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(54) **FACTOR IX POLYPEPTIDE MUTANT, ITS USES AND A METHOD FOR ITS PRODUCTION**

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(57) **ABSTRACT**

Disclosed are a modified FIX (factor IX) polypeptide comprising a leucine, cysteine, aspartic acid, glutamic acid, histidine, lysine, asparagine, glutamine or tyrosine in position 338; pharmaceutical preparations containing said modified FIX polypeptide; a nucleotide sequence coding for the modified FIX polypeptide; and a method for producing the modified FIX polypeptide.

7 Claims, 3 Drawing Sheets

Specification includes a Sequence Listing.

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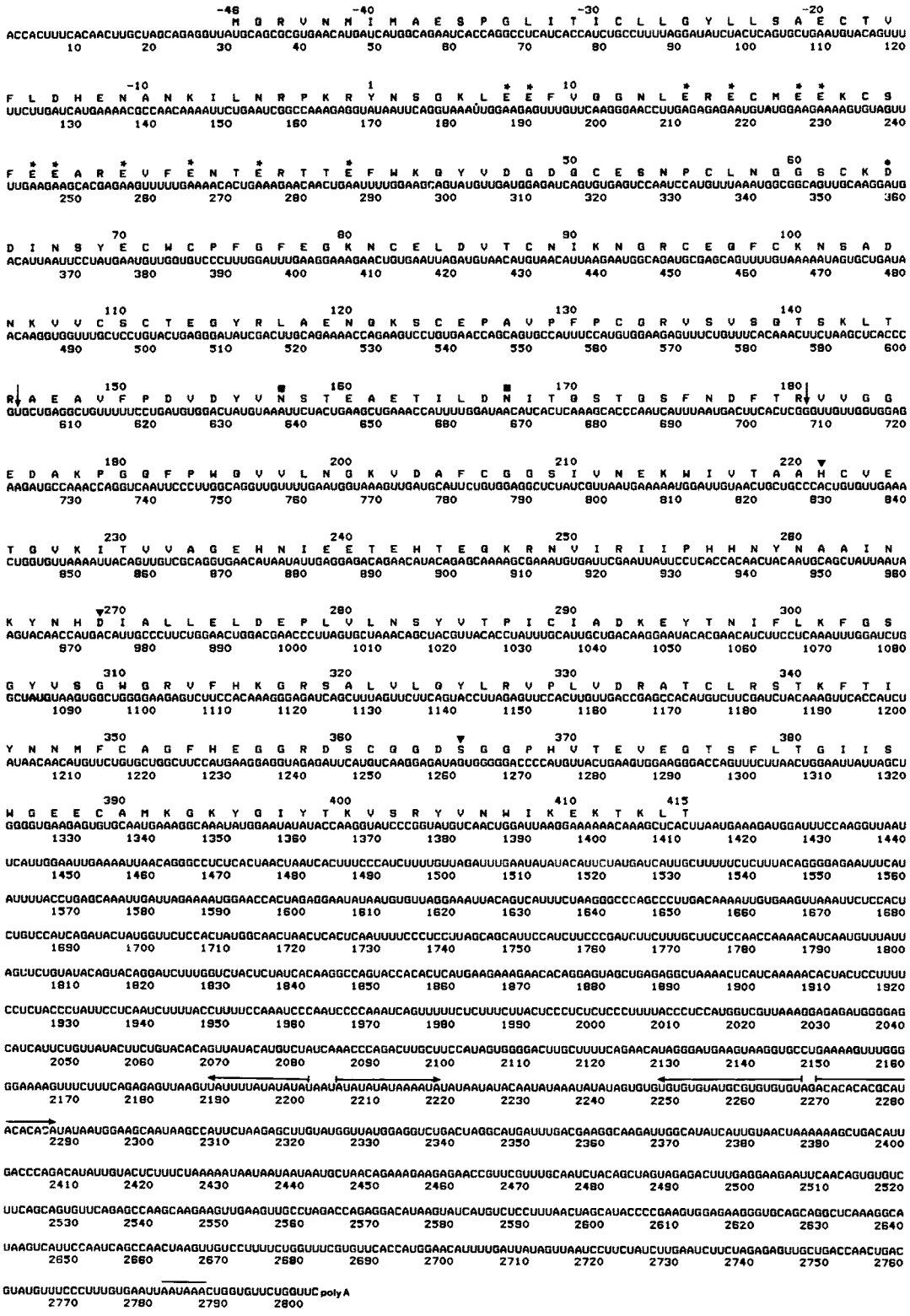

