

Isolation of cDNA sequences coding for a part of human tissue plasminogen activator

(mixed sequence oligonucleotides/overlapping oligonucleotides/colony screening)

THOMAS EDLUND*, TOR NY*†, MATS RÅNBY‡, LARS-OLOF HEDÉN§, GUNNAR PALM¶, ERIK HOLMGREN§, AND STAFFAN JOSEPHSON¶

*Department of Applied Cell and Molecular Biology and ‡Department of Physiological Chemistry, University of Umeå, S-901 87, Umeå, Sweden; and §Department of Molecular Biology and ¶Department of Chemistry, Kabigen AB, 112 87, Stockholm, Sweden

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ABSTRACT We have isolated a cDNA sequence coding for a part of human tissue plasminogen activator. mRNA coding for tissue plasminogen activator was partially purified, copied into double-stranded cDNA, and cloned into *Escherichia coli*. Two sets of partially overlapping oligodeoxynucleotide mixtures corresponding to all possible coding sequences for a known portion of the tissue plasminogen activator gene were prepared. One set was used as a probe to screen cDNA containing bacterial clones and both were used as probes in hybridization against purified plasmid DNA. Of 4,200 bacterial clones examined, 1 carried a plasmid that hybridized to both sets of oligonucleotides. This plasmid contained a 370-base-pair cDNA insert, which was shown by nucleotide sequence analysis to code for the cleavage site region in the one-chain form of the human tissue plasminogen activator.

Plasminogen activators (PAs) convert the proenzyme plasminogen to plasmin, an active serine protease, which degrades fibrin clots (for review, see ref. 1). In addition, PA activity is correlated with cell migration, tissue remodeling, and tissue destruction, as well as with cellular transformation, tumorigenicity, metastasis, and tumor promotion (for review, see refs. 2 and 3). Steroids, polypeptide hormones, cyclic nucleotides, retinoids, tumor promoters, and transforming genes all alter PA activity in various cell types (ref. 4 and references therein).

In humans at least two types of immunologically unrelated PAs have been characterized: urokinase (M_r 55,000), found primarily in urine (5), and tissue PA (M_r 70,000), found in many tissue homogenates (6). Tissue PA is identical most likely to the vascular activator, which is assumed to be synthesized by the endothelial cells and released into circulating blood by certain stimuli (7). Tumor cell lines often produce PAs (8) and it has been shown that the human Bowes melanoma cell line produces an activator very similar or identical to the vascular activator (8, 9). Tissue PA is an active serine protease composed of a single polypeptide chain (10). When digested with plasmin or trypsin, one-chain tissue PA is cleaved at a single site in the central part of the molecule, converting it to the two-chain form (A and B chains) (10). The one-chain activator and the separated A and B chains of the two-chain activator (from the Bowes cell line) have been characterized by NH_2 -terminal sequence analysis by Wallén *et al.* (11); the sequence data show that the B chain originates from the COOH-terminal part of the molecule. The partial amino acid sequence obtained from the B chain was used to design synthetic oligodeoxyribonucleotides. These oligonucleotides were used as hybridization probes for screening of cDNA carrying bacterial clones by employing a colony hybrid-

ization technique. Here we report on the isolation of a cDNA sequence corresponding to a part of the tissue PA gene.

MATERIALS AND METHODS

General Methods. Plasmid DNA was isolated by a clear lysate procedure (12), followed by two consecutive ethidium bromide/CsCl equilibrium centrifugations. Restriction enzyme reactions were performed according to the conditions suggested by the manufacturer (New England BioLabs). Oligodeoxyribonucleotides were labeled at the 5' end by transfer of [γ - ^{32}P]ATP (Amersham) by using polynucleotide kinase (Boehringer Mannheim) as described (13). Nick-translation was carried out according to the conditions suggested by the manufacturer of the nick-translation kit (New England Nuclear).

Partial Purification of Tissue PA mRNA. Cytoplasmic RNA was extracted (14) from Bowes melanoma cell line (8) and was partially purified by oligo(dT) cellulose (Collaborative Research, Waltham, MA) chromatography (15) and sucrose gradient centrifugation (16).

