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(71) Applicant(s)
Centro de Ingenieria Genetica y Biotecnologia

(72) Inventor(s)
Isis del Carmen Torrens Madrazo; Jose de la Fuente Garcia;
Ariana Garcia Ojalvo; Alina Seralena Menendez; Elder
Pupo Escalona; Julio Raul Fernandez Masso; Marta de
Jesus Gonzalez Griego

(74) Agent/Attorney
Baldwin Shelston Waters,Level 21,60 Margaret Street,SYDNEY NSW
2000

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ABSTRACT

The present invention is related to the field of biotechnology and genetic engineering techniques, particularly to a method for obtaining mutants obtain from streptokinase, to the molecules obtained from this method, as well as the expression vectors and microorganisms for recombinant obtaining.

5 The object of the present invention is to achieve streptokinase mutants from modifications of skc-2 gene coding for streptokinase SKC-2 (Heberkinase[®]), such that the obtained mutants conserve their capacity for plasminogen activator complex formation having reduced antigenicity, that could constitute preferred alternatives to native streptokinase for thrombolytic

10 therapy.

The molecules obtained from present invention can be used in the treatment of disorders as myocardial infarct, pulmonary thromboembolism, surgical complications and other cases of thrombosis.



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COMPLETE SPECIFICATION

FOR A STANDARD PATENT

ORIGINAL

Name of Applicant/s:	Centro de Ingenieria Genetica y Biotecnologia
Actual Inventor/s:	Isis del Carmen Torrens Madrazo and Jose de la Fuente Garcia and Ariana Garcia Ojalvo and Alina Seralena Menendez and Elder Pupo Escalona and Julio Raul Fernandez Masso and Martha de Jesus Gonzalez Griego
Address for Service:	BALDWIN SHELSTON WATERS 60 MARGARET STREET SYDNEY NSW 2000
Invention Title:	"STREPTOKINASE MUTANTS"

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

File: 22594.00

DESCRIPTION

STREPTOKINASE MUTANTS

The present invention is related to the field of biotechnology and genetic engineering techniques, particularly to a method for obtaining mutants obtain from streptokinase, to the molecules
5 obtained from this method, as well as the expression vectors and microorganisms for recombinant obtaining.

The streptokinase is a polypeptide of 414 amino acids residues. This is an extracellular protein produced by various strains of beta haemolytic streptococci, with molecular weight about 47.000 dalton and is a potent activator of the fibrinolytic enzyme system in humans (Tillet, W.S. and
10 Garner, R.L. (1933) Exp. Med. 58, 485-502; Tillet, W.S.; Edwards, E.D. and Garner, R.L. (1934) J. Clin. Invest 13, 47-78).

Unlike other plasminogen activators, streptokinase does not possess the intrinsic protease activity necessary to activate plasminogen to plasmin. Streptokinase activates plasminogen by the formation of 1:1 molar complex of streptokinase-plasminogen, which serves as the activator
15 of free plasminogen to form plasmin (Schick, L.A. and Castellino, F.J. (1974) Biochem. Biophys. Res. Commun. 57, 47-54).

Streptokinase, urokinase and tissue-type plasminogen activator are at present used as thrombolytic agents in the treatment of disorders which collectively represent one of the greatest causes of death in the world, such as myocardial infarct, pulmonary thromboembolism, surgical
20 complications and other cases of thrombosis.

The streptokinase is a bacterial protein and therefore, antigenic in humans. Antibodies to streptokinase are found in most individuals as a result of recurrent streptococcal infection (Tillet, W.S. and Garner, R.L. (1934) J. Clin. Invest. 13, 47-78). These antibodies are harmful for the use of streptokinase as thrombolytic, because high antibodies titers might neutralize
25 streptokinase activity preventing effective thrombolysis (Urdahl, K.B.; Mathews, J.D.; Currie, B. (1996) Australian and New Zealand J. Med. 26, 49-53; Spottl, F. and Kaiser, R. (1974) Thromb. Diath. Haemorrh. 32, 608). Patients are also immunized with streptokinase as a result of thrombolytic therapy and anti-streptokinase antibody titers exponentially rise post-treatment. These high anti-streptokinase antibody titers could neutralize a standard dose of streptokinase if
30 it is administered a second time in therapy (Rao, A.K.; Pratt, C.; Berke, A.; Jaffe, A.; Ockene, L.; Schreiber, T.L.; Bell, W.R.; Knaterund, G.; Robertson, T.L. and Terrin, M.L. (1988) J. Am. Coll. Cardiol. 11,1). One of the most common side effects of streptokinase therapy are allergic reactions, which have been noted in up to 15% of treated patients (McGrath, K.G.; Zeffren, B.; Alexander, J.; Kaplan, K. and Patterson, R. (1985) J. Allergy Clin. Immunol. 76, 453; Sorber,

- W.A. and Herbst, V. (1988) *Cutis* 42, 57; Davies, K.A.; Mathieson, P.; Winearis, C.G.; Rees, A.J.; and Walport, M.J. (1990) *Clin.Exp.Immunol.* 80, 83; Schweitzer, D.H.; Van der Wall, E.E.; Bosker, H.A.; Scheffer, E. and Macfarlane, J.D. (1991) *Cardiology* 78, 68; Bruserund, O.L.; Sollid, L. and Foyn-Jorgensen, P. (1986) *J. Clin. Lab. Immun.* 20, 69-74). The streptokinase also
- 5 induces a strong cellular immune response (Bruserund, O. (1990) *APMIS* 98, 1077-1084; Bruserund, O.; Elsayed, S. and Pawelec, G. (1992) *Mol. Immunol.* 29,1097-1104; Youkeles, L.H.; Soliman, M.Y. and Rosenstreich, D.L. (1991) *J. Allergy Clin. Immunol.* 88, 166-171; Randall, K.; Gelfond, D.H.; Stoffel, S.; Scharf, S.; Higuchi, R.; Horn, G.T.; Mullis, K.B. and Erlich, H.A. (1988) *Science* 239, 487-491).
- 10 The widespread use of streptokinase in humans makes its antigenicity an important clinical problem.
- Despite the rich clinical information about on the immunogenicity of streptokinase, little is known about the structural basis for its antigenicity. There is no X ray crystallographic data on the structure of streptokinase and it is not known whether certain regions of the molecule are
- 15 more immunogenic than others, nor have there been studies of the molecular mechanisms responsible for antibody-mediated neutralization of streptokinase activity.
- Previous reports have shown different antigenic regions in streptokinase mapped with murine anti-streptokinase monoclonal antibodies, soluble recombinant streptokinase fragment and anti streptokinase antibodies from human sera from patients treated with streptokinase (Reed, G.L.;
- 20 Kussie, P. and Parhami-Seren, B. (1993) *J. Immunol.* 150, 4407-4415; Parhami-Seren, B.; Lynch, M.; White, H.D. and Reed, G.L. (1995) *Mol. Immunol.* 32, 717-724; Parhami-Seren, B.; Keel, T. and Reed, G.L. (1996) *Hybridoma* 15, 169-176; Gonzalezgronow, M.; Enghild, J.J.; Pizzo, S.V. (1993) *Biochimica et Biophysica Acta* 1180, 283-288; Patente US No. 5,240,845).
- The object of the present invention is to achieve streptokinase mutants from modifications of
- 25 *skc-2* gene previously described (European Patent No. EP 0 489 201 B1; Estrada et al (1992) *Biotechnology* 10, 1138-1142) and coding for streptokinase SKC-2 (Heberkinase[®], Heber Biotec SA, Havana, Cuba), such that the obtained mutants conserve their capacity for plasminogen activator complex formation and having reduced antigenicity that could constitute a preferred
- alternatives to native streptokinase for thrombolytic therapy. Heberkinase[®] contains a
- 30 recombinant SKC-2 obtained after the expression of the *skc-2* gene in *E. coli* (European Patent No. EP 0 489 201 B1; Estrada et al (1992) *Biotechnology* 10, 1138-1142).
- The present invention relates to the mapping of antigenic regions located on SKC-2 using cellulose-bound peptide scans and human total sera from patients treated with Heberkinase[®].

The present invention also relates to the immunological features of a synthetic 42 amino acids peptide resembling amino acids 373-414 from the SKC-2 C-terminal region using a panel of sera collected from patients before and after Heberkinase[®] therapy and tested in anti-SKC-2(373-414) peptide ELISA and SKC-2 (373-414) direct binding assay.

- 5 The present invention relates to a method for the cloning and expression of SKC-2 mutants corresponding to the fragments 40-1245 and 1-1119 from the skc-2 gene, which codes for SKC-2, previously described in the european patent No. EP 0 489 201 B1, which products are proteins presenting:

- a deletion of the first 13 amino acids residues at the N-terminal region, called SKC-2-N13, which sequence corresponds to the Seq. Ident. No. 1.
- a deletion of the first 13 amino acids residues at the N-terminal region with Asp-Ile-Val-Asp-Gly-Gly-6xHis tail fused at the C-terminus of the protein, called SKC-2-N13-Asp-Ile-Val-Asp-Gly-Gly-6xHis which sequence corresponds to the Seq. Ident. No. 2.
- A deletion of the last 42 amino acids residues at the C-terminal region from position 373 to 414, called SKC-2-C42, which sequence corresponds to the Seq. Ident. No. 3.
- A deletion of the last 42 amino acids residues at the C-terminal region from position 373 to 414 with Asp-Ile-Val-Asp-Gly-Gly-6xHis tail fused at the C-terminus of the protein, called SKC-2-C42-Asp-Ile-Val-Asp-Gly-Gly-6xHis, which sequence corresponds to the Seq. Ident. No. 4.

- 20 The present invention also relates to these mutant proteins, which molecular weight is approximately 46.000 dalton for SKC-2-N13, 47.000 dalton for SKC-2-N13-Asp-Ile-Val-Asp-Gly-Gly-6xHis, 42.000 dalton for SKC-2-C42 and 43.000 dalton for SKC-2-C42-Asp-Ile-Val-Asp-Gly-Gly-6xHis, which amino acids sequences corresponds to the Seq. Ident. No. 1-4.

- The fragments of nucleotide sequence from skc-2 gene were obtained from pEKG3 plasmid (european patent No. EP 0 489 201 B1), by genetic amplification using the polymerase chain reaction (PCR) with 6 synthetic oligonucleotides denominated sk1, sk2, sk3, sk4, sk5 and sk6, having sequences identified with the Seq. Ident. No. 5-10.

- The present invention also relates to recombinant DNA including the nucleotide fragments 40-1245 and 1-1119 from skc-2 gene, such as vectors pEMI-1 (Figure 2), pSKH-11 (Figure 3), pIJ-4 (Figure 4) and pMC-8 (Figure 5) for the expression of these fragments in bacteria. For expression in *E. coli* these fragments were cloned under the tryptophan promoter and with the transcription termination signal from phage T4. pSKH-11 and pMC8 vectors also having a coding sequence for the Asp-Ile-Val-Asp-Gly-Gly-6x His amino acids fused at the 3' end from the respective DNA fragments and translation termination codon TAA.

The present invention relates to the microorganisms resulting from transformation of *E. coli* strain W 3110 with vectors pEMI-1, pSKH-11, pIJ-4 and pMC8. The transformants *E. coli* clones were called WSK-N13, WSK-N13-H, WSK-C42 and WSK-C42-H respectively.

Another aspect of this method is the possibility to express the DNA fragments 40-1245 and 1-1119 from *skc-2* gene in bacteria, reaching high levels of expression, around 350 mg/l from both mutant proteins, which were called mut-N13 and mut-C42, respectively.

The method described in the present invention, given the expression levels obtained for these products, makes it possible to reach optimum purity thereof for its administration to human beings and animals, without the need to develop a complex and expensive purification process.

The present invention relates to biological activity of mutant protein mut-N13, which showed a dramatic diminution of their activity and of mut-C42, which conserved similar activity as native protein.

The present invention also relates to the mutant proteins mut-N13 and mut-C42, which present reduced antigenicity with respect to the native SKC-2 protein. These mutant proteins were subjected to evaluation of their antigenicity in a direct binding assay and competition experiment between mutant and native proteins, using human sera collected from patients after Heberkinase® treatment.

The present invention also relates to the mut-C42 activity which, when compared to the SKC-2 activity, was less affected by SKC-2 neutralizing antibodies present in sera from 15 patients treated with Heberkinase®, which was evidenced by "in vitro" neutralizing assay.

The present invention relates to the slightly lower anti-SKC-2 antibodies generation in monkeys treated with mut-C42 in comparison with those treated with the native protein SKC-2.

The present invention relates to the neutralizing capacity developed by monkeys treated with SKC-2, which was significantly higher against SKC-2 than against mut-C42, indicating that the 42 C-terminal residues of SKC-2 contain one or more important epitopes for induction of neutralizing antibodies.

According to the first aspect, the present invention provides a method for producing streptokinase mutants by expression of a gene encoding said mutant streptokinase which comprises the steps of transforming a host cell with an expression vector containing a streptokinase gene skc-2 fragment (40-1245) consisting essentially of the nucleotide

5 sequence (SEQ. Ident. No. 11):

ATGAACAACA	GCCAATTAGT	TGTTAGCGTT	GCTGGTACTG	TTGAGGGGAC	GAATCAAGAC	60
ATTAGTCTTA	AATTTTTTGA	AATTGACCTA	ACATCAGCAG	CTGCTCATGG	AGGAAAGACA	120
GAGCAAGGCT	TAAGTCCAAA	ATCAAAACCA	TTTGCTACTG	ATAGTGGCGC	GATGCCACAT	180
AAACTTGAAA	AAGCTGACTT	ACTAAAGGCT	ATTCAGAAGC	AATTGATCGC	TAACGTCCAC	240
10 AGTAACGACG	ACTACTTTGA	GGTCATTGAT	TTTGCAAGCG	ATGCAACCAT	TACTGATCGA	300
AACGGCAAGG	TCTACTTTGC	TGACAAAGAT	GGTTCGGTAA	CCTTGCCGAC	CCAACCTGTC	360
CAAGAATTTT	TGCTAAGCGG	ACATGTGCGC	GTTAGACCAT	ATAAAGAAAA	ACCAATACAA	420
AATCAAGCGA	AATCTGTTGA	TGTGGAATAT	ACTGTACAGT	TTACTCCCTT	AAACCCTGAT	480
GACGATTTC	GACCAGGTCT	CAAAGATACT	AAGCTATTGA	AAACACTAGC	TATCGGTGAC	540
15 ACCATCACAT	CTCAAGAATT	ACTAGCTCAA	GCACAAAGCA	TTTTAAACAA	AACCCACCCA	600
GGCTATACGA	TTTATGAACG	TGACTCCTCA	ATCGTCACTC	ATGACAATGA	CATTTTCCGT	660
ACGATTTTAC	CAATGGATCA	AGAGTTTACT	TACCATGTCA	AAAATCGGGA	ACAAGCTTAT	720
GAGATCAATA	AAAAATCTGG	TCTGAATGAA	GAAATAAACA	ACACTGACCT	GATCTCTGAG	780
AAATATTACG	TCCTTAAAAA	AGGGGAAAAG	CCGTATGATC	CCTTTGATCG	CAGTCACTTG	840
20 AAATGTTTCA	CCATCAAATA	CGTTGATGTC	AACACCAACG	AATTGCTAAA	AAGCGAGCAG	900
CTCTTAACAG	CTAGCGAACG	TAACCTAGAC	TTGAGAGATT	TATACGATCC	TCGTGATAAG	960
GCTAAACTAC	TCTACAACAA	TCTCGATGCT	TTTGGTATTA	TGGACTATAC	CTTAACCTGGA	1020
AAAGTAGAGG	ATAATCACGA	TGACACCAAC	CGTATCATAA	CCGTTTATAT	GGGCAAGCGA	1080
CCCGAAGGAG	AGAATGCTAG	CTATCATTTA	GCCTATGATA	AAGATCGTTA	TACCGAAGAA	1140
25 GAACGAGAAG	TTTACAGCTA	CCTGCGTTAT	ACAGGGACAC	CTATACCTGA	TAACCCTAAC	1200
GACAAATAA						1209