FIG. 1

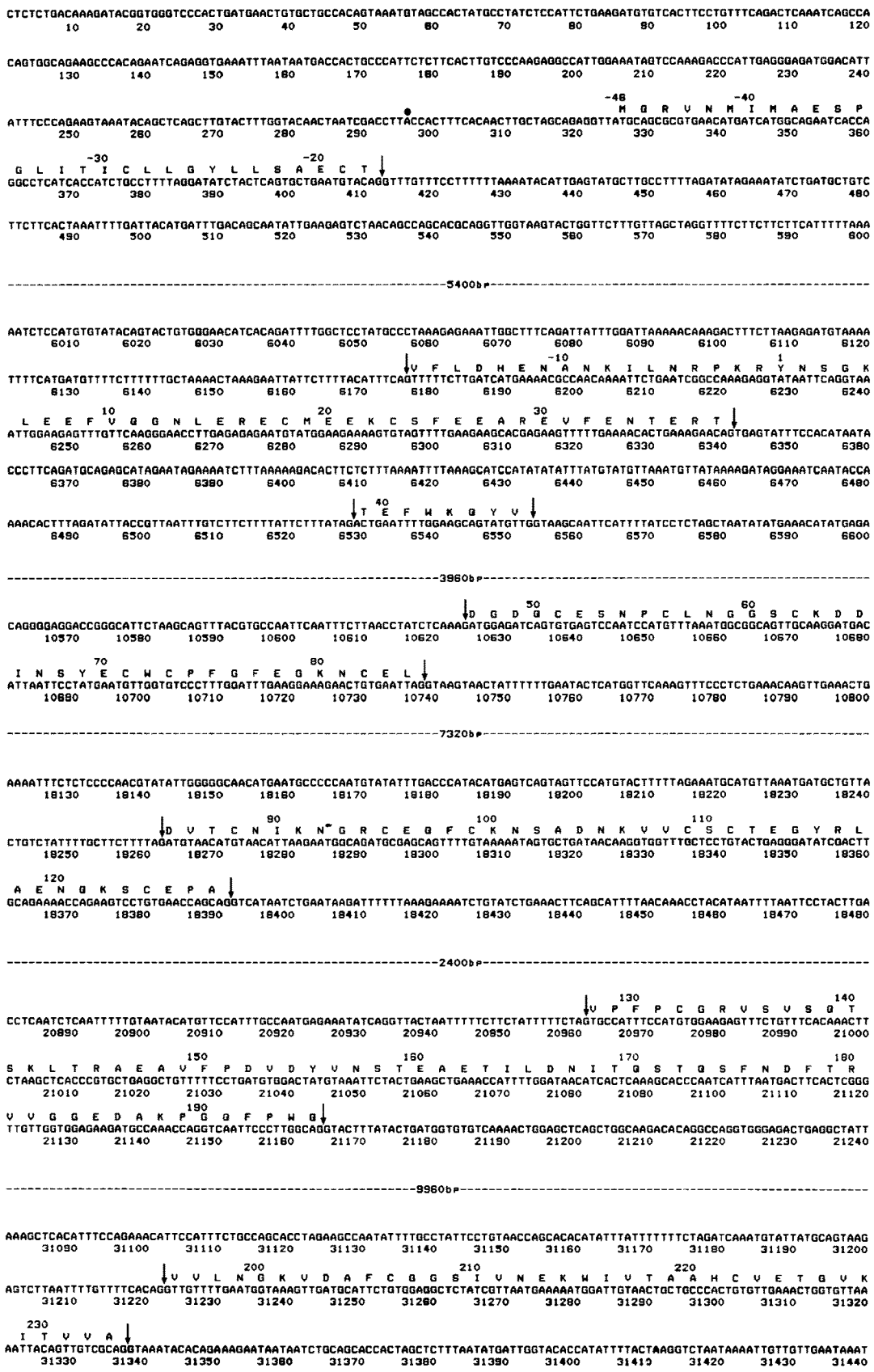

FIG. 2

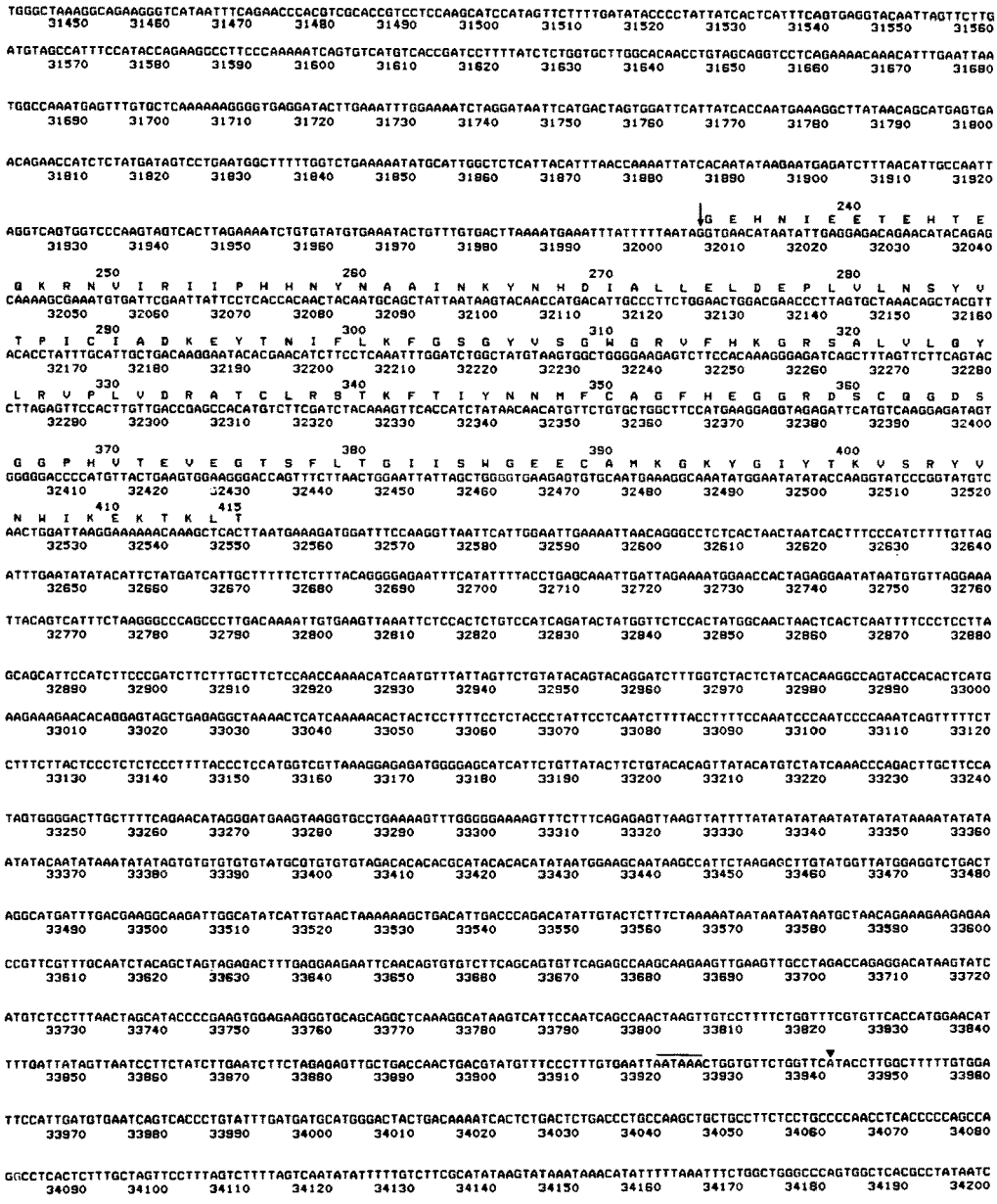

FIG. 2 - Continued

FACTOR IX POLYPEPTIDE MUTANT, ITS USES AND A METHOD FOR ITS PRODUCTION

Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue; a claim printed with strikethrough indicates that the claim was canceled, disclaimed, or held invalid by a prior post-patent action or proceeding.

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is the US national stage of International Application PCT/EP2009/061935 filed on Sep. 15, 2009 which, in turn, claims priority to Italian Patent Application BO2008A000564 filed on Sep. 15, 2008 and Italian Patent Application BO2009A000275 filed on May 6, 2009.

FIELD OF THE INVENTION

The present invention relates to a modified FIX (factor IX) polypeptide, a nucleotide sequence, a vector comprising said nucleotide sequence and a method for producing the modified FIX polypeptide.

The present invention also relates to pharmaceutical preparations and uses of modified factor FIX and of the nucleotide sequence.

PRIOR ART

FIX is a vitamin K-dependent glycoprotein belonging to the serine-protease family, and is synthesized in the liver of man and other animals, including mammals, playing a fundamental role in both intrinsic and extrinsic pathways of the coagulation cascade. Human FIX circulates in plasma as a single chain zymogen composed of 415 amino acids. Human FIX has a molecular weight of 56 kD and a plasma concentration of about 5 μg/ml. The zymogen is activated both by activated factor XI (FXIa), and tissue factor complex (TF)—activated factor VII (FVIIa). The structural organization of FIX is similar to that of other vitamin K-dependent coagulation proteins such as factor VII (FVII), factor X (FX) and protein C (PC). The amino-terminal portion of the molecule comprises the "Gla" domain, a region rich in gamma-carboxy-glutamic residues whose carboxylation is dependent on the presence of vitamin K. The main physiological function of FIX, once activated, is to convert factor X (FX) into activated factor X (FXa) in a process that requires the presence of a phospholipid surface, calcium ions and a protein with cofactor effect, namely activated factor VIII (FVIIIa). FXa itself is able to convert prothrombin into thrombin which transforms fibrinogen into soluble fibrin which, on polymerization, forms the clot. The action of FXa is enhanced by the presence of activated factor V (FVa).