Tissue PA mRNA Assay. Stage V *Xenopus laevis* oocytes (17), in groups of 20, were microinjected (18) with 50 nl of RNA (1 mg/ml) per oocyte. After incubation for 40 hr at 19°C in Barth's Hepes/saline medium (19), the oocytes were disrupted, delipidated with acetone, and extracted with 100 μl of 1 M sodium acetate buffer at pH 4.2. The extract was neutralized to pH 7.3 with 0.1 M Na_2HPO_4 and was assayed for tissue PA activity by using the functional parabolic rate assay as described (20). The assay was performed in the presence or absence of fibrin and in the presence of goat antitissue PA or normal goat immunoglobulins (20). A homogeneous preparation of human tissue PA (10, 11) was used as standard in the assay.

Synthesis of Mixed Oligodeoxyribonucleotides. The oligodeoxyribonucleotides in Fig. 1 were synthesized by using the solid-phase phosphite method (21). The concentrations of the four 5'-O-dimethoxytrityl-2'-deoxynucleoside-3'-O-methyl phosphochloridite reagents were measured (22) prior to making the mixtures so as to get 1:1 ratios between reagents.

Isolation and Screening of cDNA Clones. Sucrose density gradient fractions enriched for tissue PA mRNA were transcribed into double-stranded DNA by using the conditions described by Wickens *et al.* (23). (The reverse transcriptase was a generous gift of J. W. Beard, National Institutes of Health.) Nuclease S1 treatment of the cDNA and size fractionation on a 5–23% sucrose gradient were as outlined by Hoieijmakers *et al.* (24). cDNA molecules longer than 400 base pairs were tailed with deoxycytidine (25) and annealed with *Pst* I-digested, deoxyguanosine-elongated pBR322 (26, 27). The resulting hybrid

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Abbreviation: PA, plasminogen activator.

† To whom reprint requests should be addressed.

molecules were used to transform *Escherichia coli* strain 294 (27). Competent cells were prepared according to Dogert and Ehrlich (28). After transformation recombinant clones were selected on L agar plates containing tetracycline at 10 μ g/ml. Individual clones were picked in an ordered array, allowed to grow overnight, transferred to Whatman 541 filter paper, amplified with chloramphenicol at 250 μ g/ml, and prepared for hybridization as described by Gergen *et al.* (29). The filters were prehybridized, hybridized with ^{32} P-labeled PA14 mix, and washed as described by Wallace *et al.* (30).

Dot-Blot Hybridization. Plasmid DNA prepared (31) from 2-ml cultures was dissolved in 340 μ l of 100 mM Tris-HCl at pH 7.4. After addition of 60 μ l of 2 M NaOH and 200 μ l of 3 M NaCl/0.3 M sodium citrate, the mixture was incubated at 80°C for 20 min. The denatured DNA was chilled, neutralized with 80 μ l of 2 M Tris-HCl, and sucked through a nitrocellulose filter (7-mm² spots). Hybridization and washing conditions were according to the Southern hybridization protocol described in refs. 32 and 33.

Nucleotide Sequence Determination. Relevant fragments were cloned into phage M13 cloning vector Mp 701. Single-stranded template DNA was isolated from the phage essentially as described by Messing (34). Part of the DNA sequence was determined by the dideoxy method of Sanger *et al.* (35) by using the PA14 mix and the PA11 mix as specific primers. From the sequence obtained, two pentadecamers were synthesized and were used as primers to extend the sequence. The complete sequence of the plasmid pPA1 insert was confirmed by sequence analysis of both strands by using the method of Maxam and Gilbert (36).

RNA Transfer Blot Analysis. Oligo(dT)-purified RNA [from the Bowes melanoma cell line (8) and from the CCRF T-cell line (37)] was separated by agarose gel (1.2%) electrophoresis in the presence of formaldehyde (38). RNA was transferred to nitrocellulose filters (39) and was hybridized against the nick-translated cDNA insert of plasmid pPA1. Prehybridization, hybridization, and washing conditions were according to Thomas (39).

RESULTS

Strategy for Designing Tissue PA-Specific Oligodeoxyribonucleotide Probes. Plasmin-digested tissue PA consists of two disulfide-linked polypeptides, designated A and B, with M_r of $\approx 40,000$ and $\approx 33,000$, respectively (10, 11). To allow the synthesis of oligonucleotide sequences suitable as probes in hybridization experiments the amino acid sequence of the NH₂-terminal part of the B chain was determined (11). Two sets of oligonucleotides, complementary to all possible coding sequences for amino acid residues 12–17, were synthesized. As shown in Fig. 1, the sets were a mixture of 16 tetradecamers (PA14 mix) and a partially overlapping mixture containing 8 undecamers (PA11 mix). Both sets of probes were synthesized as a mixture of sequences (21, 22).

