting an amino acid after one or both of amino acid positions 304 and 305; or

(c) deleting one or more amino acids at amino acid positions 297 to 305, inclusive; of wild-type human t-PA.

In other aspects, the invention supplies a human tissue plasminogen activator (t-PA) variant capable of exhibiting one or more of the following biological activities: a larger differential activity between the one-chain form and the two-chain form than wild-type recombinant t-PA, a higher ratio of fibrin-dependent specific activity to fibrinogen-dependent specific activity in a S-2251 assay than wild-type rt-PA, or a higher ratio of plasma clot-dependent specific activity to plasma-dependent specific activity in a S-2251 assay than wild-type rt-PA, characterized in that it contains at least one amino acid substitution in its protease domain as compared with the corresponding wild-type t-PA (from amino acid 264 to amino acid 527), which substitution is responsible for said biological activity, provided that the variants d296-302 t-PA, R304E t-PA, R304S t-PA and A473 t-PA are excluded and said substitution excludes substitutions solely in the regions of 270-280, 448-450, and 502-527, and substitutions in the regions 411 to 416, 416 to 423 or 423 to 430; the numbering of amino acid positions corresponding to that of figure 1.

In another aspect, the invention provides a method for identifying tissue plasminogen activator variants with specified biological activity comprising:

(a) introducing at least one amino acid substitution into the protease domain of tissue plasminogen activator (t-PA) (amino acid positions 264 to 527) the substitution excluding substitutions solely in the regions of 270-280, 448-450, and 502-527 of the corresponding wild-type t-PA; and

(b) screening the resultant t-PA variant for its capability of exhibiting one or more of the following biological activities: (a) a larger differential activity between the one-chain form and the two-chain form than wild-type recombinant t-PA, (b) a higher ratio of fibrin-dependent specific activity to fibrinogen-dependent specific activity in a S-2251 assay than wild-type rt-PA, or (c) a higher ratio of plasma clot-dependent specific activity to plasma-dependent specific activity in a S-2251 assay than wild-type rt-PA; with the proviso that screening for activity (a) is excluded where the substitution is in the region of t-PA from position 411 to position 416, from position 416 to position 423, or from position 423 to position 430; the numbering of amino acid positions corresponding to that of figure 1.

In further embodiments the invention provides DNA sequences and replicable vectors encoding the above-described variants and host cells transformed with them.

In yet another embodiment, the invention is directed to a composition for treating a vascular condition or disease comprising a therapeutically effective amount of a variant herein in admixture with a pharmaceutically acceptable carrier. Also encompassed herein is a composition for preventing fibrin deposition or adhesion formation or reformation comprising a therapeutically effective amount of a variant herein in admixture with a pharmaceutically acceptable carrier.

The first aspect of the present invention is based, inter alia, upon specific successful research demonstrating that certain t-PA molecules are zymogens in the presence of a stimulator of t-PA activity, such as plasmin-degraded fibrinogen fragments, and thus can have their fibrinolytic activity turned off when generally in the plasma and activated when proximate to plasmin at the site of the clot. Thus, the zymogen is activated on demand for specific localized clot therapy. The zymogens herein are expected to be fibrinogen sparing and are generally useful in higher doses than their non-zymogenic counterparts, resulting in faster clot lysis and lysis of more clots.

The second aspect of this invention is to obtain a t-PA molecule that is more fibrin (or plasma clot) specific so that it will act more preferentially at the site of the clot than unmodified t-PA.

Figure 1 depicts the primary structure of t-PA showing the location of the five domains, the disulfide bridging, and the activation site where the molecule is clipped into a two-chain molecule.

Figures 2 and 3 are schematic representations of a suitable method for the preparation of pCISt-PA, together with a description of certain of its prominent restriction sites.

Figure 4 is a schematic representation of a suitable method for the preparation of p7-1H, together with a description of certain of its prominent restriction sites.

Figure 5 shows fibrin binding of predominantly one-chain F305H (closed triangles), two-chain F305H (closed circles with double lines), one-chain wild-type t-PA (closed squares), two-chain wild-type t-PA (closed diamonds), and a mixture of one-chain and two-chain wild-type t-PA (open triangles), at a t-PA concentration of 10 ng/ml.

Figures 6-9 show plots of the kinetics of the conversion of plasminogen to plasmin in the presence of plasmin-degraded fibrinogen by the predominantly one-chain wild-type t-PA (Fig. 6), the two-chain wild-type t-PA (Fig. 7), the predominantly one-chain F305H t-PA (Fig. 8), and the two-chain F305H t-PA (Fig. 9). The squares, lines, and circles represent various concentrations of plasminogen and t-PA in the assay buffer, which are specified at the end of Example I. In these Figures, the ordinate is the absorbance at 405 nm and the abscissa is the square of the number of minutes at which the absorbance was taken.

As used herein, the terms "human tissue plasminogen activator," "human t-PA," and "t-PA" denote human extrinsic (tissue-type) plasminogen activator having two functional regions consisting of a protease domain that is capable of converting plasminogen to plasmin and an N-terminal region believed to be responsible for fibrin binding. These three terms therefore include polypeptides containing these functional domains as part of the overall sequence. t-PA is suitably produced, e.g., by recombinant cell culture systems, in bioactive forms comprising the protease portion and portions of t-PA otherwise native to the source of the t-PA. It will be understood that natural allelic variations exist and occur from individual to individual, demonstrated by (an) amino acid difference(s) in the overall sequence.

As used herein, the term "wild-type t-PA" refers to the t-PA encoded by the cDNA reported by U.S. Pat. No. 4,766,075. The t-PA thus encoded is suitably a t-PA molecule from any native source or any recombinant expression system, including 293 or 294 cells, Chinese hamster ovary cells, etc.

As used herein, the term "protease domain" refers to the region of the mature form of wild-type t-PA from amino acid 264 to amino acid 527, inclusive.

As used herein, the terms "zymogen," "zymogenic," and "zymogenic activity" used to describe the t-PA herein must meet the definition given below.

In the definition, a "zymogen" specifically refers to a t-PA molecule that in an assay of enzymatic activity exhibits a larger differential activity between the one-chain form and the two-chain form than wild-type recombinant t-PA (rt-PA). The differential activity of the zymogen is preferably at least approximately

1.5 times that of wild-type rt-PA. This activity can be obtained by lowering the activity of the one-chain form to a greater extent than that of the two-chain form relative to wild-type rt-PA; raising the activity of the two-chain form to a greater extent than that of the one-chain form relative to wild-type rt-PA; or any combination of events described above that yield the described effect. The zymogenic character of wild-type t-PA is described in Loscalzo, J. Clin. Invest., 82: 1391-1397 (1988) and Ranby et al., Thrombosis Research, 27: 175-183 (1982).

The expression "fibrin specificity" refers to the activity of a mutant that exhibits a higher ratio of fibrin-dependent specific activity to fibrinogen-dependent specific activity in a S-2251 assay (in either the one-chain or two-chain form) than wild-type rt-PA, and preferably a ratio of at least 1.5.

The expression "plasma clot specificity" refers to the activity of a mutant that exhibits a higher ratio of plasma clot-dependent specific activity to plasma-dependent specific activity in a S-2251 assay (in either the one-chain or two-chain form) than wild-type rt-PA, and preferably a ratio of at least 1.5.

As used herein, "transient expression system" denotes a cell culture containing cells transfected with a t-PA variant-encoding vector that expresses the DNA sequence encoding the variant transiently, i.e., in a manner that may not be stable. Such cells are deemed "capable of transient expression."

For purposes of discussing the variants herein, reference is made to Figure 1, which illustrates the primary structure of t-PA. In Figure 1, the letters in the circles are single-letter amino acid codes, the connecting lines between chains indicate disulfide bridging, the open circles indicate glycosylation sites, and the designations F, GF, K1, K2, and SP indicate, respectively, the finger, growth factor, kringle 1, kringle 2, and serine protease domains.

For purposes of shorthand designation of t-PA variants described herein, it is noted that numbers refer to the amino acid residue/position along the amino acid sequences of putative mature t-PA (EPO Publ. No. 93,619). The numbering is also shown in figure 1. Amino acid identification uses the single-letter alphabet of amino acids, i.e.,

Asp	D	Aspartic acid	Ile	I	Isoleucine
Thr	T	Threonine	Leu	L	Leucine
Ser	S	Serine	Tyr	Y	Tyrosine
Glu	E	Glutamic acid	Phe	F	Phenylalanine
Pro	P	Proline	His	H	Histidine
Gly	G	Glycine	Lys	K	Lysine
Ala	A	Alanine	Arg	R	Arginine
Cys	C	Cysteine	Trp	W	Tryptophan
Val	V	Valine	Gln	Q	Glutamine
Met	M	Methionine	Asn	N	Asparagine

The designation for a substitution variant herein consists of a letter followed by a number followed by a letter. The first (leftmost) letter designates the amino acid in the wild-type, mature t-PA. The number refers to the amino acid position where the amino acid substitution is being made, and the second (right-hand) letter designates the amino acid that is used to replace the wild-type amino acid. The designation for an insertion variant consists of the letter i followed by a number designating the position of the residue in wild-type, mature t-PA before which the insertion starts, followed by one or more capital letters indicating, inclusively, the insertion to be made. The designation for a deletion variant consists of the letter d followed by the number of the start position of the deletion to the number of the end position of the deletion, with the positions being based on the wild-type, mature t-PA. Multiple mutations are separated by a comma in the notation for ease of reading them.

Examples of the nomenclature are as follows: a substitution variant where the phenylalanine at position 305 of the wild-type t-PA is replaced with a histidine residue is designated F305H. A substitution variant with multiple substitutions at consecutive positions 296-299 of AAAA for KHRR is designated K296A,H297A,R298A,R299A. An insertion variant where cysteine and valine are inserted after position 305 of wild-type t-PA is designated i305CV. A deletion variant where the amino acids at positions 300 to 305 are deleted from the wild-type, mature t-PA is designated d300-305. The notation 't-PA' follows after each mutant.

One preferred class of zymogens herein are those that have a substitution, deletion, or insertion in or around position 305 of wild-type t-PA. These variants include those with an amino acid other than phenylalanine at position 305 of the corresponding wild-type t-PA. More preferably, such variants are those with an amino acid at position 305 having a side chain that can act or does act as a hydrogen bond donor,

such as one containing a hydroxyl group or nitrogen atom. Still more preferably, such amino acids are arginine, lysine, tyrosine, asparagine, glutamine, and histidine, most preferably histidine. Preferred insertional zymogenic variants of this type include those with one or more, preferably one, amino acid inserted after the amino acid at position 304 or 305, such as the amino acids described above, i.e., those with a side chain that can act or act as a hydrogen bond donor, such as one containing a hydroxyl group or nitrogen atom, e.g., arginine, lysine, tyrosine, asparagine, glutamine, and histidine, most preferably histidine. Preferred deletion zymogenic variants include those with deletions in the region of 297 to 305, inclusive, of the wild-type t-PA, including d297 t-PA, d298 t-PA, etc. and combinations thereof, such as d297-299 t-PA or d297,d305 t-PA.

Particular embodiments of the above-noted zymogen variants include: F305H t-PA; F305T t-PA; F305N t-PA; F305K t-PA; F305R t-PA; F305Q t-PA; i304H t-PA; i304T t-PA; i304N t-PA; i304K t-PA; i304R t-PA; i304Q t-PA; i304HH t-PA; i305H t-PA; i305T t-PA; i305N t-PA; i305K t-PA; i305R t-PA; i305Q t-PA; i304H,i305H t-PA; i305HH t-PA; d297 t-PA; d298 t-PA; d299 t-PA; d300 t-PA; d301 t-PA; d302 t-PA; d303 t-PA; d304 t-PA; d305 t-PA; d297-298 t-PA; d297-299 t-PA; d297-300 t-PA; d297-301 t-PA; d297-302 t-PA; d297-303 t-PA; d297-304 t-PA; d297-305 t-PA; d300-301 t-PA; d300-302 t-PA; d300-303 t-PA; d300-304 t-PA; d300-305 t-PA; d304-305 t-PA; d297,d300 t-PA; d297,d305 t-PA; dI-44,N184D,F305H t-PA; dI-44,F305H t-PA; dI-44,I210R,G211A,K212R,V213R,F305H t-PA; dI-44,I210R,G211A,K212R,V213K,F305H t-PA; dI-44,V213K,F305H t-PA; dI-44,T252R,F305H t-PA; dI-44,V213K,T252R,F305H t-PA; dI-44,I210K,F305H t-PA; dI-44,I210R,G211H,K212Q,V213K,F305H t-PA; I210R,G211H,K212Q,V213K,F305H t-PA; I210R, G211A, K212R, V213R, F305H t-PA; dI-44, N184D, I210R, G211A, K212R, V213R, T252R, F305H t-PA; N184D,I210R,G211A,K212R,V213R,T252R,F305H t-PA; d92-179,F305H t-PA; d92-179, I210R, G211A, K212R, V213R, F305H t-PA; d92-179, N184D, I210R, G211A, K212R, V213R, T252R, F305H t-PA; d92-179,I210R,G211A,K212R,V213R,T252R,F305H t-PA; Y67N,F305H t-PA; dI-44, Y67N,F305H t-PA; and the T252R or N184S analogues thereof or combinations thereof. (The changes other than those in the protease domain are described further below.)

Of these, the preferred zymogen variants are F305H t-PA; F305T t-PA; F305N t-PA; F305K t-PA; F305R t-PA; F305Q t-PA; i304H t-PA; dI-44, F305H t-PA; d92-179,F305H t-PA; dI-44,N184D,F305H t-PA; dI-44,I210R,G211A,K212R, V213R,F305H t-PA; dI-44, I210R, G211A, K212R, V213K, F305H, t-PA; I210R,G211A,K212R, V213R,F305H t-PA; d92-179,I210R,G211A,K212R, V213R,F305H t-PA; d92-179,N184D,I210R,G211A,K212R, V213R,F305H t-PA; d92-179,N184D,I210R,G211A,K212R, V213R,T252R,F305H t-PA; dI-44,N184D,I210R,G211A,K212R, V213R,T252R,F305H t-PA; and N184D,I210R,G211A,K212R,V213R,T252R,F305H t-PA.

More preferred variants of the first class of zymogens are F305H t-PA; F305T t-PA; F305N t-PA; F305Q t-PA; i304H t-PA; dI-44, F305H t-PA; d92-179, F305H t-PA, and the most preferred F305H t-PA.

Another preferred class of zymogens herein, as well as a class of variants that may alternatively or additionally be fibrin (or plasma clot) specific, are variants with one or more alterations in small regions of the protease domain identified as having charged amino acid side chains, which regions and/or regions adjacent thereto may be responsible for the interaction of t-PA with other substances that might affect its various activities.

The regions identified for testing for activity by this method are at residue numbers 267, 283-287, 296-299, 303-304, 322, 326-327, 331-332, 339-342, 347-351, 353-356, 360-362, 364-366, 369-371, 378-383, 387-392, 400-405, 408, 410, 416-418, 426-430, 432-434, 440, 445-449, 449-453, 460-462, 471-472, 477, 487-489, 505-506, 513, 519-523, and 523-526 of the corresponding wild-type t-PA. One or more of these regions, or subunits thereof, are altered to determine if the desired biological property or properties will be obtained. The charged residues (Arg, Asp, His, Lys, and Glu) are suitably identified using a technique known as alanine-scanning mutagenesis, disclosed in Cunningham and Wells, Science, 244: 1081 - 1085 (1989), and replaced with a neutral or negatively charged amino acid to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell.

The mutants found to be in this second preferred class of zymogens and fibrin-specific molecules are those wherein the amino acids replaced are at position(s) 267, 283+287, 296-299, 303-304, 331-332, 339+342, 347-349+351, 364-366, 408, 410, 416-418, 426-427+429-430, 432-434, 440, 445+449, 449+453, 460+462, and/or 477 of the corresponding wild-type t-PA, where the "+" indicates replacements only at the positions designated, and the "-" indicates replacements at all positions designated.

For the alanine scanning mutagenesis, it is preferable that an amino acid be substituted that will neutralize the charge of the corresponding amino acid of the wild-type t-PA, rather than confer an opposite charge on the molecule. Any hydrophobic, essentially uncharged or oppositely charged amino acid can be used, including, as preferred, alanine, glycine, serine, threonine, asparagine, glutamine, valine, leucine, isoleucine, phenylalanine, or tyrosine. Among these, small amino acids, such as alanine, serine and

threonine, are preferred over larger amino acids such as valine, leucine, and isoleucine. Charged amino acids such as aspartic acid or glutamic acid are less preferred.

More preferably, the amino acid used for replacement is either alanine, serine, threonine, asparagine, glutamine, phenylalanine, or tyrosine, and more preferably still, alanine, serine, or threonine. Alanine is the most preferred amino acid for this purpose because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the wild-type t-PA molecule. Further, alanine is frequently found in both buried and exposed positions (Creighton, T.E., in The Proteins (eds. W.H. Freeman & Co., N.Y.); Chothia, C. (1976) J. Mol. Biol., 150: 1).

Preferred variants of this latter class of zymogens or fibrin (or plasma clot) specific variants are those that are inclusive of modifications within the protease domain of t-PA with the exception of the variants D365A t-PA, R462L t-PA, A473S t-PA, d296-304 t-PA, d296-302 t-PA, R298E t-PA, R299E t-PA, R304E t-PA, R304S t-PA, d296-299 t-PA, and K296E,R298E,R299E t-PA.

More specifically, they are those that have one or more substitutions at position(s) 267, 283, 287, 296, 297, 298, 299, 303, 304, 331, 332, 339, 342, 347, 348, 349, 351, 364, 365, 366, 408, 410, 416, 417, 418, 426, 427, 429, 430, 432, 434, 440, 445, 449, 453, 460, 462, or 477, or combinations thereof, of the corresponding wild-type t-PA.

More preferably, the protease domain variants have substitutions at position(s) 267, 283 + 287, 296-299, 303-304, 331-332, 339 + 342, 347-349 + 351, 364-366, 408, 410, 416-418, 426-427 + 429-430, 432 + 434, 440, 445 + 449, 449 + 453, 460 + 462, and 477 of the corresponding wild-type t-PA, where the " + " indicates alterations only at the positions designated, not positions in between, and the "-" indicates alterations at all positions designated, including those in between.

Still more preferably, the protease domain variants of the latter class of zymogens or fibrin (or plasma clot) specific variants are R267A t-PA, D283A,H287A t-PA, K296A,H297A,R298A,R299A t-PA, E303A,R304A t-PA, H331A,H332A t-PA, R339A,R342A t-PA, E347A,E348A,E349A,K351A t-PA, D364A,D365A,D366A t-PA, E408A t-PA, E410A t-PA, K416A,H417A,E418A t-PA, E426A,R427A,K429A,E430A t-PA, H432A,R434A t-PA, R440A t-PA, H445A,R449A t-PA, R449A,D453A t-PA, D460A,R462A t-PA, and D477A t-PA.

Of these, the most preferred substitution is an alanine residue in place of each of the existing residues at 296-299 of wild-type t-PA, i.e., K296A,H297A,R298A,R299A t-PA.

The preferred insertional variants that may exhibit zymogenic or fibrin-specific activity are those wherein one or more amino acids are inserted after the amino acids at positions 296, 297, 298, and/or 299. Also preferred for this purpose are those protease domain variants with an insertion consisting of either tyrosine, asparagine, lysine, arginine, or glutamine.

Other variants with one or more amino acid alterations (deletions, substitutions, or insertions, but preferably substitutions) within the protease domain (amino acids 264-527) of the native t-PA molecule are identifiable that exhibit zymogenic properties as compared to the wild-type t-PA, using one or more of the screening tests provided below.

The t-PA variants herein, in addition to being altered from the native sequence at one or more protease domain sites so as to display zymogenic and/or fibrin (or plasma clot) specific properties, also optionally contain substitutions, deletions, or insertions of residues in other regions of the native sequence to improve certain properties of the molecule, provided that changes are not made that prevent the cleavage of the one-chain form of t-PA to its two-chain form or otherwise alter a desirable biological property conferred on the molecule by the alteration(s) in the protease domain of the present invention. The preferred alterations in these other domains are provided above in the lists of the most preferred zymogen variants of the first type.

For example, the variants herein are suitably devoid of at least a portion of the finger domain, the growth factor domain, and/or the kringle 1 domain, and/or devoid of glycosylation potential at the glycosylation site surrounding amino acid 184, and suitably contain amino acid modifications in the putative lysine binding site of kringle 1 or 2.

In addition, fibrin binding of t-PA can be modulated, most preferably restored or increased, by appropriate substitutions of positively or negatively charged amino acid residues on the opposite edges of the putative ligand binding pocket of the kringle 2 domain of t-PA. The variants herein are generally prepared by site-directed mutagenesis or by excision/ligation techniques described further hereinbelow.

Specific examples of such variants include a molecule devoid of amino acids 1 to 44 (designated dl-44) and a molecule having aspartic acid at position 184 (designated N184D). Variants devoid of amino acids 1 to 44 are described more fully in WO 89/00197, *supra*.

All of the above variants are optionally modified in various other regions of the molecule, if such modifications still satisfy the criteria expressed herein for zymogenic and/or fibrin (or plasma clot) specific characteristics. Such modifications include, for example:

1. Kringle 1 modifications, for example, deletion of about 92 to 179, and/or
2. Kringle 2 modifications, for example, deletion of about 174-261 or modification in the region of amino acids about 205-215, especially 210-213, and/or
3. Amino acids about 244-255, especially 252 or its site, and/or
4. Amino acids about 233-242, especially 236-238, and/or
5. Known glycosylation sites such as amino acid 184, and/or
6. Glycosylation within the growth factor domain. Briefly, the t-PA molecule is N-or O-linked glycosylated within its growth factor domain, preferably at position 67-69, where the tyrosine at position 67 is replaced with an asparagine residue, to alter the half-life of the t-PA molecule.

Many of these modifications may significantly alter clearance rates and fibrin binding relative to native t-PA. The practitioner skilled in the art will be able to determine by the appropriate assay what the optimum properties of each variant are that are desired in any particular instance.

The modification to change or insert the appropriate amino acid(s) in the native molecule to effect the above sequence variations is accomplished by any means known in the art, such as, e.g., site-directed mutagenesis or ligation of the appropriate sequence into the DNA encoding the relevant protein, as described below.

Preparation of t-PA variants in accordance herewith is preferably achieved by site-specific mutagenesis of DNA that encodes an earlier prepared variant or a nonvariant version of the protein. Site-specific mutagenesis allows the production of t-PA variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications such as Adelman et al., DNA, 2: 183 (1983).