The human FIX gene is located on chromosome X in position Xq27.1 and contains 8 exons of lengths varying from 25 base pairs (bp) to 2000 bp. Human FIX mRNA is about 3 kb in length and comprises 205 bases which form the 5' UTR region, 1386 bases which encode the FIX polypeptide and 1392 bases of the 3' UTR region. This mRNA encodes the synthesis of 461 amino acids which form the human FIX precursor. This precursor (SEQ ID NO: 1) comprises the following segments and domains: a hydrophobic signal peptide (amino acids 1-28), a propeptide (amino acids 29-46), a Gla-domain (amino acids 47 to 92), an EGF-like 1 domain (amino acids 93 to 129), an EGF-like 2 domain (amino acids 130 to 171), an activation peptide (amino acids 192 to 226) and a serine-protease domain (amino acids 227 to 461). The mature form of human FIX (SEQ ID NO: 2) loses the hydrophobic signal peptide and the propeptide. Consequently the corresponding amino acid positions of the aforementioned domains become the following: a Gla-domain (amino acids 1 to 46), an EGF-like 1 domain (amino acids 47 to 83), an EGF-like 2 domain (amino acids 84 to 125), an activation peptide (amino acids 146 to 180) and a serine-protease domain (amino acids 181 to 415). SEQ ID NO: 1 (from which SEQ ID NO: 2 is derived) corresponds to the sequence on PubMed ("Protein" category) found by entering accession number AAB59620; this amino acid sequence comprises the signal peptide (46 AA), followed by the amino acid sequence of the mature protein.

A genetic deficiency in FIX can cause a number of coagulation diseases (coagulopathies), for example the haemorrhagic disease known as haemophilia B in affected males (sex linked genetic disease). Haemophilia B can be classified into three classes, each of which is characterized by the presence of different plasma concentrations of FIX. In severe haemophilia B the plasma levels of FIX activity are below 1% of normal; in the moderate form, levels are between 1% and 5%; in the mild form, between 5 and 25% of normal levels. There are also healthy carrier individuals who have medium FIX activity levels, between 25% and 50% of normal, but many carriers can have levels even exceeding 50%. Patients affected by severe haemophilia B present serious haemorrhagic manifestations which can be controlled or avoided by administering FIX concentrates of extractive (from human plasma) or of recombinant origin, currently only available in a single commercial formulation.

Attempts to correct the genetic defect by means of gene therapy have so far been fruitless because of various problems. These include firstly those connected to the low efficiency of expression in man of FIX levels in plasma i.e. around 1%, hence not sufficient to correct the disease; those connected to the immunogenicity of treatment with viral vectors; finally those connected to the side effects of gene therapy itself which include hepatitis, myositis and others.

An increase in plasma FIX to higher than normal levels (normal range of FIX in plasma being 70-120% i.e. 70-120 U/dl, where a unit is the quantity of FIX contained in 1 milliliter of normal plasma, equal to about 5 μg) has been associated with an increased risk in humans of developing thrombotic manifestations in the venous system. In particular, for values above 150 U/dl, a 4.8 fold increase in thrombotic risk has been noted (corrected O. R. 4.8; 95% CI, from 2.3 to 10.1). However, the genetic basis for the increased FIX levels in plasma of these individuals has never been identified.

In vitro mutagenesis studies of mutated recombinant FIX expression have demonstrated the possibility of reproducing the alterations in FIX synthesis and activity encountered in vivo in patients with haemophilia B. Vice-versa, by site-specific mutagenesis in certain positions on the FIX molecule, FIX mutants have been produced with "gain-of-function" (increased activity relative to the normal molecule) by altering their specificity for physiological substrates and/or modifying their other functions. In WO 99/03496 is disclosed the recombinant FIX arginine 338 alanine mutant which resulted in a gain-of-function whose activity levels are 2-3 folds higher than that found in wild

type FIX. These gain-of-function mutants (in particular with increased protease activity towards the physiological substrate, i.e. FX, or with an increased capacity for interaction with FVIIIa, a cofactor of FIXa) have not as yet been found to exist in nature, nor have they been tested in man. More explicitly, there is no evidence of: 1) the existence of a human carrier of mutated FIX (natural FIX mutant) with gain-of-function characterized by increased functional activity as compared to normal FIX (WT) with any gain-of-function in functional activity; 2) tests conducted in vivo in man with administrations of modified recombinant FIX; 3) tests conducted in vivo in man with administrations of modified recombinant FIX with gain-of-function for the prophylaxis and treatment of patients affected by haemophilia (genetic or acquired) or other coagulopathies; 4) tests conducted in vivo in man with administrations of modified recombinant FIX which show the absence of side effects.

SUMMARY OF THE INVENTION

The object of the present invention is to provide a modified FIX polypeptide, a nucleotide sequence, a vector comprising said nucleotide sequence, and a method for producing the modified FIX polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Sequence of factor IX mRNA (SEQ ID NO: 7) and its encoded protein (SEQ ID NO: 8). The symbols 1-415 define the mature protein and -46 to -1 the precursor region. The latter may be further subdivided into a hydrophobic signal region -46 to -21, and a hydrophilic precursor region -20 to -1 containing three basic amino acids between residues -4 to -1. Vertical arrows indicate the peptide bonds cleaved during activation in clotting. Post-translational modifications are marked (*=12λ-carboxyglutamyl residues, ●=f-hydroxyaspartyl and ■=two Asn-linked carbohydrate residues). The AAUAAA consensus sequence is overlined. His (221), Asp (269) and Ser (365) are marked (▼). Local potential hairpin loops are shown by horizontal arrows.

FIG. **2**. Sequence of the eight exon regions of the factor IX gene including the promoter and some 3'-terminal flanking sequence (SEQ ID NO: 9 and SEQ ID NO: 10). The arrows mark splice junctions and the symbol (●) marks the proposed mRNA start point (residue 296). The symbol (▼) marks the position of poly(A) addition site (residue 33 941) in the mRNA. The dashed lines indicate the approximate length of those introns not shown.

A further object of the present invention is to provide pharmaceutical preparations and uses for modified factor FIX and the nucleotide sequence.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention are provided polypeptides, nucleotide sequences, vectors, a method of production, uses of the polypeptides and nucleotide sequences and the pharmaceutical preparations, according to that described in the following independent claims and preferably in any one of the claims that depend directly or indirectly on the independent claims.

The modified FIX polypeptides herein described show a gain-of-function of at least 5 folds higher than that of the wild-type FIX molecule. This increase in the activity level is unexpectedly even higher than that disclosed for the known recombinant FIX arginine 338 alanine mutant.

The contents of the references (articles, textbooks, GenBank sequences etc.) cited in the present text are fully included herein for descriptive completion. In particular, the references (articles, textbooks, GenBank sequences etc.) cited in the present text are incorporated herein for reference.