Tissue PA mRNA. The injection of mRNA isolated from the melanoma cells into oocytes of *X. laevis* produced PA activity.

	12	13	14	15	16	17
Amino acid sequence	His	Pro	Trp	Gln	Ala	Ala
Possible codons	5' CA ^U	CCN	UGG	CA ^G	GCN	GCN 3'
Probe PA14 mix	3' GT ^A	GGN	ACC	GT ^C	CG	5'
Probe PA11 mix	3'		ACC	GT ^C	CGN	CG 5'

FIG. 1. Oligonucleotide probes for the isolation of tissue PA cDNA clones. N = A, C, G, or T (U in RNA).

This activity could be completely inhibited by antitissue PA immunoglobulins and was stimulated by fibrin to the same extent as was purified tissue PA. About 85% of the activity was associated with the oocytes (acetate buffer extract) and the remaining activity was found in the incubation medium. In control experiments purified tissue PA added to disrupted oocytes could be recovered quantitatively. Typically, after injection of 50 ng of mRNA, 10 pg of tissue PA was produced per oocyte during 40 hr of incubation. Essentially the same amount of tissue PA was synthesized upon injection of mRNA from exponentially growing or confluent melanoma cells. A 5-fold enrichment of tissue PA-specific mRNA was accomplished by sucrose density gradient centrifugation. Tissue PA mRNA was found to have a sedimentation coefficient of ≈ 20 S (data not shown).

Preparation and Screening of cDNA Clones. Sucrose gradient fractions enriched for tissue PA mRNA were used to synthesize double-stranded cDNA. The cDNA was inserted at the *Pst* I site of pBR322 with poly(dC)-poly(dG) connectors. After transformation 4,200 clones were obtained. These clones were transferred to Whatman 541 paper and were hybridized with ^{32} P-labeled PA14 mix probe. Of the 4,200 clones, 8 showed strong hybridization to the PA14 mix and several others showed weak hybridization (data not shown). From all of these clones cleared lysates were prepared and the plasmid DNA was bound to two sheets of nitrocellulose filter. One filter was hybridized with the PA11 mix and the other was hybridized with the PA14 mix. Under these conditions, only the eight previous strongly hybridizing clones showed positive hybridization against the PA14 mix, and only one of these hybridized to the PA11 mix. The result of a typical experiment is shown in Fig. 2. Plasmids 5, 6, 8, 9, and 10 all hybridized to the PA14 mix, but only plasmid 10 hybridized to both sets of oligonucleotides; this plasmid was subsequently designated pPA1.

Nucleotide Sequence Analysis of the cDNA Insert in Plasmid pPA1. Plasmid pPA1 was digested with *Pst* I and was shown to carry a 370-base-pair insert. This fragment was subcloned into M13 vector MP 701 (34) and the nucleotide sequence was determined with the dideoxy termination method (35) by using the PA14 mix and the PA11 mix as specific primers. With this strategy the sequence of DNA that should correspond to the NH₂-terminal part of the B chain could be analyzed directly.

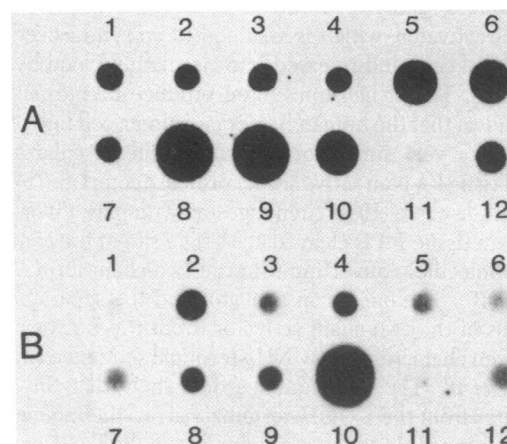


FIG. 2. Hybridization of some potential tissue PA cDNA recombinant plasmids with ^{32} P-labeled PA14 mix and PA11 mix. Plasmid DNA was isolated by a clear lysate method and was applied to two sheets of nitrocellulose filters. Filter A was hybridized at 40°C for 16 hr with PA14 mix at 32 ng/ml and filter B was hybridized at 20°C for 16 hr with PA11 mix at 16 ng/ml. Filters A and B were washed at 37°C and 20°C, respectively.

In one reading frame, the DNA sequence obtained did correspond to amino acids 1–9 of the B chain (Fig. 3). In addition, two pentadecanucleotides complementary to the cDNA insert were synthesized chemically (21, 22) and were used as primers to determine additional DNA sequences. In one reading frame the obtained DNA sequences correspond to all 19 well-established positions of the B-chain amino acid sequence. The nonestablished residue at position 11 corresponds to serine. Furthermore, as expected for a plasmin cleavage site, an arginine residue was found to precede the NH₂-terminal isoleucine of the B chain.