As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing et al., Third Cleveland Symposium on Macromolecules and Recombinant DNA, Editor A. Walton, Elsevier, Amsterdam (1981). These phage are readily commercially available and their use is generally well known to those skilled in the art. Alternatively, plasmid vectors that contain a single-stranded phage origin of replication (Veira et al., Meth. Enzymol., 153: 3 (1987)) may be employed to obtain single-stranded DNA.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant t-PA. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea et al., Proc. Natl. Acad. Sci. (USA), 75: 5765 (1978). This primer is then annealed with the single-stranded t-PA sequence-containing vector, and subjected to DNA-polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells such as JM101 cells and clones are selected, via hybridization to a radioactive probe consisting of the ^{32}P-labeled mutageneis primer, that include recombinant vectors bearing the mutated sequence arrangement.

After such a clone is selected, the mutated t-PA region may be removed and placed in an appropriate vector for t-PA production, generally an expression vector of the type that typically is employed for transformation of an appropriate eukaryotic host. In the context of the present invention, Chinese hamster ovary (CHO) cells or 293 (human kidney cells described by Graham et al., J. Gen. Virol., 36: 59 (1977)) are preferred for the preparation of long-term stable t-PA producers. However, the invention is not limited to CHO production, as it is known that numerous other cell types are suitably employed, particularly where one desires only transient production of the enzyme for test purposes. For example, described below is a transient system employing 293 cells that provides a convenient system for production of t-PA variants for analytical purposes.

Another method for making mutations in the DNA sequence encoding the t-PA involves cleaving the DNA encoding the t-PA at the appropriate position by digestion with restriction enzymes, recovering the properly cleaved DNA, synthesizing an oligonucleotide encoding the desired amino acid and flanking regions such as polylinkers with blunt ends (or, instead of using polylinkers, digesting the synthetic oligonucleotide with the restriction enzymes also used to cleave the t-PA-encoding DNA, thereby creating cohesive termini), and ligating the synthetic DNA into the remainder of the t-PA-encoding structural gene.

Although Chinese hamster ovary (CHO) expression ultimately is preferred for t-PA production, the vectors and methods disclosed herein are suitable for use in host cells over a wide range of eukaryotic organisms.

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31,446) and *E. coli* strain W3110 (ATCC No. 27,325) are particularly useful. Other suitable microbial strains include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATCC No. 31,537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes also are useful for expression. The aforementioned strains, as well as bacilli such as Bacillus subtilis, and other enterobacteriaceae such as, e.g., Salmonella typhimurium or Serratia marcesans and various Pseudomonas species are examples of useful hosts for expression.

In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., Gene, 2: 95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, must also contain, or be modified to contain, promoters that can be used by the microbial organism for expression of its own proteins.

Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al., Nature, 375: 615 (1978); Itakura et al., Science, 198: 1056 (1977); Goeddel et al., Nature, 281: 544 (1979)) and a tryptophan (trp) promoter system (Goeddel et al., Nucl. Acids Res., 8: 4057 (1980); EPO Appl. Publ. No. 36,776), and the alkaline phosphatase systems. While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (see, e.g., Siebenlist et al., Cell, 20: 269 (1980)).

In addition to prokaryotes, eukaryotic microbes, such as yeasts, also are suitably used herein. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For example, for expression in Saccharomyces, the plasmid YRp7 (Stinchcomb et al., Nature, 282: 39 (1979); Kingsman et al., Gene, 7: 141 (1979); Tschemper et al., Gene, 10: 157 (1980)) is commonly used. This plasmid already contains the trp1 gene that provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44,076 or PEP4-1 (Jones, Genetics, 85: 12 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem., 255: 2073 (1980)) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg., 7: 149 (1968); Holland et al., Biochemistry, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In the construction of suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters that have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of such useful host cell lines are VERO and HeLa cells, CHO cell lines, and W138, BHK, COS-7, 293, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment that also contains the SV40 viral origin of replication (Fiers et al., Nature, 273: 113 (1978)). Smaller or larger SV40 fragments are also suitably used, provided there is included the approximately 250-bp sequence extending from the HindIII site toward the BglI site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication typically is provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) source, or by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Satisfactory amounts of human t-PA are produced by cell cultures; however, refinements, using a secondary coding sequence, serve to enhance production levels even further. The secondary coding sequence comprises dihydrofolate reductase (DHFR) that is affected by an externally controlled parameter, such as methotrexate (MTX), thus permitting control of expression by control of the MTX concentration.

In the selection of a preferred host cell for transfection by the vectors of the invention that comprise DNA sequences encoding both variant t-PA and DHFR protein, it is appropriate to consider the type of DHFR protein employed. If wild-type DHFR protein is employed, it is preferable to select a host cell that is deficient in DHFR, thus permitting the use of the DHFR coding sequence as a marker for successful transfection in selective medium that lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the CHO cell line deficient in DHFR activity, prepared and propagated, as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. (USA) 77: 4216 (1980).

On the other hand, if DHFR protein with low binding affinity for MTX is used as the controlling sequence, it is not necessary to use DHFR-deficient cells. Because the mutant DHFR is resistant to MTX, MTX-containing media can be used as a means of selection, provided that the host cells are themselves MTX sensitive. Most eukaryotic cells that are capable of absorbing MTX appear to be sensitive to MTX. One such useful cell line is a CHO line, CHO-K1 (ATCC No. CCL 61).

If mammalian cells are used as host cells, transfection generally is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, Virology, 52: 546 (1978). However, other methods for introducing DNA into cells such as nuclear injection, electroporation, or protoplast fusion are also suitably used.

If yeast are used as the host, transfection is generally accomplished using polyethylene glycol, as taught by Hinnen, Proc. Natl. Acad. Sci. U.S.A., 75: 1929-1933 (1978).

If prokaryotic cells or cells that contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium as described by Cohen et al., Proc. Natl. Acad. Sci. (USA) 69: 2110 (1972), or more recently electroporation.

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required.

Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1 µg plasmid or DNA fragments is used with about 1 unit of enzyme in about 20 µl of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about one hour at 37 °C are workable. After incubation, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation may be treated for 15 minutes at 15 °C with 10 units of the Klenow fragment of DNA Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel et al., Nucleic Acids Res., 8: 4057 (1980).

For ligation, approximately equimolar amounts of the desired components, suitably end tailored to provide correct matching, are treated with about 10 units T4 DNA ligase per 0.5 µg DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

As discussed above, t-PA variants are preferably produced by means of specific mutation. Variants useful in the practice of the present invention are formed most readily through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient

number of adjacent nucleotides, to provide a sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the mutation being traversed.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are typically used to transform *E. coli* K12 strain 294 (ATCC 31,446) or other suitable *E. coli* strains, and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared and analyzed by restriction mapping and/or DNA sequencing by the method of Messing et al., Nucleic Acids Res., 9: 309 (1981) or by the method of Maxam et al., Methods of Enzymology, 65: 499 (1980).

After introduction of the DNA into the mammalian cell host and selection in medium for stable transformants, amplification of DHFR-protein-coding sequences is effected by growing host cell cultures in the presence of approximately 20,000-500,000 pM concentrations of MTX, a competitive inhibitor of DHFR activity. The effective range of concentration is highly dependent, of course, upon the nature of the DHFR gene and protein and the characteristics of the host. Clearly, generally defined upper and lower limits cannot be ascertained. Suitable concentrations of other folic acid analogs or other compounds that inhibit DHFR could also be used. MTX itself is, however, convenient, readily available, and effective.

In order to simplify the examples certain frequently occurring methods will be referenced by shorthand phrases.

"Plasmids" are designated by a low case p followed by an alphanumeric designation. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes, and the sites for which each is specific is called a restriction site. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements as established by the enzyme suppliers were used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme infrequently is followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional (T. Maniatis et al., 1982, Molecular Cloning pp. 133-134).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see R. Lawn et al., 1981, Nucleic Acids Res. 9:6103-6114, and D. Goeddel et al., 1980, Nucleic Acids Res. 8:4057.

"Southern Analysis" is a method by which the presence of DNA sequences in a digest or DNA-containing composition is confirmed by hybridization to a known, labelled oligonucleotide or DNA fragment. For the purposes herein, unless otherwise provided, Southern analysis shall mean separation of digests on 1 percent agarose, denaturation and transfer to nitrocellulose by the method of E. Southern, 1975, J. Mol. Biol. 98: 503-517, and hybridization as described by T. Maniatis et al., 1978, Cell 15: 687-701.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or chromosomal integrant. Unless otherwise provided, the method used herein for transformation of *E. coli* is the $CaCl_2$ method of Mandel et al., 1970, J. Mol. Biol. 53: 154.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (T. Maniatis et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

"Preparation" of DNA from transformants means isolating plasmid DNA from microbial culture. Unless otherwise provided, the alkaline/SDS method of Maniatis et al., Id., p. 90, may be used.

"Oligonucleotides" are short length single or double stranded polydeoxynucleotides that are chemically synthesized by known methods and then purified on polyacrylamide gels.

C. Pharmaceutical Compositions

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the t-PA product hereof is combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable carrier vehicles and their formulation, inclusive of other human proteins, e.g.. human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Such compositions will typically contain an effective amount of the variant herein, for example, from about 0.5 to about 5 mg/ml, together with a suitable amount of carrier vehicle to prepare pharmaceutically acceptable compositions suitable for effective administration to the host. The t-PA variant herein may be administered parenterally to subjects suffering from cardiovascular diseases or conditions, or by other methods that ensure its delivery to the bloodstream in an effective form.

Compositions particularly well suited for the clinical administration of variant t-PA products employed in the practice of the present invention include, for example, sterile aqueous solutions, or sterile hydratable powders such as lyophilized protein. It is generally desirable to include further in the formulation an appropriate amount of a pharmaceutically acceptable salt, generally in an amount sufficient to render the formulation isotonic. A pH regulator such as arginine base, and phosphoric acid, are also typically included in sufficient quantities to maintain an appropriate pH, generally from 5.5 to 7.5. Moreover, for improvement of shelf-life or stability of aqueous formulations, it may also be desirable to include further agents such as glycerol. In this manner, variant t-PA formulations are rendered appropriate for parenteral administration, and, in particular, intravenous administration.

Dosages and desired drug concentrations of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. For example, in the treatment of deep vein thrombosis or peripheral vascular disease, "bolus" doses, on the order of about 0.05 to about 0.2 mg/kg, will typically be preferred with subsequent administrations, on the order of about 0.1 to about 0.2 mg/kg, being given to maintain an approximately constant blood level, preferably on the order of about 3 µg/ml.

However, for use in connection with emergency medical care facilities where infusion capability is generally not available and due to the generally critical nature of the underlying disease (e.g., embolism, infarct), it will generally be desirable to provide somewhat larger initial doses, such as an intravenous bolus on the order of about 0.3 mg/kg.

For example, the t-PA variant hereof is suitably administered parenterally to subjects suffering from cardiovascular diseases or conditions. Dosage and dose rate may be parallel to or higher than that currently in use in clinical investigations of other cardiovascular, thrombolytic agents, e.g., about 1-2 mg/kg body weight as an intravenous or intra-arterial dose over 1.5 to 12 hours in human patients suffering from myocardial infarction, pulmonary embolism, etc. Higher doses may be tolerated because the variants herein have lower side effects than wild-type t-PA, leading to faster and more complete clot lysis.

As one example of an appropriate dosage form, a vial containing 50 mg t-PA, arginine, phosphoric acid, and polysorbate 80 is reconstituted with 50 ml sterile water for injection and mixed with a suitable volume of 0.9 percent sodium chloride injection.

The t-PA variants herein also are useful to prevent fibrin deposition or adhesion formation or reformation. One embodiment of this use is described in PCT WO 89/00049 published January 12, 1989. Generally, such treatment involves topical administration of a composition to a site of potential fibrin or adhesion formation wherein the composition comprises a therapeutically effective amount of the t-PA variant in a sparingly soluble form that is continuously released at that site for a period of time of about from three days to two weeks. Typically, the t-PA variant is administered at a dosage sufficient to prevent fibrin deposition or formation of adhesions following surgery, infection, trauma, or inflammation. Typically, this amount is from 0.02 mg/g of gel to 25 mg/g of gel, with preferred amounts from 0.20 mg/g gel to about 2.5 mg/g of gel, most preferably from 0.25 mg/g to about 1.0 mg/g of gel.

The vehicle in which the t-PA is typically formulated for preventing adhesion formation is a semisolid, mucilaginous pharmaceutically inert carrier for positioning the enzyme at the site of potential adhesion formation. Such a carrier includes long-chain hydrocarbons or vegetable oils and waxes composed of mixtures of saturated and unsaturated fatty acid glycerides or mixtures of modified saturated and unsaturated fatty acid glycerides. Examples include semisolid vehicles such as petroleum jelly or semi-synthetic glycerides, polyhydroxy solvents such as glycerol, long-chain hydrocarbons, bioerodable polymers, or liposomes.

The following examples are intended merely to illustrate the best mode now known for practicing the invention, but the invention is not to be considered limited thereto.

EXAMPLE I

A. Preparation and Utilization of Expression Vector for Recombinant Production of the t-PA Variants Hereof

1. Construction of Plasmid p7-1H

a) Plasmid pCISt-PA

Plasmid pCISt-PA was prepared as described as follows. In recapitulation, the vector pCIHt-PA containing the cytomegalovirus enhancer and promoter, the cytomegalovirus splice donor site and intron, the Ig variable region splice acceptor site, the cDNA-encoding t-PA (Pennica et al., Nature, 301: 214 (1983)) and the hepatitis surface antigen polyadenylation and transcription termination site was constructed first:

The vector pF8CIS containing the cytomegalovirus enhancer (Boshart et al., Cell, 41: 520 (1985)) and promoter (Thomsen et al., Proc. Natl. Acad. Sci. (U.S.A.) 81: 659 (1984)), the cytomegalovirus splice donor site and a portion of an intron (Sternberg et al., J. of Virol., 49: 190 (1984)), the Ig variable region intron and splice acceptor site, the cDNA encoding factor VIII, and the SV40 polyadenylation site was constructed. The three parts of the construction are detailed below.

1. The ampicillin resistance marker and replication origin of the final vector was derived from the starting plasmid pUC13pML, a variant of the plasmid pML (Lusky et al., Nature, 293: 79 (1981)). pUC13pML was constructed by transferring the polylinker of pUC13 (Veira et al., Gene, 19: 259 (1982)) to the EcoRI and HindIII sites of pML. A second starting plasmid pUC8CMV was the source of the CMV enhancer, promoter and splice donor sequence. pUC8CMV was constructed by inserting nucleotides 1 through 732 for the CMV enhancer, promoter and splice donor sequence into the blunted PstI and SphI sites of pUC8 - Veira et al., *supra*. Synthetic BamHI-HindIII linkers (commercially available from New England Biolabs) were ligated to the cohesive BamHI end, creating a HindIII site. Following this ligation a HindIII-HincII digest was performed. This digest yielded a fragment of approximately 800 bp that contained the CMV enhancer, promoter and splice donor site. Following gel isolation, this 800-bp fragment was ligated to a 2900-bp piece of pUC13pML. The fragment required for the construction of pF8CIS was obtained by digestion of the above intermediate plasmid with SalI and HindIII. This 3123-bp piece contained the resistance marker for ampicillin, the origin of replication from pUC13pML, and the control sequences for the CMV, including the enhancer, promoter, and splice donor site.

2. The Ig variable region intron and splice acceptor sequence was constructed using a synthetic oligomer. A 99-mer and a 30-mer were chemically synthesized having the following sequence for the IgG intron and splice acceptor site (Bothwell et al., Cell, 24: 625 (1981)):

```
 1   5'-AGTAGCAAGCTTGACGTGTGGCAGGCTTGA...
31     GATCTGGCCATACACTTGAGTGACAATGA...
60     CATCCACTTTGCCTTTCTCTCCACAGGT...
88     GTCCACTCCCAG-3'
 1   3'-CAGGTGAGGGTGCAGCTTGACGTCGTCGGA-5'
```

DNA polymerase I (Klenow fragment) filled in the synthetic piece and created a double-stranded fragment (Wartell et al., Gene, 9: 307 (1980)). This was followed by a double digest of PstI and HindIII. This synthetic linker was cloned into pUC13 (Veira et al., *supra*) at the PstI and HindIII sites. The clone containing the synthetic oligonucleotide, labeled pUCIg.10, was digested with PstI. A ClaI site was added to this fragment by use of a PstI-ClaI linker. Following digestion with HindIII a 118- bp piece containing part of the Ig intron and the Ig variable region splice acceptor was gel isolated.

3. The third part of the construction scheme replaced the hepatitis surface antigen 3' end with the polyadenylation site and transcription termination site of the early region of SV40. A vector, pUC.SV40, containing the SV40 sequences was inserted into pUC8 at the BamHI site described in Veira et al., *supra*. pUC.SV40 was then digested with EcoRI and HpaI. A 143-bp fragment containing only the SV40 polyadenylation site was gel isolated from this digest. Two additional fragments were gel isolated following digestion of pSVE.8c1D (EPO Pub. No. 160,457). The 4.8-kb fragment generated by EcoRI and

ClaI digest contains the SV40-DHFR transcription unit, the origin of replication of pML, and the ampicillin resistance marker. The 7.5-kb fragment produced following digestion with ClaI and HpaI contains the cDNA for Factor VIII. A three-part ligation yields pSVE.8c24D. This intermediate plasmid was digested by ClaI and SalI to give a 9611-bp fragment containing the cDNA for Factor VIII with the SV40 polyadenylation and transcription termination sites followed by the SV40 DHFR transcription unit.

The final three-part ligation to yield pF8CIS used: a) the 3123-bp SalI-HindIII fragment containing the origin of replication, the ampicillin resistance marker and the CMV enhancer, promoter and splice donor; b) the 118-bp HindIII-ClaI fragment containing the Ig intron and splice acceptor; and c) a 9611-bp ClaI-SalI fragment containing the cDNA for Factor VIII, SV40 polyadenylation site, and the SV40 DHFR transcription unit.

Next, the completion of the construction of plasmid pCIHt-PA from intermediate plasmid pCla t-PA and plasmid pF8CIS (above) was undertaken:

The t-PA cDNA was first cloned into pML to provide a ClaI site at the 5' end of the gene. To do this, a 3238-bp HindIII fragment from pSVpa-DHFR (otherwise referred to as pETPFR, *supra*) was inserted into the HindIII site of pML (Lusky et al., *supra*). Colonies were screened for clones that have the 5' end of the cDNA juxtaposed to the ClaI site. The intermediate plasmid was labeled pCLAt-PA. A t-PA cDNA followed by the 3'-polyadenylation regions was isolated as a ClaI-KpnI fragment of 2870 bp. This fragment was ligated to the 5146-bp fragment of pF8CIS. This ClaI-KpnI fragment of the CIS vector provided the 5' control region, a SV40-DHFR transcriptional unit, the ampicillin resistance gene, and the origin region from pML. See Figure 2.

Expression levels of t-PA were obtained by transfecting CHO or 293 cells with pCIHt-PA, in accordance with methods generally known per se and described *supra*. Media from the transfected 293 cells, for example, were assayed, demonstrating that pCIHt-PA produced 420 ng/ml of t-PA.

The vector pCISt-PA containing the cytomegalovirus enhancer and promoter, the cytomegalovirus splice donor site and intron, the Ig variable region splice acceptor site, the cDNA encoding t-PA, and the pSV40 polyadenylation sequence was finally constructed as follows:

The starting vectors for this construction were pCIHt-PA and pF8CIS (*supra*). The latter vector has the same 5' controls as pCIHt-PA, but includes the cDNA for Factor VIII and the SV40 polyadenylation site. SacII was used to cleave 3' of the t-PA cDNA. The resultant 3' overhang was blunted by T4 polymerase. pCIHt-PA was then cut with ClaI. This site separates the chimeric intron cleaving between the CMV intronic sequences and the Ig variable region intron. An 2870-bp fragment was gel isolated from the ClaI treatment. The SV40 polyadenylation site, DHFR, transcription control, bacterial origin of replication, and amp^r gene, as well as the CMV enhancer and promoter and splice donor were isolated from pF8CIS. These elements were isolated into fragments as a 2525-bp SalI-BamHI fragment and a HpaI-SalI and 3113-bp fragment. A three-part ligation of the KpnI (blunt)-ClaI fragment with the HpaI-SalI fragment and SalI to BamHI fragment yields pCISt-PA, which was expressed in both CHO and 293 cells as discussed above for plasmid pCIHt-PA, giving 55 and 3000 ng/ml of t-PA, respectively. See Figure 3.

b) Final Construction of p7-1H

The plasmid pCISt-PA was digested with SpeI, then treated with *E. coli* DNA polymerase I large fragment (Klenow) and deoxyribonucleoside triphosphates to create blunt ends. The resulting linear fragment was ligated, using T4 DNA ligase, to the 0.45kb-RsaI/AhaIII fragment containing the + strand origin from the single-stranded DNA phage, f1, as described in Zinder et al., Microbiol. Rev., 49: 101 (1985). Ligation products were isolated with the f1 origin inserted in both possible orientations at the SpeI site of the pCISt-PA fragment. A plasmid containing this origin, in such an orientation that the anti-sense strand of the t-PA gene was packaged into virions in the presence of helper phage, was chosen and termed p7- 1H. See Figure 4.

2. Mutagenesis of Expression Plasmid

a) Template Preparation

Plasmid p7-1H was introduced into *E. coli* strain JM101 (ATCC No. 33,876) via $CaCl_2$-mediated transformation. These cells were then infected with the helper virus M13K07 and single-stranded p7-1H DNA was prepared as described by Veira et al., Meth. Enzymol., 153: 3 (1987). Briefly, to 0.3 ml of a saturated culture of transformed cells in 2YT broth was added 10^9-10^{10} pfu of M13K07 and the mixture was incubated for 15 min. at 37 °C. 1.5 ml of fresh 2YT broth, containing 50 µg/ml carbenicillin, was added and

the culture was gently shaken for 16 hours at 37 °C. After the cells were pelleted, phage and packaged plasmid DNA were harvested, and single-stranded DNA was prepared as described by Anderson, Nucl. Acids. Res., 9: 3015 (1981).

b) Site-directed *in vitro* Mutagenesis

Mutagenesis on p7-1H was carried out using the oligodeoxyribonucleotide, 5'-CGGAGAGCGG-CACCTGTGCGGGG-3', essentially as described by Zoller et al., Meth. Enzymol., 100: 468 (1983), except that the mutant, with the mutation phe305 - --> his305, was identified by colony hybridization rather than plaque hybridization. Mutations were verified by DNA sequencing directly on the single-stranded plasmid DNA using the dideoxynucleotide chain termination method (Sanger et al., Proc. Natl. Acad. Sci. (U.S.A.) 74: 5463 (1977)).