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According to a second aspect, the present invention provides a method for producing streptokinase mutants by expression of a gene encoding said mutant streptokinase which comprises the steps of transforming a host cell with an expression vector containing a streptokinase gene skc-2 fragment (40-1245) fashioned to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence (Seq. Ident. No. 12):

AACAACAGCC AATTAGTTGT TAGCGTTGCT GGTACTGTTG AGGGGACGAA TCAAGACATT 60
AGTCTTAAT TTTTGAAAT TGACCTAACA TCACGACCTG CTCATGGAGG AAAGACAGAG 120
CAAGGCTTAA GTCCAAAATC AAAACCATTT GCTACTGATA GTGGCGCGAT GCCACATAAA 180
10 CTTGAAAAAG ACGACTTACT AAAGGCTATT CAAGAACAAT TGATCGCTAA CGTCCACAGT 240
AACGACGACT CATTGAGGT CATTGATTTT GCAAGCGATC GAACCATTAC TGATCGAAAC 300
GGCAAGGTCT ACTTTGCTGA CAAAGATGGT TCGGTAACCT TGCCGACCCA ACCTGTCCAA 360
GAATTTTTC TAAGCGGACA TGTGCGCGTT AGACCATATA AAGAAAAACC AATACAAAAT 420
CAAGCGAAAT CTGTTGATGT GGAATATACT GTACAGTTTA CTCCTTAAA CCCTGATGAC 480
15 GATTCAGAC CAGGTCTCAA AGATACTAAG CTATTGAAA CACTAGCTAT CGGTGACACC 540
ATCAGATCTC AAGAATTACT AGCTCAAGCA CAAAGCATTT TAAACAAAAC CCACCCAGGC 600
TATACGATTT ATGAACGTGA CTCCTCAATC GTCACATG ACAATGACAT TTTCCGTACG 660
ATTTTACCAA TGGATCAAGA GTTTACTTAC CATGTCAAAA ATCGGGAACA AGCTTATGAG 720
ATCAATAAAA AATCTGGTCT GAATGAAGAA ATAAACAACA CTGACCTGAT CTCTGAGAAA 780
20 TATTACGTCC TAAAAAAGG GGAAGAGCCC TATGATCCCT TTGATCGCAG TCACTTGAAA 840
CTGTTACCA TCAAATACGT TGATGTCAAC ACCAACGAAT TGCTAAAAAG CGAGCAGCTC 900
TTAACAGCTA GCGAACGTAA CTTAGACTTC AGAGATTTAT ACGATCCTCG TGATAAGGCT 960
AAACTACTCT ACAACAATCT CGATGCTTTT GGTATTATGG ACTATACCTT AACTGGAAAA 1020
GTAGAGGATA ATCAGCATGA CACCAACCGT ATCATAACCG TTTATATGGG CAAGCGACCC 1080
25 GAAGGAGAGA ATGCTAGCTA TCATTAGCC TATGATAAAG ATGCTTATAC CGAAGAAGAA 1140
CGAGAAGTTT ACAGCTACCT GCGTTATACA GGGACACCTA TACCTGATAA CCCTAACGAC 1200
AAATAAGATA TCGTCGACGG GGGGCACCAC CACCACCACC ACTAA 1245

According to a third aspect, the present invention provides a method for producing streptokinase mutants by expression of a gene encoding said mutant streptokinase which comprises the steps of transforming a host cell with an expression vector containing a streptokinase gene skc-2 fragment (1-1119) consisting essentially of the nucleotide sequence (Seq. Ident. No. 13):

ATGATTGCTG GACCTGAGTG GCTGCTAGAC CGTCCATCTG TCAACAACAG CCAATTAGTT 60
GTTAGCGTTG CTGGTACTGT TGAGGGGACG AATCAAGACA TTAGTCTTAA ATTTTGTGAA 120
ATTGACCTAA CATCACGACC TGCTCATGGA GGAAAGACAG AGCAAGGCTT AAGTCCAAAA 180
TCAAAACCAT TTGCTACTGA TAGTGGCGCG ATGCCACATA AACTTGAAAA AGCTGACTTA 240
10 CTAAAGGCTA TTCAAGAACA ATTGATCGCT AACGTCCACA GTAACGACGA CTACTTTGAG 300
GTCATTGATT TTGCAAGCGA TGCAACCAAT ACTGATCGAA ACGGCAAGGT CTACTTTGCT 360
GACAAAGATG GTTCGGTAAC CTTGCCGACC CAACCTGTCC AAGAATTTT GCTAAGCGGA 420
CATGTGCGCG TTAGACCATA TAAAGAAAAA CCAATACAAA ATCAAGCGAA ATCTGTTGAT 480
GTGGAATATA CTGTACAGTT TACTCCCTTA AACCTGATG ACGATTTTCA ACCAGGTCTC 540
15 AAAGATACTA AGCTATTGAA AACACTAGCT ATCGGTGACA CCATCACATC TCAAGAATTA 600
CTAGCTCAAG CACAAAGCAT TTAAACAAA ACCCACCAG GCTATACGAT TTATGAACGT 660
GACTCCTCAA TCGTCACTCA TGACAATGAC ATTTTCCGTA CGATTTTACC AATGGATCAA 720
GAGTTTACTT ACCATGTCAA AAATCGGGA CAAGCTTATG AGATCAATAA AAAATCTGGT 780
CTGAATGAAG AAATAACAA CACTGACCTG ATCTCTGAGA AATATTACGT CCTTAAAAAA 840
20 GGGGAAAAGC CGTATGATCC CTTTGATCGC AGTCACTTGA AACTGTTTAC CATCAAATAC 900
GTTGATGTCA ACACCAACGA ATTGCTAAAA AGCGAGCAGC TCTTAACAGC TAGCGAACGT 960
AACTTAGACT TCAGAGATT ATACGATCCT CGTGATAAGG CTAAGTACT CTACAACAAT 1020
CTCGATGCTT TTGGTATTAT GGACTATACC TTAAGTGGAA AAGTAGAGGA TAATCAGAT 1080
GACACCAACC GTATCATAAC CGTTTATATG GGCAAGCGAT AA 1122

According to a fourth aspect, the present invention provides a method for producing streptokinase mutants by expression of a gene encoding said mutant streptokinase which comprises the steps of transforming a host cell with an expression vector containing a streptokinase gene skc-2 fragment (1-1119) fused to the sequence
5 tail Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence (Seq. Ident. No. 14):

ATGATTGCTG GACCTGAGTG GCTGCTAGAC CGTCCATCTG TCAACAACAG CCAATTAGTT 60
GTTAGCGTTG CTGGTACTGT TGAGGGGACG AATCAAGACA TTAGTCTTAA ATTTTTTGAA 120
ATTGACCTAA CATCACGACC TGCTCATGGA GGAAAGACAG AGCAAGGCTT AAGTCCAAAA 180
TCAAAACCAT TTGCTACTGA TAGTGGCGCG ATGCCACATA AACTTGAAAA AGCTGACTTA 240
10 CTAAGGCTA TTCAAGAACA ATTGATCGCT AACGTCCACA GTAACGACGA CTACTTTGAG 300
GTCATTGATT TTGCAAGCGA TGCAACCATT ACTGATCGAA ACGGCAAGGT CTACTTTGCT 360
GACAAAGATG GTTCGGTAAC CTTGCCGACC CAACCTGTCC AAGAATTTT GCTAAGCGGA 420
CATGTGCGCG TTAGACCATA TAAAGAAAA CCAATACAAA ATCAAGCGAA ATCTGTTGAT 480
GTGGAATATA CTGTACAGTT TACTCCCTTA AACCTGATG ACGATTTTCA ACCAGGTCTC 540
15 AAAGATACTA AGCTATTGAA AACACTAGCT ATCGGTGACA CCATCACATC TCAAGAATTA 600
CTAGCTCAAG CACAAAGCAT TTAAACAAA ACCCACCAG GCTATACGAT TTATGAACGT 660
GACTCCTCAA TCGTCACTCA TGACAATGAC ATTTTCCGTA CGATTTTACC AATGGATCAA 720
GAGTTTACTT ACCATGTCAA AAATCGGGAA CAAGCTTATG AGATCAATAA AAAATCTGGT 780
CTGAATGAAG AAATAACAA CACTGACCTG ATCTCTGAGA AATATTACGT CCTTAAAAAA 840
20 GGGGAAAAGC CGTATGATCC CTTTGATCGC AGTCACTTGA AACTGTTTAC CATCAAATAC 900
GTTGATGTCA ACACCAACGA ATTGCTAAAA AGCGAGCAGC TCTTAACAGC TAGCGAACGT 960
AACTTAGACT TCAGAGATT ATACGATCCT CGTGATAAGG CTAAACTACT CTACAACAAT 1020
CTCGATGCTT TTGGTATTAT GGACTATACC TTAAGTGGAA AAGTAGAGGA TAATCACGAT 1080
GACACCAACC GTATCATAAC CGTTTATATG GGCAAGCGAG ATATCGTCGA CGGGGGGCAC 1140
CACCACCACC ACCACTAA 1158

According to a fifth aspect, the present invention provides an isolated and purified nucleic acid consisting essentially of the nucleotide sequence shown in the first aspect.

According to a sixth aspect, the present invention provides an isolated and purified nucleic acid consisting essentially of the nucleotide sequence shown in the second
5 aspect.

According to a seventh aspect, the present invention provides an isolated and purified nucleic acid consisting essentially of the nucleotide sequence shown in the third aspect.

According to an eighth aspect, the present invention provides an isolated and
10 purified nucleic acid consisting essentially of the nucleotide sequence shown in the fourth aspect.

According to a ninth aspect, the present invention provides an expression vector for the expression of streptokinase mutant SKC-2-N13 in a host cell, said vector containing a streptokinase gene skc-2 fragment 40-1245 consisting essentially of the
15 nucleotide sequence shown in the first aspect operably linked to an effective promoter and transcription terminator.

According to a tenth aspect, the present invention provides an expression vector for the expression of streptokinase mutant SKC-2-N13 in a host cell, said vector containing a streptokinase gene skc-2 fragment 40-1245 fused to the sequence tail
20 Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence shown in the first aspect operably linked to an effective promoter and transcription terminator.

According to an eleventh aspect, the present invention provides expression vector for the expression of streptokinase mutant SKC-2-C42 in a host cell, said vector containing a streptokinase gene skc-2 fragment 1-1119 consisting essentially of the

nucleotide sequence shown in the first aspect operably linked to an effective promoter and transcription terminator.

According to a twelfth aspect, the present invention provides an expression vector for the expression of streptokinase mutant SKC-2-C42 in a host cell, said vector
5 containing a streptokinase gene skc-2 fragment 1-1119 fusioned to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence shown in the first aspect operably linked to an effective promoter and transcription terminator.

According to a thirteenth aspect, the present invention provides a host cell for producing streptokinase mutant by expression of a gene encoding said streptokinase
10 mutant, said host cell being transformed with an expression vector containing a streptokinase gene skc-2 fragment 40-1245 consisting essentially of the nucleotide sequence shown in the first aspect operably linked to an effective promoter and transcription terminator.

According to a fourteenth aspect, the present invention provides a host cell for
15 producing streptokinase mutant by expression of a gene encoding said streptokinase mutant, said host cell being transformed with an expression vector containing a streptokinase gene skc-2 fragment 40-1245 fusioned to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence shown in the second aspect operably linked to an effective promoter and transcription terminator.

According to a fifteenth aspect, the present invention provides a host cell for
20 producing streptokinase mutant by expression of a gene encoding said streptokinase mutant, said host cell being transformed with an expression vector containing a streptokinase gene skc-2 fragment 1-1119 consisting essentially of the nucleotide sequence shown in the third aspect operably linked to an effective promoter and
25 transcription terminator.

According to a sixteenth aspect, the present invention provides a host cell for producing streptokinase mutant by expression of a gene encoding said streptokinase mutant, said host cell being transformed with an expression vector containing a streptokinase gene skc-2 fragment 1-1119 fused to the sequence tail Asp-Ile-Val-
5 Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence shown in the fourth aspect operably linked to an effective promoter and transcription terminator.

According to a seventeenth aspect, the present invention provides an isolated and purified streptokinase mutant consisting essentially of the amino acid sequence encoded by the nucleotide sequence shown in the first aspect.

10 According to an eighteenth aspect, the present invention provides an isolated and purified streptokinase mutant consisting essentially of the amino acid sequence encoded by the nucleotide sequence shown in the second aspect.

According to a nineteenth aspect, the present invention provides an isolated and purified streptokinase mutant consisting essentially of the amino acid sequence encoded
15 by the nucleotide sequence shown in the third aspect.

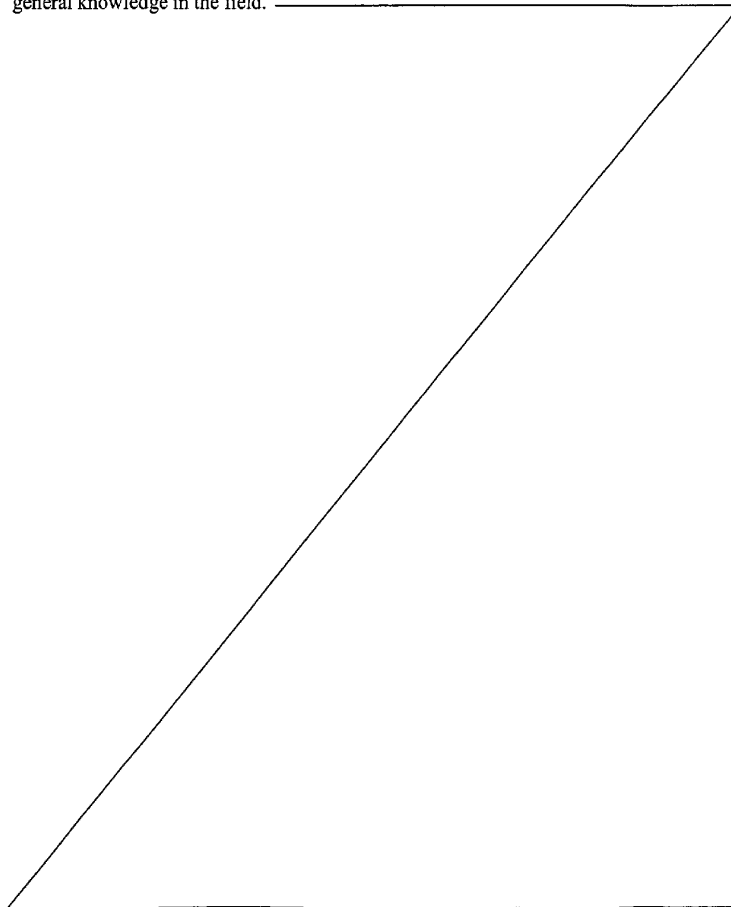
According to a twentieth aspect, the present invention provides an isolated and purified streptokinase mutant consisting essentially of the amino acid sequence encoded by the nucleotide sequence shown in the fourth aspect.

According to a twenty-first aspect, the present invention provides a pharmaceutical
20 composition comprising an isolated and purified streptokinase mutant as defined in the nineteenth aspect and a pharmaceutically acceptable diluent, carrier or excipient.

According to a twenty-second aspect, the present invention provides a pharmaceutical composition comprising an isolated and purified streptokinase mutant as defined in the twentieth aspect and a pharmaceutically acceptable diluent, carrier or
25 excipient.

Unless the context clearly requires otherwise, throughout the description and the claims, the words 'comprise', 'comprising', and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

- 5 Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.



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EXAMPLES

Example 1: Study of SKC-2 antigenic regions.