Hybridization to mRNA Blots. In RNA transfer blot analysis with mRNA from the Bowes melanoma cell line and the nick-translated cDNA insert of plasmid pPA1 as probe, a single hybridizing band with a mobility corresponding to 22 S was observed (Fig. 4).

DISCUSSION

This paper reports on the isolation of a bacterial clone carrying a cDNA sequence coding for a part of human tissue PA. This was accomplished by using synthetic oligonucleotides as hybridization probes. Recently Suggs *et al.* (33) reported on the isolation of cDNA sequences coding for β_2 -microglobulin. They used one mixture of 16 pentadecamers as a colony hybridization probe and 1 positive clone was found out of 535 examined. In the present study, two sets of partially overlapping oligonucleotides (see Fig. 1) were used and 1 clone carrying cDNA sequences for tissue PA was found out of 4,200 examined. The two sets of oligonucleotides together correspond to a 17-base-long

5' -	CT	GCA	GGG	GGG	GGG	GGG	GGG	GGG	GGG	TAC	ACA	
Ala	Gln	Asn	Pro	Ser	Ala	Gln	Ala	Leu	Gly			
GCA	CAG	ANC	CCC	AGT	GCC	CAG	GCA	CTG	GGC			
Leu	Gly	Lys	His	Asn	Tyr	Cys	Arg	Asn	Pro			
CTG	GGC	AAA	CAT	AAT	TAC	TGC	CGG	AAT	CCT			
Asp	Gly	Asp	Ala	Lys	Pro	Trp	Cys	His	Val			
GAT	GGG	GAT	GCC	AAG	CCC	TGG	TGC	CAC	GTG			
Leu	Lys	Asn	Arg	Arg	Leu	Thr	Trp	Glu	Tyr			
CTG	AAG	ANC	CAC	AGG	CTG	ACG	TGG	GAG	TAC			
Cys	Asp	Val	Pro	Ser	Cys	Ser	Thr	Cys	Gly			
TGT	GAT	GTG	CCC	TCC	TGC	TCC	ACC	TGC	GGC			
Leu	Arg	Gln	Tyr	Ser	Gln	Pro	Gln	Phe	Arg			
CTG	AGA	CAG	TAC	AGC	CAG	CCT	CAG	TTT	CGC			
1	2	3	4	5	6	7	8	9	10			
Ile	Lys	Gly	Gly	Leu	Phe	Ala	Asp	Ile	Ala			
ATC	AAA	GGA	GGG	CTC	TTC	GCC	GAC	ATC	GCC			
-	12	13	14	15	16	17	18	19	20			
Ser	His	Pro	Trp	Gln	Ala	Ala	Ile	Phe	Ala			
TCC	CAC	CCC	TGG	CAG	GCT	GCC	ATC	TTT	GCC			
Lys	His	Arg	Arg	Ser	Pro	Gly	Glu	Arg	Phe			
AAG	CAC	AGG	AGG	TGG	CCC	GGA	GAG	CGG	TTC			
Leu	Cys	Gly	Gly	Ile	Leu	Ile	Ser	Ser	Cys			
CTG	TGC	GGG	GGC	ATA	CTC	ATC	AGC	TCC	TGC			
Trp	Ile	Leu	Ser	Ala	Ala	His	Cys					
TGG	ATT	CTC	TCT	GCC	GCC	CAC	TGC	TTC	CCC			
CCC	CCC	CCC	CTG	CAG	3'							

FIG. 3. The nucleotide sequence of the pPA1 insert and the predicted amino acid sequence. The numbered amino acids correspond to the known NH₂-terminal amino acid sequence of the tissue PA B chain (unpublished data). The arrow indicates the plasmin cleavage site, and the boxes indicate regions homologous to kringle structures in plasminogen and prothrombin.