Unless otherwise explicitly specified, the following terms have the meanings indicated below.

In the present text the term "percentage identity" and "% identity" between two amino acid (peptide) or nucleic acid (nucleotide) sequences means the percentage of identical amino acid or nucleotide residues in corresponding positions in the two optimally aligned sequences.

To determine the "percentage identity" of the two amino acid or nucleic acid sequences, the sequences are aligned together. To achieve an optimal match, gaps can be introduced into the sequence (i.e. deletions or insertions which can also be placed at the sequence ends). Amino acid and nucleotide residues in the corresponding positions are then compared. When a position in the first sequence is occupied by the same amino acid or nucleotide residue that occupies the corresponding position in the second sequence, the molecules are identical in that position. The percentage identity between two sequences is a function of the number of identical positions divided by the sequences [i.e. % identity=(number of identical positions/total number of positions)×100]

According to an advantageous embodiment, the sequences have the same length. Advantageously, the compared sequences do not have gaps (or insertions).

The percentage identity can be obtained by using mathematical algorithms. A non-limiting example of an algorithm used for comparing two sequences is the Karlin and Altschul algorithm [Proc. Natl. Acad. Sci. USA 87 (1990) 2264-2268] modified by Karlin and Altschul [Proc. Natl. Acad. Sci. USA 90 (1993) 5873-5877]. Said algorithm is incorporated in the BLASTn and BLASTp programmes of Altschul [Altschul et al, J. Mol. Bio. 215 (1990) 403-410].

With the purpose of achieving alignments even in the presence of one or more gaps (or insertions) methods may be used which assign a relatively high penalty for each gap (or insertion) and a lower penalty for each additional amino acid or nucleotide residue in the gap (this additional amino acid or nucleotide residue is defined as gap extension). High penalties will obviously lead to the alignments being optimized with the least number of gaps.

An example of a programme able to achieve this type of alignment is the BLAST programme as described in Altschul et al., Nucleic Acids Res. 25 (1997) 3389-3402. For this purpose the BLASTn and BLASTp programmes can be used with the default parameters. When using the BLAST programme the BLOSUM62 matrix is typically employed.

An advantageous and non-limiting example of a programme for achieving an optimal alignment is GCG Wisconsin Bestfit package (University of Wisconsin, USA; Devereux et. al., 1984, Nucleic Acid Research 12:387). The default parameters are again used i.e. for an amino acid sequence they allow a penalty of −12 for a gap and a penalty of −4 for each extension.

In the present text the term "percentage homology" and "% homology" between two amino acid or nucleotide sequences means the percentage of homologous amino acid or nucleotide residues in corresponding positions in the two optimally aligned sequences.

The percentage homology between two sequences is determined in a substantially identical manner to that described above for determining percentage identity except for the fact that homologous positions and not only identical positions are considered in the calculation.

With regard to a nucleotide sequence, two homologous positions present two different nucleotides but which, within their codon, code for the same amino acid. With regard to an amino acid sequence, two homologous positions present two homologous amino acids, that is to say amino acids possessing similar physico-chemical properties, for example amino acids belonging to the same groups such as: aromatic (Phe, Trp, Tyr), acids (Glu, Asp), polar (Gln, Asn), basic (Lys, Arg, His), aliphatic (Ala, Leu, Ile, Val), with a hydroxyl group (Ser, Thr), with a short side chain (Gly, Ala, Ser, Thr, Met). It is expected that substitutions between these homologous amino acids would not change the phenotype of the proteins (conservative amino acid substitutions). Specific examples of conservative is substitutions are known in this technical field and are described in the various literature (e.g. Bowie et al., Science, 247:1306-1310 (1990)).

Further examples of programmes and/or articles relating to the determination of alignments and percentage homologies and/or identities are indicated in, for example, US2008003202, US2007093443, WO06048777.

In the present text the term "corresponding position" means a position in a polypeptide or nucleic acid sequence which, following an alignment, corresponds to (or faces), a precise position in a reference sequence. For example, a position corresponding to a precise position on the FIX polypeptide presenting SEQ ID NO: 2 can be determined by aligning the SEQ ID NO: 2 with a polypeptide of interest; the alignment can be carried out manually or as explained above in relation to percentage identity determination.

In the present text the term "naked chain" means a polypeptide which has not been chemically modified but contains only covalently bound amino acids.

In the present text the term "promoter" means a DNA portion of a gene that controls (activates) the transcription of a nucleotide sequence to which it is operatively linked (but not necessarily flanking it). The promoter includes one or more DNA sequences, which are recognized by RNA polymerase and bind RNA polymerase so that RNA polymerase itself initiates transcription.

In the present text the term "treat" or "treatment" of a pathology means the prophylaxis and/or therapy and/or cure of this pathology. The term prophylaxis means advantageously to at least partially arrest the development of a potential disease and/or to prevent the worsening of symptoms or progression of a disease. Advantageously, the term therapy means a partial or total alleviation of the disease symptoms.

In the present text the term "vector" means an element used to introduce a nucleic acid into a cell for the expression or replication of said nucleic acid. An example of vectors are episomes, which are capable of extra-chromosomal replication. The vectors can also be integrated into host chromosomes. Vectors are often in the form of plasmids, generally circular double-helical DNA.

In the present text "vehicle presenting a nucleic acid" means: a vector which includes nucleic acid; a cell which includes nucleic acid; or a pharmaceutically acceptable excipient combined with the nucleic acid by mixing. Advantageously the vehicle is chosen from a vector or a cell.

According to a first aspect of the present invention, a modified FIX polypeptide is provided comprising an amino acid chosen from the group consisting of: leucine, cysteine, aspartic acid, glutamic acid, histidine, lysine, asparagine, glutamine, tyrosine in a position corresponding to position 338.

According to other embodiments, the amino acid is chosen from the group consisting of: leucine, aspartic acid, glutamine.

According to other embodiments, the amino acid is chosen from the group consisting of: aspartic acid, glutamine.

According to other embodiments, the amino acid is aspartic acid.

According to other embodiments, the amino acid is glutamine.

According to other embodiments, the amino acid is chosen from the group consisting of: aspartic acid, leucine.

According to other embodiments, the amino acid is chosen from the group consisting of: leucine, glutamine.

According to other embodiments, the amino acid is leucine.

The modified FIX polypeptide must be able to carry out its function within the coagulation cascade and can be of synthetic or natural origin, for example human or animal origin.

Examples of FIX polypeptides include (but are not limited to) unmodified wild-type FIX (such as the polypeptide of SEQ ID NO: 2), precursors of said wild-type FIX (such as the polypeptide of SEQ ID NO: 1), natural polymorphic variants (such as: a polypeptide presenting an alanine in a position corresponding to position T148 or to a precursor polypeptide thereof).

In the present text the loci (positions) of the modified or unmodified amino acid sequences are identified by reference to the amino acid numbering in the corresponding positions of an unmodified mature FIX polypeptide, as identified by SEQ ID NO: 2. Corresponding positions can be determined by alignment of unmodified residues (see above). By way of example we report hereinafter the sequences and relative numberings of the mature FIX polypeptide (SEQ ID NO: 2) and of the FIX polypeptide precursor (SEQ ID NO:1).