3. Expression and Purification

a) Plasmid Preparation

Transformed cells were grown to saturation in 500-ml LB broth containing 50 µg/ml carbenicillin. Cells were pelleted by centrifugation and resuspended in 40 ml of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0). To this suspension was added 60 ml of 1% sodium dodecyl sulfate, 0.07 M NaOH, and the mixture was incubated for 2 min at 25 °C, then at 10 min. at 0 °C. To this 52 ml of 4 M acetic acid, 3 M sodium acetate was added and the mixture was incubated for 30 min. at 0 °C. This was then centrifuged at 11,500 rpm for 20 min., the supernatant mixed with two volumes of 100% cold ethanol, and the resulting precipitate harvested by centrifugation. The pellet, containing plasmid DNA and RNA, was dried and redissolved in 100 mM Tris (pH 8.0), 10 mM EDTA, 1 µg/ml RNase A. After the resulting solution was clarified by centrifugation, it was adjusted to 0.5 mg/ml in ethidium bromide and an equal weight of CsCl gas added. The DNA was then centrifuged in a Beckman VT165 rotor for 16 hours at 55,000 rpm at 18 °C. The DNA band was harvested by side puncture, extracted with n-butanol to remove the ethidium bromide, diluted with H_2O, and precipitated by ethanol. DNA was redissolved in 10 mM Tris (pH 8.0), 1 mM EDTA, to a final concentration of 1 mg/ml.

b) Transfection and Expression

293 cells were grown to confluence. Ten µg of t-PA plasmid DNA mutant was mixed with 1 µg of DNA encoding the VA RNA gene (Thimmappaya et al., Cell, 31: 543 (1982)) and dissolved in 500 µl of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M $CaCl_2$. Added to this (dropwise while vortexing) was 500 µl of 50 mM HEPES (pH 7.35), 280 mM NaCl, 1.5 mM $NaPO_4$, and the precipitate was allowed to form for 10 min. at 25 °C. The suspended precipitate was then added to the cells (in 100 mM plate) and allowed to settle for four hours in the incubator. The medium was then aspirated off and 2 ml of 20% glycerol in phosphate-buffered saline (PBS) was added for 30 sec. The cells were washed twice with 5 ml of serum-free medium, then fresh medium was added and the cells were incubated for five days.

For the creation of stable CHO cell lines expressing the t-PA variant, the 1.4 kb BglII/ApaI fragment containing the bulk of the t-PA coding sequences (the BglII site spans codons - 1 to 1 of full-length t-PA-encoding DNA and the ApaI site spans codons 465 to 466 of full-length t-PA-encoding DNA) may be ligated to the 6.0-kb BglII/ApaI fragment from the vector pPADHFR-6 (described in EPO Pat. Publn. No. 93,619). The resultant plasmid is then introduced into CHO cells and induced to over-express the t-PA variants by amplifying the coding sequence by means of selection in methotrexate-containing media.

c) Purification

Purification of the t-PA product was accomplished by passing the conditioned medium over a column (1-ml bed volume) of controlled glass beads to which an anti-t-PA goat polyclonal A6 antibody (prepared according to standard methods known per se) had been coupled. Before the medium was loaded, the column was equilibrated with PBS and, after loading, the column was equilibrated with 0.1 M Tris-HCl (pH 7.5), 1 M NaCl. The t-PA was eluted with 0.1 M acetic acid, 0.15 M NaCl, 0.02 M arginine, 0.01% Tween 80 (pH 2.0), and fractions were immediately neutralized with Tris-base. Fractions were adjusted to 0.01% Tween 80 before pooling. The t-PA was found on a reducing SDS gel to be predominantly (80%) single chain.

B. Biological Assays

1. t-PA Quantitation

Protein concentrations were routinely determined by an ELISA standardized to native-sequence t-PA (See EPO Pat. Publ. 93,619, *supra*). Protein purity and homogeneity were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (PAGE-SDS) with the buffer system of Laemmli, Nature, 227: 680 (1970). Typically, 7 to 17% gradient gels were used and proteins were visualized with the silver-staining technique of Morrissey, Anal. Biochem., 117: 307 (1981). The t-PA variant prepared as described above was found to be pure and homogeneous by this method.

2. S-2251 Assay

Results for clot lysis and S-2251 assays show averages of several independent observations (clot lysis, two determinations; S-2251, three determinations).

The ability of t-PA to activate plasminogen can be measured in an *in vitro* assay by preincubating t-PA and plasminogen and then adding the plasmin-specific substrate H-D-valyl-H-leucyl-H-lysine-paranitroanilide (S-2251). The maximum rate of this reaction is observed in the presence of fibrin(ogen) or fragments of fibrin(ogen) that act as stimulators of the reaction.

The plasmin-specific substrate S-2251 was used in a two-stage assay to measure the ability of the sample to activate plasminogen. Fibrinogen could be used as a stimulator by incubating the sample with 0.02 ml of a 20 mg/ml fibrinogen solution in a total volume of 0.12 ml of 0.05 M Tris-HCl, 0.12 M NaCl, 0.01% Tween 80, pH 7.4.

Glu-plasminogen solution (commercially available), 0.03 ml of a 2.0 mg/ml solution in 0.05M Tris, 0.12 M NaCl buffer, pH 8, was then added. After ten min. at 37 °C, 0.35 ml of 0.86 mM S-2251 in 0.037 M Tris, 0.086 NaCl, 0.007% Tween 80, pH 7.4 was added. This mixture was incubated for five minutes; then the reaction was stopped by the addition of 0.1 ml of 50% glacial acetic acid. Absorbance at 405 nm was measured. The activity was expressed as the change in absorbance per nanogram per minute in the presence of substrate.

The results are that the F305H variant, with fibrinogen, has 78% of the wild-type specific activity, which may be due to the lag before the A405 increases.

3. Clot Lysis

Wild-type and F305H t-PA were assayed for their ability to lyse fibrin in the presence of saturating concentrations of plasminogen, according to the method of Carlsen et al., Anal. Biochem., 168: 428-435 (1988). The *in vitro* clot lysis assay measures the activity of t-PAs by turbidimetry using a microcentrifugal analyzer. A mixture of thrombin and t-PA test samples is centrifuged into a mixture of fibrinogen and plasminogen to initiate clot formation and subsequent clot dissolution. The resultant profile of absorbance versus time is analyzed to determine the assay endpoint. Activities of the t-PA variants were compared to a standard curve of rt-PA (EPO Publ. No. 93,619, *supra*). The buffer used throughout the assay was 0.06 M sodium phosphate, pH 7.4, containing 0.01% (v/v) Tween 80 and 0.01% (w/v) sodium azide. Human thrombin was at a concentration of 33 units/ml. Fibrinogen (at 2.0 mg/ml clottable protein) was chilled on wet ice to precipitate fibronectin and then gravity filtered. Glu-plasminogen was at a concentration of 1 mg/ml. The analyzer chamber temperature is set at 37 °C The loader is set to dispense 20 µl of rt-PA (about 62.5 ng/ml to 1.0 µg/ml) as the sample for the standard curve, or 20 µl of variant rt-PA at a concentration to cause lysis within the range of the standard curve. Twenty µl of thrombin was used as the secondary reagent, and 200 µl of a 50:1 (v/v) fibrinogen: plasminogen mixture as the primary reagent. The absorbance/time program was used with a five-minute incubation time, 340-nm filter, and 90-interval readings.

The results indicate that the F305H variant, using this assay, has about 46% of the clot lysis activity of normal wild-type t-PA.

4. Fibrin Binding

The method for fibrin binding is a modification of the method described by Rijken et al., J. Biol. Chem., 257: 2920 (1982). The t-PA sample to be tested is added to a solution containing 0.05 M Tris (pH 7.4), 0.12 M NaCl, 0.01% Tween 80, 1 mg/ml human serum albumin, and various concentrations of plasminogen-free

fibrin (0, 0.05, 0.1, 0.25, and 0.5 mg/ml). The final volume of the reaction mixture was 1 ml, and the t-PA concentration was 10 ng/ml for each sample. The samples were incubated at 37 °C for 5 min., followed by the addition of 1 unit of thrombin. The samples were then incubated for one hour at 37 °C. The clot was removed by centrifugation, and the amount of t-PA remaining unbound in the supernatant was determined by ELISA.

The results (Figure 5) show that predominantly single-chain F305H t-PA (closed triangles) binds to fibrin under the assay conditions employed almost as well as one-chain wild-type t-PA (closed squares) and the mixture of one-chain and two-chain wild-type t-PA (open triangles). Also, two-chain F305H t-PA (closed circles) binds fibrin at least as well as two-chain wild-type t-PA (closed diamonds).

5. Zymogenic Kinetics by S-2251

a) Preparation of Fibrinogen

Human fibrinogen (Calbiochem) was made plasminogen free by applying it to a lysine-Sepharose column and collecting the flow-through. The resulting fibrinogen pool was degraded by treatment with plasmin-Sepharose at room temperature overnight. The resulting clottability was 7%. The concentration was then adjusted to 1.51 mg/ml.

b) Procedure

The kinetics of the conversion of plasminogen to plasmin by the wild-type t-PA and the F305H t-PA were determined using the chromogenic plasmin substrate S-2251 in the presence of fibrinogen. The wild-type and F305H t-PA molecules were used both in the predominantly one-chain form (obtained by purification as described previously) and in the two-chain, clipped form (obtained by incubation of the predominantly one-chain form plasmin-Sepharose for one hour at 37 °C).

In the presence of 1.2 μM plasmin-degraded fibrinogen fragments prepared as described above, the reactions were carried out at plasminogen concentrations from 0.08 to 0.89 μM and t-PA concentrations of 2.3 to 9.0 nM in 0.12 M NaCl, 0.05 M Tris, 0.01% Tween 80, pH 7.4. The plasminogen, fibrinogen, and buffer were pre-incubated for three hours at room temperature. The S-2251 was added for a final concentration of 0.9 mM, and the samples were warmed to 37 °C for about 5 minutes. At time zero, the t-PA samples were added, and the absorbance of each sample was read in intervals of 30 seconds for ten minutes at 37 °C.

The results are shown in Figures 6 and 7 (for wild-type, one-chain and two-chain t-PA, respectively) and in Figures 8 and 9 (for F305H, one-chain and two-chain t-PA, respectively). In the figures, the ordinate is the absorbance at 405 nm and the abscissa is the square of the number of minutes at which the absorbances were taken. The closed circles represent 0.09 μM of plasminogen (and 15.7 nM wild-type two-chain t-PA, 10.8 nM F305H two-chain t-PA, 16.1 nM wild-type one-chain t-PA, and 17.2 nM F305H one-chain t-PA), the closed triangles represent 0.11 μM of plasminogen (and 11.8 nM wild-type two-chain t-PA, 8.1 nM F305H two-chain t-PA, 12.1 nM wild-type one-chain t-PA, 12.9 nM F305H one-chain t-PA), the closed squares represent 0.16 μM of plasminogen (and 9.8 nM wild-type two-chain t-PA, 6.7 nM F305H two-chain t-PA, 10.1 nM wild-type one-chain t-PA, 10.8 nM F305H one-chain t-PA), the open circles represent 0.22 μM of plasminogen (and 7.9 nM wild-type two-chain t-PA, 5.4 nM F305H two-chain t-PA, 8.1 nM wild-type one-chain t-PA, 8.6 nM F305H one-chain t-PA), the open triangles represent 0.44 μM of plasminogen (and 3.9 nM wild-type two-chain t-PA, 2.7 nM F305H two-chain t-PA, 4.0 nM wild-type one-chain t-PA, 4.3 nM F305H one-chain t-PA), and the open squares represent 0.9 μM of plasminogen (and 3.9 nM wild-type two-chain t-PA, 2.7 nM F305H two-chain t-PA, 4.0 nM wild-type one-chain t-PA, 4.3 nM F305H one-chain t-PA).

The graphs show that only with the predominantly one-chain F305H variant does the absorbance exhibit a pronounced lag at early times in the reactions, and a rise in activity thereafter. Two-chain F305H variant does not exhibit this lag, but rather appears to have kinetic properties similar to one- or two-chain wild-type t-PA This behavior demonstrates the zymogenic nature of the F305H variant that is not observed with the wild-type t-PA.

EXAMPLE II

A strategy known as alanine-scanning mutagenesis (ALA-scan), described in Cunningham and Wells, *supra*, was employed for generation of the t-PA variants evaluated in this example. This method involved the identification of small surface regions of the t-PA protease domain that contain charged amino acid side

chains. Without limitation to any one theory, it is believed that either these regions containing clusters of charge, or neighboring regions, or both, are responsible for the interaction of the t-PA molecule with its substrate and various other compounds that may modulate its activity. The charged amino acids in each region (i.e., Arg, Asp, His, Lys, and Glu) were replaced (one region per mutant molecule) with alanine to assess the importance of the particular region to the overall activity of the t-PA molecule. The results are indicated below.

1. Construction of pRK7-t-PA

Plasmid pRK7 was used as the vector for generation of the t-PA mutants. pRK7 is identical to pRK5 (EP Pub. No. 307,247 published March 15, 1989), except that the order of the endonuclease restriction sites in the polylinker region between ClaI and HindIII is reversed. The t-PA cDNA (Pennica et al., Nature, 301: 214 (1983)) was prepared for insertion into the vector by cutting with restriction endonuclease HindIII (which cuts 49 base pairs 5' of the ATG start codon) and restriction endonuclease BalI (which cuts 276 base pairs downstream of the TGA stop codon). This cDNA was ligated into pRK7 previously cut with HindIII and SmaI using standard ligation methodology (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982). This construct was named pRK7-t-PA.

2. Site-Directed Mutagenesis of pRK7-t-PA

Site-directed mutagenesis of t-PA cDNA was performed by the method of Taylor et al., Nucl. Acids. Res., 13: 8765 (1985) using a kit purchased from the Amersham Corporation (catalog number RPN 1253). For generation of the desired mutants, oligonucleotides of sequences coding for the desired amino acid substitutions were synthesized and used as primers. These oligonucleotides were annealed to single-stranded pRK7-t-PA that had been prepared by standard procedures (Viera et al., Meth. Enz., 143: 3 (1987)-).

A mixture of three deoxyribonucleotide triphosphates, deoxyriboadenosine triphosphate (dATP), deoxyriboguanosine triphosphate (dGTP), and deoxyribothymidine triphosphate (dTTP), was combined with a modified thio-deoxyribocytosine called dCTP(αS) provided in the kit by the manufacturer of the kit, and added to the single-stranded pRK7-t-PA to which was annealed the oligonucleotide.

Upon addition of DNA polymerase to this mixture, a strand of DNA identical to pRK7-t-PA except for the mutated bases was generated. In addition, this new strand of DNA contained dCTP(αS) instead of dCTP, which served to protect it from certain restriction endonuclease digestion. After the template strand of the double-stranded heteroduplex was nicked with an appropriate restriction enzyme, the template strand was digested with ExoIII nuclease past the region that contained the mutagenic oligomer. The reaction was then stopped to leave a molecule that was only partly single-stranded. A complete double-stranded DNA homoduplex molecule was then formed by DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase.

The following oligonucleotides were prepared to use as primers to generate pRK7-t-PA molecules using the ALA-scan methodology described above:

5'-GGCTGTACTGGGCCAGGCCGCA-3' (R267A)

5'-GGCAGCCTGCCAGGGGGCGGAGGCGATGGCGGCGAAGAG-3'(D283A,H287A)

5'-CCGCTCTCCGGGCGACGCCGCGGCCGCGGCAAAGAT-3'(K296A,H297A,R298A,R299A)

5'-GCCCCCGCACAGGAACGCCGCTCCGGGCGA-3' (E303A,R304A)

5'-CTCCTGGAAGCAGGCGGCGGCAGA-3' (H322A)

5'-GTGGTGGGGCGGAAACGCCGCCTGGAACGA-3' (E326A,R327A)

5'-CAAGATCACCGTCAGGGCGGCGGGCGGAAA-3' (H331A,H332A)

5'-CTCGCCAGGGACCACCGCGTATGTTGCGCCCAAGAT-3' (R339A,R342A)

5'-TTTTTCGACTTCAAATGCCTGCGCCGCCGCGCCAGGGAC-3'(E347A,E348A,E349A,K351A)

5'-CTTATGGACAATGTATGCTGCGACTGCAAATTTCTG-3'(E353A,E355A,K356A)

5'-AGTGTCATCATCGAATGCCGCAGCGACAATGTA-3' (H360A,K361A,E362A)

5'-GTCATTGTCGTAAGTGGCAGCAGCGAATTCCTT-3' (D364A,D365A,D366A)

5'-CTGCAGCAGCGCAATGGCATTGGCGTAAGTGTC-3' (D369A,D371A)

5'-CTCCTGGGCACAGGCGGACGAAGCCGATGCCAGCTGCAG-3' (K378A,D380A,R383A)

5'-AAGGCACACAGTGGCGACCACGCTGCTCGCCTGGGCACA-3' (E387A,R392A)

5'-ACACTCCGTCCAGGCCGGCAGCTGCAGGGCCGCCGGGGG-3' (D400A,D405A)

5'-GGAGAGCTCACAGGCCGTCCAGTC-3' (E408A)

5'-GTAGCCGGAGAGGGCACACTCCGT-3' (E410A)

5'-AGGAGACAAGGCCGCAGCCGCGCCGTAGCC-3' ((K416A,H417A,E418A)

5'-TCTGACATGAGCCGCCGCCAGCGCCGCCGAATAGAA-3' (E426A,R427A,K429A,E430A)

5'-GGATGGGTACAGTGCGACAGCAGCCTCCTT-3' (H432A,R434A)

5'-TTGTGATGTGCAGGCGCTGGATGG-3' (R440A)

5'-GTCGGTGACTGTTGCGTTAAGTAAAGCTTGTGATGT-3' (H445A,R449A)

5'-ACACAGCATGTTGGCGGTGACTGTTGCGTTAAGTAA-3' (R449A,D453A)

5'-GGGCCCGCCGCTCGCAGTGGCTCCAGCACA-3' (D460A,R462A)

5'-GCCCTGGCAGGCGGCGGCCAAGTTTGC-3' (H471A,D472A)

5'-GGGGCCTCCCGAAGCGCCCTGGCA-3' (D477A)

5'-CACCAAAGTCATGGCGCCAGCGTTCAGACA-3' D487A,R489A)

5'-CACACCCGGGACAGCCGCCTGTCCACA-3' (K505A,D506A)

5'-GTAGTTGGTAACGGCTGTGTACAC-3' (K513A)

5'-CGGTCGCATGTTGGCAGCAATCCAGGCTAGGTAGTT-3'(D519A,R522A,D523A)

5'-TCCTGGTCACGGTGCCATGTTGGCACGAATCCA-3' (D523A,R526A)

3. Bacterial Transformation and DNA Preparation

The mutant t-PA constructs generated using the protocol above were transformed into *E. coli* host strain MM294tonA using the standard $CaCl_2$ procedure (Maniatis et al., *supra*) for preparation and transformation of competent cells. MM294tonA (which is resistant to T1 phage) was prepared by the insertion and subsequent imprecise excision of a Tn10 transposon into the tonA gene. This gene was then inserted, using transposon insertion mutagenesis (Kleckner et al., J. Mol. Biol., 116: 125-159 (1977)), into *E. coli* host MM294 (ATCC 31,446).

DNA was extracted from individual colonies of bacterial transformants using the standard miniprep procedure of Maniatis et al., *supra*. The plasmids were further purified by passage over a Sephacryl CL6B spin column, and then analyzed by sequencing and by restriction endonuclease digestion and agarose gel electrophoresis.

One of these transformants containing the plasmid encoding the K296A,H297A,R298A,R299A mutant, and designated pTPA33-2, was deposited with the American Type Culture Collection on July 18, 1989 as ATCC No. 68,059. 4. Transfection of Human Embryonic Kidney 293 Cells (Small-Scale)

293 cells were grown to 70% confluence in 6-well plates. 2.5 µg of t-PA plasmid DNA mutant was dissolved in 150 µl of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M $CaCl_2$. Added to this (dropwise while vortexing) was 150 µl of 50 mM MEPES buffer (pH 7.35), 280 mM NaCl, 1.5 mM $NaPO_4$, and the precipitate was allowed to form for ten min. at 25 °C. The suspended precipitate was then added to the cells in the individual wells in a 6-well plate and allowed to settle for four hours in the incubator. The medium was then aspirated off and 1 ml of 20% glycerol in PBS was added for 30 sec. The cells were washed twice, first with 3 ml, then with 1 ml, of serum-free medium. Then 3 ml of fresh medium was added and the cells were incubated for five days. The medium was then collected and assayed.

When single-chain t-PA was required, the procedure was as described above except that plasminogen-depleted serum was used during the growth phase of the cells. 5. Transfection of Human Embryonic Kidney 293 Cells (Large Scale)

For large-scale purification of the K296A,H297A,R298A,R299A variant, useful for production in significant quantities, the transfection procedure used was obtained from Current Protocols in Molecular Biology, Ausubel et al., eds. (Wiley Interscience, 1988) and modified slightly as follows: A suspension of human embryonic kidney 293 cells was grown in a cell culture medium and concentrated by pelleting. The pellet was resuspended to a concentration of about 10^8 cells per milliliter and the cells were washed as necessary in serum-free media. The DNA-dextran solution was added at a concentration of about 250 µg of DNA per 500 ml of cells, and this mixture was incubated with mild agitation at 37 °C for up to 90 minutes. DMSO was added to a final concentration of ten percent and, after about two minutes, fresh medium was added to dilute the cells to about 10^6 per milliliter. Cells were then incubated for up to seven days, after which time the supernatant was collected.

Purification of this mutant was accomplished by passage of the supernatant over a column of glass beads coupled to anti-t-PA goat polyclonal A6 antibody. The column had been preconditioned with PBS. After the supernatant was loaded, the column was equilibrated with a Tris-saline buffer [0.1M Tris.HCl (pH 7.5) and 1M NaCl]. The t-PA variant was then eluted with 0.1M acetic acid, 0.15 M NaCl, 0.02 M arginine, and 0.01% Tween 80. Fractions were immediately neutralized with Tris base and adjusted to 0.01% Tween 80.