- To identify the regions of SKC-2 involved in anti-SKC-2 antibodies binding, the peptide spot synthesis approach as previously described by Frank, R. (1992) Tetrahedron 48, 9217-9232 was used. A cellulose-bound set of 41 overlapping 20-mer peptides (10 overlapping amino acids) spanning the primary sequence of SKC-2 (amino acids 1-414) (European patent No. EP 0 489 201 B1; Estrada et al (1992) Biotechnology 10, 1138-1142) was elaborated. The cellulose sheet was probed with human sera collected from ten patients at 10 days after Heberkinase® therapy. Cellulose sheet was soaked in ethanol to prevent possible hydrophobic interactions between the peptides. Ethanol was exchanged against Tris-buffered saline (TBS) (10 mM Tris, pH 7.6, 150 mM NaCl) by sequential washing, and nonspecific binding was blocked by incubating overnight in 10 ml of T-TBS blocking buffer (0.05% Tween 20 in TBS). The sheet was subsequently incubated for 3 h at room temperature with serum samples obtained from ten patients 10 days after Heberkinase® therapy, diluted in 10 ml of T-TBS blocking buffer. Serum samples were diluted according to the predetermined anti-SKC-2 Ab titers. Sera with 5×10^5 , 10^5 and 5×10^4 Ab titers, were diluted 1:1000, 1:500 and 1:300, respectively. Cellulose sheets were washed three times with T-TBS. Then, an alkaline phosphatase-conjugated anti-human Ab (Sigma) was added at 1:2500 dilution in T-TBS blocking buffer for 2h. Sheets were washed three times with T-TBS. Detection of bound anti-SKC-2 Abs was achieved by incubating the sheets with 0.3 mg/ml 5-Bromo 4-chloro 3-Indolyl Phosphate (BCIP) (Sigma), 4.5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) in substrate buffer (100 mM Tris, pH 8.9, 100 mM NaCl, 2 mM $MgCl_2$). Positive spots developed a violet color. Washing with PBS stopped staining. Cellulose sheets carrying the peptides were finally regenerated for the next test.
- Several distinct binding areas were observed for the ten tested sera (Figure 1). However, there are in the SKC-2 molecule binding sequences that are common for most of the patients. Eight out of ten sera recognized spot 14 comprising amino acids 130-149. Seventy percent of patients recognized spot 18 comprising residues 170-189. Six out of ten samples bound at spot 1 comprising amino acids 1-20 of the SKC-2 N-terminal region. Other six patients recognized spot 39 comprising residues 380-399. Fifty percent of tested sera recognized spot 40 comprising amino acids 390-409 within the C-terminal region.
- The simultaneous recognition of the spots 6 and 7 indicates the presence of a continuous epitope comprised between residues 60-69 (SKPFATDSGA). Likewise, for spots 39 and 40, the existence of a continuous epitope comprising residues 390-399 (TEEEREVYSY) was delineated. The recognition of spots 27, 28 and 29 indicated the presence of one or more

continuous epitopes comprised between residues 270-289 (ISEKYYVLKKGEKPYDPFDR). Spots showing isolated positive signals, without recognition of adjacent positions, suggested the existence of continuous epitopes including more than ten amino acids.

Example 2: Study of immunodominance of the SKC-2 C-terminal region.

5 a) Anti-SKC-2(373-414) peptide ELISA with patients sera.

Human total sera collected from 64 patients in different hospitals in Havana, Cuba, before (A) and ten days after (B) Heberkinase® therapy were tested in an anti-SKC-2 ELISA. Samples before therapy showed anti-SKC-2 Ab titers between 1:10 and 1:10⁴, while after therapy Ab titer range increased to 1:10³ – 1:5x10⁵. These samples were assayed in an anti-SKC-2(373-414) peptide ELISA in order to assess the recognition rate for the C-terminal region of SKC-2. In order to know the immunodominance of SKC-2 C-terminus, a peptide corresponding to the sequence 373-414 of SKC-2, containing 42 amino acid residues (PEGENASYHLAYDKDRYTEEEREVYSYLYTG TPIPDNPNDK) was synthesized. Polyvinyl plates (High Binding, Costar, Cambridge, Massachusetts, U.S.A.). Plates were coated with 1µg/ml SKC-2(373-414) peptide, and incubated overnight at 4°C. After washing three times with PBS-Tween, plates were blocked using 2% bovine serum albumin (BSA) (Sigma), and 100µl of 1:50 dilution of each human serum were added. The binding of human Abs to SKC-2(373-414) peptide was measured using a horseradish peroxidase-conjugated anti-human Ab (Sigma). The reaction was developed using 100µl per well of 1 mg/ml o-phenylenediamine (Sigma), 0.03% H₂O₂ in substrate buffer (0.1M citric acid, 0.2M Na₂HPO₄, pH 5.0). After 30 min, the reaction was stopped with 50µl of 4M H₂SO₄. Results were measured on a Multiskan system (Titertek, Helsinki, Finland) at 492nm. Each sample was tested by duplicated. Different degrees were considered for positive samples according to the sample/ background ratio: Samples showing absorbance values two, three and four or more times higher than the background were classified as +, ++ and +++, respectively. The results are shown in the Table 1. Before therapy (A), 39% of patients recognized the SKC-2(373-414) peptide. As it was expected, the recognition increased to 64% after therapy (B). This increase was not only due to a larger number of positive samples, but also to higher intensity of these positive signals.

Table 1: Anti-SKC-2(373-414) peptide ELISA with patient sera.

Patient	A	B	Patient	A	B
SA 01	+	+++	SA 41	-	+++
SA 03	+	+	SA 42	-	-
SA 05	+	++	SA 45	-	+++
SA 06	-	+	SA 46	-	-
SA 07	-	+	SA 47	++	+
SA 08	-	-	SA 48	+	++
SA 09	-	+	SA 49	-	+
SA 10	-	+	SA 50	-	+++
SA 11	-	++	SA 51	+	-
SA 12	++	+++	SA 52	-	+++
SA 13	+	+++	SA 53	-	+++
SA 14	-	++	SA 54	-	+++
SA 15	+	-	SA 55	-	+++
SA 17	+	+	SA 56	-	-
SA 18	-	-	SA 58	+	++
SA 19	-	-	SA 59	+	-
SA 20	+	+++	SA 60	-	++
SA 23	-	-	SA 61	-	-
SA 24	-	++	SA 64	-	++
SA 25	-	-	SA 65	-	-
SA 26	-	-	EC 04	-	++
SA 28	+	+++	EC 05	++	+
SA 29	+	-	EC 06	-	+
SA 30	+	+++	EC 10	-	+
SA 31	-	+++	EC 23	++	-
SA 32	+	+++	EC 25	-	-
SA 33	+	+++	CG 05	+	+++
SA 34	+	+++	CG 06	-	-
SA 35	-	+	CG 07	+++	+++
SA 37	+	-	CG 10	-	-
SA 39	-	-	LD 01	-	-
SA 40	+	++	LD 03	-	-
			Total	64	64
			(+)	25	41
			% (+)	39.063	64.063

b) SKC-2(373-414) direct binding assay with patients sera

In order to assess the proportion of the anti-SKC-2(373-414) recognition with respect to the total anti-SKC-2 Ab response, we performed a direct binding assay with 21 out of 64 patient sera obtained after Heberkinase[®] therapy. Experimental conditions were determined by titration of samples against native SKC-2 and SKC-2(373-414) peptide in order to select those dilution conditions (dln.1 for SKC-2(373-414) and dln.2 for SKC-2) in which there is not excess of Ab directed to each molecule.

Polyvinyl plates (High Binding, Costar, Cambridge, Massachusetts, U.S.A.) Plates were divided in two sections and coated with 10µg/ml SKC-2 and 1µg/ml SKC-2(373-414) peptide, respectively. After washing three times with PBS-Tween, plates were blocked with 2% BSA. One hundred µl of human sera collected from patients ten days after Heberkinase[®] therapy were added at previously determined optimal dilutions. After incubation for 1 h at 37°C, the binding of human anti-SKC-2 Abs to molecules on solid phase was measured using a horseradish peroxidase-conjugated anti-human Ab (Sigma). The reaction was developed using 100µl per well of 1 mg/ml *o*-phenylenediamine (Sigma), 0.03% H₂O₂ in substrate buffer (0.1M citric acid, 0.2M Na₂HPO₄, pH 5.0). After 30 min, the reaction was stopped with 50µl of 4M H₂SO₄. Results were measured on a Multiskan system (Titertek, Helsinki, Finland) at 492nm. Each sample was tested by duplicated. Percent direct binding of human anti-SKC-2 Abs to SKC-2(373-414) peptide was determined from the following formula:

$$100 \times \frac{(\text{Absorbance binding to SKC-2(373-414)}) \times \text{dln.1}}{(\text{Absorbance binding to SKC-2}) \times \text{dln.2}}$$

Percent Ab binding to SKC-2(373-414) ranged between 0.14 and 10.68% with respect to anti-SKC-2 Ab recognition (Table 2). The mean value from 21 samples was 2.96% (St. dev.=3.30).

Table 2: SKC-2(373-414) direct binding assay with patient sera

Patient	SKC-2			SKC-2(373-414)			% Direct Binding
	Absorbance	dilution factor	Abs x dilution	Absorbance	dilution factor	Abs x dilution	
SA 01	0.239	6400	1528.53	0.255	160	40.77	2.67
SA 11	0.290	12800	3709.87	0.267	20	5.35	0.14
SA 12	0.244	3200	781.87	0.284	80	22.75	2.91
SA 13	0.260	6400	1662.93	0.289	80	23.11	1.39
SA 20	0.232	6400	1486.93	0.265	40	10.59	0.71
SA 24	0.267	6400	1707.73	0.233	20	4.66	0.27
SA 28	0.272	12800	3479.47	0.292	320	93.55	2.69
SA 30	0.259	6400	1656.53	0.283	80	22.67	1.37
SA 31	0.228	12800	2922.67	0.230	80	18.43	0.63
SA 32	0.251	6400	1608.53	0.268	20	5.37	0.33
SA 33	0.304	12800	3889.07	0.290	1280	370.99	9.54
SA 34	0.263	12800	3370.67	0.279	320	89.23	2.65
SA 41	0.262	12800	3351.47	0.265	80	21.23	0.63
SA 45	0.236	6400	1512.53	0.252	640	161.49	10.68
SA 50	0.243	12800	3108.27	0.289	80	23.11	0.74
SA 52	0.311	12800	3985.07	0.298	320	95.47	2.40
SA 53	0.297	12800	3799.47	0.274	40	10.95	0.29
SA 54	0.256	12800	3274.67	0.256	1280	327.47	10.00
SA 55	0.221	3200	706.67	0.284	80	22.75	3.22
CG 05	0.242	6400	1547.73	0.280	160	44.77	2.89
CG 07	0.223	800	178.27	0.267	40	10.67	5.99
Total						21	
Mean						2.96	
St. Dev.						3.30	

c) Study of the recognition of SKC-2(373-414) peptide by sera from normal donors.

Antibodies directed against streptokinase are found in most individuals as a result of recurrent streptococcal infections. Regarding the immunodominance of SKC-2 C-terminal region, part of this antibody response is likely to direct against amino acids 373-414 from the C-terminus of the molecule. In order to assess the proportion of this recognition in normal population, 1008 normal donor sera were tested using an anti-SKC-2(373-414) peptide Ultra-Micro-ELISA. Plates (Greiner, Frankfurt, Germany) were coated with 15 μ L per well of 2 μ g/mL SKC-2(373-414) in coating buffer (50mM Na₂CO₃, 50mM NaHCO₃, pH 9.6), and incubated at 37°C for 4h. After washing with Tris-buffered saline, 0.05% Tween 20 (TBS-Tween), plates were blocked with 2% BSA (Sigma) at room temperature, overnight. Blocking solution was removed and plates were dried at 37°C for 1h. Ten μ L of 1:20 dilution of each human serum in TBS, 0.05% Tween 20, 1% BSA were added. Plates were incubated at 37°C for 30min and washed four

times. Binding of human Abs to SKC-2(373-414) peptide was measured using 1:5000 dilution of an alkaline phosphatase-conjugated anti-human IgGAb (Sigma). Plates were incubated at 37°C for 30min and washed four times. Reactions were developed by addition of 100µL per well of substrate solution (0.13mg/mL 4-methylumbelliferyl phosphate in 3M diethanolamine-HCl buffer, pH 9.8) and plates were incubated at room temperature for 30min. Fluorescence was measured using an Ultra-Micro-ELISA plates reader PR-521 (SUMA Technology, Havana, Cuba). Each sample was tested by duplicated. The experiment was validated by positive, negative and blank controls. In order to homogenize the results, the sample/positive control ratio was determined for each tested serum using the following formula:

$$\text{Sample/Positive} = \frac{(\text{Sample fluorescence}) - (\text{Blank fluorescence})}{(\text{Positive control fluorescence}) - (\text{Blank fluorescence})}$$

Sample/Positive ratio of the 1008 tested samples ranged between 0.005 and 1.970. The mean value was 0.369 (St. Dev. 0.499). A frequency distribution was made according to 40 classes defined by Sample/Positive ratio (Figure 2).

- 15 In order to determine the cut off for the assay an auxiliary experiment was performed. Inhibition of anti-SKC-2(373-414) Ab binding by previous adsorption of samples with the same peptide was studied. This experiment was performed with 140 samples randomly selected from 1008 previously tested. A 1:4 dilution of each sample was mixed with SKC-2(373-414) peptide at a final concentration of 5µg/mL and incubated at room temperature with agitation, overnight.
- 20 Samples were centrifuged at 12000 rpm for 10min in order to precipitate immunocomplexes. Plates were coated with 2µg/mL SKC-2(373-414), as described above. Adsorbed samples were diluted 1:5 to reach 1:20 final dilution. Each one was accompanied by 1:20 dilution of intact serum as a control. Plates were incubated at 37°C for 30min and washed four times. Next steps were performed as described above. Each sample was tested by duplicated. The proportion of
- 25 each adsorbed sample with respect to its intact control (Adsorbed/Intact) was determined. Positive sample was considered when Adsorbed/Intact ratio was no higher than 0.6. Figure 3 shows plots of Adsorbed/Intact ratio versus Sample/Positive ratio. For small Sample/Positive ratio values there is a high concentration of samples over 0.6. However, as this ratio increases, negative samples decrease. Based on these results a Sample/Positive ratio value of 0.3 was
- 30 selected as cut off because it assures to take the highest number of positive individuals with a minimum unespecificity.

Regarding a cut off of 0.3, the analysis of the results showed that 306 out of 1008 tested samples recognize SKC-2(373-414) peptide, representing 30.36% from total (Figure 4).

Example 3: Cloning and expression of SKC-2 mutants protein.

For subcloning of skc-2 mutants in bacteria, DNA from plasmid pEKG-3 (containing skc-2 gene; European patent No. EP 0 489 201 B1; Estrada et al (1992) Biotechnology 10, 1138-1142) was taken and the fragments were amplified by PCR using oligonucleotides sk1, sk2, sk3, sk4, sk5 and sk6. Oligonucleotides sk1 and sk4 have an EcoRI restriction site, oligonucleotides sk2 and sk5 have a BamHI restriction site, and oligonucleotides sk3 and sk6 have an EcoRV restriction site. Oligonucleotides sk1 and sk4 have an ATG codon for the translation initiation. Oligonucleotides sk2 and sk5 have a TAA codon for translation termination.

One μ g of pEKG-3 was taken and the gene coding for SKC-2 was amplified by PCR (Dagert, M., and Erlich, S.L. (1974) Gene 6: 23-28) using the oligonucleotides sk1, sk2 and sk3 for cloning the mutant gene cloning with a 39 bp deletion at the 5' end, corresponding to the nucleotide fragment 40-1245 from skc-2 gene; and oligonucleotides sk4, sk5 and sk6 for the cloning mutant gene with a 126 bp deletion at the 3' end, corresponding to the nucleotide fragment 1-1119 from skc-2 gene.

For each reaction 100 pmol of each oligonucleotide, 2 units of Taq polymerase (Enzibiot) and 200 μ mol of each dNTP were used. Reactions were performed in 10 mM $MgCl_2$, 100mM dTT, 10mM NaCl and 100 μ l mineral oil. Twenty five amplification cycles were performed, wherein each one the reaction was incubated at 95°C for 1 minute for denaturisation, at 50°C for 45 seconds for oligonucleotide annealing at 70°C for 80 seconds for DNA chains extension. An amplification efficacy higher than 5% was obtained.