SEQ ID NO: 1

```
MQRVNMIMAE  SPGLITICLL  GYLLSAECTV  FLDHENANKI

LNRPKRYNSG  KLEEFVQGNL  ERECMEEKCS  FEEAREVFEN

TERTTEFWKQ  YVDGDQCESN  PCLNGGSCKD  DINSYECWCP

FGFEGKNCEL  DVTCNIKNGR  CEQFCKNSAD  NKVVCSCTEG

YRLAENQKSC  EPAVPFPCGR  VSVSQTSKLT  RAEAVFPDVD

YVNSTEAETI  LDNITQSTQS  FNDFTRVVGG  EDAKPGQFPW

QVVLNGKVDA  FCGGSIVNEK  WIVTAAHCVE  TGVKITVVAG

EHNIEETEHT  EQKRNVIRII  PHHNYNAAIN  KYNHDIALLE

LDEPLVLNSY  VTPICIADKE  YTNIFLKFGS  GYVSGWGRVF

HKGRSALVLQ  YLRVPLVDRA  TCLRSTKFTI  YNNMFCAGFH

EGGRDSCQGD  SGGPHVTEVE  GTSFLTGIIS  WGEECAMKGK

YGIYTKVSRY  VNWIKEKTKL  T
```

SEQ ID NO: 2

```
YNSGKLEEFV  QGNLERECME  EKCSFEEARE  VFENTERTTE

FWKQYVDGDQ  CESNPCLNGG  SCKDDINSYE  CWCPFGFEGK
```

-continued

NCELDVTCNI	KNGRCEQFCK	NSADNKVVCS	CTEGYRLAEN
QKSCEPAVPF	PCGRVSVSQT	SKLTRAETVF	PDVDYVNSTE
AETILDNITQ	STQSFNDFTR	VVGGEDAKPG	QFPWQVVLNG
KVDAFCGGSI	VNEKWIVTAA	HCVETGVKIT	VVAGEHNIEE
TEHTEQKRNV	IRIIPHHNYN	AAINKYNHDI	ALLELDEPLV
LNSYVTPICI	ADKEYTNIFL	KFGSGYVSGW	GRVFHKGRSA
LVLQYLRVPL	VDRATCLRST	KFTIYNNMFC	AGFHEGGRDS
CQGDSGGPHV	TEVEGTSFLT	GIISWGEECA	MKGKYGIYTK
VSRYVNWIKE	KTKLT		

Likewise, the positions of the modified or unmodified nucleotide sequences are identified, unless otherwise indicated, by reference to the nucleotide numbering in the corresponding positions of the nucleotide sequence identified by accession number K02402 (GenBank). The nucleotide sequence K02402 codes for the FIX polypeptide precursor (SEQ ID NO: 1) and includes some intron regions (in this regard see Anson D S, Choo K H, Rees D J, Giannelli F, Gould K, Huddleston J A, Brownlee G G. The gene structure of human anti-haemophilic factor IX. The EMBO Journal 1984; 3:1053-1060).

Included within the definition of a modified FIX polypeptide are chimeric variants which can be produced by replacing amino acids or entire domains of the FIX with amino acids or sequences of other factors belonging to the coagulation factor family (for example factor VII or factor X).

According to other embodiments, the modified FIX polypeptide presented herein is either a naked chain or exhibits post-transcriptional modifications. Examples of modifications include one or more chemical modifications, which comprise (but are not limited to): glycosylation, acylation, methylation, phosphorylation, sulphation, carboxylation, salification, vitamin C-dependent modifications such as hydrolysis of proline, aspartic acid, lysine, or carboxy-terminal amidation; vitamin K-dependent modifications such as carboxylation of glutamic acid residues; incorporation of selenium to form one or more selenocysteine(s); incorporation of a PEG moiety (polyethylene glycol).

In addition to the possible modifications disclosed herein, the modified FIX polypeptide can contain one or more variants known in the state of the art such as hyperglycosylation, deimmunization and others (see for example: U.S. Pat. No. 6,277,618, U.S. Pat. No. 6,315,995, U.S. Pat. No. 6,531,298, US2004/0102388, US2004/0110675, US2004/0254106, US2005/0100982, US2006/0040856).

Non-limiting examples of modified FIX polypeptide variants can be deduced from one or more of the following references: US2006/040856, Friedler et al (2000) J. Biol Chem. 275:23783-23789, US2004/102388, WO2006/018201, Lim et al. (1990) J. Biol Chem. 265(1):144-150, Cheung et al. (1992) J. Biol. Chem. 267(29): 20529-20531, Gui et al. (2002) Blood 100(1):153-158, Schuettrumpf et al. (2005) Blood 105(6): 2316-2323, US2004/110675, U.S. Pat. No. 6,315,995.

According to some alternative embodiments, the modified FIX polypeptide has at least 50%, 60%, 70%, 80%, 85%, 90%, 94%, 97%, 99%, 100% homology (or, advantageously, identity) with a peptide sequence chosen from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

Advantageously, the modified FIX polypeptide has at least 60% homology (or, advantageously, identity) with a peptide sequence chosen from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

Advantageously, the modified FIX polypeptide has at least 80% homology (or, advantageously, identity) with a peptide sequence chosen from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

Advantageously, the modified FIX polypeptide has at least 90% homology (or, advantageously, identity) with a peptide sequence chosen from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

Advantageously, the peptide sequence is SEQ ID NO: 2.

According to a second aspect of the present invention, a nucleotide sequence is provided which codes for the FIX polypeptide of the first aspect of the present invention.

According to some alternative embodiments, the nucleotide sequence has at least 50%, 60%, 70%, 80%, 85%, 90%, 94%, 97%, 99%, 100% homology (or, advantageously, identity) with the sequence having accession number K02402 (Gen Bank).

Advantageously, the nucleotide sequence has at least 70% homology (or, advantageously, identity) with the sequence having accession number K02402 (GenBank).

Advantageously, the nucleotide sequence has at least 90% homology (or, advantageously, identity) with the sequence having accession number K02402 (Gen Bank).

Advantageously, the nucleotide sequence has at least 100% homology (or, advantageously, identity) with the sequence having accession number K02402 (Gen Bank).

According to some alternative embodiments, the nucleotide sequence is a RNA sequence and has at least 50%, 60%, 70%, 80%, 85%, 90%, 94%, 97%, 99%, 100% homology (or, advantageously, identity) with the sequence from position 31 to position 1411 (SEQ ID NO: 3) (advantageously from position 169 to position 1411—SEQ ID NO: 5) of the polynucleotide of FIG. **2** in the article by Anson D S, Chao K H, Rees O J, Giannelli F, Gould K, Huddleston J A and Brownlee G G The gene structure of human anti-hemophilic factor IX. The EMBO Journal 1984; 3: 1053-1060, also reported in FIG. **1** of the application. In this case (that is, with reference to SEQ ID NO: 3 and SEQ ID NO: 5, also in the above-mentioned article Anson D S et al.), the position numbers refer to the numbering reported in the aforementioned FIG. **2** of the article Anson D S et al and in the corresponding FIG. **1** of the instant application.