6. Biological Assays

A. t-PA Quantitation

The amount of t-PA present in the cell culture supernatants was determined by the ELISA procedure using polyclonal antibodies prepared against wild-type t-PA.

B. S-2288 Assay

The S-2288 assay was used to measure the proteolytic activity of the mutants in both the one- and two-chain forms. This assay is a direct assay for t-PA proteolytic activity; t-PA cleaves the bond between the small peptide and the paranitroanilide chromophore.

Standard curve samples were prepared by diluting wild-type rt-PA with cell culture media. The standard curve samples and rt-PA mutant samples were added to the wells of a microtiter plate. If the assay was used to measure the activity of two-chain rt-PA, an incubation step with human plasmin was included in the procedure. Human plasmin (KabiVitrum) was added to a final concentration of 0.13 CU (casein units)/ml. The samples were incubated for 90 minutes at room temperature. For assaying the samples in the single-chain form, the plasmin solution was replaced by PBS and the 90-minute incubation was omitted.

Aprotinin [Sigma, approximately 14 TIU (trypsin ihhibitor unit)/mg] was added to a final concentration of 72 µg/ml to inhibit the plasmin activity, and the samples were incubated at room temperature for 15 minutes. A 2.16 mM solution of S-2288 was diluted to 1.45 mM with 0.1 M Tris, 0.106 mM NaCl, 0.02% sodium azide, pH 8.4, and 100 µl of this solution was added to each well of the microtiter plate (final volume in each well was 200 µl). Color development was monitored at 405 nm. The slope of the absorbance vs. time curve for each standard and sample was determined. A standard curve was prepared by plotting the slope of the absorbance vs. time curve as a function of rt-PA concentration for the rt-PA standards. The relative activity concentration of the mutants was then determined from the standard curve. The activity concentration of each mutant was divided by the concentration for the mutant obtained in the rt-

PA ELISA, and the resulting specific activities were expressed relative to wild-type t-PA, which was assigned a value of 1.0.

The data are averages of two assays and are presented as activity relative to wild-type rt-PA in Table I. The results show that for all mutants presented, the two-chain form is more active (at least 1.5-fold, up to nearly 60-fold) than the one-chain form, relative to wild-type rt-PA, indicating that each of these mutants may be considered zymogenic in this assay.

TABLE I

Zymogens in the S-2288 Assay			
Mutation	Activity Relative to wt rt-PA (where wt is 1.0; () indicates experimental error)		
	one-chain	two-chain	fold difference
R267A	0.33 (0.03)	0.85 (0.13)	2.6
D283A,H287A	0.08 (0.11)	0.60 (0.23)	7.5
R339A,R342A	0.01 (0.01)	0.52 (0.01)	52
E347A,E348A,E349A,K351A	0.01 (0.01)	0.49 (0.18)	49
K416A,H417A,E418A	0.01 (0.01)	0.59 (0.01)	59
E426A,R427A,K429A,E430A	0.01 (0.01)	0.33 (0.04)	33
H432A,R434A	0.08 (0.01)	0.71 (0.08)	8.9
R440A	0.53 (0.03)	0.78 (0.04)	1.5

C. S-2251 Assay

This assay is an indirect assay for t-PA activity. In this assay, plasminogen is converted to plasmin by the action of t-PA, and plasmin cleaves the S-2251 substrate to release the paranitroanilide chromophore. Production of this chromophore is then measured over time.

1. Fibrin-Stimulated S-2251 Assay

Standard curve samples were prepared as described for the S-2288 assay. Samples assayed in the two-chain form were incubated with plasmin-Sepharose. Plasmin-Sepharose was prepared by coupling approximately 20.8 CU of human plasmin (KabiVitrum) to 1 ml of cyanogen bromide activated Sepharose (Pharmacia). The plasmin-Sepharose (50 µl of a 5% slurry) was incubated with shaking for 90 min. at room temperature with 150 µl of sample. Following the incubation, the resin was removed by centrifugation, and 10 µl of sample were added to the wells of a microtiter plate.

For samples assayed in the one-chain form, 50 µl of cell culture media were added in place of resin, and the incubation step was omitted. Human thrombin (10 µl of a 42 unit/ml solution) was added to each well. The reaction in each well was started by the addition of a cocktail (130 µl) composed of 28 µl of human Glu-plasminogen (5.3 µM); 10 µl of plasminogen-free human fibrinogen (10 µM); 30 µl of 3mM S-2251 (KabiVitrum); and 62 µl of PBS. Color development was monitored at 405 nm, and the absorbance at the reference wavelength of 492 nm was subtracted from each time point. The slope of the absorbance vs. time squared curve was determined for each standard and mutant sample. A standard curve was prepared by plotting the slope of the absorbance vs. time squared curve as a function of rt-PA concentration for the rt-PA standards. The determination of the relative specific activity for the mutants was as described for the S-2288 assay.

2. Fibrinogen-Stimulated S-2251 Assay

This assay was performed as described for the fibrin-stimulated S-2251 assay except that PBS was substituted for the thrombin.

3. Plasma Clot S-2251 Assay

The standard curve sample preparation and the conversion of one-chain rt-PA to two-chain rt-PA using plasmin-Sepharose were as described for the fibrin-stimulated S-2251 assay. Human thrombin (10 µl of a

31 µg/ml solution) was added to each well of the microtiter plate. The standard and mutant samples (40 µl) were added to the plate and the reaction was started by adding 100 µl of a mixture of 90 µl of acid citrate dextrose human plasma and 10 µl of 9.1 mM S-2251 (KabiVitrum). Color development was monitored at 405 nm and the absorbance at the reference wavelength of 492 nm was subtracted from each time point. The analysis of the data was as described for the fibrin-stimulated S-2251 assay.

4. Plasma S-2251 Assay

This assay was performed as described for the plasma clot S-2251 assay except that PBS was substituted for the thrombin.

Mutants were assayed for zymogenic qualities using the fibrin-dependent and plasma clot-dependent assays, and the results, relative to wild-type, are shown in Tables II and III, respectively. Values for mutants in the single-chain form are averages of two determinations. Values for mutants in the two-chain form are averages of four determinations.

TABLE II

Zymogens in the Fibrin-Dependent S-2251 Assay			
Mutation	Activity Relative to wt rt-PA (where wt is 1.0; () indicates experimental error)		
	one-chain	two-chain	fold difference
K296A,H297A,R298A,R299A	1.26 (0.02)	2.82 (0.32)	2.2
E303A,R304A	1.38 (0.04)	2.13 (0.29)	1.5
H331A,H332A	1.29 (0.04)	2.19 (0.68)	1.7
R339A,R342A	0.38 (0.07)	1.04 (0.11)	2.7
K416A,H417A,E418A	0.21 (0.04)	0.96 (0.09)	4.6
E426A,R427A,K429A,E430A	0.14 (0)	0.76 (0.07)	5.4
H432A,R434A	0.16 (0.08)	0.99 (0.13)	6.2
D460A,R462A	0.68 (0.04)	1.04 (0.08)	1.5

TABLE III

Zymogens in the Plasma Clot-Dependent S-2251 Assay			
Mutation	Activity Relative to wt rt-PA (where wt is 1.0; () indicates experimental error)		
	one-chain	two-chain	fold difference
D283A,H287A	0.53 (0.01)	0.82 (0.04)	1.6
K296A,H297A,R298A,R299A	0.42 (0)	1.29 (0.20)	3.1
E303A,R304A	0.90 (0.03)	1.34 (0.10)	1.5
H331A,H332A	1.09 (0.09)	1.61 (0.68)	1.5
R339A,R342A	0.19 (0.03)	0.79 (0.10)	4.2
E347A,E348A,E349A,K351A	0.38 (0.05)	0.97 (0.10)	2.6
D364A,D365A,D366A	0.88 (0.03)	1.46 (0.15)	1.7
K416A,H417A,E418A	0.19 (0.05)	0.77 (0.05)	4.1
E426A,R427A,K429A,E430A	0.11 (0.01)	0.66 (0.12)	6
H432A,R434A	0.05 (0.01)	0.38 (0.05)	7.6
H445A,R449A	0.80 (ND)*	1.19 (0.11)	1.5
R449A,D453A	0.79 (0.01)	1.22 (0.13)	1.5
D460A,R462A	0.21 (0.01)	0.32 (0.02)	1.5
D477A	0.11 (0.02)	0.25 (0.08)	2.3

*ND = Not Determined

A summary of the data in Tables I-III, indicating for which assay each mutant displays zymogenicity, is shown below, in Table IV:

TABLE IV

Mutation	S-2288	Fn*	Zymogen in Plasma Clot 2251	Zymogen in Plasma Clot 2251
R267A	X			
D283A,H287A	X			X
K296A,H297A,R298A,R299A			X	X
E303A,R304A			X	X
H331A,H332A			X	X
R339A,R342A	X		X	X
E347A,E348A,E349A,K351A	X			X
D364A,D365A,D366A				X
K416A,H417A,E418A	X		X	X
E426A,R427A,K429A,E430A	X		X	X
H432A,R434A	X		X	X
R440A	X			
H445A,R449A				X
R449A,D453A				X
D460A,R462A			X	X
D477A				X

*Fn=fibrin.

The zymogenic t-PA variants listed in Table IV were analyzed in the S-2251 fibrin specificity assay and/or S-2251 plasma clot specificity assay in both the one-chain and two-chain forms. The results for one-chain and two-chain t-PA variants are shown in Tables V and VI, respectively.

A summary of the data in Tables V and VI is shown below in Table VII. It can be seen that each of the zymogenic rt-PA variants from Table IV is also fibrin- and/or plasma clot-specific relative to wild-type t-PA. An X indicates a ratio of fibrin to fibrinogen or plasma clot to plasma of >1.5 as measured in the S-2251 assay and reported in Tables V and VI.

TABLE V

FIBRIN- AND PLASMA CLOT- SPECIFICITY OF rt-PA VARIANTS (ONE-CHAIN)

The activities are relative to wt rt-PA (where wt is 1.0).

	Mutant	Fg	Fb	Fb/Fg	Pl	PC	PC/Pl
	R267A						
AVE		0.53	0.92	1.74	0.24	0.7	3.00
SD			(0.00)	(0.01)		(0.01)	(0.09)
	D283A, H287A						
AVE		0.59	0.78	1.32	0.19	0.53	2.76
SD			(0.02)	(0.05)		(0.07)	(0.01)
	K296A, H297A, R298A, R299A						
AVE		0.29	1.26	4.40	0.14	0.42	3.00
SD			(0.05)	(0.02)		(0.03)	(0.00)
	E303A, R304A						
AVE		0.68	1.38	2.04	0.35	0.90	2.61
SD			(0.09)	(0.04)		(0.01)	(0.03)
	H331A, H332A						
AVE		1.19	1.29	1.08	1.12	1.09	0.97
SD			(0.06)	(0.04)		(0.13)	(0.09)
	R339A, R342A						
AVE		0.34	0.38	1.12	0.02	0.19	9.50
SD			(0.00)	(0.07)		(0.03)	(0.03)
	E347A, E348A, E349A, K351A						
AVE		0.50	0.87	1.75	0.13	0.38	2.88
SD			(0.09)	(0.15)		(0.04)	(0.05)
	D364A, D365A, D366A						
AVE		0.66	1.50	2.27	0.28	0.88	3.14
SD			(0.03)	(0.04)		(0.04)	(0.03)
	K416A, H417A, E418A						
AVE		0.34	0.21	0.60	0.10	0.19	1.95
SD			(0.01)	(0.04)		(0.04)	(0.05)

TABLE V (CONTINUED)

Mutant		Fg	Fb	Fb/Fg	Pl	PC	PC/Pl
E426A, R427A, K429A, E430A							
	AVE	0.21	0.14	0.68	0.07	0.11	1.50
	SD	(0.01)	(0.00)		(0.01)	(0.01)	
H432A, R434A							
	AVE	0.16	0.16	1.00	0.08	0.05	0.60
	SD	(0.01)	(0.08)		(0.01)	(0.01)	
R440A							
	AVE	0.62	1.02	1.64	0.48	0.86	1.81
	SD	(0.08)	(0.18)		(0.04)	(0.10)	
H445A, R449A							
	AVE	0.52	1.12	2.15	0.24	0.80	3.33
	SD						
R449A, D453A							
	AVE	0.58	1.16	2.00	0.28	0.79	2.87
	SD	(0.01)	(0.11)		(0.01)	(0.01)	
D460A, R462A							
	AVE	0.13	0.68	5.19	0.10	0.21	2.16
	SD	(0.03)	(0.04)		(0.05)	(0.01)	
D477A							
	AVE	0.08	0.09	1.06	0.03	0.11	4.20
	SD	(0.00)	(0.01)		(0.04)	(0.02)	

Fg = Fibrinogen
Fb = Figrin
Pl = Plasma
PC = Plasma clot
AVE= Average
SD = Standard deviation

TABLE VI

FIBRIN AND PLASMA CLOT SPECIFICITY OF rt-PA VARIANTS (TWO-CHAIN)

The activities are relative to wt rt-PA (where wt is 1.0).

Mutant		Fg	Fb	Fb/Fg	Pl	PC	PC/Pl
R267A							
AVE		0.85	0.97	1.14	0.70	0.86	1.23
SD			(0.21)	(0.15)		(0.08)	(0.11)
D283A, H287A							
AVE		0.77	1.00	1.31	0.68	0.82	1.19
SD			(0.12)	(0.08)		(0.14)	(0.04)
K296A, H297A, R298A, R299A							
AVE		0.31	2.82	9.24	0.24	1.29	5.50
SD			(0.14)	(0.32)		(0.15)	(0.20)
E303A, R304A							
AVE		0.56	2.13	3.79	0.26	1.34	5.10
SD			(0.23)	(0.29)		(0.08)	(0.10)
H331A, H332A							
AVE		1.03	2.19	2.12	1.03	1.61	1.56
SD			(0.86)	(0.68)		(0.67)	(0.68)
R339A, R342A							
AVE		0.55	1.04	1.89	0.23	0.79	3.38
SD			(0.18)	(0.11)		(0.05)	(0.10)
E347A, E348A, E349A, K351A							
AVE		0.76	1.33	1.76	0.44	0.97	2.20
SD			(0.24)	(0.12)		(0.11)	(0.10)
D364A, D365A, D366A							
AVE		0.73	1.77	2.44	0.26	1.46	5.68
SD			(0.17)	(0.23)		(0.12)	(0.15)
K416A, H417A, E418A							
AVE		0.77	0.96	1.24	0.42	0.77	1.82
SD			(0.13)	(0.09)		(0.08)	(0.05)

TABLE VI (CONTINUED)

Mutant		Fg	Fb	Fb/Fg	Pl	PC	PC/Pl
E426A, R427A, K429A, E430A							
	AVE	0.38	0.76	2.01	0.24	0.66	2.80
	SD	(0.27)	(0.07)		(0.07)	(0.12)	
H432A, R434A							
	AVE	0.16	0.99	6.40	0.16	0.38	2.31
	SD	(0.05)	(0.13)		(0.05)	(0.05)	
R440A							
	AVE	0.51	0.92	1.81	0.51	0.90	1.78
	SD	(0.11)	(0.10)		(0.08)	(0.09)	
H445A, R449A							
	AVE	0.62	1.46	2.36	0.29	1.19	4.06
	SD	(0.16)	(0.10)		(0.09)	(0.11)	
R449A, D453A							
	AVE	0.74	1.50	2.04	0.36	1.22	3.36
	SD	(0.14)	(0.23)		(0.12)	(0.13)	
D460A, R462A							
	AVE	0.18	1.04	5.91	0.12	0.32	2.59
	SD	(0.12)	(0.08)		(0.02)	(0.02)	
D477A							
	AVE	0.12	0.10	0.89	0.11	0.25	2.24
	SD	(0.07)	(0.02)		(0.03)	(0.08)	

Fg = Fibrinogen
Fb = Figrin
Pl = Plasma
PC = Plasma clot
AVE= Average
SD = Standard deviation

TABLE VII

Mutant	Fibrin Specificity		Plasma Clot Specificity	
	1-chain	2-chain	1-chain	2-chain
R267A	X		X	
D283A,H287A			X	
K296A,H297A,R298A,R299A	X	X	X	X
E303A,R304A	X	X	X	X
H331A,H332A		X		X
R339A,R342A		X	X	X
E347A,E348A,E349A,K351A	X	X	X	X
D364A,D365A,D366A	X	X	X	X
K416A,H417A,E418A			X	X
E426A,R427A,K429A,E430A		X	X	X
H432A,R434A		X		X
R440A	X	X	X	X
H445A,R449A	X	X	X	X
R449A,D453A	X	X	X	X
D460A,R462A	X	X	X	X
D477A			X	X

There was a striking correlation between those variants of rt-PA exhibiting fibrin and/or plasma clot specificity and those that meet the criteria of zymogenicity specified above. In addition, two variants of rt-PA were found to exhibit fibrin specificity but were not zymogenic relative to wild-type rt-PA. Table VIII shows the S-2251 assay data for the fibrin-specific and plasma clot-specific variants.

TABLE VIII

FIBRIN- AND PLASMA CLOT- SPECIFIC, NON-ZYMOGENIC VARIANTS OF rt-PA							
	Mutant	Fa/1ch	Fb/1ch	Fb/Fg1ch	Pl/1ch	PC/1ch	PC/Pl1ch
AVE	E408A	0.35	0.79	2.28	0.19	0.47	2.51
SD		(0.01)	(0.06)		(0.04)	(0.01)	
AVE	E410A	0.61	0.90	1.47	0.51	0.74	1.44
SD		(0.08)	(0.04)		(0.11)	(0.05)	
	Mutant	Fg/2ch	Fb/2ch	Fb/Fg2ch	Pl/2ch	PC/2ch	PC/Pl2ch
AVE	E408A	0.34	0.89	2.61	0.24	0.64	2.72
SD		(0.18)	(0.11)		(0.08)	(0.04)	
AVE	E410A	0.55	0.92	1.67	0.48	0.88	1.84
SD		(0.13)	(0.09)		(0.10)	(0.08)	

Fg = Fibrinogen
Fb = Figrin
Pl = Plasma
PC = Plasma clot
1ch = 1 chain
2ch = 2 chain
AVE = Average
SD = Standard deviation

Deposit of Materials

The following culture has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

Strain	ATCC Dep. No.	Deposit Date
pTPA33-2 in *E. coli* MM294tonA	68,059	July 18, 1989

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from the date of deposit. The organism will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC § 122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if the culture on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Claims

1. A tissue plasminogen activator (t-PA) variant that in an assay of enzymatic activity exhibits a larger differential activity between the one-chain form and the two-chain form than wild-type recombinant t-PA by virtue of:
   (a) an amino acid other than phenylalanine substituted at amino acid position 305; or
   (b) an amino acid inserted after one or both of amino acid positions 304 and 305; or
   (c) one or more amino acids deleted at amino acid positions 297 to 305, inclusive;
   of wild-type human t-PA;
   the numbering of amino acid positions corresponding with that of figure 1.

2. The variant of claim 1 wherein the amino acid substituted or inserted has a side chain that can act or acts as a hydrogen bond donor.

3. The variant of claim 2 wherein the amino acid is histidine, tyrosine, asparagine, lysine, arginine, or glutamine.

4. The variant of claim 3 wherein the amino acid is histidine.

5. The variant of claim 1 wherein the deletion is at position 297, 300, 304, or 305.

6. The variant of claim 1 selected from the group consisting of F305H t-PA; F305T t-PA; F305N t-PA; F305K t-PA; F305R t-PA; F305Q t-PA; i304H t-PA; i304T t-PA; i304N t-PA; i304K t-PA i304R t-PA; i304Q t-PA; i304HH t-PA; i305H t-PA; i305T t-PA; i305N t-PA; i305K t-PA; i305R t-PA; i305Q t-PA; i304H,i305H t-PA; i305HH t-PA; d297 t-PA; d298 t-PA; d299 t-PA; d300 t-PA; d301 t-PA; d302 t-PA; d303 t-PA; d304 t-PA; d305 t-PA; d297-298 t-PA; d297-299 t-PA; d297-300 t-PA; d297-301 t-PA; d297-302 t-PA; d297-303 t-PA; d297-304 t-PA; d297-305 t-PA; d300-301 t-PA; d300-302 t-PA; d300-303 t-PA; d300-304 t-PA; d300-305 t-PA; d304-305 t-PA; d297,d300 t-PA; d297,d305 t-PA; dl-44,N184D,F305H t-PA; dl-44,F305H t-PA; dl-44,I210R,G211A,K212R,V213R,F305H t-PA; dl-44,I210R, G211A, K212R, V213K,F305H t-PA; dl-44,V213K,F305H t-PA; dl-44,T252R, F305H t-PA; dl-44,V213K,T252R,F305H t-PA; dl-44, I210K,F305H t-PA; dl-44,I210R,G211H,K212Q,V213K,F305H t-PA; I210R,G211H,K212Q,V213K, F305H t-PA; I210R,G211A,K212R, V213R,F305H t-PA; dl-44,N184D, I210R,G211A,K212R, V213R,T252R, F305H t-PA; N184D,I210R,G211A, K212R,V213R, T252R,F305H t-PA; d92-179,F305H t-PA; d92-179, I210R,G211A,K212R,V213R,F305H t-PA; d92-179,N184D,I210R, G211A, K212R,V213R,T252R,F305H t-PA; d92-179, I210R,G211A, K212R,V213R, T252R,F305H t-PA; Y67N,F305H t-PA; and dl-44,Y67N,F305H t-PA.

7. The variant of claim 1 selected from the group consisting of F305H t-PA; F305T t-PA; F305N t-PA; F305K t-PA; F305R t-PA; F305Q t-PA; i304H t-PA; dl-44,F305H t-PA; d92-179,F305H t-PA; dl-44,N184D,F305H t-PA; dl-44,I210R, G211A,K212R,V213R,F305H t-PA; dl-44,I210R,G211A,K212R, V213K,F305H t-PA; I210R,G211A,K212R, V213R,F305H t-PA; d92-179,I210R,G211A,K212R,V213R,F305H t-PA; d92-179,N184D, I210R,G211A,K212R,V213R,F305H t-PA; d92-179,N184D,I210R, G211A,K212R,V213R,T252R,F305H t-PA; dl-44,N184D,I210R,G211A, K212R,V213R,T252R,F305H t-PA; and N184D,I210R,G211A,K212R, V213R,T252R,F305H t-PA.

