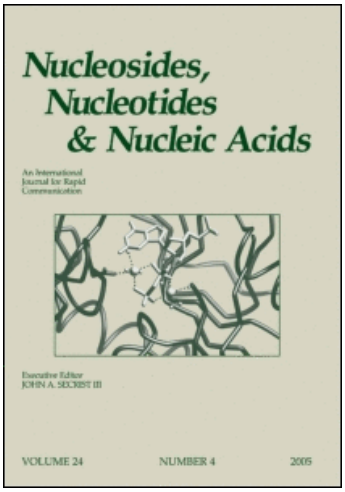

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DESIGN AND CHEMOENZYMATIC SYNTHESIS OF A TISSUE PLASMINOGEN ACTIVATOR GENE WITH UNIQUE RESTRICTION SITES: A MODEL FOR STUDYING PROTEIN DOMAIN FUNCTION

by N. Theriault Hatzenbuhler*[1], E.F. Rehberg[2], J.B. Carter[1], D.P. Palermo[2], E.V. Hubert[4], P.W. Bergum[3], L.A. Erickson[3] and K.R. Marotti[2]
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ABSTRACT. We have designed and constructed a cassette tPA gene with unique restriction sites in the interdomain regions. Each domain was chemoenzymatically assembled, cloned and sequenced. We have constructed and expressed the full-length molecule and some deletion mutants.

Tissue Plasminogen activator (tPA) is a glycosylated serine protease of 527 amino acids residues in length produced by vascular endothelial cells[1]. It is a single polypeptide chain[2] consisting of five putative domains: the fibronectin finger domain (F), the epidermal growth factor domain (G), the kringle 1 (K_1) and kringle 2 (K_2) domains, and the protease domain (P). Its mechanism of action involves conversion of the inactive zymogen plasminogen to plasmin which in turn degrades the fibrin network of a clot[3,4]. Tissue PA is itself a substrate for plasmin and thus is rapidly converted to a two-chain disulfide linked form (heavy(F,G,K_1,K_2) and light(P) chains) by cleavage at a single site (Arg275-Ile276)[5]. It is also known that tPA becomes activated upon binding to fibrin[6]. The investigation of human tPA as a possible thrombolytic agent has been hampered by its low concentration in blood, tissue extracts and cell cultures.

A few years ago[1], the tPA cDNA sequence was cloned and expressed and its amino acid sequence determined. We then constructed a cassette tPA gene with convenient restriction sites flanking the regions of the DNA encoding the various domains of tPA, thus simplifying the manipulation of these domains as shown schematically in figure 1. Amino acid changes in the interdomain regions were kept to a minimum and a few changes in codon

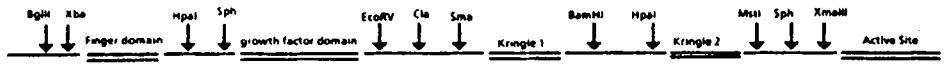

FIG. 1: Restriction enzyme map in the interdomain region of the tPA cassette gene

usage inside domains were deemed necessary eliminating problems related to dyad symmetry and self-complementarity of the component oligonucleotides as shown in figure 2. These modifications increased the size of the cDNA by six nucleotides and consequently increased the size of the protein by two amino acids. This system not only provides for the construction and expression of tPA mutants having single domain deletions but also facilitates the site-directed mutagenesis and expression of individual domains.

A double-stranded 1095 base-pair DNA sequence corresponding to F,G,K_1,K_2 and part of the protease domain was divided in 96 oligonucleotide fragments varying in length from a 17-mer to a 36-mer. Some of the oligonucleotides were ordered from the outside and some were chemically synthesized using the solid-phase methoxy-phosphoramidite triester coupling approach developed by Beaucage and Caruthers[7] using a 380A ABI DNA synthesizer or a SAM ONE BioSearch DNA synthesizer. After deprotection and purification following the usual procedure[8], these oligonucleotides were enzymatically assembled in four major blocks corresponding to the domains of the cassette tPA gene using T4 DNA ligase according to a protocol recently published[9]. Each individual block was cloned in either pKC7 or pBR322 and its sequence confirmed by either Maxam and Gilbert or dideoxysequencing methodology[10,11]. A tPA cDNA clone isolated at Upjohn was a convenient source of the active site portion of the nucleic acid sequence starting at Glu-362 (EcoR1 site).