Advantageously, the RNA sequence has at least 80% homology (or advantageously, identity) with the sequence SEQ ID NO: 3 (advantageously, SEQ ID NO: 5). Advantageously, the RNA sequence has at least 90% homology (or, advantageously, identity) with the sequence SEQ ID NO: 3 (advantageously, SEQ ID NO: 5). Advantageously, the RNA sequence has at least 95% homology (or, advantageously, identity) with the sequence SEQ ID NO: 3 (advantageously, SEQ ID NO: 5).

The RNA sequence can be linked, at the head and/or tail, to additional nucleotide chains that are either not translated or translated separately.

According to some alternative embodiments, the nucleotide sequence is a DNA sequence and comprises (in particular, consists of) intron and exon portions, which present an overall sequence (that is to say exon portions without gaps and linked together in order) having at least 50%, 60%, 70%, 80%, 85%, 90%, 94%, 97%, 99%, 100% homology (or, advantageously, identity) with the overall sequence of exon regions in the sequence (SEQ ID NO: 7) of FIG. **4** in the article by Anson D S, Chao K H, Rees O J, Giannelli F,

Gould K, Huddleston J A and Brownlee G G. The gene structure of human anti-hemophilic factor IX. The EMBO Journal 1984; 3:1053-1060, corresponding to FIG. **2** of the instant application.

Advantageously, the exon portions are separate from each other and placed in order (arranged relative to each other) as are the respective exon regions in the sequence SEQ ID NO: 7. Advantageously, the overall sequence of the exon portions has at least 80% homology (or, advantageously, identity) with the overall sequence of the exon regions. Advantageously, the overall sequence of the exon portions has at least 90% homology (or, advantageously, identity) with the overall sequence of the exon regions. Advantageously, the overall sequence of the exon portions has at least 95% homology (or, advantageously, identity) with the overall sequence of the exon regions.

According to some embodiments, the nucleotide sequence comprises a thymine in a position corresponding to position 34099 (or in the corresponding position 32318 according to the numbering given in SEQ ID NO: 7; or in the corresponding position 31134 according to the numbering given in the Database of mutations of Hemophilia B (Gianneili et al., Hemophilia B: Database of point mutations and short additions and deletions, Nucleic Acids Research 1990; 18:4053-9); or a uracil in the corresponding position 11180 of SEQ ID NO: 3 or SEQ ID NO: 5).

In other words, the aforementioned nucleotide sequence differs from the sequence having accession number K02402 (GenBank) by at least the fact of bearing a mutation from guanine to thymine in position 34099 (G34099T) or in a corresponding position (for example position 32318 according to the numbering of SEQ ID NO: 7; or from guanine to uracil in the corresponding position 11180 of SEQ ID NO: 3 or SEQ ID NO: 5).

In this case the nucleotide sequence codes for a leucine in a position corresponding to position 338.

According to some embodiments, the nucleotide sequence in the positions corresponding to 34098, 34099 and 34100, presents a triplet chosen from the group consisting of: TTA, UUA, TTG, UUG, CTT, CUU, CTC, CUC, CTA, CUA, CTG, CUG, GAT, GAU, GAC, CAA, CAG. In particular, when the nucleotide sequence is a DNA sequence, the triplet is chosen from the group consisting of TTA, TTG, CTT, CTC, CTA, CTG, GAT, GAC, CAA, CAG.

According to some embodiments, the nucleotide sequence, in the positions corresponding to 34098, 34099 and 34100, presents a triplet chosen from the group consisting of: TTA, UUA, TTG, UUG, CTT, CUU, CTC, CUC, CTA, CUA, CTG, CUG, CAA, CAG. In particular, when the nucleotide sequence is a DNA sequence, the triplet is chosen from the group consisting of TTA, TTG, CTT, CTC, CTA, CTG, CAA, CAG. In these cases, the sequence codes for a leucine or a glutamine in a position corresponding to position 338.

According to some embodiments, the nucleotide sequence, in the positions corresponding to 34098, 34099 and 34100, presents a triplet chosen from the group consisting of: TTA, UUA, TTG, UUG, CTT, CUU, CTC, CUC, CTA, CUA, CTG, CUG. In particular, when the nucleotide sequence is a DNA sequence, the triplet is chosen from the group consisting of TTA, TTG, CTT, CTC, CTA, CTG. Advantageously, the triplet is CTA. In these cases, the sequence codes for a leucine in a position corresponding to position 338.

According to some embodiments, the nucleotide sequence, in the positions corresponding to 34098, 34099 and 34100, presents a triplet chosen from the group consisting of: CAA, CAG. In these cases, the sequence codes for a glutamine in a position corresponding to position 338. Advantageously, the triplet is CAA. To obtain the CAA triplet, an adenine is inserted in place of the guanine in position 34099.

According to some embodiments, the nucleotide sequence, in the positions corresponding to 34098, 34099 and 34100, presents a triplet chosen from the group consisting of: GAT, GAU, GAC, CAA, CAG. In particular, when the nucleotide sequence is a DNA sequence, the triplet is chosen from the group consisting of GAT, GAC, CAA, CAG. In these cases, the sequence codes for an aspartic acid or a glutamine in a position corresponding to position 338.

According to some embodiments, the nucleotide sequence, in the positions corresponding to 34098, 34099 and 34100, presents a triplet chosen from the group consisting of: GAT, GAU, GAC. In particular, when the nucleotide sequence is a DNA sequence, the triplet is chosen from the group consisting of GAT, GAC. In these cases, the sequence codes for an aspartic acid in a position corresponding to position 338. Advantageously, the triplet is GAT. To obtain the GAT triplet, a guanine is inserted in place of the adenine in position 34098, an adenine in place of the guanine in position 34099 and a thymine in place of the adenine in position 34100.

The aforesaid homology (or identity) percentages are calculated without considering the specific mutated positions indicated. In other words, for example, the sequence SEQ ID NO: 2 modified with a leucine in position 338 is considered as having 100% homology (and identity) with the unmodified sequence SEQ ID NO: 2.

According to a third aspect of the present invention, a nucleic acid is provided which comprises a nucleotide sequence according to the second aspect of the present invention.

According to some embodiments, the nucleic acid comprises a promoter in operational linkage with the nucleotide sequence.

According to a fourth aspect of the present invention, a vector is provided comprising a nucleic acid as aforedefined in relation to the third aspect of the present invention. In particular, the vector comprises a nucleotide sequence according to the second aspect of the present invention.

According to some embodiments, the vector is chosen from: a prokaryote vector, a eukaryote vector or a viral vector.

Advantageously, the vector is a viral vector. In particular, the vector is chosen from: an adenovirus, a retrovirus, a herpesvirus, a lentivirus, a poxvirus, a cytomegalovirus.

According to a fifth aspect of the present invention, a method for the production of a modified FIX polypeptide is provided, whereby the modified FIX polypeptide is expressed by means of a nucleic acid according to the third aspect of the present invention.