8. A human tissue plasminogen activator (t-PA) variant capable of exhibiting one or more of the following biological activities: a larger differential activity between the one-chain form and the two-chain form than wild-type recombinant t-PA, a higher ratio of fibrin-dependent specific activity to fibrinogen-dependent specific activity in a S-2251 assay than wild-type rt-PA, or a higher ratio of plasma clot-dependent specific activity to plasma-dependent specific activity in a S-2251 assay than wild-type rt-PA, characterized in that it contains at least one amino acid substitution in its protease domain as compared with the corresponding wild-type t-PA (from amino acid 264 to amino acid 527), which substitution is responsible for said biological activity, provided that the variants d296-302 t-PA, R304E t-PA, R304S t-PA and A473S t-PA are excluded and said substitution excludes substitutions solely in the regions of 270-280, 448-450, and 502-527, and substitutions in the regions 411 to 416, 416 to 423 or 423 to 430; the numbering of amino acid positions corresponding to that of figure 1.

9. The variant of claim 8 wherein the substitution involves replacing an Arg, Asp, His, Glu, or Lys with a neutral or negatively charged amino acid.

10. The variant of claim 9 wherein the amino acid used for replacement is alanine, glycine, serine, threonine, asparagine, glutamine, valine, leucine, isoleucine, phenylalanine, or tyrosine.

11. The variant of claim 9 wherein the amino acid used for replacement is alanine, serine, threonine, asparagine, glutamine, valine, leucine, isoleucine, phenylalanine, or tyrosine.

12. The variant of claim 9 wherein the amino acid used for replacement is alanine, serine, or threonine.

13. The variant of claim 12 wherein the substitution is at position 267, 283, 287, 296, 297, 298, 299, 303, 304, 331, 332, 339, 342, 347, 348, 349, 351, 364, 365, 366, 408, 410, 432, 434, 440, 445, 449, 453, 460, 462, or 477, or combinations thereof, of the corresponding wild-type t-PA.

14. The variant of claim 13 wherein the substitution is at position(s) 267, 283 + 287, 296-299, 303-304, 331-332, 339 + 342, 347-349 + 351, 364-366, 408, 410, 432 + 434, 440, 445 + 449, 449 + 453, 460 + 462, or 477 of the corresponding wild-type t-PA, where the " + " indicates alterations only at the positions designated, and the "-" indicates alterations at all positions designated.

15. The variant of claim 14 that is R267A t-PA, D283A,H287A t-PA, K296A,H297A,R298A,R299A t-PA, E303A,R304A t-PA, H331A,H332A t-PA, R339A,R342A t-PA, E347A,E348A,E349A,K351A t-PA, D364A,D365A,D366A t-PA, E408A t-PA, E410A t-PA, H432A,R434A t-PA, R440A t-PA, H445A,R449A t-PA, R449A,D453A t-PA, D460A,R462A t-PA, or D477A t-PA.

16. The variant of claim 14 having a substitution at any of amino acid positions 296-299, inclusive.

17. The variant of claim 16 having an alanine substituted at any of amino acid positions 296-299, inclusive.

18. The variant of claim 16 having an alanine substituted at each of amino acid positions 296-299, inclusive.

19. The variant of claim 12 wherein the substitution is at positions 416-418 or 426-427 + 429-430 of the corresponding wild-type t-PA, where the " + " indicates alterations only at the positions designated, and the "-" indicates alterations at all positions designated.

20. The variant of claim 19 that is K416A,H417A,E418A t-PA or E426A,R427A,K429A,E430A t-PA.

21. The variant of any one of claims 8 to 20 that is devoid of at least a portion of the finger domain of the corresponding wild-type t-PA.

22. The variant of any one of claims 8 to 21 that has an amino acid substitution, insertion or deletion at a position 117-119, 184-186 or 448-450 of corresponding wild-type human t-PA.

23. The variant of claim 22 that is devoid of functional carbohydrate structure at amino acid 184.

24. The variant of any one of claims 8 to 23 that is glycosylated within the growth factor domain of the corresponding wild-type t-PA.

25. The variant of claim 24 that contains an asparagine at position 67 of the corresponding wild-type t-PA.

26. The variant of any one of claims 8 to 25 that is devoid of amino acids 92-179, inclusive, of the corresponding wild-type t-PA.

27. A tissue plasminogen-activator (t-PA) variant according to claim 8 which has alanine substituted at each of positions 296-299.

28. A method for identifying tissue plasminogen activator (t-PA) variants which have a larger differential activity between the one-chain form and the two-chain form than wild-type recombinant t-PA, compris-

ing:

(a) substituting an amino acid other than phenylalanine at amino acid position 305; or

(b) inserting an amino acid after one or both of amino acid positions 304 and 305; or

(c) deleting one or more amino acids at amino acid positions 297 to 305, inclusive;

of wild-type human t-PA; the numbering of amino acid positions corresponding to that of figure 1.

29. The method of claim 28 wherein the amino acid substituted or inserted has a side chain that can act or acts as a hydrogen bond donor.

30. The method of claim 28 wherein the amino acid is histidine.

31. A method for identifying tissue plasminogen activator variants with specified biological activity comprising:

(a) introducing at least one amino acid substitution into the protease domain of tissue plasminogen activator (t-PA) (amino acid positions 264 to 527) the substitution excluding substitutions solely in the regions of 270-280, 448-450, and 502-527 of the corresponding wild-type t-PA; and

(b) screening the resultant t-PA variant for its capability of exhibiting one or more of the following biological activities: (a) a larger differential activity between the one-chain form and the two-chain form than wild-type recombinant t-PA, (b) a higher ratio of fibrin-dependent specific activity to fibrinogen-dependent specific activity in a S-2251 assay than wild-type rt-PA, or (c) a higher ratio of plasma clot-dependent specific activity to plasma-dependent specific activity in a S-2251 assay than wild-type rt-PA; with the proviso that screening for activity (a) is excluded where the substitution is in the region of t-PA from position 411 to position 416, from position 416 to position 423, or from position 423 to position 430; the numbering of amino acid positions corresponding to that of figure 1.

32. The method of claim 31 wherein the t-PA variant being screened is produced in a transient expression system.

33. The method of claim 31 wherein the substitution involves replacing an Arg, Asp, His, Glu, or Lys of wild-type t-PA with a neutral or negatively charged amino acid.

34. The method of claim 33 wherein the replacement is at position(s) 267, 283 + 287, 296-299, 303-304, 322, 331-332, 339 + 342, 347-349 + 351, 353 + 355-356, 360-362, 364-366, 408, 410, 416-418, 426-427 + 429-430, 432-434, 440, 445 + 449, 449 + 453, 460 + 462, 471-472, or 477, or combinations thereof, of the corresponding wild-type t-PA, where the " + " indicates replacements only at the position designated, and the "-" indicates replacements at all positions designated.

35. The method of claim 34 wherein the amino acid used for replacement is alanine, serine, threonine, asparagine, glutamine, valine, leucine, isoleucine, phenylalanine, or tyrosine.

36. The method of claim 35 wherein the amino acid used for replacement is alanine.

37. The method of claim 36 wherein the replacement is at position(s) 267, 283 + 287,296-299, 303-304, 331-332, 339 + 342, 347-349 + 351, 364-366, 408, 410, 416-418, 426-427 + 429-430, 432 + 434, 440, 445 + 449, 449 + 453, 460 + 462, or 477 of the corresponding wild-type t-PA, where the " + " indicates replacements only at the positions designated, and the "-" indicates replacements at all positions designated.

38. A DNA molecule encoding the variant of claim 1.

39. A DNA molecule encoding the variant of claim 8.

40. A DNA molecule according to claim 39 wherein the variant has alanine substituted at each of positions 296-299.

41. A DNA molecule according to claim 39 which encodes R267A t-PA, D283A,H287A t-PA, K296A,H297A,R298A,R299A t-PA, E303A,R304A t-PA, H331A,H332A t-PA, R339A,R342A t-PA, E347A,E348A,E349A,K351A t-PA, D364A,D365A,D366A t-PA, E408A t-PA, E410A t-PA,

K416A,H417A,E418A t-PA, E426A,R427A,K429A,E430A t-PA, H432AR434A t-PA, R440A t-PA, H445A,R449A t-PA, R449A,D453A t-PA, D460A,R462A t-PA, or D477A t-PA.

42. A replicable expression vector capable, in a transformant host cell, of expressing the DNA sequence of claim 38.

43. A replicable expression vector according to claim 42 wherein the transformant host cell is eukaryotic.

44. A replicable expression vector capable, in a transformant host cell, of expressing the DNA sequence of claim 39.

45. A replicable expression vector according to claim 44 wherein the transformant host cell is prokaryotic.

46. A replicable expression vector according to claim 44 wherein the variant has alanine substituted at each of positions 296-299.

47. The vector of claim 46 that is pTPA33-2, available from deposit number ATCC 68,059.

48. Host cells transformed with the vector of claim 42.

49. Eukaryotic host cells transformed with the vector of claim 43.

50. The cells of claim 49 that are yeast cells.

51. The cells of claim 49 that are mammalian cells.

52. Host cells transformed with the vector of claim 44.

53. The host cells of claim 52 that are eukaryotic cells.

54. The host cells of claim 53 that are capable of transient expression of the DNA sequence encoding the variant.

55. Prokaryotic host cells transformed with the vector of claim 45.

56. Host cells according to claim 52 wherein the variant has alanine substituted at each of positions 296-299.

57. *E. coli* host cells transformed with the vector of claim 47.

58. A composition for treating a vascular condition or disease comprising a therapeutically effective amount of the variant of claim 1 in admixture with a pharmaceutically acceptable carrier.

59. A composition for treating a vascular condition or disease comprising a therapeutically effective amount of the variant of claim 8 in admixture with a pharmaceutically acceptable carrier.

60. A composition according to claim 59 wherein the variant has alanine substituted at each of positions 296-299.

61. A composition for preventing fibrin deposition or adhesion formation or reformation comprising a therapeutically effective amount of the variant of claim 1 in admixture with a pharmaceutically acceptable carrier.

62. A composition for preventing fibrin deposition or adhesion formation or reformation comprising a therapeutically effective amount of the variant of claim 8 in admixture with a pharmaceutically acceptable carrier.

63. A composition according to claim 62 wherein the variant has alanine substituted at each of positions 296-299.

Patentansprüche

1. Gewebeplasminogenaktivator-(t-PA)-Variante, die in einem Assay auf enzymatische Aktivität eine größere Differenzaktivität zwischen der einkettigen Form und der zweikettigen Form als rekombinanter Wildtyp-t-PA aufweist, aufgrund von:
   (a) einer anderen Aminosäure als Phenylalanin, die an Aminosäure-Position 305 des Wildtyp-t-PAs substituiert ist; oder
   (b) einer Aminosäure, die nach einer oder beiden der Aminosäure-Positionen 304 und 305 des Wildtyp-t-PAs eingefügt ist; oder
   (c) einer oder mehrerer Aminosäuren, die an den Aminosäure-Positionen 297 bis einschließlich 305 des Wildtyp-t-PAs gelöscht sind; wobei die Numerierung der Aminosäure-Positionen jener in Fig. 1 entspricht.

2. Variante nach Anspruch 1, worin die substituierte oder eingefügte Aminosäure eine Seitenkette besitzt, die als Wasserstoffbindungs-Donor wirken kann oder wirkt.

3. Variante nach Anspruch 2, worin die Aminosäure Histidin, Tyrosin, Asparagin, Lysin, Arginin oder Glutamin ist.

4. Variante nach Anspruch 3, worin die Aminosäure Histidin ist.

5. Variante nach Anspruch 1, worin die Löschung an Position 297, 300, 304 oder 305 erfolgt.

6. Variante nach Anspruch 1, ausgewählt aus der Gruppe, bestehend aus
   F305H t-PA; F305T t-PA; F305N t-PA; F305K t-PA; F305R t-PA; F305Q t-PA; i304H t-PA; i304T t-PA; i304N t-PA; i304K t-PA; i304R t-PA; i304Q t-PA; i304HH t-PA; i305H t-PA; i305T t-PA; i305N t-PA; i305K t-PA; i305R t-PA; i305Q t-PA; i304H, i305H t-PA; i305HH t-PA; d297 t-PA; d298 t-PA; d299 t-PA; d300 t-PA; d301 t-PA; d302 t-PA; d303 t-PA; d304 t-PA; d305 t-PA; d297-298 t-PA; d297-299 t-PA; d297-300 t-PA; d297-301 t-PA; d297-302 t-PA; d297-303 t-PA; d297-304 t-PA; d297-305 t-PA; d300-301 t-PA; d300-302 t-PA; d300-303 t-PA; d300-304 t-PA; d300-305 t-PA; d304-305 t-PA; d297,d300 t-PA; d297,d305 t-PA; dl-44,N184D,F305H t-PA; dl-44,F305H t-PA; dl-44,I210R,G211A,K212R,V213R,F305H t-PA; dl-44,I210R, G211A, K212R, V213K,F305H t-PA; dl-44,V213K,F305H t-PA; dl-44,T252R, F305H t-PA; dl-44,V213K,T252R,F305H t-PA; dl-44, I210K,F305H t-PA; dl-44,I210R,G211H,K212Q,V213K,F305H t-PA; I210R,G211H,K212Q,V213K, F305H t-PA; I210R,G211A,K212R, V213R,F305H t-PA; dl-44,N184D, I210R,G211A,K212R, V213R,T252R, F305H t-PA; N184D,I210R,G211A, K212R,V213R, T252R,F305H t-PA; d92-179,F305H t-PA; d92-179, I210R,G211A,K212R,V213R,F305H t-PA; d92-179,N184D,I210R, G211A, K212R,V213R,T252R,F305H t-PA; d92-179, I210H,G211A, K212R,V213R, T252R,F305H t-PA; Y67N,F305H t-PA; und dl-44,Y67N,F305H t-PA.

7. Variante nach Anspruch 1, ausgewählt aus der Gruppe, bestehend aus
   F305H t-PA; F305T t-PA; F305N t-PA; F305K t-PA; F305R t-PA; F305Q t-PA; i304H t-PA; dl-44, F305H t-PA; d92-179, F305H t-PA; dl-44, N184D, F305H t-PA; dl-44, I210R, G211A, K212R, V213R, F305H t-PA; dl-44, I210R, G211A, K212R, V213K, F305H t-PA; I210R, G211A, K212R, V213R, F305H t-PA; d92-179, I210R, G211A, K212R, V213R, F305H t-PA; d92-179, N184D, I210R, G211A, K212R, V213R, F305H t-PA; d92-179, N184D, I210R, G211A, K212R, V213R, T252R, F305H t-PA; dl-44, N184D, I210R, G211A, K212R, V213R, T252R, F305H t-PA; und N184D, I210R, G211A, K212R, V213R, T252R, F305H t-PA.

8. Menschliche Gewebeplasminogenaktivator-(t-PA)-Variante, die in der Lage ist, eine oder mehrere der folgenden biologischen Aktivitäten aufzuweisen: eine größere Differenzaktivität zwischen der einkettigen Form und der zweikettigen Form als rekombinanter Wildtyp-t-PA, ein größeres Verhältnis von fibrin-abhängiger spezifischer Aktivität zu fibrinogen-abhängiger spezifischer Aktivität in einem S-2251-Assay als Wildtyp-rt-PA, oder ein größeres Verhältnis von plasmagerinnungs-abhängigerspezifischer Aktivität zu plasma-abhängiger spezifischer Aktivität in einem S-2251-Assay als Wildtyp-rt-PA, dadurch gekennzeichnet, daß sie zumindest eine Aminosäure-Substitution in ihrer Proteasen-Domäne im Vergleich zum

entsprechenden Wildtyp-t-PA enthält (von Aminosäure 264 bis Aminosäure 527), wobei die Substitution für diese biologische Aktivität verantwortlich ist, vorausgesetzt, daß die Varianten d296-302 t-PA, R304E t-PA, R304S t-PA und A473S t-PA ausgeschlossen sind, und wobei die Substitution alleinige Substitutionen in den Bereichen 270 - 280, 448 - 450 und 502 - 527 und Substitutionen in den Bereichen 411 - 416, 416 - 423 oder 423 - 430 ausschließt; wobei die Numerierung der Aminosäure-Positionen jener in Fig. 1 entspricht.

9. Variante nach Anspruch 8, worin die Substitution das Ersetzen einer Arg, Asp, His, Glu oder Lys durch eine neutrale oder negativ geladene Aminosäure umfaßt.

10. Variante nach Anspruch 9, worin die zum Ersetzen verwendete Aminosäure Alanin, Glycin, Serin, Threonin, Asparagin, Glutamin, Valin, Leucin, Isoleucin, Phenylalanin oder Tyrosin ist.

11. Variante nach Anspruch 9, worin die zum Ersetzen verwendete Aminosäure Alanin, Serin, Threonin, Asparagin, Glutamin, Valin, Leucin, Isoleucin, Phenylalanin oder Tyrosin ist.

12. Variante nach Anspruch 9, worin die zum Ersetzen verwendete Aminosäure Alanin, Serin oder Threonin ist.

13. Variante nach Anspruch 12, worin die Substitution an Position 267, 283, 287, 296, 297, 298, 299, 303, 304, 331, 332, 339, 342, 347, 348, 349, 351, 364, 365, 366, 408, 410, 432, 434, 440, 445, 449, 453, 460, 462 oder 477 oder an Kombinationen davon des entsprechenden Wildtyp-t-PAs erfolgt.

14. Variante nach Anspruch 13, worin die Substitution an (den) Position(en) 267, 283 + 287, 296 - 299, 303 - 304, 331 - 332, 339 + 342, 347 - 349 + 351, 364 - 366, 408, 410, 432 + 434, 440, 445 + 449, 449 + 453, 460 + 462 oder 477 des entsprechenden Wildtyp-t-PAs erfolgt, wobei das "+" Änderungen nur an den angegebenen Positionen und das "-" Änderungen an allen angegebenen Positionen angibt.

15. Variante nach Anspruch 14, d.h. R267A t-PA, D283A, H287A t-PA, K296A, H297A, R298A, R299A t-PA, E303A, R304A t-PA, H331A, H332A t-PA, R339A, R342A t-PA, E347A, E348A, E349A, K351A t-PA, D364A, D365A, D366A t-PA, E408A t-PA, E410A t-PA, H432A, R434A t-PA, R440A t-PA, H445A, R449A t-PA, R449A, D453A t-PA, D460A, R462A t-PA, oder D477A t-PA.

16. Variante nach Anspruch 14 mit einer Substitution an jeder der Aminosäure-Positionen 296 bis einschließlich 299.

17. Variante nach Anspruch 16 mit einem substituierten Alanin an jeder der Aminosäure-Positionen 296 bis einschließlich 299.

18. Variante nach Anspruch 16 mit einem substituierten Alanin an jeder der Aminosäure-Positionen 296 bis einschließlich 299.

19. Variante nach Anspruch 12, worin die Substitution an den Positionen 416 - 418 oder 426 - 427 + 429 - 430 des entsprechenden Wildtyp-t-PAs erfolgt, wobei das "+" Änderungen nur an den angegebenen Positionen und das "-" Änderungen an allen angegebenen Positionen angibt.

20. Variante nach Anspruch 19, d.h. K416A, H417A, E418A t-PA oder E426A, R427A, K429A, E430A t-PA.

21. Variante nach irgendeinem der Ansprüche 8 - 20, der zumindest ein Abschnitt der Fingerdomäne des entsprechenden Wildtyp-t-PAs fehlt.

22. Variante nach irgendeinem der Ansprüche 8 - 21, die eine Aminosäure-Substitution,-Einfügung oder -Löschung an einer Position 117 - 119, 184 - 186 oder 448 - 450 des entsprechenden menschlichen Wildtyp-t-PAs aufweist.

23. Variante nach Anspruch 22, der die funktionelle Kohlenhydrat-Struktur bei Aminosäure 184 fehlt.

24. Variante nach irgendeinem der Ansprüche 8 - 23, die innerhalb der Wachstumsfaktor-Domäne des entsprechenden Wildtyp-t-PAs glykosyliert ist.

25. Variante nach Anspruch 24, die ein Asparagin an Position 67 des entsprechenden Wildtyp-t-PAs enthält.

26. Variante nach irgendeinem der Ansprüche 8 - 25, der die Aminosäuren 92 bis einschließlich 179 des entsprechenden Wildtyp-t-PAs fehlen.

27. Gewebeplasminogenaktivator-(t-PA)-Variante nach Anspruch 8, die an jeder der Positionen 296 - 299 ein substituiertes Alanin aufweist.

28. Verfahren zur Identifizierung von Gewebeplasminogenaktivator-(t-PA)-Varianten, die eine größere Differenzaktivität zwischen der einkettigen Form und der zweikettigen Form als rekombinanter Wildtyp-t-PA aufweisen, umfassend:
(a) Substituieren einer anderen Aminosäure als Phenylalanin an Aminosäure-Position 305 des Wildtyp-t-PAs; oder
(b) Einfügen einer Aminosäure nach einer oder beiden Aminosäure-Positionen 304 und 305 des Wildtyp-t-PAs; oder
(c) Löschen einer oder mehrerer Aminosäuren an den Aminosäure-Positionen 297 bis einschließlich 305 des Wildtyp-t-PAs; wobei die Numerierung der Aminosäure-Positionen jener in Fig. 1 entspricht.

29. Verfahren nach Anspruch 28, worin die substituierte oder eingefügte Aminosäure eine Seitenkette besitzt, die als Wasserstoffbindungs-Donor wirken kann oder wirkt.

30. Verfahren nach Anspruch 28, worin die Aminosäure Histidin ist.

31. Verfahren zur Identifizierung von Gewebeplasminogenaktivator-Varianten mit spezifizierter biologischer Aktivität, umfassend:
(a) Einbringen zumindest einer Aminosäure-Substitution in die Proteasen-Domäne von Gewebeplasminogenaktivator (t-PA) (Aminosäure-Positionen 264 - 527), wobei die Substitution alleinige Substitutionen in den Bereichen 270 - 280, 448 - 450 und 502 - 527 des entsprechenden Wildtyp-t-PAs ausschließt; und
(b) Screenen der resultierenden t-PA-Variante auf ihre Fähigkeit, eine oder mehrere der folgenden biologischen Aktivitäten aufzuweisen: (a) eine größere Differenzaktivität zwischen der einkettigen Form und der zweikettigen Form als rekombinanter Wildtyp-t-PA, (b) ein größeres Verhältnis von fibrin-abhängiger spezifischer Aktivität zu fibrinogen-abhängiger spezifischer Aktivität in einem S-2251-Assay als Wildtyp-rt-PA, oder (c) ein größeres Verhältnis von plasmagerinnungs-abhängiger spezifischer Aktivität zu plasma-abhängiger spezifischer Aktivität in einem S-2251-Assay als Wildtyp-rt-PA; vorausgesetzt, daß das Screenen auf Aktivität (a) ausgeschlossen ist, falls die Substitution im t-PA-Bereich von Position 411 bis 416, 416 - 423 oder 423 - 430 liegt; wobei die Numerierung der Aminosäure-Positionen jener in Fig. 1 entspricht.