For cloning in bacteria (*E. coli*), a genetic construct containing the tryptophan promoter of *E. coli* and the termination signal of bacteriophage T4 terminator was used. Fragments amplified by PCR using combinations of primer-oligonucleotides sk1-sk2 and sk4-sk5 were digested with EcoRI and BamHI, and ligated with the EcoRI-BamHI digested vector. Fragments amplified by PCR using combinations of primer-oligonucleotides sk1-sk3 and sk4-sk6 were digested with EcoRI and EcoRV, and ligated with the EcoRI-EcoRV digested vector containing a coding sequence for the amino acids tail Asp-Ile-Val-Asp-Gly-Gly-6XHis that was fused to the 3' end of both fragments. These constructions were transformed into a preparation of competent cells ((Hanahan, D. (1983) J. Mol. Biol. 166, 557-580) of *E. coli* strain MC1061 (F⁺ ara D 139 (ara-leu) 7696 (lac) X74 gal u galk hsd R2(rk⁺mk⁺) mcrB1 rpsL (Str^r)), having a frequency higher than 10⁷ transformants per DNA μ g.

Resultant colonies were applied to LB plates (10gr/l trypton, 5gr/l yeast extract, 10gr/l NaCl and 50 μ g/ml ampicillin), and subjected to hybridization (Maniatis, T.; Frisch, E.F. and Sambrook, J. (1982) Cold Spring Harbor Laboratory, USA), using the fragment resulting from PCR

amplification as a probe, labelled with dATP³² (Amersham, R.U.) and the Klenow fragment of DNA- polymerase I of *E.coli* for 30 minutes at 37°C. The reaction was stopped by EDTA and heat. The hybridization was performed in Whatman 541 filters, 8% of the colonies were positive clones, which were examined by restriction analysis and had the same pattern of digestion with more than 10 restriction enzymes. Moreover, positive clones were checked by double chain DNA sequencing (Sanger, F.; Nickler, S. and Coulson, A.K. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467), using an oligonucleotide of 17 bases (5' ATCATCGAAGTAGTAA 3') which annels at the 3' promoter end, corroborating that 39 bp deletion at the 5' end of the gene and joining to the promoter were correct; and an oligonucleotide of 22 bases (5' GGTCATTCAAAGGTCATCCAC 3') which annels at the 5' end of the T4 terminator, corroborating that 126 bp deletion of at the 3' end of the gene and fusion to the coding sequence for Asp-Ile-Val-Asp-Gly-Gly-6xHis tail was correct.

The selected clones (Figures 5, 6, 7 and 8) were called pEMI-1 (mutant with 39 bp deletion the at the 5' end having the *skc-2* gene fragment 40-1245), pSKH-11 (mutant with 39 bp deletion at the 5' end having the *skc-2* gene fragment 40-1245 fused to the coding sequence for Asp-Ile-Val-Asp-Gly-Gly-6xHis tail at the 3' end), pIJ-4 (mutant with 126 bp deletion at the 3' end having the *skc-2* gene fragment 1-1119) and pMC-8 (mutant with 126 bp deletion at the 3' end having the *skc-2* gene fragment 1-1119 fused to the coding sequence for Asp-Ile-Val-Asp-Gly-Gly-6xHis tail at the 3' end). These clones were transformed in the *E.coli* strain W3110 and were subjected to a fermentation process, wherein stable expression levels higher than 10% of the total protein content of the cells were obtained, and 150-200 mg of SKC-2 mutants mut-N13 and mut-C42 per litre of culture medium were obtained.

Example 4: Purification of SKC-2 mutant proteins.

E. coli cells were suspended in the disruption buffer that containing 50 mM Tris-HCl, 0.5 M NaCl, 3mM EDTA, pH 7.0 at a concentration of 40 % (w/v) and mechanically disrupted by using a French Pressure (Ohtake, Japan). Cells were passed twice through the French Pressure in order to achieve an optimal cell disruption. Disrupted cells were homogenized and centrifuged at 15000 rpm for 1 h at 4°C by using a RPR 20-2 rotor (Hitachi, Japan). The supernatant, containing the recombinant streptokinase, was collected for protein purification.

The supernatant was loaded into a Sephadex G-25 gel filtration column (2.6 X 27.5; I.D. x L., in cm) (Pharmacia, Sweden) which was previously equilibrated with 0.02 M Tris-HCl pH 6.0 at a flow rate of 5 ml/min. Proteins eluted from the gel filtration support were loaded into a Q-Sepharose Fast Flow anion exchange column (2.6 x 5.5; I.D. x L., in cm) (Pharmacia, Sweden), previously equilibrated with 0.02 M Tris-HCl pH 6.0 at a flow rate of 10 ml/min. The non-

bound protein was washed from the column with this equilibrium buffer. Elution of proteins was carried out with a linear gradient of increasing NaCl concentrations, which was produced by using an FPLC system (Pharmacia, Sweden). The recombinant streptokinase was eluted at 0.12 M NaCl in the equilibrium buffer.

- 5 The pH of the eluate from the ion exchange support was increased from 6.0 to 8.0 by adding a 1 M Tris solution. Ammonium sulfate was added to this sample up to 10 % saturation of this salt. This sample was loaded into a column (1.6 x 5; I.D. x L., in cm) containing a TSK-butyl (Tosohas Technical Center, USA) hydrophobic interaction chromatography support. This column was equilibrated with 0.02 M Tris-HCl, ammonium sulfate at 10 % saturation, pH 8.0, at
10 a flow rate of 4 ml/min. After washing the non-bound protein with equilibrium buffer, the recombinant streptokinase was eluted by using an FPLC system (Pharmacia, Sweden) which produced a linear gradient of decreasing ammonium sulfate concentration in the equilibrated buffer. The recombinant streptokinase was eluted at a concentration of ammonium sulfate of 3
15 % saturation. The material obtained was sterilized by filtration through a 0.22- μ m Millipore filter.

Example 5: Determination of biological activity of SKC-2 mutant proteins.

- The *in vitro* biological activity of mutant proteins mut-N13 and mut-C42 was determined by agarose-fibrin plates assay (Astrup, T. and Mullertz, S. (1952) Arch. Biochem. Biophys 40, 346-351), chromogenic substrate (Fiberger, P. (1982) J. Clin. Lab. Invest. 42, Suppl. 162, 49-54) and
20 clot lysis (Westlund, L.E. and Anderson, L.O. (1985) Thrombosis Research 37, 213-223). mut-C42 showed a specific activity of 50 000-100 000 IU/mg similar to that obtained for native SKC-2, and mut-N13 showed a dramatic diminution of its specific activity with values of 2000-4000 IU/mg.
- mut-C42 *in vivo* fibrinolytic activity was verified in clinical test on animals, wherein there was
25 success in dissolving clots in the femoral arteries of rabbits and coronary arteries of dogs. Blood parameters maintained similar to those obtained for native SKC-2 and those reported in the literature for this type of product.

Example 6: Immunological characterization of SKC-2 mutant proteins. *In vitro* assays.

a) mut-N13 and mut-C42 direct binding assay by human anti-SKC-2 antibodies.

- 30 A direct binding assay was performed in order to compare mut-N13 and mut-C42 mutant proteins with native SKC-2 regarding their capacity for binding human anti-SKC-2 Abs present in sera from patients after Heberkinasa® therapy. Polyvinyl plates (Medium binding, Costar, Cambridge, Massachusetts, U.S.A) were divided in three sections and coated with 10 μ g/ml of full length SKC-2, mut-N13 and mut-C42, respectively. Then, plates were washed three times

with PBS-Tween. One hundred μ l of human sera collected from eight patients ten days after Heberkinase[®] therapy were added at a previously determined optimal dilution. Samples were diluted according to the predetermined anti-SKC-2 Ab titers. For sera with 5×10^5 , 10^5 and 5×10^4 Ab titers, dilutions were of $1:3.2 \times 10^4$, $1:1.6 \times 10^4$ and $1:2 \times 10^3$, respectively. After incubation for 1 h at 37°C, the binding of human anti-SKC-2 Abs to molecules on solid phase was measured using a horseradish peroxidase-conjugated anti-human Ab (Sigma). The reaction was developed using 100 μ l per well of 1 mg/ml o-phenylenediamine (Sigma), 0.03% H₂O₂ in substrate buffer (0.1M citric acid, 0.2M Na₂HPO₄, pH 5.0). After 30 min, the reaction was stopped with 50 μ l of 4M H₂SO₄. Each sample was tested by duplicated. Percent direct binding of human anti-SKC-2 Abs to deletion mutants (Table 3) was determined from the following formula:

$$100 \times (\text{Absorbance binding to mutant proteins} / (\text{Absorbance binding to SKC-2}))$$

Table 3: Direct binding assay of human anti-SKC-2 antibodies to mutant proteins mut-N13 and mut-C42.

Sera	mut-N13	mut-C42
SA06B	86.0335	43.3892
SA07B	96.0191	55.414
SA12B	95.2128	51.0638
SA14B	92.0415	55.1903
SA17B	88.8889	49.537
SA28B	88.8631	48.7239
SA32B	87.8238	50.7772
SA42B	84.4828	51.7241
Mean	89.9207	50.7275
St. Dev.	4.15685	3.82233
n	8	8
t	6.85822	36.4605
P	0.00012	1.5E-09

All eight tested sera showed a similar binding pattern. Binding of human anti-SKC-2 Abs to mut-N13 was 89.92% (P=0.00012) and to mut-C42 was 50.73% (P=1.52 x 10⁻⁹) of their binding to native SKC-2 (Figure 9).

b) Competition assay of proteins mut-N13, mut-C42 and SKC-2.

Similar results were obtained from the same eight samples using a competition assay in which native and mutant proteins mut-N13 and mut-C42 competed with a biotinylated SKC-2 for binding human anti-SKC-2 Abs. Plates (Costar) were coated with 5 μ g/ml of goat anti-human Abs in coating buffer. After washing three times with PBS-Tween, plates were blocked using 2% BSA (Sigma). One hundred μ l of human sera collected from eight patients

ten days after Heberkinase® therapy were added at a previously determined optimal dilution. Samples were diluted according to the predetermined anti-SKC-2 Ab titers. For sera with 5×10^5 , 10^5 and 5×10^4 Ab titers, dilutions were of $1:10^4$, $1:5 \times 10^3$ and $1:10^3$, respectively. This way, human anti-SKC-2 Abs were immobilized on the coated plates. After washing, 100µl of a solution of 1µg/ml of biotinylated SKC-2 mixed with different concentrations of non-labeled full length SKC-2 or deletion mutants (4 – 0.25 µg/ml, two-fold dilutions) were added. The binding of biotinylated SKC-2 to human anti-SKC-2 Abs, after competition with non-labeled molecules, was measured using horseradish peroxidase-conjugated streptavidin. The reaction was developed using 100µl per well of 1 mg/ml *o*-phenylenediamine (Sigma), 0.03% H₂O₂ in substrate buffer (0.1M citric acid, 0.2M Na₂HPO₄, pH 5.0). After 30 min, the reaction was stopped with 50µl of 4M H₂SO₄. Each sample was tested by duplicate. The effective dose 50% (ED50) values for mutant and native proteins were determined from plots of absorbance versus concentration of non-labeled molecules using a Probit transformation in order to obtain 50% inhibition (Table 4; Figures 10 and 11).

Table 4: ED 50 inhibición (□g/ml) of SKC-2, mut-N13 and mut-C42 for each patient treated with Heberkinase®.

Sera	SKC-2	mut-N13	mut-C42
6	0.2865	0.39447	0.6079
7	0.2653	0.33091	0.5579
12	0.1625	0.22202	0.2007
14	0.4338	0.56878	0.6071
17	0.1519	0.20047	0.2136
28	0.2775	0.32181	0.3411
32	0.1556	0.23464	0.235
42	0.3678	0.32266	0.492
Mean	0.2626	0.32447	0.4069
St. Dev.	0.1035	0.11867	0.179

Statistical significance of differences was determined by Student's *t* test (Tables 5 and 6) for paired values, evidencing the existence of significant differences between each mutant and native protein (P= 0.0066 for mut-N13 and P= 0.0036 for mut-C42).

Table 5: Results of Student's "t" test for paired values.

	mut-N13	SKC-2
Mean	0.3245	0.2626
Variance	0.0141	0.0107
n	8	8
PC	0.8946	
HMD	0	
df	7	
t	3.295	
P	0.0066	

Table 6: Results of Student's "t" test for paired values.

	mut-C42	SKC-2
Mean	0.40692	0.26262
Variance	0.03204	0.0107
n	8	8
PC	0.83236	
HMD	0	
df	7	
t	3.73891	
P	0.0036	

We expressed mut-N13 and mut-C42 ED50 values in terms of percent with respect to SKC-2. Binding of mut-N13 and mut-C42 to human anti-SKC-2 Abs was 80.57% (P=0.0036) and 67.57% (P= 0.0001) of reactivity to native SKC-2, respectively (Table 7; Figure 12).

Table 7: ED50 values in terms of percent with respect to SKC-2.

Sera	mut-N13	mut-C42
6	72.633	47.131
7	80.173	47.55
12	73.194	80.972
14	76.267	71.455
17	75.763	71.104
28	86.243	81.37
32	66.294	66.188
42	114.01	74.76
Mean	80.572	67.566
St. Dev.	14.702	13.469
n	8	8
t	3.7377	6.8111
P	0.0036	0.0001

c) Neutralizing activity assays using sera from patients treated with Heberkinase®.

Neutralizing activity titers (NAT) against mut-C42 and native SKC-2 proteins were determined for 15 patients, ten days after Heberkinase® therapy. The chromogenic substrate (S-2251) reaction was performed in polyvinyl plates (Costar, Cambridge, Massachusetts, U.S.A.). Serial dilutions of SKC-2 and mut-C42 (128 – 2 IU, two-fold dilutions in 20 mM Tris-HCl pH8 / 0.5 M NaCl) were prepared in a volume of 25µl. Curves were mixed with 25µl of 1:10 dilutions of each patient serum, and a negative control consisting of a human serum having low anti-SKC-2 Ab titer and preabsorbed with SKC-2. Fifty µl of 25µg/ml human Plg were added and allowed to mix for 10 min at room temperature. The reaction was developed by addition of 50µl of chromogenic substrate S-2251 (Chromogenix, Antwerp, Belgium). After incubation for 30 min, the reaction was stopped with 25µl of 20% acetic acid. Results were measured on a Multiskan system (Titertek, Helsinki, Finland) at 405nm. The experiment was validated by a standard curve of each protein. All samples were tested by duplicated. The activity required to obtain an absorbance of 0.7 was determined from plots of absorbance versus activity. Neutralizing activity titer (NAT) was determined as the difference between tested serum and negative control values and was expressed as microgrammes of protein neutralized per milliliter of tested serum (Figure 13). Results were statistically analyzed by the Student's *t* test for paired values (Table 8).

Table 8: Neutralizing Activity Titers (NAT) against SKC-2 and mut-C42 for 15 patients treated with Heberkinase®.

Patient	SKC-2	mut-C42
5	2.5928	0
6	211.33	234.6
7	274.16	235.28
12	97.526	61.632
14	363.43	277.24
15	128.42	63.13
17	146.36	94.586
18	428.27	215.4
28	284.47	113.92
30	210.96	95.655
32	196.41	64.875
42	244.19	221.93
46	193.54	88.788
LD1	196.02	59.081
NR	214	110.89
Mean	212.78	129.13
St. Dev.	103.14	84.307
P(test T)	0.0002	

NAT values ranged between 61.63 and 428.27 µg of protein neutralized per ml of tested serum. For most of the individuals mut-C42-NAT decreased with respect to SKC-2-NAT, ranging from 30 to 91% of the native protein value (P=0.0013).

Example 7: Immunological characterization of mut-C42 compared to SKC-2. Animals study.

Fourteen monkeys (*Cercopithecus aethiops*) of either sex, between two to three years old, weighing 1.8-2.5 kg, were selected for the study. Sera from these monkeys were tested in an anti-SKC-2 ELISA and animals were divided in two groups according to the results:

Group A: eight monkeys without previous anti-SKC-2 Ab titers

Group B: six monkeys with previous anti-SKC-2 Ab titers, probably due to previous contact with streptococcus.