According to some embodiments the method comprises the steps of: introducing a vector of the fourth aspect of the present invention into a cell; and culturing the cell such that the FIX polypeptide is expressed.

Alternatively, the modified FIX polypeptide can be produced by a host animal or in vitro from the aforementioned nucleotide sequence.

According to a particular aspect of the present invention, the method comprises the steps of: introducing the nucleotide sequence of the second aspect of the present invention into a cell-free system; expressing the modified polypeptide in the cell-free system.

According to a further particular aspect of the present invention, the method allows the modified FIX polypeptide to be expressed in a transgenic animal comprising a nucleic acid in accordance with the third aspect of the present invention (in particular, the nucleotide sequence of the second aspect of the present invention). Useful hosts for expression of the modified FIX polypeptide include: E. coli, yeasts, plants, insect cells, mammalian cells (Pham et al. (2003) Biotechnol. Bioeng. 84:332-42; Bon et al. (1998) Semin Hematol. 35 (2 Suppl 2): 11-17; Wahij et al., J. Biol. Chem. 280 (36) 31603-31607) and transgenic animals.

The hosts can vary as to their levels of protein production and also the types of modifications induced in the modified FIX polypeptide subsequent to transcription. Eukaryote hosts can include yeasts such as Saccharomyces cerevisiae and Pichia pastoris (Skoko et al. (2003) Biotechnol. Appl. Biochem. 38 (Pt 3): 257-65), insect cells (Muneta et al. (2003) J. Vet. Med. Sci. 65(2): 219-23), plants and cells from plants such as tobacco, rice, algae (Mayfield et al. (2003) PNAS 100:438-442) etc. The plants are typically modified by direct transfer of DNA and agrobacterium-mediated transformations. Advantageously usable vectors comprise promoter sequences and transcription termination and control elements.

Yeasts are usually modified by replicating episomal vectors or by a stable chromosomal integration by homologous recombination. Advantageously, promoters are used to regulate gene expression. Examples of promoters include GAL1, GAL7, GALS, CUP1. Proteins produced by yeasts are usually soluble; alternatively, proteins expressed in yeasts can be secreted.

Expression in eukaryotic hosts also includes production in animals, for example in serum, milk and eggs. Transgenic animals for the production of FIX polypeptides are known (for example US2002/0166130 and US2004/0133930) and can be adapted for producing the modified FIX polypeptide as aforedefined.

Prokaryote cells in particular E. coli can be advantageously utilized to produce large quantities of modified FIX polypeptide as aforedefined (Platis et al. (2003) Protein Exp. Purif. 31(2):222-30; Khalizzadeh et al. (2004) J. Ind. Microbiol. Biotechnol. 31(2): 63-69).

The vectors used with E. coli advantageously contain promoters able to induce high levels of protein expression and to express proteins that show some toxicity towards the host cells. Examples of promoters are T7 and SP6 RNA.

Reducing agents such as β-mercaptoethanol can be utilized to solubilise polypeptides which may precipitate in the cytoplasmic environment of E. coli.

According to a sixth aspect of the present invention, a modified FIX polypeptide is also provided in accordance with the first aspect of the present invention, for use as a medicament.

The modified FIX polypeptide can be used for disease treatments either alone or in combination with other active compounds.

The modified FIX polypeptide is useful for treating coagulopathies (congenital or acquired), haematological diseases (congenital or acquired), haemorrhagic disorders (such as haemorrhagic gastritis and/or uterine bleeding), other cardiovascular diseases

According to some embodiments, the modified FIX polypeptide is provided for the treatment of at least one coagulopathy.

According to some embodiments, the modified FIX polypeptide is provided for the treatment of haematological diseases.

According to some embodiments, the modified FIX polypeptide is provided for the treatment of haemorrhagic disorders.

According to some embodiments, the modified FIX polypeptide is administered to patients periodically for relatively long time periods or before, during and/or after surgical procedures to reduce and/or prevent haemorrhages.

The use of modified FIX polypeptide for the treatment of coagulopathies is particularly effective.

Advantageously, modified FIX polypeptide is used for the treatment of haemophilia, and in particular haemophilia A and haemophilia B.

According to advantageous embodiments, the modified FIX polypeptide is provided for treating haemophilia B, and advantageously severe and/or moderate haemophilia B.

Advantageously, modified FIX polypeptide is used for the treatment of mammals, in particular human patients.

According to a seventh and an eighth aspect of the present invention, the following are provided: use of the modified FIX polypeptide in accordance with the first aspect of the present invention for preparing a drug (pharmaceutical preparation) advantageously for treating a coagulopathy; and a pharmaceutical preparation comprising the modified FIX polypeptide and, advantageously, at least one pharmaceutically acceptable excipient.

According to some embodiments, the pharmaceutical preparation is for the treatment of a pathology chosen from the group consisting of: coagulopathies (congenital or acquired), haematological diseases (congenital or acquired), haemorrhagic disorders (such as haemorrhagic gastritis and/or uterine bleeding), haemophilia (haemophilia A or haemophilia B). According to specific embodiments, the pharmaceutical preparation is for treating a coagulopathy. According to specific embodiments, the pharmaceutical preparation is for treating haemophilia.

According to a further aspect of the present invention, a method is provided for treating at least one coagulopathy, this method allowing the administration of an effective quantity of a modified FIX polypeptide as aforedefined.

The modified FIX polypeptide can be administered as a pure compound, but is advantageously presented in the form of a pharmaceutical preparation. Non-limiting examples of pharmaceutical preparations if needed for this purpose are explained below.

The modified FIX polypeptide can be formulated for oral, parenteral or rectal administration, or in forms suited to administrations by inhalation or insufflation (either via the mouth or nose). Formulations for oral or parenteral administration are advantageous.

For oral administrations, the pharmaceutical preparations are in the form of, for example, tablets or capsules prepared by known methods with pharmaceutically acceptable excipients such as binders (for example pregelatinized maize starch, polyvinylpyrrolidone, or methyl cellulose); fillers (for example lactose, microcrystalline cellulose or calcium hydrogen phosphate); additives (for example magnesium stearate, talc, silica); disintegrants (for example potato starch); and/or lubricants (for example sodium lauryl sulphate). The tablets can be coated using known methods. Liquid preparations for oral administration have the form, for example, of solutions, syrups or suspensions, or can be in the form of a dry product that can be dissolved in water or another liquid prior to use. Said preparations are prepared by known methods with pharmaceutically acceptable additives such as suspending agents (for example sorbitol, cellulose derivatives, edible hydrogenated fats); emulsifying agents (for example lecithin or acacia); non-aqueous liquids

(for example almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and/or preservatives (for example methyl or propylhydroxybenzoates, sorbic acid or ascorbic acid). The preparations can also contain, in appropriate cases, buffering salts, colouring agents, flavouring agents and/or sweeteners.

Preparations for oral administration are formulated in a known manner, in order to provide a controlled release of the active compound.