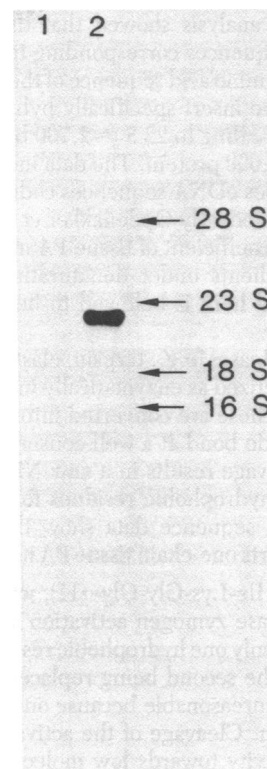


FIG. 4. Detection of tissue PA mRNA. Poly(A)⁺ RNA was isolated, subjected to agarose gel electrophoresis, and, after blotting to nitrocellulose, was hybridized to pPA1 ³²P-labeled DNA. The autoradiograph is shown. Eukaryotic (28 S and 18 S) and prokaryotic (23 S and 16 S) RNAs were used as markers. Lane 1, control poly(A)⁺ RNA from the human CCRF T-cell line; lane 2, poly(A)⁺ RNA from the human Bowes melanoma cell line.

DNA sequence. When using a mixture of 16 tetradecamers (PA14 mix) as colony hybridization probe, 8 positive clones were found out of 4,200 examined. As a second screening step, the PA14 mix and a mixture of eight undecamers (PA11 mix) were used as "dot-blot" probes. Only one clone (pPA1) was found to hybridize to both sets of oligonucleotides. The amino acid sequence used to design the oligonucleotides contains at least three residues conserved in many serine proteases (40); thus, the nontissue PA clones identified with the PA14 mix may code for other serine proteases.

When the original 4,200 clones were rescreened by using the nick-translated pPA1 cDNA insert as colony hybridization probe, no clones, except pPA1, were positive. This demonstrates the reliability of the method. Generally, the approach of using partially overlapping oligonucleotide mixtures may prove valuable, especially when limited amino acid sequence data are available. Longer oligonucleotides are more discriminatory, but with the amino acid sequence data at hand, penta-, hexa-, or heptadecamers required mixtures of 64 oligonucleotides. Mixtures of more than 16 oligonucleotides contain <6% of an individual sequence, resulting in unfavorable signal/noise ratios in hybridization experiments.

This study supports the notion of Suggs *et al.* (33) that, in cloning of rare sequences, the direct use of oligonucleotides as hybridization probes is superior to the method employing the oligonucleotides as primers in the synthesis of longer cDNA probes. With the latter approach we observed several extension products of the expected size but, upon sequence analysis, no band corresponding to tissue PA mRNA was found (data not shown).

DNA sequence analysis showed that the cDNA insert of pPA1 contained sequences corresponding to all 19 positions of the NH₂-terminal amino acid sequence of the tissue PA B chain.

Furthermore, the insert specifically hybridizes to a mRNA with a size corresponding to 22 S ($\approx$ 2,700 bases), which is reasonable for a M_r 70,000 protein. The data indicate strongly that plasmid pPA1 carries cDNA sequences coding for a part of the tissue PA protein. Recently Opdenakker *et al.* (41) determined the sedimentation coefficient of tissue PA mRNA to be 19 S on linear sucrose gradients under denaturing conditions. The difference observed here is believed to be of methodological origin.

Many serine proteases (e.g., trypsin, elastase, thrombin, and plasmin) are synthesized as enzymatically inactive zymogens or proenzymes (42). These are converted into active enzymes by cleavage of a peptide bond at a well-conserved activation site. The activation cleavage results in a new NH₂ terminus that is composed of two hydrophobic residues followed by two glycines (40). Protein sequence data show that the proteolytic cleavage that converts one-chain tissue PA into a two-chain form occurs at site $\downarrow$ Arg-Ile-Lys-Gly-Gly- (11), which is homologous to the serine protease zymogen activation site. However, the sequence contains only one hydrophobic residue (Ile) preceding the two glycines, the second being replaced by a lysine. This irregularity is not unreasonable because one-chain tissue PA is not a true zymogen. Cleavage of the activation site results in an enhanced reactivity towards low molecular substrates and inhibitors (10, 43) but the fibrinolytic activity is hardly influenced (10, 43, 44). The physiological significance of the cleavage is not clear. Regulatory functions have been proposed (10), but subsequent studies have not produced conclusive evidence (43, 45).

Plasminogen and prothrombin both bind to fibrin. The fibrin binding sites of these proteins have been located to triple-loop structures (kringles) containing characteristic amino acid sequences (46). Tissue PA also binds to fibrin (47) and the amino acid sequence (Fig. 3) shows that the A chain of tissue PA contains these characteristic sequences. Thus, the kringel structure found in tissue PA explains its fibrin binding property, which is of great importance for the localization and regulation of the fibrinolytic process (48).

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