The comparative antigenicity of mutant protein mut-C42 versus native SKC-2 was studied after 850 µg (425 µg/kg of corporal weight) subcutaneous administrations in groups A and B. In each group half of monkeys were treated with mut-C42 and the other half with SKC-2.

Humoral response was quantified at week 8 after 4 administrations, for group A; and at week 2 after one administration, for group B. Titration was performed by an anti-SKC-2 ELISA. Polyvinyl plates (Costar, Cambridge, Massachusetts, U.S.A.) were coated with 10µg/ml SKC-2 in coating buffer (0.1M Na₂CO₃, 0.1M NaHCO₃, pH 9.6), and incubated overnight at 4°C. Then, plates were washed three times with 0.05% Tween 20 in PBS (PBS-Tween). One hundred µl of serial dilutions (1:2-1:4096, two-fold dilutions in 3% fat-free milk, PBS, 0.05% Tween 20) of each monkey serum were added. After incubation for 1 h at 37°C, plates were incubated with a biotinylated protein A solution at 1:3000 dilution. After incubation for 1 h at 37°C, the binding of monkey Abs to SKC-2 was measured using a horseradish peroxidase-conjugated streptavidin (Sigma). The reaction was developed using 100µl per well of 1 mg/ml o-phenylenediamine (Sigma), 0.03% H₂O₂ in substrate buffer (0.1M citric acid, 0.2M Na₂HPO₄, pH 5.0). After 30 min, the reaction was stopped with 50µl of 4M H₂SO₄. Results were measured on a Multiskan system (Titertek, Helsinki, Finland) at 492nm. The anti-SKC-2 Ab titer was determined as the maximal dilution in which positive signal was obtained. Positive signal was considered when the value was at least two-fold the background.

Anti-SKC-2 Ab titers rose post-treatment, but animals from group B developed titers notably higher than those from group A (Table 9). Ab titers from group A were slightly lower for monkeys treated with mut-C42 compared with those treated with SKC-2. There are two particular monkeys (33 and 85) showing very low Ab titers. Ab titers generated by animals from group B showed no differences between treatments.

Table 9: Anti-SKC-2 antibody titers in monkeys treated with SKC-2 or mut-C42

Treatment	Grup A (week 8)		Grup B (week 2)	
	Animal	Titer	Animal	Titer
SKC-2	18	64	3	640
	21	50	42	640
	73	256	66	240
	321	256		
	Mean	156.5	Mean	506.66
	St. Dev.	115.03	St. Dev.	230.94
mut-C42	6	100	23	640
	33	16	26	260
	78	256	79	520
	85	16		
	Mean	97	Mean	473.33
	St. Dev.	113.15	St. Dev.	194.25

Animal sera were also subjected to a neutralization assay in order to determine their neutralizing activity titer (NAT).

- 15 Serial dilutions of SKC-2 and mut-C42 (128 – 2 IU, two-fold dilutions in 20 mM Tris-HCl pH8 / 0.5 M NaCl) were prepared in a volume of 25µl in polyvinyl plates (Costar, Cambridge, Massachusetts, USA). SKC-2 and mut-C42 curves were mixed with 25µl of each monkey diluted serum and a negative control. For monkeys without previous anti-SKC-2 Ab titer a 1:2 dilution was used, and sera from monkeys with previous anti-SKC-2 Ab titer were diluted 1:5.
- 20 The negative control was a monkey serum without anti-SKC-2 Ab titer. Fifty µl of 25µg/ml human pasminogen were added and allowed to mix for 10 min at room temperature. The reaction was developed by addition of 50µl of chromogenic substrate S-2251 (Chromogenix, Antwerp, Belgium). After incubation for 30 min, the reaction was stopped with 25µl of 20% acetic acid. Results were measured on a Multiskan system (Titertek, Helsinki, Finland) at 405nm. The experiment was validated by a standard curve of each protein. All samples were tested by duplicate. The activity required to obtain an absorbance of 0.87 for grup A and 0.37 for grup B, was determined from plots of absorbance versus activity. The neutralizing activity titer (NAT) was determined as the difference between the tested serum and negative control values and was expressed as microgrammes of protein neutralized per milliliter of tested serum (Table 10; Figure 14).
- 30

Abs from most of the monkeys inhibited the formation of SKC-2-Plg and mut-C42-Plg activator complexes *in vitro*. SKC-2-NAT developed by monkeys from group A were considerably lower than SKC-2 neutralizing capacity exhibited by group B. However, mut-C42-NAT values were similar for both groups.

Monkeys from group A treated with SKC-2 showed NAT values ranging between 35.43 and 54.17 μg (45.3 ± 8.33) of SKC-2 and between 0 and 19.3 μg (9.13 ± 8.47) of mut-C42 moiety neutralized per ml of tested serum. Sera from monkeys treated with mut-C42 elicited NAT values ranging between 6.79 and 44 μg (24.3 ± 20) of SKC-2 and between 0 and 14.12 μg (7.5 ± 8.69) of mut-C42 moiety neutralized per ml of tested serum. Interestingly, animals 33 and 85, showing low anti-SKC-2 Ab titers, exhibited insignificant or none NAT against both proteins.

Table 10. Neutralizing Activity Titers (NAT) of monkey sera against SKC-2 and mut-C42 proteins.

Treatment	Grup A (week 8)				Grup B (week 2)			
	Animal #	SKC-2	mut-C42	P	Animal #	SKC-2	mut-C42	P
SKC-2	18	35.430	12.371	0.0042	3	241.897	6.580	0.0369
	21	41.806	0.000		42	89.256	0.000	
	73	54.176	4.866		66	151.102	21.708	
	321	49.797	19.304					
	Mean	45.302	9.135		Mean	160.752	9.429	
	St. Dev.	8.339	8.477		St. Dev.	76.777	11.131	
mut-C42	6	44.032	14.121	0.0621	23	374.112	33.905	0.0394
	33	6.792	0.000		26	184.289	24.357	
	78	39.084	15.881		79	313.603	15.554	
	85	7.382	0.000					
	Mean	24.322	7.500		Mean	290.668	24.605	
	St. Dev.	20.005	8.690		St. Dev.	96.967	9.178	
P		0.1247	0.7967			0.1467	0.1451	

Monkeys from group B showed a considerable increase in Ab titers after one administration of the proteins. However, there were no differences in anti-SKC-2 Ab titers between monkeys treated with native and mutant proteins. These animals showed no SKC-2 neutralizing capacity before the treatment. After only one administration of the studied proteins, Abs from most of the monkeys inhibited the formation of SKC-2-Plg and mut-C42-Plg activator complexes *in vitro*. Monkeys treated with SKC-2 showed NAT values ranging between 89.25 and 241.9 μg (160.75 ± 76.77) of SKC-2 and between 0 and 21.7 μg (9.42 ± 11.13) of mut-C42 moiety neutralized per ml of tested serum. Sera from monkeys treated with mut-C42 elicited NAT values ranging between 184.29 and 374.11 μg (290.67 ± 96.96) of SKC-2 and between 15.55 and 33.9 μg (24.6 ± 9.17) of mut-C42 moiety neutralized per ml of tested serum.

Statistical analyses supported the following results: (a) mut-C42 was significantly less affected than SKC-2 by neutralizing Abs from monkeys treated with the native protein ($P=0.0042$ for group A and $P=0.0369$ for group B), (b) the same result was obtained for group B animals treated with mut-C42 ($P=0.0394$), (c) in contrast, monkeys from group A receiving mut-C42 treatment showed no significant differences between SKC-2- and mut-C42-neutralizing activities ($P=0.0621$), and (d) within each group, no statistical significance was obtained from comparison between SKC-2 and mut-C42 treatments.

Statistical significance of the differences for neutralizing activities in monkeys treated with SKC-2 was determined using the Student's *t* test for paired values, one-tailed distribution.

- 10 Neutralizing activities of monkeys treated with mut-C42 were analyzed using the Student's *t* test for paired values, two-tailed distribution. Statistical significance of the differences between SKC-2 and mut-C42 treatments was determined by a Student's *t* test for two-samples with unequal variances ($P<0.05$).

BRIEF DESCRIPTION OF FIGURES

15 STREPTOKINASE MUTANTS

Figure 1: Schematic representation of the reactivity of patient sera to a spot-synthesized peptide library spanning the whole sequence of SKC-2 protein.

N_1 : number of patients whose sera reacted with each individual spot.

N_2 : number of spots recognized by each patient serum.

- 20 **Figure 2:** Frequency distribution of 1008 samples tested by anti-SKC-2(373-414) Ultra-Micro-ELISA according to their Sample/Positive ratio.

Figure 3: Plots of Adsorbed/Intact ratio versus Sample/Positive ratio for 140 samples tested in an auxiliary inhibition assay based on previous adsorption with SKC-2(373-414) peptide.

- 25 **Figure 4:** Recognition of SKC-2(373-414) peptide by sera from normal donors. Schematic representation of the results.

Figure 5: Plasmid pEM-1, carrying the sequence of the mutant *skc-2-N13* gene under the *E. coli* tryptophan promoter, and displaying bacteriophage T4 termination signal at the 3' end of the gene in order to provide higher stability to expression.

Figure 6: Plasmid pSKH-11, carrying the sequence of the mutant skc-2-N13 gene fused to the coding sequence for Asp-Ile-Val-Asp-Gly-Gly-6xHis tail under the *E. coli* tryptophan promoter, and displaying bacteriophage T4 termination signal at the 3' end of the gene in order to provide higher stability to expression.

- 5 **Figure 7:** Plasmid pIJ-4, carrying the sequence of the mutant skc-2-C42 under the *E. coli* tryptophan promoter, and displaying bacteriophage T4 termination signal at the 3' end of the gene in order to provide higher stability to expression.

- 10 **Figure 8:** Plasmid pMC-8, carrying the sequence of the mutant skc-2-C42 gene fused to the coding sequence Asp-Ile-Val-Asp-Gly-Gly-6xHis tail under the *E. coli* tryptophan promoter, and displaying bacteriophage T4 termination signal at the 3' end of the gene in order to provide higher stability to expression.

Figure 9: mut-N13 and mut-C42 direct binding assay by anti-SKC-2 human antibodies from patients treated with Heberkinase®.

- 15 **Figure 10:** 50% inhibition ($\square$ g/ml) of SKC-2, mut-N13 and mut-C42 for each patient treated with Heberkinase®.

Figure 11: Mean and standard deviation of values represented in Figure 10.

Figure 12: Mean and standard deviation of values represented in Figure 10 expressed in terms of percent with respect to SKC-2.

- 20 **Figure 13:** Neutralizing Activity Titer (NAT) against SKC-2 and mut-C42 proteins in patients receiving Heberkinase® therapy.

Figure 14: Neutralizing Activity Titer (NAT) of monkey sera against SKC-2 and mut-2 proteins.

Sequence Listing:

(1) GENERAL INFORMATION:

- (i) APPLICANT:
(A) ADDRESSEE: CENTRO DE INGENIERIA GENETICA Y BIOTECNOLOGIA.
(B) STREET: Ave. 31 entre 158 y 190, Cubanacan, Playa.
(C) CITY: C. Habana.
(D) STATE: Ciudad Habana.
(E) COUNTRY: Cuba.
(F) ZIP: 12100
(G) TELEPHONE: 53 7 216013
(H) TELEFAX: 53 7 336008
(ii) TITLE OF INVENTION: Mutantes de Estreptoquinasa.
(iii) NUMBER OF SEQUENCES: 14
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) LOGIC SOPORT: Patentin Release #1.0, Version #1.30 (EPO)

(2) INFORMACIÓN FOR NO. IDENTIF. SEQ. 1:

- (i) SEQUENCE CHARACTERISTICS :
(A) LENGHT: 401 amino acidos
(B) TYPE: amino acids
(C) STRANDEDNESS: unknow
(D) TOPOLOGY: unknow
(ii) MOLECULE TYPE: protein
(iii) HIPOTÉTIC: NO
(iv) ANTI-SENSE: NO
(v) FRAGMENT TYPE: C-terminal
(vi) ORIGIN SOURCE OF:
(A) ORGANISM: Streptococcus equisimilis
(B) STRAIN: ATCC 9542
(C) AISLADE INDIVIDUAL: AISLADE
(vii) IMMEDIATE SOURCE:
(B) CLON: pEMI-1

(x) INFORMATION ABOUT PUBLICATION:

- (H) NUMBER OF DOCUMENT: EP 0 489 201 B1
(I) DATA OF PRESENTATION: 17-JUL-1990
(J) DATA OF PUBLICATION: 10-JUN-1992

(xi) SEQUENCE DESCRIPTION: NO. IDENTIF. SEQ. 1:

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- 24 -

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35 40 45

Pro Phe Ala Thr Asp Ser Gly Ala Met Pro His Lys Leu Glu Lys Ala
50 55 60

Asp Leu Leu Lys Ala Ile Gln Glu Gln Leu Ile Ala Asn Val His Ser
65 70 75 80

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85 90 95

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Asp Phe Arg Pro Gly Leu Lys Asp Thr Lys Leu Leu Lys Thr Leu Ala
165 170 175

Ile Gly Asp Thr Ile Thr Ser Gln Glu Leu Leu Ala Gln Ala Gln Ser
180 185 190

Ile Leu Asn Lys Thr His Pro Gly Tyr Thr Ile Tyr Glu Arg Asp Ser
195 200 205

Ser Ile Val Thr His Asp Asn Asp Ile Phe Arg Thr Ile Leu Pro Met
210 215 220

Asp Gln Glu Phe Thr Tyr His Val Lys Asn Arg Glu Gln Ala Tyr Glu
225 230 235 240

Ile Asn Lys Lys Ser Gly Leu Asn Glu Glu Ile Asn Asn Thr Asp Leu
245 250 255

Ile Ser Glu Lys Tyr Tyr Val Leu Lys Lys Gly Glu Lys Pro Tyr Asp
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Pro Phe Asp Arg Ser His Leu Lys Leu Phe Thr Ile Lys Tyr Val Asp
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Val Asn Thr Asn Glu Leu Leu Lys Ser Glu Gln Leu Leu Thr Ala Ser
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Glu Arg Asn Leu Asp Phe Arg Asp Leu Tyr Asp Pro Arg Asp Lys Ala
305 310 315 320

Lys Leu Leu Tyr Asn Asn Leu Asp Ala Phe Gly Ile Met Asp Tyr Thr
325 330 335

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Leu Thr Gly Lys Val Glu Asp Asn His Asp Asp Thr Asn Arg Ile Ile
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Thr Val Tyr Met Gly Lys Arg Pro Glu Gly Glu Asn Ala Ser Tyr His
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Leu Ala Tyr Asp Lys Asp Arg Tyr Thr Glu Glu Glu Arg Glu Val Tyr
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385 390 395 400
Lys
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2) INFORMATION FOR NO. IDENTIF. SEQ. 2:

(i) SEQUENCE CHARACTERISTICS :

- (A) LENGHT: 413 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: unknow
(D) TOPOLOGY: unknow

(ii) MOLECULE TYPE: protein

(iii) HIPOTÉTIC: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: C-terminal

(v) TIPO DE FRAGMENTO: C-terminal

(vi) ORIGIN SOURCE OF:

- (A) ORGANISM: Streptococcus equisimilis
(B) STRAIN: ATCC 9542
(C) AISLADE INDIVIDUAL: AISLADE

(vii) IMMEDIATE SOURCE:

- (B) CLON: pSKH-11

(x) INFORMATION ABOUT PUBLICATION:

(H) NÚMBER OF DOCUMENT: EP 0 489 201 B1

(I) DATA OF PRESENTATION: 17-JUL-1990

(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) DESCRIPCIÓN DE LA SECUENCIA: NO. IDENTIF. SEQ. 2:

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Asn Gln Asp Ile Ser Leu Lys Phe Phe Glu Ile Asp Leu Thr Ser Arg
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 50 55 60
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 65 70 75 80
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 100 105 110
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 115 120 125
 Arg Val Arg Pro Tyr Lys Glu Lys Pro Ile Gln Asn Gln Ala Lys Ser
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 325 330 335
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 340 345 350
 Thr Val Tyr Met Gly Lys Arg Pro Glu Gly Glu Asn Ala Ser Tyr His
 355 360 365
 Leu Ala Tyr Asp Lys Asp Arg Tyr Thr Glu Glu Glu Arg Glu Val Tyr
 370 375 380
 Ser Tyr Leu Arg Tyr Thr Gly Thr Pro Ile Pro Asp Asn Pro Asn Asp
 385 390 395 400
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 405 410

(2) INFORMACIÓN PARA NO. IDENTIF. SEQ. 3:

(i) SEQUENCE CHARACTERISTICS :

- (A) LENGTH: 372 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknow
- (D) TOPOLOGY: unknow

(ii) MOLECULE TYPE: protein

(iii) HIPOTÉTICA: NO

(iii) HIPOTÉTIC: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGIN SOURCE OF:

- (A) ORGANISM: Streptococcus equisimilis
- (B) STRAIN: ATCC 9542
- (C) AISLADE INDIVIDUAL: AISLADE

(vii) IMMEDIATE SOURCE:

- (B) CLON: pIJ-4

(x) INFORMATION ABOUT PUBLICATION:

(H) NUMBER OF DOCUMENT: EP 0 489 201 B1

(I) DATA OF PRESENTATION: 17-JUL-1990

(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) DESCRIPTION OF SEQUENCE: NO. IDENTIF. SEQ. 3:

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Gln Leu Val Val Ser Val Ala Gly Thr Val Glu Gly Thr Asn Gln Asp
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Lys Ala Ile Gln Glu Gln Leu Ile Ala Asn Val His Ser Asn Asp Asp
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Tyr Asn Asn Leu Asp Ala Phe Gly Ile Met Asp Tyr Thr Leu Thr Gly
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Lys Val Glu Asp Asn His Asp Asp Thr Asn Arg Ile Ile Thr Val Tyr
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Met Gly Lys Arg
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(2) INFORMATION FOR NO. IDENTIF. SEQ. 4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGHT: 384 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknow
- (D) TOPOLOGY: unknow

- (ii) MOLECULE TYPE: protein
(iii) HIPOTETIC: NO
(iv) ANTI-SENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGIN SOURCE OF:
(A) ORGANISM: Streptococcus equisimilis
(B) STRAIN: ATCC 9542
(C) AISLADE INDIVIDUAL: AISLADE

- (vii) IMMEDIATE SOURCE:
(B) CLON: pMC-8
x) INFORMATION ABOUT PUBLICATION:
(H) NUMBER OF DOCUMENT: EP 0 489 201 B1
(I) DATA OF PRESENTATION: 17-JUL-1990
(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 4:

Ile Ala Gly Pro Glu Trp Leu Leu Asp Arg Pro Ser Val Asn Asn Ser
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Gln Leu Val Val Ser Val Ala Gly Thr Val Glu Gly Thr Asn Gln Asp
20 25 30
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35 40 45
Gly Gly Lys Thr Glu Gln Gly Leu Ser Pro Lys Ser Lys Pro Phe Ala
50 55 60
Thr Asp Ser Gly Ala Met Pro His Lys Leu Glu Lys Ala Asp Leu Leu
65 70 75 80
Lys Ala Ile Gln Glu Gln Leu Ile Ala Asn Val His Ser Asn Asp Asp
85 90 95
Tyr Phe Glu Val Ile Asp Phe Ala Ser Asp Ala Thr Ile Thr Asp Arg
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Asn Gly Lys Val Tyr Phe Ala Asp Lys Asp Gly Ser Val Thr Leu Pro
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Pro Tyr Lys Glu Lys Pro Ile Gln Asn Gln Ala Lys Ser Val Asp Val
145 150 155 160

- 30 -

Glu Tyr Thr Val Gln Phe Thr Pro Leu Asn Pro Asp Asp Asp Phe Arg
165 170 175

Pro Gly Leu Lys Asp Thr Lys Leu Leu Lys Thr Leu Ala Ile Gly Asp
180 185 190

Thr Ile Thr Ser Gln Glu Leu Leu Ala Gln Ala Gln Ser Ile Leu Asn
195 200 205

Lys Thr His Pro Gly Tyr Thr Ile Tyr Glu Arg Asp Ser Ser Ile Val
210 215 220

Thr His Asp Asn Asp Ile Phe Arg Thr Ile Leu Pro Met Asp Gln Glu
225 230 235 240

Phe Thr Tyr His Val Lys Asn Arg Glu Gln Ala Tyr Glu Ile Asn Lys
245 250 255

Lys Ser Gly Leu Asn Glu Glu Ile Asn Asn Thr Asp Leu Ile Ser Glu
260 265 270

Lys Tyr Tyr Val Leu Lys Lys Gly Glu Lys Pro Tyr Asp Pro Phe Asp
275 280 285

Arg Ser His Leu Lys Leu Phe Thr Ile Lys Tyr Val Asp Val Asn Thr
290 295 300

Asn Glu Leu Leu Lys Ser Glu Gln Leu Leu Thr Ala Ser Glu Arg Asn
305 310 315 320

Leu Asp Phe Arg Asp Leu Tyr Asp Pro Arg Asp Lys Ala Lys Leu Leu
325 330 335

Tyr Asn Asn Leu Asp Ala Phe Gly Ile Met Asp Tyr Thr Leu Thr Gly
340 345 350

Lys Val Glu Asp Asn His Asp Asp Thr Asn Arg Ile Ile Thr Val Tyr
355 360 365

Met Gly Lys Arg Asp Ile Val Asp Gly Gly His His His His His His
370 375 380

(2) INFORMATION FOR NO. IDENTIF. SEQ. 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGHT: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: sigle
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE : another nucleic acid

- (A) DESCRIPCIÓN = "oligonucleotide"

(iii) HIPOTETIC: NO

(iv) ANTI-SENSE: NO

(x) INFORMATION ABOUT PUBLICATION:

(H) NÚMBER OF DOCUMENT: EP 0 489 201 B1

(I) DATA OF PRESENTATION: 17-JUL-1990

(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 5:

GGAATTCATG AACAACAGCC AATTAGTTGT

30

(2) INFORMATION FOR NO. IDENTIF. SEQ. 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGHT::30 base pairs

(B) TYPE: nucleic acid

(C)) STRANDEDNESS: sigle

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE : another nucleic acid

(A) DESCRIPCIÓN: "oligonucleotide"

(iii) HIPOTETIC: NO

(iv) ANTI-SENSE: NO

(x) INFORMATION ABOUT PUBLICATION:

(H) NÚMBER OF DOCUMENT: EP 0 489 201 B1

(I) DATA OF PRESENTATION: 17-JUL-1990

(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 6:

CGGATCCTTA TTTGTCGTTA GGGTATCAG

30

(2) INFORMATION FOR NO. IDENTIF. SEC. 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGHT: 27 base pairs

(B) TYPE: nucleic acid

(C)) STRANDEDNESS: sigle

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE : another nucleic acid

(A) DESCRIPCIÓN: "oligonucleotide"

(iii) HIPOTETIC: NO
(iv) ANTI-SENSE: NO

(x) INFORMATION ABOUT PUBLICATION:
(H) NUMBER OF DOCUMENT: EP 0 489 201 B1
(I) DATA OF PRESENTATION: 17-JUL-1990
(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 7:
CGATATCTTT GTCGTTAGGG TTATCAG

27

(2) INFORMATION FOR NO. IDENTIF. SEQ. 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGHT: 29 base pairs
(B) TYPE: nucleic acid
(C)) STRANDEDNESS: sigle
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE : another nucleic acid
(A) DESCRIPCIÓN: "oligonucleotide"

(iii) HIPOTETIC: NO
(iv) ANTI-SENSE: NO

(x) INFORMATION ABOUT PUBLICATION:
(H) NUMBER OF DOCUMENT: EP 0 489 201 B1
(I) DATA OF PRESENTATION: 17-JUL-1990
(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 8:
GGAATTCATG ATTGCTGGAC CTGAGTGGC

29

(2) INFORMATION FOR NO. IDENTIF. SEQ. 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGHT: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: sigle
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE : another nucleic acid
(A) DESCRIPCIÓN: "oligonucleotide"

(iii) HIPOTETIC: NO
(iv) ANTI-SENSE: NO

(x) INFORMATION ABOUT PUBLICATION:
(H) NUMBER OF DOCUMENT: EP 0 489 201 B1
(I) DATA OF PRESENTATION: 17-JUL-1990

(J) DATA OF PUBLICATION: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 9:

CGGATCCTTA TCGCTTGCCC ATATAAACGG

30

(2) INFORMATION FOR NO. IDENTIF. SEQ. 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGHT: 27 base pairs

(B) TYPE: nucleic acid

(C)) STRANDEDNESS: sigle

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE : another nucleic acid

(A) DESCRIPCIÓN: "oligonucleotide"

(iii) HIPOTETIC: NO

(iv) ANTI-SENSE: NO

(x) INFORMATION ABOUT PUBLICATION:

(H) NÚMBER OF DOCUMENT: EP 0 489 201 B1

(I) DATA OF PRESENTATION: 17-JUL-1990

(J) DATA OF PUBLICATION: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 10:

CGATATCTCG CTTGCCCATATAAACGG

27

(2) INFORMATION FOR NO. IDENTIF. SEQ. 11:

(i) SEQUENCE CHARACTERISTICS

(A) LENGHT: 1209 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: sigle

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HIPOTETIC: NO

(iv) ANTI-SENSE: NO

(v) TIPO DE FRAGMENTO: C-terminal

(vi) ORIGIN SOURCE OF:

(A) ORGANISM: Streptococcus equisimilis

(B) STRAIN: ATCC 9542

(C) AISLADE INDIVIDUAL: AISLADE

(vii) IMMEDIATE SOURCE:

(B) CLON: pEMI-1

(x) INFORMATION ABOUT PUBLICATION:

(H) NÚMBER OF DOCUMENT: EP 0 489 201 B1

(I) DATA OF PRESENTATION: 17-JUL-1990

(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 11:

ATGAACAACA GCCAATTAGT TGTAGCGTT GCTGGTACTG TTGAGGGGAC GAATCAAGAC	60
ATTAGTCTTA AATTTTTTGA AATTGACCTA ACATCAGCAG CTGCTCATGG AGGAAAAGACA	120
GAGCAAGGCT TAAGTCCAAA ATCAAAACCA TTGCTACTG ATAGTGGCGC GATGCCACAT	180
AACTTGAAA AAGCTGACTT ACTAAAGGCT ATTCAAGAAC AATTGATCGC TAACGTCCAC	240
AGTAACGACG ACTACTTTGA GGTCATTGAT TTTGCAAGCG ATGCAACCAT TACTGATCGA	300
AACGGCAAGG TCTACTTTGC TGACAAAGAT GGTTCGGTAA CCTTGCCGAC CCAACCTGTC	360
CAAGAATTTT TGCTAAGCGG ACATGTGCGC GTTAGACCAT ATAAAGAAAA ACCAATACAA	420
AATCAAGCGA AATCTGTTGA TGTGGAATAT ACTGTACAGT TTAATCCCTT AAACCCGTGAT	480
GAGGATTICA GACCAGGTCT CAAAGATACT AAGCTATTGA AAACACTAGC TATCGGTGAC	540
ACCATCACAT CTCAGAATTT ACTAGCTCAA GCACAAAGCA TTTTAAACAA AACCCACCCA	600
GGCTATACGA TTTATGAACG TGACTCCTCA ATCGTCACTC ATGACAATGA CATTTTCCGT	660
ACGATTTTAC CAATGGATCA AGAGTTTACT TACCATGTCA AAAATCGGGA ACAAGCTTAT	720
GAGATCAATA AAAATCTGG TCTGAATGAA GAAATAACA AACTGACCT GATCTCTGAG	780
AAATATTACG TCCTTAAAA AGGGGAAAAG CCGTATGATC CCTTTGATCG CAGTCACTTG	840
AACTGTTC CAATCAAATA CGTTGATGTC AACACCAACG AATTGCTAAA AAGCGAGCAG	900
CTCTTAACAG CTAGCGAACG TAACTTAGAC TTCAGAGATT TATACGATCC TCGTGATAAG	960
GCTAAACTAC TCTACAACAA TCTCGATGCT TTTGGTATTA TGGACTATAC CTTAACTGGA	1020
AAAGTAGAGG ATAATCACGA TGACACCAAC CGTATCATAA CCGTTTATAT GGGCAAGCGA	1080
CCCGAAGGAG AGAATGCTAG CTATCATTTA GCCTATGATA AAGATCGTTA TACCGAAGAA	1140
GAACGAGAAG TTTACAGCTA CCTGCGTTAT ACAGGGACAC CTATACCTGA TAACCCTAAC	1200
GACAAATAA	1209

(2) INFORMATION FOR NO. IDENTIF. SEQ.: 12:

(i) SEQUENCE CHARACTERISTICS

(A) LENGHT: 1245 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: sigle

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (iii) HIPOTETIC: NO
(iv) ANTI-SENSE: NO
(v) TIPO DE FRAGMENTO: C-terminal
(vi) ORIGIN SOURCE OF:
(A) ORGANISM: Streptococcus equisimilis
(B) STRAIN: ATCC 9542
(C) AISLADE INDIVIDUAL: AISLADE
(vii) INMEDIATE SOURCE
(B) CLON: pSKH-11
(x) INFORMATION ABOUT PUBLICATION:
(H) NUMBER OF DOCUMENT: EP 0 489 201 B1
(I) DATA OF PRESENTATION: 17-JUL-1990
(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) SEQUENCE DESCRIPTION: NO. IDENTIF. SEC. 12:

ATGAACAACA GCCAATTAGT TGTAGCGTT GCTGGTACTG TTGAGGGGAC GAATCAAGAC	60
ATTAGTCTTA AATTTTTTGA AATTGACCTA ACATCAGGAC CTGCTCATGG AGGAAAGACA	120
GAGCAAGGCT TAAGTCCAAA ATCAAAACCA TTGCTACTG ATAGTGGCGC GATGCCACAT	180
AAACTTGAAA AAGCTGACTT ACTAAAGGCT ATTCAAGAAC AATTGATCGC TAACGTCCAC	240
AGTAACGACG ACTACTTTGA GGTCATTGAT TTTGCAAGCG ATGCAACCAT TACTGATCGA	300
AACGGCAAGG TCTACTTTGC TGACAAAGAT GGTTCGGTAA CCTTGCCGAC CCAACCTGTC	360
CAAGAAATTT TGCTAAGCGG ACATGTGCGC GTTAGACCAT ATAAAGAAAA ACCAATACAA	420
AATCAAGCGA AATCTGTTGA TGTGGAATAT ACTGTACAGT TTAATCCCTT AAACCTGAT	480
GACGATTTCA GACCAGTCT CAAAGATACT AAGCTATTGA AAACACTAGC TATCGGTGAC	540
ACCATCACAT CTCAAGAATT ACTAGCTCAA GCACAAAGCA TTTTAAACAA AACCCACCCA	600
GGCTATACGA TTTATGAACG TGACTCCTCA ATCGTCACTC ATGACATGA CATTTTCCGT	660
ACGATTTTAC CAATGGATCA AGAGTTTACT TACCATGTCA AAAATCGGGA ACAAGCTTAT	720
GAGATCAATA AAAAATCTGG TCTGAATGAA GAAATAACA AACTGACCT GATCTCTGAG	780
AAATATTACG TCCTTAAAAA AGGGGAAAAG CCGTATGATC CCTTTGATCG CAGTCACTTG	840
AAACTGTTCA CCATCAAATA CGTTGATGTC AACACCAACG AATTGCTAAA AAGCGAGCAG	900
CTCTTAACAG CTAGCGAACG TAACTTAGAC TTCAGAGATT TATACGATCC TCGTGATAAG	960
GCTAAACTAC TCTACAACAA TCTCGATGCT TTTGGTATTA TGGACTATAC CTTAACTGGA	1020
AAAGTAGAGG ATAATCACGA TGACACCAAC CGTATCATAA CCGTTTATAT GGGCAAGCGA	1080
CCCGAAGGAG AGAATGCTAG CTATCATTTA GCCTATGATA AAGATCGTTA TACCGAAGAA	1140
GAACGAGAAG TTTACAGCTA CCTGCGTTAT ACAGGGACAC CTATACCTGA TAACCCTAAC	1200