32. Verfahren nach Anspruch 31, worin die zu screenende t-PA-Variante in einem transienten Expressionssystem produziert wird.

33. Verfahren nach Anspruch 31, worin die Substitution das Ersetzen eines Arg, Asp, His, Glu oder Lys von Wildtyp-t-PA durch eine neutrale oder negativ geladene Aminosäure umfaßt.

34. Verfahren nach Anspruch 33, worin das Ersetzen an (den) Position(en) 267, 283 + 287, 296 - 299, 303 - 304, 322, 331 - 332, 339 + 342, 347 - 349 + 351, 353 + 355 - 356, 360 - 362, 364 - 366, 408, 410, 416 - 418, 426 - 427 + 429 - 430, 432 - 434, 440, 445 + 449, 449 + 453, 460 + 462 oder 477 oder Kombinationen davon des entsprechenden Wildtyp-t-PAs erfolgt, wobei das "+" Änderungen nur an den angegebenen Positionen und das "-" Änderungen an allen angegebenen Positionen angibt.

35. Verfahren nach Anspruch 34, worin die zum Ersetzen verwendete Aminosäure Alanin, Serin, Threonin, Asparagin, Glutamin, Valin, Leucin, Isoleucin, Phenylalanin oder Tyrosin ist.

36. Verfahren nach Anspruch 35, worin die zum Ersetzen verwendete Aminosäure Alanin ist.

37. Verfahren nach Anspruch 36, worin das Ersetzen an (den) Position(en) 267, 283 + 287, 296 - 299, 303 - 304, 331 - 332, 339 + 342, 347 - 349 + 351, 364 - 366, 408, 410, 416 - 418, 426 - 427 + 429 - 430, 432 - 434, 440, 445 + 449, 449 + 453, 460 + 462 oder 477 des entsprechenden Wildtyp-t-PAs erfolgt, wobei das "+" Änderungen nur an den angegebenen Positionen und das "-" Änderungen an allen angegebenen Positionen angibt.

38. DNA-Molekül, das für die Variante nach Anspruch 1 kodiert.

39. DNA-Molekül, das für die Variante nach Anspruch 8 kodiert.

40. DNA-Molekül nach Anspruch 39, worin die Variante an jeder der Positionen 296 - 299 ein substituiertes Alanin aufweist.

41. DNA-Molekül nach Anspruch 39, das für
R267A t-PA, D283A, H287A t-PA, K296A, H297A, R298A, R299A t-PA, E303A, R304A t-PA, H331A, H332A t-PA, R339A, R342A t-PA, E347A, E348A, E349A, K351A t-PA, D364A, D365A, D366A t-PA, E408A t-PA, E410A t-PA, K416A, H417A, E418A t-PA, E426A, R427A, K429A, E430A t-PA, H432AR434A t-PA, R440A t-PA, H445A, R449A t-PA, R449A, D453A t-PA, D460A, R462A t-PA, oder D477A t-PA.
kodiert.

42. Replikabler Expressionsvektor, der in der Lage ist, in einer transformierten Wirtszelle die DNA-Sequenz nach Anspruch 38 zu exprimieren.

43. Replikabler Expressionsvektor nach Anspruch 42, worin die transformierte Wirtszelle eukaryotisch ist.

44. Replikabler Expressionsvektor, der in der Lage ist, in einer transformierten Wirtszelle die DNA-Sequenz nach Anspruch 39 zu exprimieren.

45. Replikabler Expressionsvektor nach Anspruch 44, worin die transformierte Wirtszelle prokaryotisch ist.

46. Replikabler Expressionsvektor nach Anspruch 44, worin die Variante an jeder der Positionen 296 - 299 ein substituiertes Alanin aufweist.

47. Vektor nach Anspruch 46, der pTPA33-2 ist, erhältlich mit der Hinterlegungsnummer ATCC 68.059.

48. Wirtszellen, transformiert mit dem Vektor nach Anspruch 42.

49. Eukaryotische Wirtszellen, transformiert mit dem Vektor nach Anspruch 43.

50. Zellen nach Anspruch 49, die Hefezellen sind.

51. Zellen nach Anspruch 49, die Säugetierzellen sind.

52. Wirtszellen, transformiert mit dem Vektor nach Anspruch 44.

53. Wirtszellen nach Anspruch 52, die eukaryotische Zellen sind.

54. Wirtszellen nach Anspruch 53, die zur transienten Expression der für die Variante kodierenden DNA-Sequenz befähigt sind.

55. Prokaryotische Wirtszellen, transformiert mit dem Vektor nach Anspruch 45.

56. Wirtszellen nach Anspruch 52, worin die Variante an jeder der Positionen 296 - 299 ein substituiertes Alanin aufweist.

57. E. coli-Wirtszellen, transformiert mit dem Vektor nach Anspruch 47.

58. Zusammensetzung zur Behandlung eines vaskulären Zustands oder Erkrankung, umfassend eine therapeutisch wirksame Menge der Variante nach Anspruch 1 in einem Gemisch mit einem pharmazeutisch annehmbaren Träger.

59. Zusammensetzung zur Behandlung eines vaskulären Zustands oder Erkrankung, umfassend eine therapeutisch wirksame Menge der Variante nach Anspruch 8 in einem Gemisch mit einem pharmazeutisch annehmbaren Träger.

60. Zusammensetzung nach Anspruch 59, worin die Variante an jeder der Positionen 296 - 299 ein substituiertes Alanin aufweist.

61. Zusammensetzung zum Verhindern von Fibrinablagerung oder Adhäsionsbildung oder -wiederbildung, umfassend eine therapeutisch wirksame Menge der Variante nach Anspruch 1 in einem Gemisch mit einem pharmazeutisch annehmbaren Träger.

62. Zusammensetzung zum Verhindern von Fibrinablagerung oder Adhäsionsbildung oder -wiederbildung, umfassend eine therapeutisch wirksame Menge der Variante nach Anspruch 8 in einem Gemisch mit einem pharmazeutisch annehmbaren Träger.

63. Zusammensetzung nach Anspruch 62, worin die Variante an jeder der Positionen 296 - 299 ein substituiertes Alanin aufweist.

Revendications

1. Variant d'activateur du plasminogène tissulaire (t-PA) qui, dans un essai d'activité enzymatique, présente une différence d'activité entre la forme à une chaîne et une forme à deux chaînes supérieure à celle du t-PA recombinant de type sauvage en raison de la présence :
   (a) d'un aminoacide autre que la phénylalanine présent comme substituant à la position d'aminoacide 305 ; ou
   (b) d'un aminoacide inséré après une des, ou les deux, positions d'aminoacides 304 et 305 ; ou
   (c) de la délétion d'un ou plusieurs aminoacides au niveau des positions d'aminoacides 297 à 305 inclus ;
   du t-PA humain de type sauvage ;
   la numérotation des positions d'aminoacides correspondant à celle de la figure 1.

2. Variant suivant la revendication 1, dans lequel l'aminoacide substitué ou inséré possède une chaîne latérale qui peut agir ou qui agit comme donneur de liaison hydrogène.

3. Variant suivant la revendication 2, dans lequel l'aminoacide est l'histidine, la tyrosine, l'asparagine, la lysine, l'arginine ou la glutamine.

4. Variant suivant la revendication 3, dans lequel l'aminoacide est l'histidine.

5. Variant suivant la revendication 1, dans lequel la délétion est en position 297, 300, 304 ou 305.

6. Variant suivant la revendication 1, choisi dans le groupe consistant en F305H t-PA ; F305T t-PA ; F305N t-PA ; F305K t-PA ; F305R t-PA ; F305Q t-PA ; i304H t-PA ; i304T t-PA ; i304N t-PA ; i304K t-PA ; i304R t-PA ; i304Q t-PA ; i304HH t-PA ; i305H t-PA ; i305T t-PA ; i305N t-PA ; i305K t-PA ; i305R t-PA ; i305Q t-PA ; i304H,i305H t-PA ; i305HH t-PA ; d297 t-PA ; d298 t-PA ; d299 t-PA ; d300 t-PA ; d301 t-PA ; d302 t-PA ; d303 t-PA ; d304 t-PA ; d305 t-PA ; d297-298 t-PA ; d297-299 t-PA ; d297-300 t-PA ; d297-301 t-PA ; d297-302 t-PA; d297-303 t-PA; d297-304 t-PA; d297-305 t-PA; d300-301 t-PA; d300-302 t-PA; d300-303 t-PA; d300-304 t-PA; d300-305 t-PA; d304-305 t-PA; d297,d300 t-PA; d297,d305 t-PA; dl-44,N184D,F305H t-PA; dl-44,F305H t-PA; dl-44,I210R,G211A,K212R,V213R,F305H t-PA; dl-44,I210R, G211A, K212R, V213K, F305H t-PA; dl-44,V213K,F305H t-PA; dl-44, T252R, F305H t-PA ; dl-44,V213K,T252R,F305H t-PA; dl-44, I210K, F305H t-PA; dl-44,I210R,G211H,K212Q,V213K,F305H t-PA ; I210R,G211H,K212Q,V213K, F305H t-PA;

I210R,G211A,K212R, V213R,F305H t-PA; dl-44,N184D,I210R,G211A,K212R,V213R,T25-2R,F305H t-PA; N184D,I210R,G211A,K212R,V213R,T252R,F305H t-PA; d92-179,F305H t-PA; d92-179,I210R,G211A,K212R,V213R, F305H t-PA ; d92-179,N184D,I210R,G211A,K212R,V213R,T252R, F305H t-PA ; d92-179,I210R,G211A,K212R,V213R,T252R,F305H t-PA ; Y67N,F305H t-PA ; et dl-44,Y67N,F305H t-PA.

7. Variant suivant la revendication 1 choisi dans le groupe consistant en F305H t-PA; F305T t-PA; F305N t-PA; F305K t-PA; F305R t-PA; F305Q t-PA; i304H t-PA; dl-44,F305H t-PA; d92-179,F305H t-PA; dl-44,N184D,F305H t-PA; dl-44, I210R,G211A,K212R,V213R,F305H t-PA; dl-44,I210R,G211A, K212R,V213K, F305H t-PA; I210R,G211A,K212R,V213R,F305H t-PA; d92-179,I210R,G211A,K212R,V213R,F305H t-PA; d92-179,N184D, I210R,G211A,K212R,V213R,F305H t-PA; d92-179,N184D,I210R, G211A,K212R,V213R,T252R,F305H t-PA; dl-44,N184D,I210R, G211A,K212R,V213R,T252R,F305H t-PA ; et N184D,I210R,G211A, K212R,V213R,T252R,F305H t-PA.

8. Variant d'activateur du plasminogène tissulaire (t-PA) humain capable de présenter une ou plusieurs des activités biologiques suivantes : une différence d'activité entre la forme à une chaîne et la forme à deux chaînes supérieure à celle du t-PA recombinant de type sauvage, un rapport de l'activité spéicifique dépendant de la fibrine à l'activité spécifique dépendant du fibrinogène dans une analyse utilisant le S-2251 supérieur à celui du rt-PA de type sauvage, ou un rapport de l'activité spécifique dépendant du caillot plasmatique à l'activité spécifique dépendant du plasma dans une analyse utilisant le S-2251 supérieur à celui du rt-PA de type sauvage, caractérisé en ce qu'il contient au moins une substitution d'aminoacide dans son domaine de protéase comparativement au t-PA de type sauvage correspondant (de l'aminoacide 264 à l'aminoacide 527), substitution qui est responsable de ladite activité biologique, sous réserve que les variants d296-302 t-PA, R304E t-PA, R304S t-PA et A473S t-PA soient exclus et que ladite substitution exclue des substitutions uniquement dans les régions 270-280, 448-450 et 502-527, et des substitutions dans les régions 411 à 416, 416 à 423 ou 423 à 430 ; la numérotation des positions d'aminoacides correspondant à celle de la figure 1.

9. Variant suivant la revendication 8, dans lequel la substitution comprend le remplacement d'un résidu Arg, Asp, His, Glu ou Lys par un aminoacide neutre ou chargé négativement.

10. Variant suivant la revendication 9, dans lequel l'aminoacide utilisé pour le remplacement est l'alanine, la glycine, la sérine, la thréonine, l'asparagine, la glutamine, la valine, la leucine, l'isoleucine, la phénylalanine ou la tyrosine.

11. Variant suivant la revendication 9, dans lequel l'aminoacide utilisé pour le remplacement est l'alanine, la sérine, la théonine, l'asparagine, la glutamine, la valine, la leucine, l'isoleucine, la phénylalanine ou la tyrosine.

12. Variant suivant la revendication 9, dans lequel l'aminoacide utilisé pour le remplacement est l'alanine, la sérine ou la thréonine.

13. Variant suivant la revendication 12, dans lequel la substitution est effectuée en position 267, 283, 287, 296, 297, 298, 299, 303, 304, 331, 332, 339, 342, 347, 348, 349, 351, 364, 365, 366, 408, 410, 432, 434, 440, 445, 449, 453, 460, 462 ou 477, ou en plusieurs de ces positions, du t-PA de type sauvage correspondant.

14. Variant suivant la revendication 13, dans lequel la substitution est effectuée en la ou les positions 267, 283 + 287, 296-299, 303-304, 331-332, 339 + 342, 347-349 + 351, 364-366, 408, 410, 432 + 434, 440, 445 + 449, 449 + 453, 460 + 462 ou 477 du t-PA de type sauvage correspondant, positions dans lesquelles le signe " + " indique des modifications seulement au niveau des positions désignées et le signe "-" indique des modifications au niveau de toutes les positions désignées.

15. Variant suivant la revendication 14, qui consiste en R267A t-PA, D283A,H287A t-PA, K296A,H297A,R298A, R299A t-PA, E303A,R304A t-PA, H331A,H332A t-PA, R339A,R342A t-PA, E347A,E348A,E349A,K351A t-PA, D364A,D365A,D366A t-PA, E408A t-PA, E410A t-PA, H432A,R434A t-PA, R440A t-PA, H455A, R449A t-PA, R449A,D453A t-PA, D460A,R462A t-PA ou D477A t-PA.

16. Variant suivant la revendication 14, possédant une substitution au niveau de n'importe laquelle des positions d'aminoacides 296 à 299 inclus.

17. Variant suivant la revendication 16, possédant une alanine comme substituant à l'une quelconque des positions d'aminoacides 296 à 299 inclus.

18. Variant suivant la revendication 16, possédant une alanine comme substituant à chacune des positions d'aminoacides 296 à 299 inclus.

19. Variant suivant la revendication 12, dans lequel la substitution est présente aux positions 416-418 ou 426-427 + 429-430 du t-PA de type sauvage correspondant, positions dans lesquelles le signe " + " indique les modifications seulement au niveau des positions désignées et le signe "-" indique des modifications au niveau de toutes les positions désignées.

20. Variant suivant la revendication 19, qui consiste en K416A,H417A,E418A t-PA ou E426A,R427A,K429A, E430A t-PA.

21. Variant suivant l'une quelconque des revendications 8 à 20, qui est dépourvu d'au moins une portion du domaine digitiforme du t-PA du type sauvage correspondant.

22. Variant suivant l'une quelconque des revendications 8 à 21, qui possède une subtitution, une insertion ou une délétion d'aminoacide à une position 117-119, 184-186 ou 448-450 du t-PA humain de type sauvage correspondant.

23. Variant suivant la revendication 22, qui est dépourvu de structure glucidique fonctionnelle au niveau de l'aminoacide 184.

24. Variant suivant l'une quelconque des revendications 8 à 23, qui est glycosylé à l'intérieur du domaine de facteur de croissance du t-PA de type sauvage correspondant.

25. Variant suivant la revendication 24, qui contient une asparagine en position 67 du t-PA de type sauvage correspondant.

26. Variant suivant l'une quelconque des revendications 8 à 25, qui est dépourvu des aminoacides 92 à 179 inclus du t-PA de type sauvage correspondant.

27. Variant d'activateur du plasminogène tissulaire (t-PA) suivant la revendication 8, qui possède une alanine présente comme substituant à chacune des positions 296 à 299.

28. Procédé pour identifier des variants d'activateur du plasminogène tissulaire (t-PA) qui possèdent une différence d'activité entre la forme à une chaîne et la forme à deux chaînes supérieure à celle du t-PA recombinant de type sauvage, comprenant :

(a) une substitution d'un aminoacide autre que la phénylalanine à la position d'aminoacide 305 ; ou

(b) une insertion d'un aminoacide après une des ou les deux, positions d'aminoacides 304 et 305 ; ou

(c) une délétion d'un ou plusieurs aminoacides aux positions d'aminoacides 297 à 305 inclus ;

du t-PA humain de type sauvage ; la numérotation des positions d'aminoacides correspondant à celle de la figure 1.

29. Procédé suivant la revendication 28, dans lequel l'aminoacide substitué ou inséré possède une chaîne latérale qui peut agir ou qui agit comme donneur de liaison hydrogène.

30. Procédé suivant la revendication 28, dans lequel l'aminoacide est l'histidine.

31. Procédé pour identifier les variants d'activateur du plasminogène tissulaire présentant une activité biologique spécifique, comprenant :

(a) l'introduction d'au moins une substitution d'aminoacide dans le domaine de protéase de l'activateur du plasminogène tissulaire (t-PA) (positions d'aminoacides 264 à 527), la substitution

excluant des substitutions uniquement dans les régions 270-280, 448-450 et 502-527 du t-PA de type sauvage correspondant ; et
(b) la sélection du variant de t-PA résultant pour sa capacité à présenter une ou plusieurs des activités biologiques suivantes : (a) une différence d'activité entre la forme à une chaîne et la forme à deux chaînes supérieure à celle du t-PA recombinant de type sauvage, (b) un rapport de l'activité spécifique dépendant de la fibrine à l'activité spécifique dépendant du fibronogène dans une analyse utilisant le S-2251 supérieur à celui du rt-PA de type sauvage, ou (c) un rapport de l'activité spécifique dépendant du caillot plasmatique à l'activité spécifique dépendant du plasma dans une analyse utilisant le S-2251 supérieur à celui du rt-PA de type sauvage ; sous réserve que la sélection pour l'activité (a) soit exclue lorsque la substitution est dans la région du t-PA allant de la position 411 à la position 416, de la position 416 à la position 423, ou de la position 423 à la position 430 ; la numérotation des positions d'aminoacides correspondant à celle de la figure 1.

32. Procédé suivant la revendication 31, dans lequel le variant de t-PA qui est sélectionné est produit dans un système d'expression transitoire.

33. Procédé suivant la revendication 31, dans lequel la substitution fait intervenir le remplacement d'un résidu Arg, Asp, His, Glu ou Lys du t-PA de type sauvage par un aminoacide neutre ou chargé négativement.

34. Procédé suivant la revendication 33, dans lequel le remplacement est effectué en la ou les positions 267, 283 + 287, 296-299, 303-304, 322, 331-332, 339 + 342, 347-349 + 351, 353 + 355-356, 360-362, 364-366, 408, 410, 416-418, 426-427 + 429-430, 432-434, 440, 445 + 449, 449 + 453, 460 + 462, 471-472 ou 477, ou des associations de ces positions, du t-PA de type sauvage correspondant, positions dans lesquelles le signe " + " indique un remplacement seulement à la position désignée et le signe "-" indique un remplacement à toutes les positions désignées.

35. Procédé suivant la revendication 34, dans lequel l'aminoacide utilisé pour le remplacement est l'alanine, la sérine, la thréonine, l'asparagine, la glutamine, la valine, la leucine, l'isoleucine, la phénylalanine ou la tyrosine.

36. Procédé suivant la revendication 35, dans lequel l'aminoacide utilisé pour le remplacement est l'alanine.

37. Procédé suivant la revendication 36, dans lequel le remplacement est effectué en la ou les positions 267, 283 + 287,296-299, 303-304, 331-332, 339 + 342, 347-349 + 351, 364-366, 408, 410, 416-418, 426-427 + 429-430, 432 + 434, 440, 445 + 449, 449 + 453, 460 + 462 ou 477 du t-PA de type sauvage correspondant, positions dans lesquelles le signe " + " indique des remplacements seulement aux positions désignées et le signe "-" indique des remplacements à toutes les positions désignées.

38. Molécule d'ADN codant pour le variant suivant la revendication 1.

39. Molécule d'ADN codant pour le variant suivant la revendication 8.

40. Molécule d'ADN suivant la revendication 39, dans laquelle le variant possède une alanine présente comme substituant à chacune des positions 296 à 299.

41. Molécule d'ADN suivant la revendication 39, qui code pour le R267A t-PA, D283A,H287A t-PA, K296A,H297A, R298A,R299A t-PA, E303A,R304A t-PA, H331A,H332A t-PA, R339A, R342A t-PA, E347A,E348A,E349A,K351A t-PA, D364A,D365A,D366A t-PA, E408A t-PA, E410A t-PA, K416A,H417A,E418A t-PA, E426A, R427A,K429A,E430A t-PA, H432AR434A t-PA, R440A t-PA, H445A, R449A t-PA, R449A,D453A t-PA, B460A,R462A t-PA ou D477A t-PA.

42. Vecteur d'expression réplicable capable, dans une cellule hôte transformante, d'exprimer la séquence d'ADN suivant la revendication 38.

43. Vecteur d'expression réplicable suivant la revendication 42, dans lequel la cellule hôte transformante est une cellule eucaryotique.

44. Vecteur d'expression réplicable capable, dans une cellule hôte transformante, d'exprimer la séquence d'ADN suivant la revendication 39.

45. Vecteur d'expression réplicable suivant la revendication 44, dans lequel la cellule hôte transformante est une cellule procaryotique.

46. Vecteur d'expression réplicable suivant la revendication 44, dans lequel le variant possède une alanine présente comme substituant à chacune des positions 296 à 299.

47. Vecteur suivant la revendication 46 qui est le pTPA33-2, disponible sous le numéro de dépôt ATTC 68 059.

48. Cellules hôtes transformées avec le vecteur suivant la revendication 42.

49. Cellules hôtes eucaryotiques transformées avec le vecteur suivant la revendication 43.

50. Cellules suivant la revendication 49, qui sont des cellules de levures.

51. Cellules suivant la revendication 49, qui sont des cellules de mammifères.

52. Cellules hôtes transformées avec le vecteur suivant la revendication 44.

53. Cellules hôtes suivant la revendication 52, qui sont des cellules eucaryotiques.

54. Cellules hôtes suivant la revendication 53, qui sont aptes à une expression transitoire de la séquence d'ADN codant pour le variant.