We have constructed and expressed the prototype, full-length molecule (FGK_1K_2P) as well as the deletion mutants GK_1K_2P, FK_1K_2P, FGK_1P and FP. We have purified each of these mutants by immuno-affinity chromatography and have assessed molecular weight by fibrin autography[12]. The specific activity was determined via [1,3-^{3}H]di-isopropyl phosphofluoridate (3HDFP) active site titration. The activity portion of the specific activity assessment was

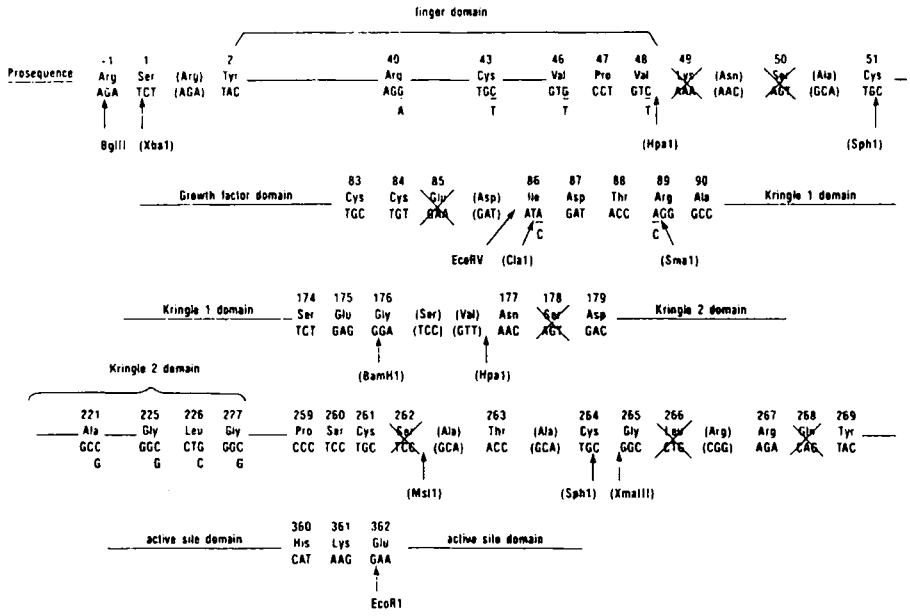

FIG. 2: Nucleotide and amino acid changes in the tPA cassette gene. Amino acids and DNA sequences deleted from the naturally occurring tPA sequence are marked with an X. Inserted amino acids and DNA sequences are indicated in parenthesis. Nucleotide changes with no amino acid change are underlined.

determined either in the presence or in the absence of fibrinogen fragments by using a coupled spetrophotometric assay[13]. It was found (data not shown) that the Bowes melanoma tPA, the cDNA derived tPA and the cassette gene tPA all have similar relative specific activities, determined in the presence of fibrinogen fragments. In the absence of fragments, the specific activity of the cassette gene tPA appears to be somewhat increased.

Steady-state kinetic analysis of the cassette gene product in comparison to the recombinant cDNA product demonstrate that the K_m and K_{cat} values are essentially the same for Bowes tPA, the cDNA derived, and the cassette gene tPA (data not shown). Since the tPA cassette protein does not appear to be significantly different from the human cDNA derived protein, we used the cassette gene analogue as a starting point for constructing deletion mutants. It was found (data not shown) that dramatic changes can be made in the

heavy chain without compromising the integrity and catalytic activity of the active site.

This study has shown that the tPA cassette gene, because of its strategically placed restriction sites, provides a powerful system for studying the functions of the individual domains within the tPA molecule. In addition, the tPA cassette gene serves as a model that may be applied to other multi-domain proteins.

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