GACAAAGATA TCGTCGACGG GGGGCACCAC CACCACCACC ACTAA

1245

(2) INFORMATION FOR NO. IDENTIF. SEQ. 13:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGHT: 1122 pares de base
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: sigle
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HIPOTETIC: NO
- (iv) ANTI-SENSE: NO
- (v) TYPE OF FRAGMENT: N-terminal
- (vi) ORIGIN SOURCE OF:
 - (A) ORGANISM: Streptococcus equisimilis
 - (B) STRAIN: ATCC 9542
 - (C) AISLADE INDIVIDUAL: AISLADE
- (vii) IMMEDIATE SOURCE:
 - (B) CLON: pIJ-4

(x) INFORMATION ABOUT PUBLICATION:

(H) NÚMBER OF DOCUMENT: EP 0 489 201 B1

(I) DATA OF PRESENTATION: 17-JUL-1990

(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 13:

ATGATTGCTG GACCTGAGTG GCTGCTAGAC CGTCCATCTG TCAACAACAG CCAATTAGTT	60
GTTAGCGTTG CTGGTACTGT TGAGGGGACG AATCAAGACA TTAGTCTTAA ATTTTTTGAA	120
ATTGACCTAA CATCACGACC TGCTCATGGA GGAAAGACAG AGCAAGGCTT AAGTCCAAAA	180
TCAAAACCAT TTGCTACTGA TAGTGGGCGG ATGCCACATA AACTTGAAAA AGCTGACTTA	240
CTAAAGGCTA TTCAAGAACA ATTGATCGCT AACGTCCACA GTAACGACGA CTACTTTGAG	300
GTCATTGATT TTGCAAGCGA TGCAACCATT ACTGATCGAA ACGGCAAGGT CTACTTTGCT	360
GACAAAGATG GTTCGGTAAC CTTGCCGACC CAACCTGTCC AAGAATTTT GCTAAGCGGA	420
CATGTGCGCG TTAGACCATA TAAAGAAAA CCAATACAAA ATCAAGCGAA ATCTGTTGAT	480
GTGGAATATA CTGTACAGTT TACTCCCTTA AACCCGTATG ACGATTTTAC ACCAGGTCTC	540
AAAGATACTA AGCTATTGAA AACACTAGCT ATCGGTGACA CCATCACATC TCAAGAATTA	600
CTAGCTCAAG CACAAAGCAT TTAAACAAA ACCCACCAG GCTATACGAT TTATGAACGT	660
GACTCCTCAA TCGTCACTCA TGACAATGAC ATTTTCCGTA CGATTTTACC AATGGATCAA	720
GAGTTTACTT ACCATGTCAA AAATCGGGAA CAAGCTTATG AGATCAATAA AAAATCTGGT	780

CTGAATGAAG AAATAAACAA CACTGACCTG ATCTCTGAGA AATATTACGT CCTTAAAAA 840
GGGGAAAAGC CGTATGATCC CTTTGATCGC AGTCACTTGA AACTGTTTAC CATCAAATAC 900
GTTGATGTCA ACACCAACGA ATTGCTAAAA AGCGAGCAGC TCTTAACAGC TAGCGAACGT 960
AACTTAGACT TCAGAGATTT ATACGATCCT CGTGATAAGG CTAACTACT CTACAACAAT 1020
CTCGATGCTT TTGGTATTAT GGACTATACC TTAAGTGGAA AAGTAGAGGA TAATCAGGAT 1080
GACACCAACC GTATCATAAC CGTTTATATG GGCAAGCGAT AA 1122

(2) INFORMATION FOR NO. IDENTIF. SEQ. 14:

(ii) SEQUENCE CHARACTERISTICS

- (A) LENGHT: 1158 pares de base
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: sigle
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HIPOETIC: NO

(iv)ANTI-SENSE: NO

(v)TYPE OF FRAGMENT: N-terminal

(vi) ORIGIN SOURCE OF:

- (A) ORGANISM: Streptococcus equisimilis
- (B) STRAIN: ATCC 9542
- (C) AISLADE INDIVIDUAL: AISLADE

(vii) IMMEDIATE SOURCE:

- (B) CLON: pMC-8

(x) INFORMATION ABOUT PUBLICATION:

(H) NÚMBER OF DOCUMENT: EP 0 489 201 B1

(I) DATA OF PRESENTATION: 17-JUL-1990

(J) DATA OF PUBLICACIÓN: 10-JUN-1992

(xi) SECUENCE DESCRIPTION: NO. IDENTIF. SEQ. 14:

ATGATTGCTG GACCTGAGTG GCTGCTAGAC CGTCCATCTG TCAACAACAG CCAATTAGTT 60
GTTAGCGTTG CTGGTACTGT TGAGGGGACG AATCAAGACA TTAGTCTTAA ATTTTGTGAA 120
ATTGACCTAA CATCACGACC TGCTCATGGA GGAAGACAG AGCAAGGCTT AAGTCCAAAA 180
TCAAAACCAT TTGCTACTGA TAGTGGCGCG ATGCCACATA AACTTGAAAA AGCTGACTTA 240
CTAAAGGCTA TTCAAGAACA ATTGATCGCT AACGTCCACA GTAACGACGA CTACTTTGAG 300
GTCATTGATT TTGCAAGCGA TGCAACCATT ACTGATCGAA ACGGCAAGGT CTACTTTGCT 360
GACAAAGATG GTTCGGTAAC CTTGCCGACC CAACCTGTCC AAGAATTTT GCTAAGCGGA 420
CATGTGCGCG TTAGACCATA TAAAGAAAAA CCAATACAAA ATCAAGCGAA ATCTGTTGAT 480

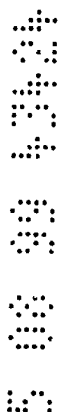
GTGGAATATA CTGTACAGTT TACTCCCTTA AACCCGTGATG ACGATTTTCAG ACCAGGTCTC 540
AAAGATACTA AGCTATTGAA AACACTAGCT ATCGGTGACA CCATCACATC TCAAGAATTA 600
CTAGCTCAAG CACAAAGCAT TTAAACAAA ACCCACCAG GCTATACGAT TTATGAACGT 660
GACTCCTCAA TCGTCACTCA TGACAATGAC ATTTCCGTA CGATTTTACC AATGGATCAA 720
GAGTTTACTT ACCATGTCAA AAATCGGGAA CAAGCTTATG AGATCAATAA AAAATCTGGT 780
CTGAATGAAG AAATAAACAA CACTGACCTG ATCTCTGAGA AATATTACGT CCTTAAAAA 840
GGGGAAAAGC CGTATGATCC CTTTGATCGC AGTCACTTGA AACTGTTTAC CATCAAATAC 900
GTTGATGTCA ACACCAACGA ATTGCTAAAA AGCGAGCAGC TCTTAACAGC TAGCGAACGT 960
AACTTAGACT TCAGAGATTT ATACGATCCT CGTGATAAGG CTAACTACT CTACAACAAT 1020
CTCGATGCTT TTGGTATTAT GGACTATACC TTAAGTGGAA AAGTAGAGGA TAATCACCAT 1080
GACACCAACC GTATCATAAC CGTTTATATG GGCAAGCGAG ATATCGTCGA CGGGGGGCAC 1140
CACCACCACC ACCACTAA 1158

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REFERENCES

- Astrup, T. and Mullertz, S. (1952) *Arch. Biochem. Biophys* 40, 346-351.
- Bruserund, O. (1990) *APMIS* 98, 1077-1084.
- Bruserund, O.; Elsayed, S. and Pawelec, G. (1992) *Mol. Immunol.* 29, 1097-1104.
- Bruserund, O.L.; Sollid, L. and Foyn-Jorgensen, P. (1986) *J. Clin. Lab. Immun.* 20, 69-74.
- Dagert, M., and Erlich, S.L. (1974) *Gene* 6: 23-28.
- Davies, K.A.; Mathieson, P.; Winearis, C.G.; Rees, A.J.; and Walport, M.J. (1990) *Clin. Exp. Immunol.* 80, 83
- Estrada, M. P., Hernández, L., Pérez, A., Rodríguez, P., Serrano, R., Rubiera, R., Pedraza, A., Padrón, G., Antuch, W., de la Fuente, J. and Herrera, L. (1992) *Biotechnology* 10, 1138-1142.
- Fiberger, P. (1982) *J. Clin. Lab. Invest.* 42, Suppl. 162, 49-54.
- Frank, R. (1992) *Tetrahedron* 48, 9217-9212.
- Gonzalezgronow, M.; Enghild, J.J.; Pizzo, S.V. (1993) *Biochimica et Biophysica Acta* 1180, 283-288
- Hanahan, D. (1983) *J. Mol. Biol.* 166, 557-580.
- Maniatis, T.; Fritsch, E.F. and Sambrook, J. (1982) Cold Spring Harbor Laboratory, USA.
- McGrath, K.G.; Zeffren, B.; Alexander, J.; Kaplan, K. and Patterson, R. (1985) *J. Allergy Clin. Immunol.* 76, 453
- Parhami-Seren, B.; Keel, T. and Reed, G.L. (1996) *Hybridoma* 15, 169-176
- Parhami-Seren, B.; Lynch, M.; White, H.D. and Reed, G.L. (1995) *Mol. Immunol.* 32, 717-724
- Patente europea No. EP 0 489 201 B1.
- Patente US No. 5,240,845.
- Randall, K.; Gelfond, D.H.; Stoffel, S.; Scharf, S.; Higuchi, R.; Horn, G.T.; Mullis, K.B. and Erlich, H.A. (1988) *Science* 239, 487-491.
- Rao, A.K.; Pratt, C.; Berke, A.; Jaffe, A.; Ockene, L.; Schreiber, T.L.; Bell, W.R.; Knaterund, G.; Robertson, T.L. and Terrin, M.L. (1988) *J. Am. Coll. Cardiol.* 11,1
- Reed, G.L.; Kussie, P. and Parhami-Seren, B. (1993) *J. Immunol.* 150, 4407-4415.
- Sanger, F.; Nickler, S. and Coulson, A.K. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.
- Schick, L.A. and Castellino, F.J. (1974) *Biochem. Biophys. Res. Commun.* 57, 47-54
- Schweitzer, D.H.; Van der Wall, E.E.; Bosker, H.A.; Scheffer, E. and Macfarlane, J.D. (1991) *Cardiology* 78, 68.
- Sorber, W.A. and Herbst, V. (1988) *Cutis* 42, 57
- Spottl, F. and Kaiser, R. (1974) *Thromb. Diath. Haemorrh.* 32, 608

- Tillet, W. S. and Garner, R.L.(1933) J. Exp. Med. 58, 485-502.
- Tillet, W.S; Edwards, E.D. and Garner, R.L. (1934) J. Clin. Invest. 13, 47-78
- Urdahl, K.B.; Mathews, J.D.; Currie, B. (1996) Australian and New Zealand J. Med. 26,49-53
- Westlund, L.E. and Anderson, L.O. (1985) Thrombosis Research 37, 213-223.
- Youkeles, L.H.; Soliman, M.Y. and Rosenstreich, D.L. (1991) J. Allergy Clin. Immunol. 88, 166-171.



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A method for producing streptokinase mutants by expression of a gene encoding said mutant streptokinase which comprises the steps of transforming a host cell with an expression vector containing a streptokinase gene skc-2 fragment (40-1245) consisting essentially of the nucleotide sequence (Seq. Ident. No. 11):

5	ATGAACAACA GCCAATTAGT TGTTAGCGTT GCTGGTACTG TTGAGGGGAC GAATCAAGAC	60
	ATTAGTCTTA AATTTTTTGA AATTGACCTA ACATCAGCAG CTGCTCATGG AGGAAAGACA	120
	GAGCAAGGCT TAAGTCCAAA ATCAAAACCA TTTGCTACTG ATAGTGGGCG GATGCCACAT	180
	AAACTTGAAA AAGCTGACTT ACTAAAGGCT ATTCAAGAAC AATTGATCGC TAACGTCCAC	240
10	AGTAAAGCAG ACTACTTTGA GGICATTGAT TTTGCAAGCG ATGCAACCAT TACTGATCGA	300
	AACGGCAAGG TCTACTTTGC TGACAAAGAT GGTTCGGTAA CCTTGCCGAC CCAACCTGTC	360
	CAAGAATTTT TGCTAAGCGG ACATGTGCGC GTTAGACCAT ATAAGAAAA ACCAATACAA	420
	AATCAAGCGA AATCTGTGTA TGTGGAATAT ACTGTACAGT TTACTCCCTT AAACCTGAT	480
	GACGATTCA GACCAGGTCT CAAAGATACT AAGCTATTGA AAACACTAGC TATCGGTGAC	540
15	ACCATCACAT CTCAGAATT ACTAGCTCAA GCACAAAGCA TTTTAAACA AACCCACCCA	600
	GGCTATACGA TTTATGAACG TGACTCCTCA ATCGTCACTC ATGACAATGA CATTTCCTGT	660
	ACGATTTTAC CAATGGATCA AGAGTTTACT TACCATGTCA AAAATCGGGA ACAAGCTTAT	720
	GAGATCAATA AAAATCTGG TCTGAATGAA GAAATAAACA ACACTGACCT GATCTCTGAG	780
	AAATATTACG TCCTTAAAAA AGGGGAAAAG CCGTATGATC CCTTTGATCG CAGTCACTTG	840
20	AAACTGTTCA CCATCAATA CGTTGATGTC AACACCAACG AATTGTAA AAGCGAGCAG	900
	CTCTTAACAG CTAGCGAACG TAACCTTAGAC TTCAGAGATT TATACGATCC TCGTGATAAG	960
	GCTAACTAC TCTACAACA TCTCGATGCT TTTGSTATTA TGGACTATAC CTTAACTGGA	1020
	AAAGTAGAGG ATAATCAGTA TGACACCAAC CGTATCATAA CCGTTTATAT GGGCAAGCGA	1080
	CCCGAAGGAG AGAATGCTAG CTATCATTTA GCCTATGATA AAGATCGTTA TACCGAAGAA	1140
25	GAACGAGAAG TTTACAGCTA CCTGCGTTAT ACAGGGACAC CTATACCTGA TAACCTAAC	1200
	GACAAATAA	1209

2. A method for producing streptokinase mutants by expression of a gene encoding said mutant streptokinase which comprises the steps of transforming a host cell with an expression vector containing a streptokinase gene *skc-2* fragment (40-1245) fusioned to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence (Seq. Ident. No. 12):