55. Cellules hôtes eucaryotiques transformées avec le vecteur suivant la revendication 45.

56. Cellules hôtes suivant la revendication 52, dans lesquelles le variant possède une alanine présente comme substituant à chacune des positions 296 à 299.

57. Cellules hôtes de E. coli transformées avec le vecteur suivant la revendication 47.

58. Composition pour le traitement d'une affection ou maladie vasculaire, comprenant une quantité à effet thérapeutique du variant suivant la revendication 1 en mélange avec un support pharmaceutiquement acceptable.

59. Composition pour le traitement d'une affection ou maladie vasculaire, comprenant une quantité à effet thérapeutique du variant suivant la revendication 8 en mélange avec un support pharmaceutiquement acceptable.

60. Composition suivant la revendication 59, dans lequel le variant possède une alanine présente comme substituant à chacune des positions 296 à 299.

61. Composition pour la prévention du dépôt de fibrine ou de la formation ou reformation d'adhérences, comprenant une quantité à effet thérapeutique du variant suivant la revendication 1 en mélange avec un support pharmaceutiquement acceptable.

62. Composition pour la prévention du dépôt de fibrine ou de la formation ou de la reformation d'adhérences, comprenant une quantité à effet thérapeutique du variant suivant la revendication 8 en mélange avec un support pharmaceutiquement acceptable.

63. Composition suivant la revendication 62, dans laquelle le variant possède une alanine présente comme substituant à chacune des positions 296 à 299.

Fig. 1.

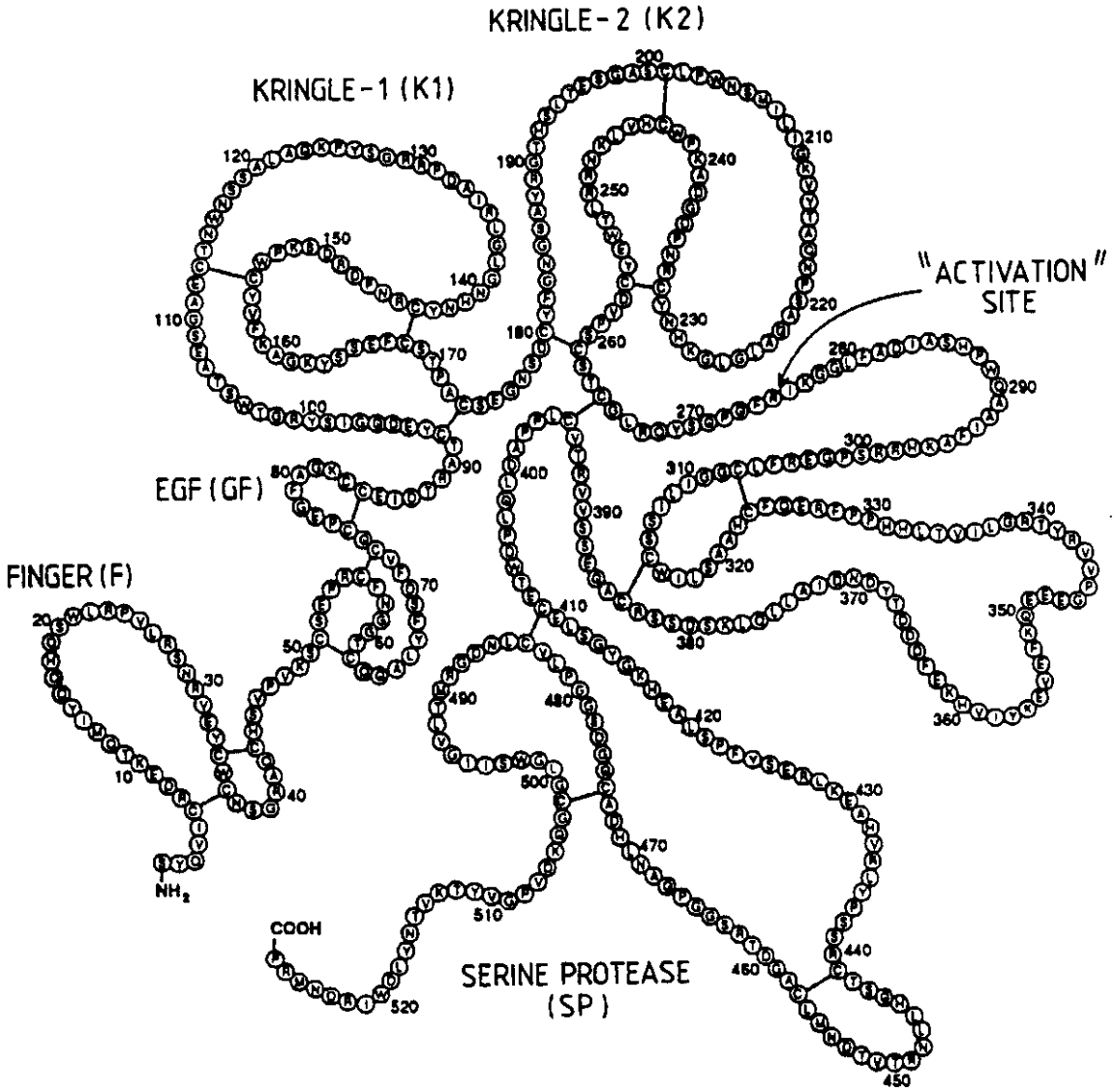

KRINGLE-2 (K2)
KRINGLE-1 (K1)
"ACTIVATION" SITE
EGF (GF)
FINGER (F)
SERINE PROTEASE (SP)
NH2
COOH

Fig.2.

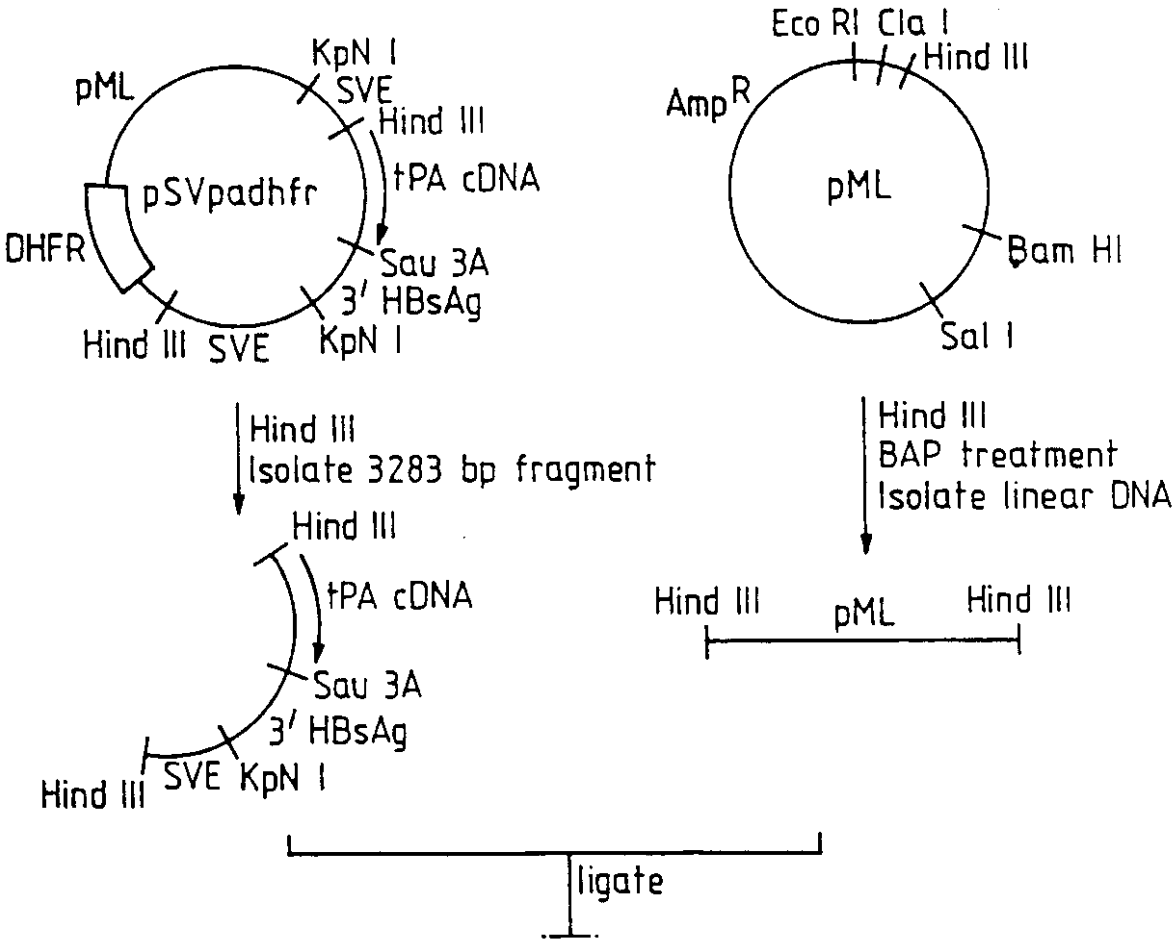

pML
KpN I
SVE
Hind III
tPA cDNA
pSVpadhfr
DHFR
Sau 3A
3' HBsAg
Hind III SVE
KpN I
Eco RI Cla I
Hind III
Amp R
pML
Bam HI
Sal I
Hind III
Isolate 3283 bp fragment
Hind III
BAP treatment
Isolate linear DNA
Hind III
tPA cDNA
Sau 3A
3' HBsAg
Hind III SVE KpN I
Hind III pML Hind III
ligate

Fig.2(cont.)

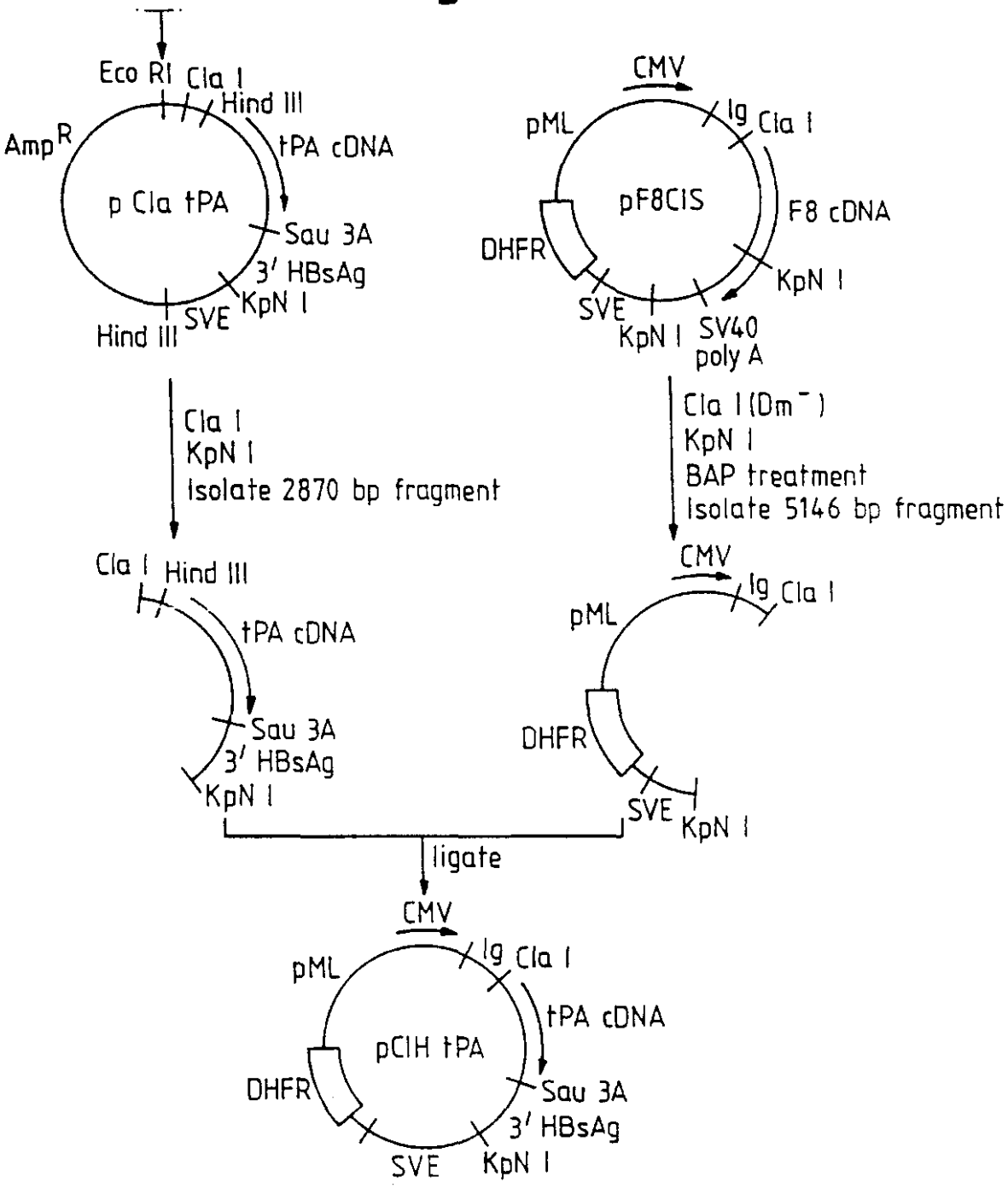

Eco RI
Cla I
Hind III
Amp R
tPA cDNA
p Cla tPA
Sau 3A
3' HBsAg
KpN I
SVE
Hind III
CMV
pML
Ig
Cla I
pF8CIS
F8 cDNA
DHFR
KpN I
SVE
KpN I
SV40
poly A
Cla I
KpN I
Isolate 2870 bp fragment
Cla I (Dm⁻)
KpN I
BAP treatment
Isolate 5146 bp fragment
Cla I
Hind III
tPA cDNA
Sau 3A
3' HBsAg
KpN I
CMV
Ig
Cla I
pML
DHFR
SVE
KpN I
ligate
CMV
pML
Ig
Cla I
tPA cDNA
pCIH tPA
DHFR
Sau 3A
3' HBsAg
SVE
KpN I

Fig.3.

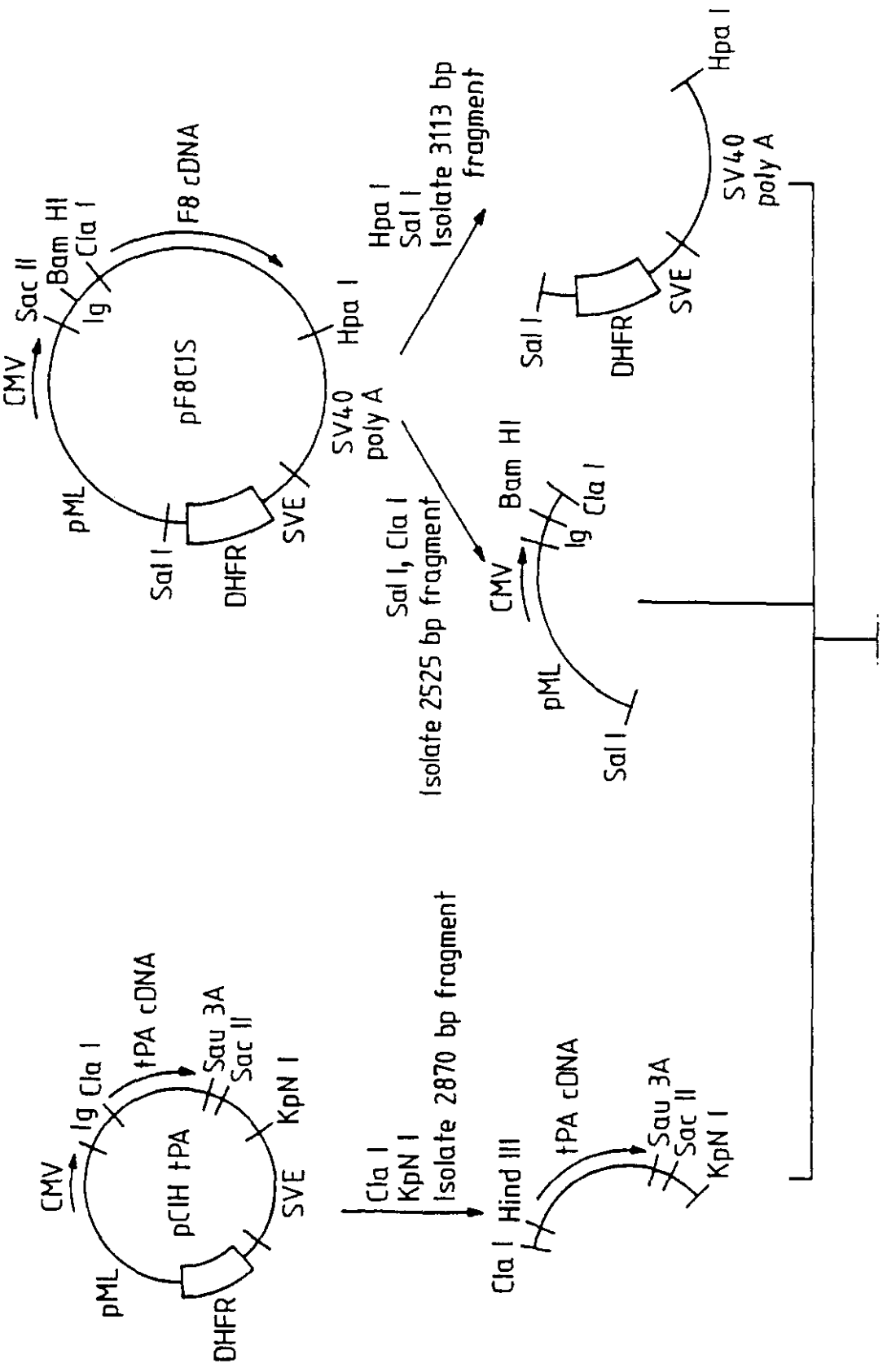

CMV
Sac II
Bam HI
Cla I
Ig
F8 cDNA
pF8CIS
pML
Sal I
DHFR
SVE
SV40
poly A
Hpa I
Hpa I
Sal I
Isolate 3113 bp
fragment
Sal I, Cla I
Isolate 2525 bp fragment
CMV
Bam HI
Ig
Cla I
pML
Sal I
Sal I
DHFR
SVE
SV40
poly A
Hpa I
CMV
Ig
Cla I
tPA cDNA
Sau 3A
Sac II
KpN I
pCIH tPA
pML
DHFR
SVE
Cla I
KpN I
Isolate 2870 bp fragment
Cla I
Hind III
tPA cDNA
Sau 3A
Sac II
KpN I

Fig. 3 (cont.)

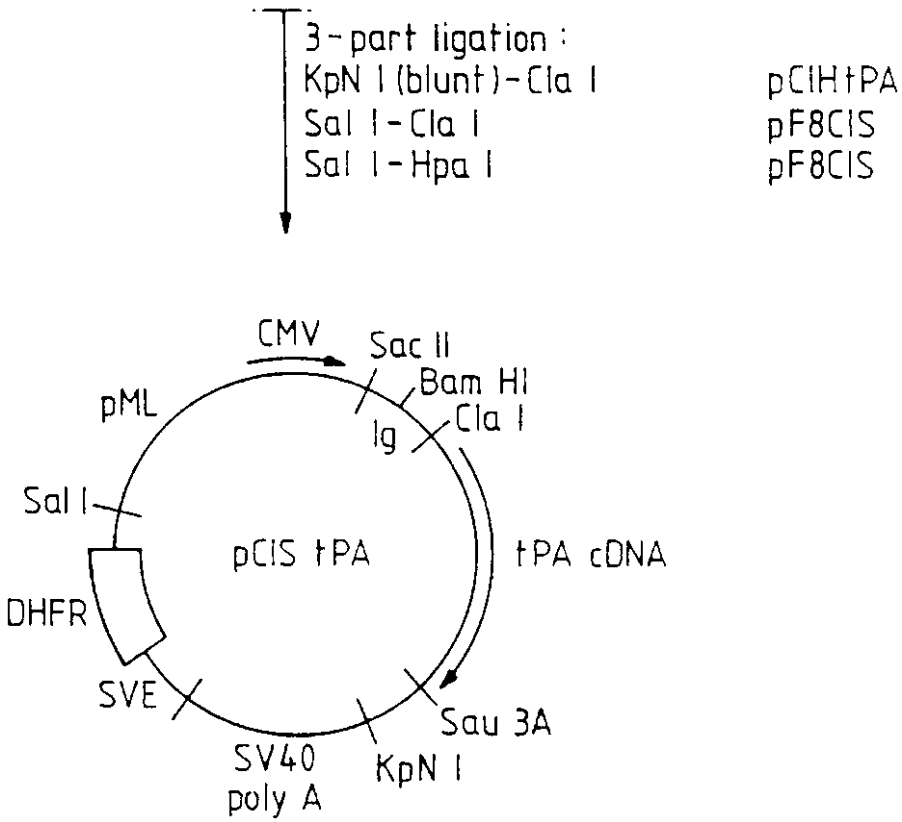

3-part ligation :
KpN I (blunt)-Cla I
Sal I-Cla I
Sal I-Hpa I
pClHtPA
pF8CIS
pF8CIS
CMV
Sac II
Bam HI
Cla I
Ig
pML
Sal I
pCIS tPA
tPA cDNA
DHFR
SVE
Sau 3A
SV40
poly A
KpN I

Fig.4.

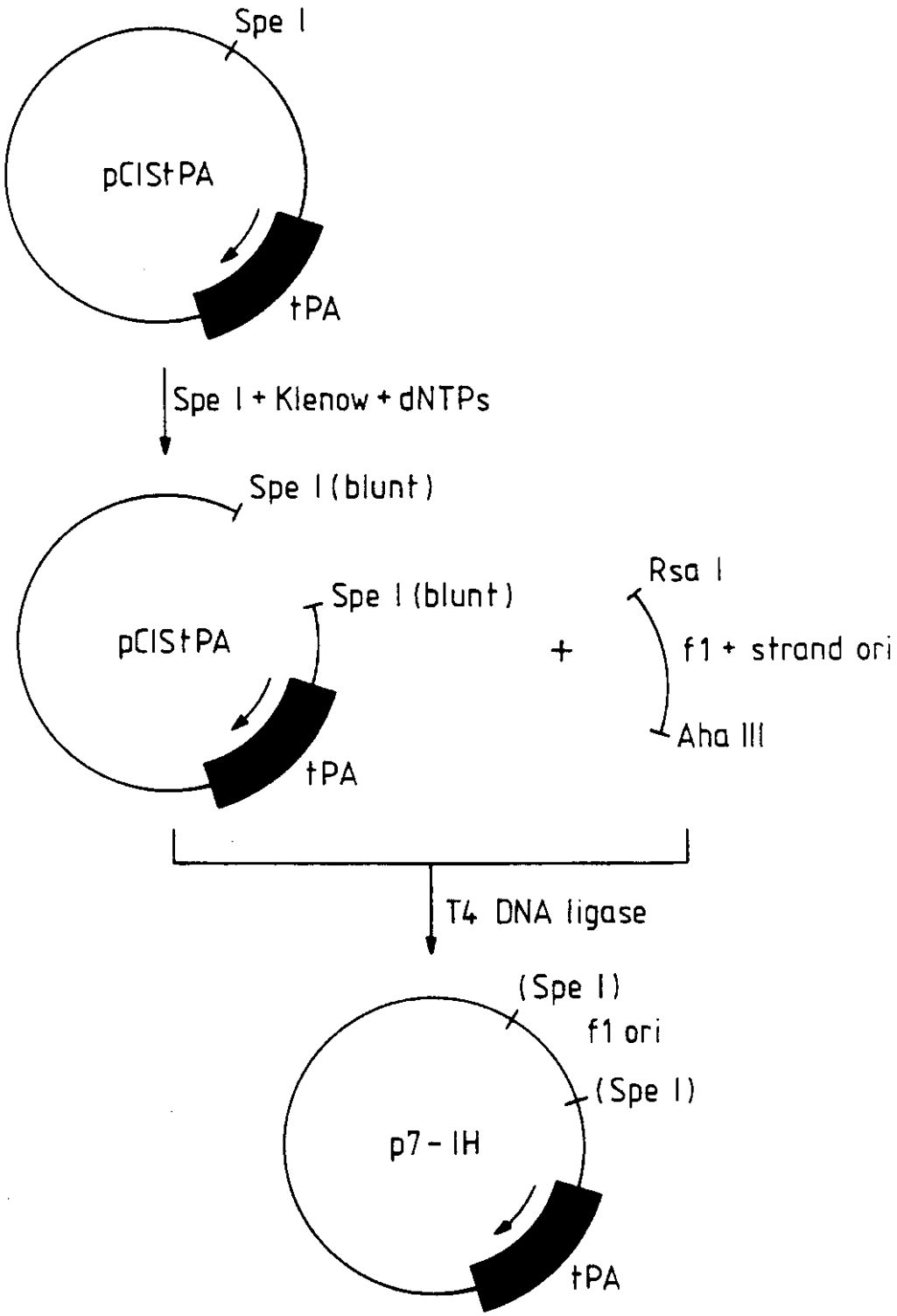

Spe I
pCISt PA
tPA
Spe I + Klenow + dNTPs
Spe I (blunt)
pCISt PA
Spe I (blunt)
tPA
+
Rsa I
f1 + strand ori
Aha III
T4 DNA ligase
(Spe I)
f1 ori
(Spe I)
p7 - IH
tPA

Fig.5.

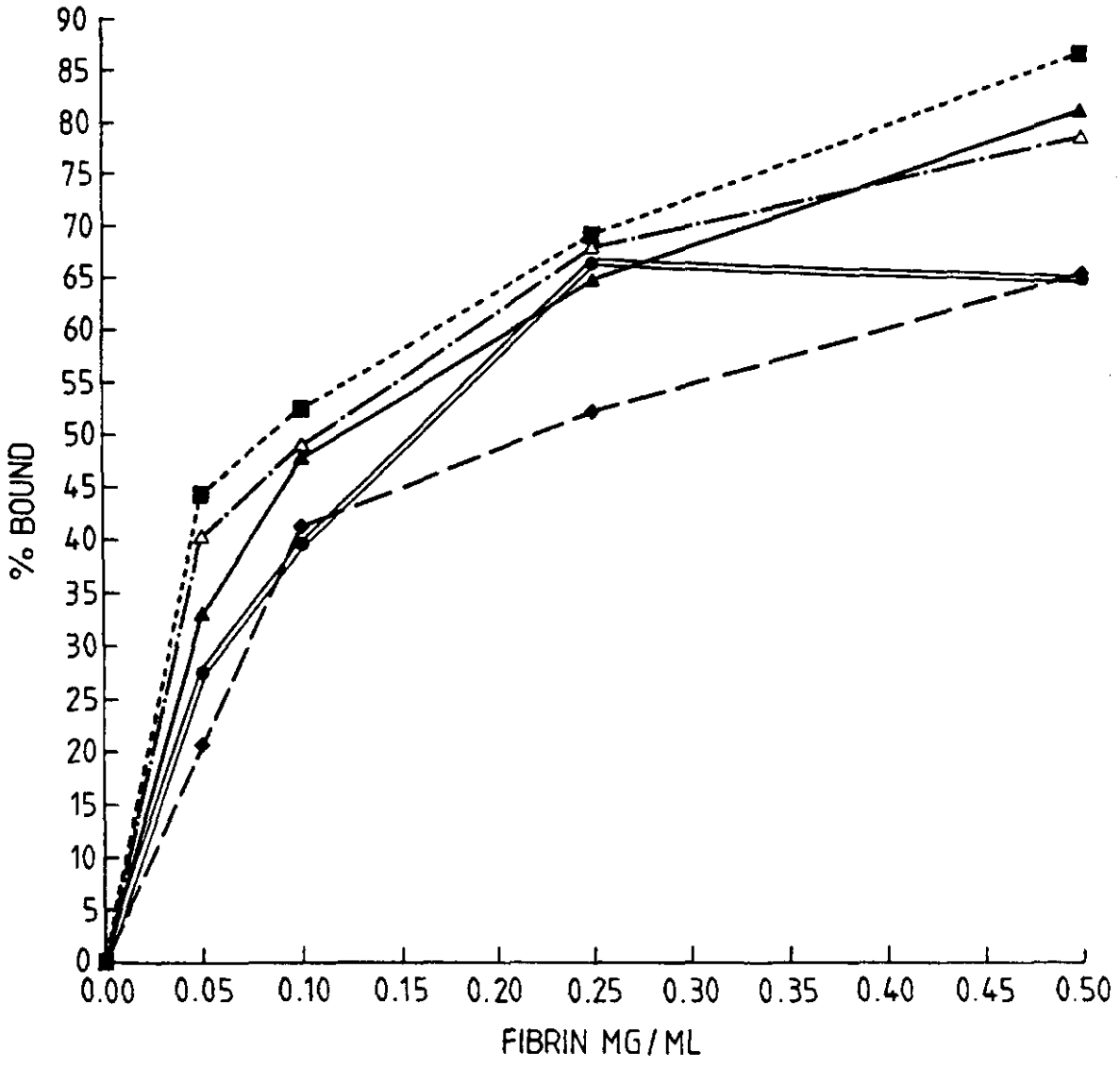

% BOUND
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
FIBRIN MG / ML

Fig.6.

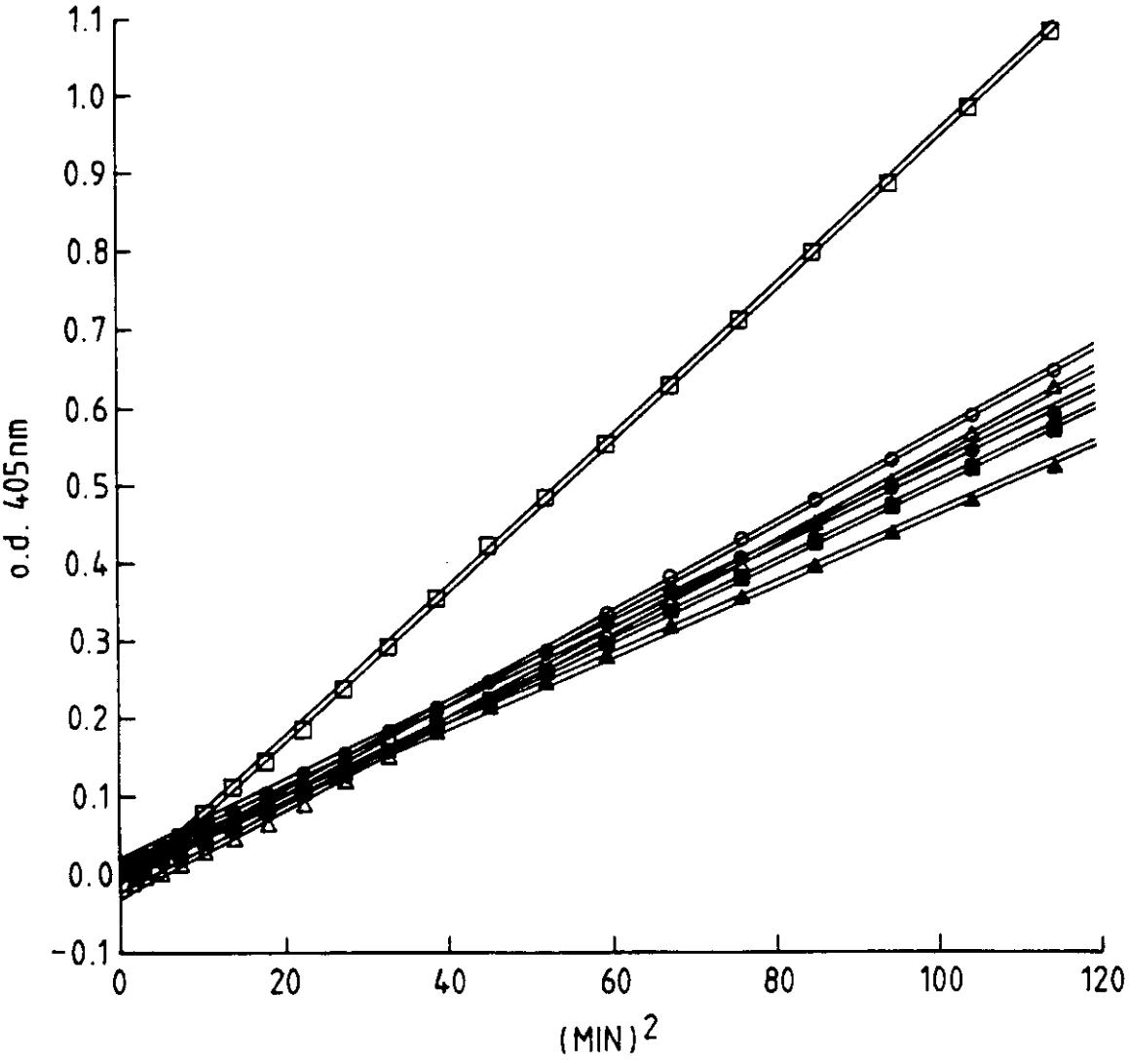

1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
o.d. 405nm
0
20
40
60
80
100
120
(MIN)2

Fig. 7.

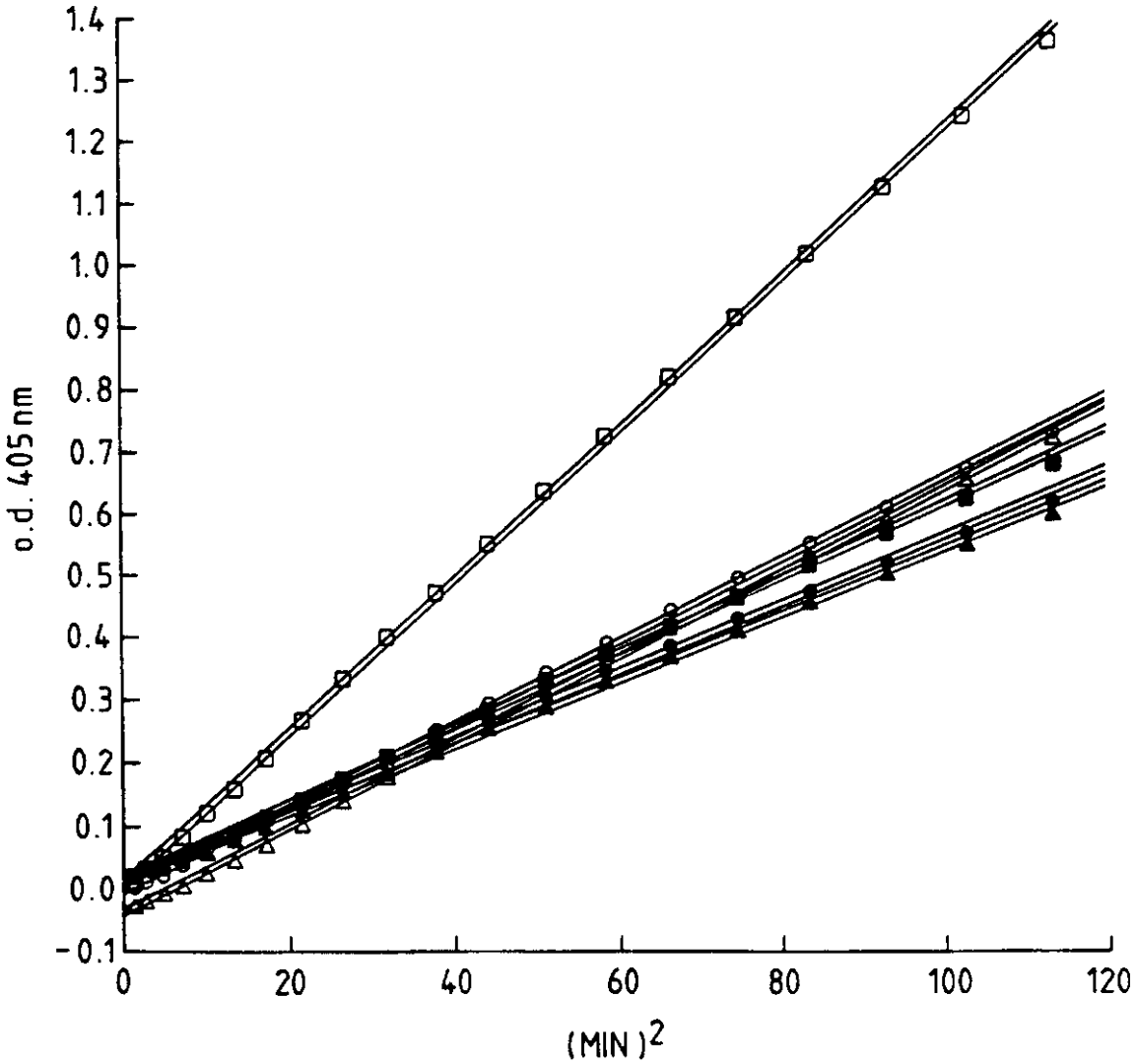

1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
o.d. 405 nm
0
20
40
60
80
100
120
(MIN)2

Fig.8.

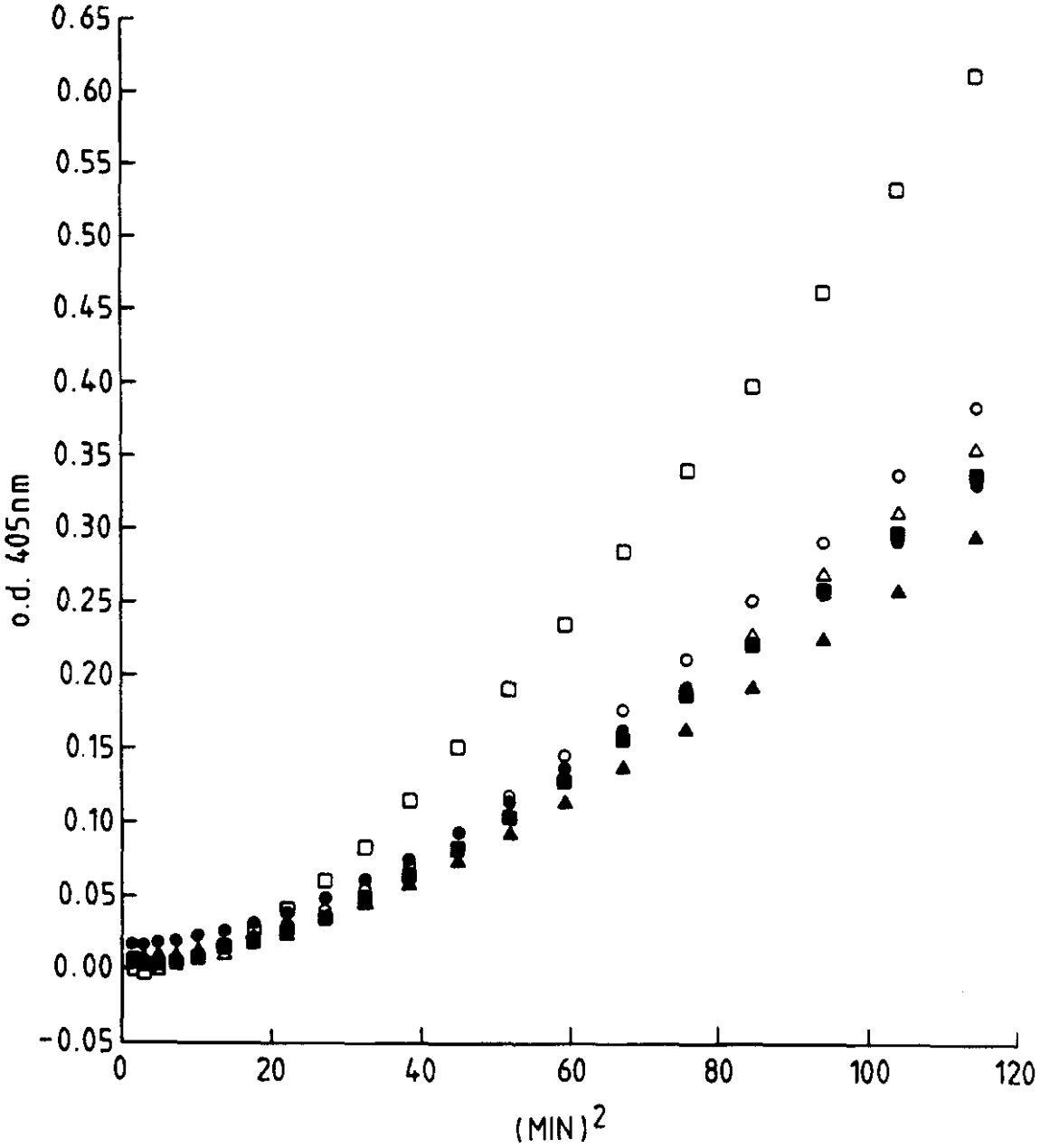
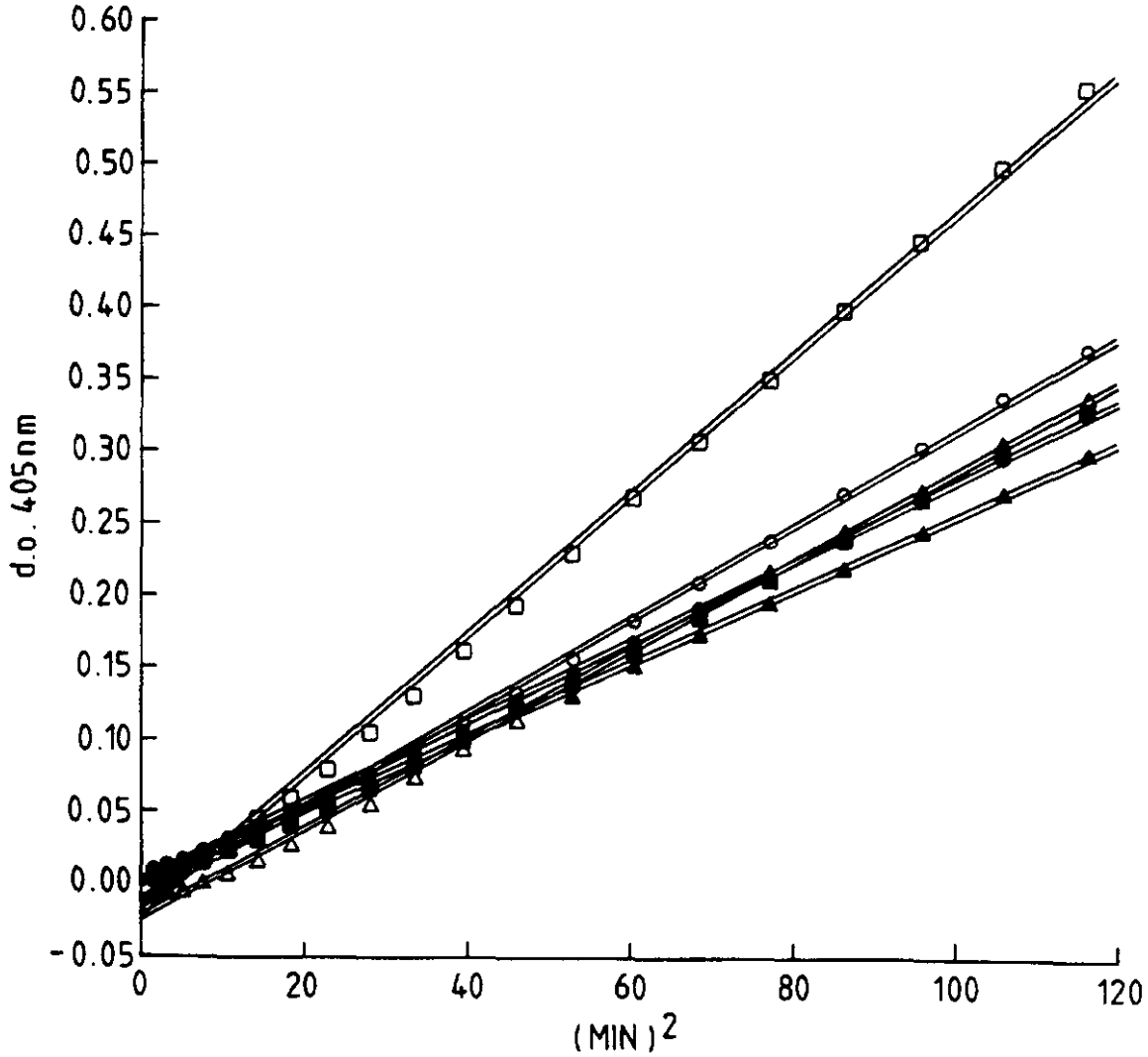

0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
o.d. 405nm
0
20
40
60
80
100
120
(MIN)2

Fig. 9.


0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
d.o. 405nm
0
20
40
60
80
100
120
(MIN)2