5	AACAACAGCC AATTAGTTGT TAGCGTTGCT GGTACTGTTG AGGGGACGAA TCAAGACATT	60
	AGTCTTAAAT TTTTGAAT TGACCTAACA TCACGACCTG CTCATGGAGG AAAGACAGAG	120
	CAAGGCTTAA GTCCAAAATC AAAACCATTT GCTACTGATA GTGGCGGAT GCCACATAAA	180
	CTTGAAAAG ACGACTTACT AAAGGCTATT CAAGAACAAT TGATCGCTAA CGTCCACAGT	240
	AACGACGACT CATTGAGGT CATTGATTTT GCAAGCGATC GAACCATAC TGATCGAAAC	300
10	GGCAAGTCT ACTTGTCTGA CAAAGATGGT TCGGTAACCT TGCCGACCA ACCTGTCCAA	360
	GAATTTTTCG TAAGCGGACA TGTGCGCGTT AGACCATATA AAGAAAAACC AATACAAAAT	420
	CAAGCGAAAT CTGTTGATGT GGAATATACT GTACAGTTTA CTCCCTTAAA CCTGATGAC	480
	GATTTGAGAC CAGGTCTCAA AGATACTAAG CTATTGAAA CACTAGCTAT CGGTGACACC	540
	ATCACATCTC AAGAATTACT AGCTCAAGCA CAAAGCATTT TAAACAAAAC CCACCCAGGC	600
15	TATACGATTT ATGAACGTGA CTCCTCAATC GTCACATG ACAATGACAT TTTCCGTACG	660
	ATTTTACCAA TGGATCAAGA GTTTACTTAC CATGICAAAA ATCGGGAACA AGCTTATGAG	720
	ATCAATAAAA AATCTGGTCT GAATGAAGAA ATAAACAACA CTGACCTGAT CTCTGAGAAA	780
	TATTACGTCC TAAAAAAGG GAAAAAGCCC TATGATCCCT TTGATCGCAG TCACTTGAAA	840
	CTGTTCACCA TCAATACGT TGATGTCAAC ACCAACGAAT TGCTAAAAAG CGAGCAGCTC	900
20	TTAACAGCTA GCGAACGTAA CTTAGACTTC AGAGATTTAT ACGATCCTCG TGATAAGGCT	960
	AAACTACTCT ACAACAATCT CGATGCTTTT GGTATTATGG ACTATACCTT AACTGGAAAA	1020
	GTAGAGGATA ATCAGATGA CACCAACCGT ATCATAACCG TTTATATGGG CAAGCGACCC	1080
	GAAGGAGAGA ATGCTAGCTA TCATTTAGCC TATGATAAAG ATGCTTATAC CGAAGAAGAA	1140
	CGAGAAGTTT ACAGCTACCT GCGTTATACA GGGACACCTA TACCTGATAA CCCTAACGAC	1200
25	AAATAAGATA TCGTCGACGG GGGGCACCAC CACCACCACC ACTAA	1245

3. A method for producing streptokinase mutants by expression of a gene encoding said mutant streptokinase which comprises the steps of transforming a host cell with an expression vector containing a streptokinase gene *skc-2* fragment (1-1119) consisting essentially of the nucleotide sequence (Seq. Ident. No. 13):

		ATGATTGCTG GACCTGAGTG GCTGCTAGAC CGTCCATCTG TCAACAACAG CCAATTAGTT	60
		GTTAGCGTTG CTGGTACTGT TGAGGGGACG AATCAAGACA TTAGTCTTAA ATTTTGTGAA	120
		ATTGACCTAA CATCACGACC TGCTCATGGA GGAAAGACAG AGCAAGGCTT AAGTCCAAAA	180
		TCAAAACCAT TTGCTACTGA TAGTGGCGCG ATGCCACATA AACTTGAAAA AGCTGACTTA	240
5		CTAAAGGCTA TTCAAGAACA ATTGATCGCT AACGTCCACA GTAACGACGA CTACTTTGAG	300
		GTCATTGATT TTGCAAGCGA TGCAACCATT ACTGATCGAA ACGGCAAGGT CTACTTTGCT	360
		GACAAAGATG GTTCGGTAAC CTTGCCGACC CAACCTGTCC AAGAATTTTT GCTAAGCGGA	420
		CATGTGCGCG TTAGACCATA TAAAGAAAAA CCAATACAAA ATCAAGCGAA ATCTGTTGAT	480
		GTGGAATATA CTGTACAGTT TACTCCCTTA AACCCGTGATG ACGATTTCAG ACCAGGTCTC	540
10		AAAGATACTA AGCTATTGAA AACACTAGCT ATCGGTGACA CCATCACATC TCAAGAATTA	600
		CTAGCTCAAG CACAAAGCAT TTTAAACAAA ACCCACCAG GCTATACGAT TTATGAACGT	660
		GACTCCTCAA TCGTCACTCA TGACAATGAC ATTTTCCGTA CGATTTTACC AATGGATCAA	720
		GAGTTTACTT ACCATGTCAA AAATCGGGAA CAAGCTTATG AGATCAATAA AAAATCTGGT	780
		CTGAATGAAG AAATAACAA CACTGACCTG ATCTCTGAGA AATATTACGT CCTAAAAAA	840
15		GGGGAAAAGC CGTATGATCC CTTTGATCGC AGTCACTTGA AACTGTTTAC CATCAAATAC	900
		GTTGATGTCA ACACCAACGA ATTGCTAAAA AGCGAGCAGC TCTTAACAGC TAGCGAACGT	960
		AACTTAGACT TCAGAGATTT ATACGATCCT CGTGATAAGG CTAACTACT CTACAACAAT	1020
		CTCGATGCTT TTGGTATTAT GGACTATACC TTAAGTGGAA AAGTAGAGGA TAATCACGAT	1080
		GACACCAACC GTATCATAAC CGTTTATATG GGCAAGCGAT AA	1122
20	4.	A method for producing streptokinase mutants by expression of a gene encoding said mutant streptokinase which comprises the steps of transforming a host cell with an expression vector containing a streptokinase gene skc-2 fragment (1-1119) fused to the sequence tail Asp-Ile-	
25	Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence (Seq. Ident. No. 14):		
		ATGATTGCTG GACCTGAGTG GCTGCTAGAC CGTCCATCTG TCAACAACAG CCAATTAGTT	60
		GTTAGCGTTG CTGGTACTGT TGAGGGGACG AATCAAGACA TTAGTCTTAA ATTTTGTGAA	120
		ATTGACCTAA CATCACGACC TGCTCATGGA GGAAAGACAG AGCAAGGCTT AAGTCCAAAA	180
30		TCAAAACCAT TTGCTACTGA TAGTGGCGCG ATGCCACATA AACTTGAAAA AGCTGACTTA	240
		CTAAAGGCTA TTCAAGRACA ATTGATCGCT AACGTCCACA GTAACGACGA CTACTTTGAG	300

GTCATTGATT TTGCAAGCGA TGCAACCATT ACTGATCGAA ACGGCAAGGT CTACTTTGCT 360
GACAAAGATG GTTCGGTAAC CTTGCCGACC CAACCTGTCC AAGAATTTT GCTAAGCGGA 420
CATGTGCGCG TTAGACCATA TAAAGAAAAA CCAATACAAA ATCAAGCGAA ATCTGTTGAT 480
GTGGAATATA CTGTACAGTT TACTCCCTTA AACCCGTGATG ACGATTTTACG ACCAGGTCTC 540
5 AAAGATACTA AGCTATTGAA AACACTAGCT ATCGGTGACA CCATCACATC TCAAGAATTA 600
CTAGCTCAAG CACAAAGCAT TTTAAACAAA ACCCACCAG GCTATACGAT TTATGAACGT 660
GACTCCTCAA TCGTCACTCA TGACAATGAC ATTTTCCGTA CGATTTTACC AATGGATCAA 720
GAGTTTACTT ACCATGTCAA AAATCGGGAA CAAGCTTATG AGATCAATAA AAAATCTGGT 780
CTGAATGAAG AAATAACAA CACTGACCTG ATCTCTGAGA AATATTACGT CCTTAAAAA 840
10 GGGGAAAAGC CGTATGATCC CTTTGATCGC AGTCACTTGA AACTGTTTAC CATCAAATAC 900
GTTGATGTCA ACACCAACGA ATTGCTAAAA AGCGAGCAGC TCTTAACAGC TAGCGAACGT 960
AACTTAGACT TCAGAGATT ATACGATCCT CGTGATAAGG CTAAACTACT CTACACAAT 1020
CTCGATGCTT TTGGTATTAT GGACTATACC TTAAGTGGAA AAGTAGAGGA TAATCACGAT 1080
GACACCAACC GTATCATAAC CGTTTATATG GGCAAGCGAG ATATCGTCGA CGGGGGGCAC 1140
15 CACCACCACC ACCACTAA 1158

5. The method of Claims 1 or 3 wherein said skc-2 gene fragments are isolated from plasmid pEKG-3.
6. The method of any one of Claims 1-4 wherein said host cell is bacterium.
7. The method of Claim 6 wherein said bacterium is Escherichia coli.
- 20 8. The method of Claim 6 wherein said is Escherichia coli strain W3110.
9. The method of Claim 1 wherein said expression vector is a plasmid containing the skc-2 gene fragment (40-1245) operably linked to the Escherichia coli tryptophan promoter and the T4 transcription terminator.
10. The method of Claim 2 wherein said expression vector is a plasmid containing the
- 25 skc-2 gene fragment (40-1245) fusioned to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis operably linked to the Escherichia coli tryptophan promoter and the T4 transcription terminator.
11. The method of Claim 3 wherein said expression vector is a plasmid containing the skc-2 gene fragment (1-1119) operably linked to the Escherichia coli tryptophan
- 30 promoter and the T4 transcription terminator.

12. The method of Claim 4 wherein said expression vector is a plasmid containing the skc-2 gene fragment (1-1119) fused to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis operably linked to the Escherichia coli tryptophan promoter and the T4 transcription terminator.
- 5 13. The method of Claim 9 wherein said expression vector is the plasmid pEMI-1.
14. The method of Claim 10 wherein said expression vector is the plasmid pSKH-11.
15. The method of Claim 11 wherein said expression vector is the plasmid pIJ-4.
16. The method of Claim 12 wherein said expression vector is the plasmid pMC-8.
17. An isolated and purified nucleic acid consisting essentially of the nucleotide sequence shown
- 10 in claim 1.
18. An isolated and purified nucleic acid consisting essentially of the nucleotide sequence shown in claim 2.
19. An isolated and purified nucleic acid consisting essentially of the nucleotide sequence shown in claim 3.
- 15 20. An isolated and purified nucleic acid consisting essentially of the nucleotide sequence shown in claim 4.
21. An expression vector for the expression of streptokinase mutant SKC-2-N13 in a host cell, said vector containing a streptokinase gene skc-2 fragment 40-1245 consisting essentially of the nucleotide sequence shown in claim 1 operably linked to an effective promoter and
- 20 transcription terminator.
22. An expression vector for the expression of streptokinase mutant SKC-2-N13 in a host cell, said vector containing a streptokinase gene skc-2 fragment 40-1245 fused to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence shown in claim 1 operably linked to an effective promoter and transcription terminator.
- 25 23. An expression vector for the expression of streptokinase mutant SKC-2-C42 in a host cell, said vector containing a streptokinase gene skc-2 fragment 1-1119 consisting essentially of the nucleotide sequence shown in claim 1 operably linked to an effective promoter and transcription terminator.
24. An expression vector for the expression of streptokinase mutant SKC-2-C42 in a host cell,
- 30 said vector containing a streptokinase gene skc-2 fragment 1-1119 fused to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence shown in claim 1 operably linked to an effective promoter and transcription terminator.

25. The expression vector of Claim 21 containing the skc-2 gene fragment 40-1245 operably linked to the Escherichia coli tryptophan promoter and the T4 transcription terminator for expression of said skc-2 gene fragment in bacteria.
26. The expression vector of Claim 22 containing the skc-2 gene fragment 40-1245 fused to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis operably linked to the Escherichia coli tryptophan promoter and the T4 transcription terminator for expression of said skc-2 gene fragment in bacteria.
27. The expression vector of Claim 23 containing the skc-2 gene fragment 1-1119 operably linked to the Escherichia coli tryptophan promoter and the T4 transcription terminator for expression of said skc-2 gene fragment in bacteria.
28. The expression vector of Claim 24 containing the skc-2 gene fragment 1-1119 fused to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis operably linked to the Escherichia coli tryptophan promoter and the T4 transcription terminator for expression of said skc-2 gene fragment in bacteria.
29. The expression vector of Claim 25, which is the plasmid pEMI-1, contained in Escherichia coli.
30. The expression vector of Claim 26, which is the plasmid pSKH-11, contained in Escherichia coli.
31. The expression vector of Claim 27, which is the plasmid pIJ-4, contained in Escherichia coli.
32. The expression vector of Claim 28, which is the plasmid pMC-8, contained in Escherichia coli.
33. A host cell for producing streptokinase mutant by expression of a gene encoding said streptokinase mutant, said host cell being transformed with an expression vector containing a streptokinase gene skc-2 fragment 40-1245 consisting essentially of the nucleotide sequence shown in Claim 1 operably linked to an effective promoter and transcription terminator.
34. A host cell for producing streptokinase mutant by expression of a gene encoding said streptokinase mutant, said host cell being transformed with an expression vector containing a streptokinase gene skc-2 fragment 40-1245 fused to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence shown in Claim 2 operably linked to an effective promoter and transcription terminator.
35. A host cell for producing streptokinase mutant by expression of a gene encoding said streptokinase mutant, said host cell being transformed with an expression vector containing a streptokinase gene skc-2 fragment 1-1119 consisting essentially of the nucleotide sequence shown in Claim 3 operably linked to an effective promoter and transcription terminator.

36. A host cell for producing streptokinase mutant by expression of a gene encoding said streptokinase mutant, said host cell being transformed with an expression vector containing a streptokinase gene skc-2 fragment 1-1119 fused to the sequence tail Asp-Ile-Val-Asp-Gly-Gly-6xHis consisting essentially of the nucleotide sequence shown in Claim 4 operably linked to an effective promoter and transcription terminator.
37. The host cell of Claim 33 which is bacterium.
38. The host cell of Claim 34 which is bacterium.
39. The host cell of Claim 35 which is bacterium.
40. The host cell of Claim 36 which is bacterium.
41. The host cell of Claim 37 which is Escherichia coli.
42. The host cell of Claim 38 which is Escherichia coli.
43. The host cell of Claim 39 which is Escherichia coli.
44. The host cell of Claim 40 which is Escherichia coli.
45. The host cell of Claim 41, which is Escherichia coli, strain W3110.
46. The host cell of Claim 42, which is Escherichia coli, strain W3110.
47. The host cell of Claim 43, which is Escherichia coli, strain W3110.
48. The host cell of Claim 44, which is Escherichia coli, strain W3110.
49. The transformed host cell of Claim 45, which is transformed clon WSK-N-13.
50. The transformed host cell of Claim 46, which is transformed clon WSK-N-13-H.
51. The transformed host cell of Claim 47, which is transformed clon WSK-C42.
52. The transformed host cell of Claim 48, which is transformed clon WSK-C42-H.
53. An isolated and purified streptokinase mutant consisting essentially of the amino acid sequence encoded by the nucleotide sequence shown in Claim 1.
54. An isolated and purified streptokinase mutant consisting essentially of the amino acid sequence encoded by the nucleotide sequence shown in Claim 2.
55. An isolated and purified streptokinase mutant consisting essentially of the amino acid sequence encoded by the nucleotide sequence shown in Claim 3.
56. An isolated and purified streptokinase mutant consisting essentially of the amino acid sequence encoded by the nucleotide sequence shown in Claim 4.
57. A pharmaceutical composition comprising an isolated and purified streptokinase mutant as defined in Claim 55 and a pharmaceutically acceptable diluent, carrier or excipient.

58. A pharmaceutical composition comprising an isolated and purified streptokinase mutant as defined in Claim 56 and a pharmaceutically acceptable diluent, carrier or excipient.
59. Method of treating a condition requiring the administration of a thrombolytic agent
- 5 comprising the step of administering to the subject requiring such treatment a streptokinase mutant of any one of Claims 53 to 56 or a pharmaceutical composition of claims 57 or 58.
60. Use of a streptokinase mutant of any one of Claims 53 to 56 in the manufacture of a medicament for treating a condition requiring the administration of a thrombolytic
- 10 agent.
61. A method for producing streptokinase mutants according to any one of Claims 1 to 16 and substantially as herein described with reference to any one of the examples.
62. An isolated and purified nucleic acid, substantially as herein described with reference to any one of the examples.
- 15 63. An expression vector for the expression of streptokinase mutant according to any one of Claims 21 to 32 and substantially as herein described with reference to any one of the examples.
64. A host cell producing streptokinase mutant according to any one of Claims 33 to 48 and substantially as herein described with reference to any one of the examples.
- 20 65. A transformed host cell according to any one of Claims 49 to 52 and substantially as herein described with reference to any one of the examples.
66. An isolated and purified streptokinase mutant according to any one of Claims 53 to 56 and substantially as herein described with reference to any one of the examples.
67. A pharmaceutical composition comprising an isolated and purified streptokinase
- 25 mutant substantially as herein described with reference to any one of the examples.

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BALDWIN SHELSTON WATERS
Attorneys for: CENTRO DE INGENIERIA GENETICA Y BIOTECNOLOGIA