The modified FIX polypeptide is formulated, in a known manner, for parenteral administration, by injection or continuous administration. Formulations for injection are, advantageously, in the form of dosage units, for example in ampoules or multi-dose containers containing preservatives. The composition can be in the form of a suspension, in aqueous or oily liquids, and can contain elements of the formulation as dispersing and stabilizing agents. Alternatively, the active compound can be in powder form to be dissolved just before use in a liquid as needed, such as sterile water.

The modified FIX polypeptide can be formulated for rectal administration as suppositories or enemas, for example, containing suppository excipients of known type such as cocoa butter or other glycerides.

The modified FIX polypeptide is also formulated, in a known manner, in extended release compositions. These extended release compositions are, for example, administered by means of an implant (for example subcutaneous or intramuscular) or an intramuscular injection. Therefore, for example, the modified FIX polypeptide is formulated with suitable polymer or hydrophobic materials (such as an emulsion or an oil) or ion exchange resins, or relatively poorly soluble derivatives, such as relatively poorly soluble salts.

For intranasal administration, the modified FIX polypeptide is formulated by administrations via a (known) device, such as in a powder with a suitable vehicle. The dosages of the modified FIX polypeptide will depend on the patient age and condition, and so the precise dosage will have to be decided each time by the 10 physician. The dosage will also depend on the mode of administration and the particular compound selected. Usable doses can be for example comprised between 0.1 μg/kg and 400 μg/kg body weight per day.

According to a further aspect of the present invention, the nucleotide sequence is provided in accordance with the second aspect of the present invention for use as a medicament (advantageously for treating a coagulopathy).

The nucleotide sequence can be used for treating a pathology either alone or in combination with other active compounds.

The nucleotide sequence is useful for treating the pathologies of the sixth aspect of the present invention.

According to particular aspects of the present invention, the following are provided: the use of the aforementioned nucleotide sequence for preparing a drug advantageously for treating a coagulopathy; and a pharmaceutical preparation containing the nucleotide sequence.

Instead of administering the modified FIX polypeptide it is possible to administer the nucleotide sequence which encodes it.

The nucleotide sequence can be inserted into cells or tissues by means of any known method. The nucleotide sequence can be incorporated into a vector for subsequent manipulations.

For example, certain cells could be engineered so as to express the modified FIX polypeptide, by integrating the aforementioned nucleotide sequence into a genomic location operatively linked with the promoter sequences. Said cells can be administered to a patient locally or systemically.

Usable viral vectors include poxvirus, herpesvirus, retrovirus, adenovirus, adeno-associated virus and other viruses suitable for gene therapy.

The vectors can remain as episomal or can be integrated into the chromosomes of the treated individual. Adenovirus serotypes are commercially available from the American Type Culture Collection (ATCC, Rockville).

The viral vectors, in particular adenovirus, are used ex vivo; for example, cells are isolated from a patient and transduced with an adenovirus expressing the modified FIX polypeptide. After a suitable period of culturing, the transduced cells are administered to the patient locally or systemically.

Alternatively, the viruses, in particular adenoviruses, which express the modified FIX polypeptide are isolated and formulated with a pharmaceutically acceptable excipient and administered to the patient. Typically, the adenoviruses are administered at doses of 1 to 1014 particles per kilogram of patient weight, generally from 106 to 1012 particles per kilogram of patient weight.

Additional examples of cell types for the expression and release of the modified FIX polypeptide are fibroblasts and endothelial cells (Palmer et al. (1989) Blood 73:483-445; Yao et al (1991) PNAS 88:8101-8105).

A vehicle which presents the aforementioned nucleotide sequence can be formulated in a similar manner to that described above for the modified FIX polypeptide.

The nucleotide sequence and/or drugs and/or vehicles presenting said nucleotide sequence can be used for treating the pathologies referred to above in relation to the modified FIX peptide.

Advantageously, the aforementioned nucleotide sequence is used for treating mammals, in particular human patients.

According to a further aspect of the present invention, a method is provided for detecting the protein of the first aspect of the present invention and/or the nucleotide sequence of the second aspect of the present invention.

Usable methods are those known in the state of the art, and can be adapted to those polymorphism under study to include for example immunoenzymatic assays, coagulation protein activity tests (including FIX activity), coagulometric and chromogenic tests.

According to some embodiments, the method comprises a step of amplifying by PCR part of a nucleic acid molecule (in which it is required to verify the presence of the nucleotide sequence of the second aspect of the present invention).

Advantageously, the amplification step is preceded by a step of purifying, in particular isolating, the nucleic acid molecule.

Advantageously the amplification step is followed by a sequencing step.

By way of example, the methods of examples 2 and 3 below can be followed to detect the aforesaid nucleotide sequence.

The method for detecting the protein and/or nucleotide sequence can be used to assist in the identification of those individuals who display a high tendency to develop blood diseases such as thrombosis.

Further characteristics of the present invention will ensue from the following description of some examples which are merely illustrative and non-limiting.

Example 1

Routine Laboratory Tests Carried Out on the Proband

Routine laboratory coagulation tests were carried out with regard to thrombophilia screening on an individual (defined as the Proband) exhibiting episodes of deep vein thrombosis but no other health problems.

In particular, the following were carried out: prothrombin time, partial thromboplastin time, factor IX levels, factor VIII and XI levels, antithrombin levels (activity and antigen), protein C levels (coagulometric and chromogenic activity, antigen), protein S levels (total antigen, free antigen and activity), activated protein C resistance, DNA analysis for factor V Leiden, DNA analysis for the prothrombin variant G20210A, antiphospholipid antibodies, plasminogen, fibrinolysis tests. The coagulation tests carried out on the Proband were all found to be within normal limits except for FIX activity (see example 4 below).

Example 2

Isolation of Mutant FIX from Plasma and from the Cell Culture Medium

Isolation of FIX from plasma or from culture medium was achieved by means of the immunoaffinity column technique, using a resin (sepharose 4B) to which the anti-FIX monoclonal antibody AHIX-5041 [Haematologic Technologies, Inc. (Essex Junction, Vt., USA)] was covalently bound (3.5 mg of monoclonal antibody per 3 ml of sepharose resin). Briefly, the column was equilibrated with buffer containing 20 mM Tris, 150 mM NaCl, 1 mM benzamidine (mM=millimolar). Starting from the plasma, vitamin K-dependent factors were precipitated by adding barium chloride. After centrifugation, the sediment was resuspended in a solution containing 0.2 M EDTA. The preparation thus obtained was extensively dialyzed (2 times, for at least 2 hours) in a solution containing 20 mM Tris, 150 mM NaCl. After dialysis, the preparation was permitted to pass through the column at a rate of 0.5 ml/min. After extensive column washing (10 column volumes) with Tris/NaCl buffer, elution was carried out using a solution of acidic glycine (pH 2.45). The eluate pH was immediately neutralized by adding 2 M Tris at pH 7.5. The eluate fractions containing protein (tested by the Bradford protein assay) were pooled and dialyzed against a Tris-NaCl solution, the FIX was then concentrated through a 200 μl microcolumn of fast-flow sepharose Q (ion exchange). The purity of the preparation was evaluated by applying the silver staining technique on the SDS-PAGE gel.

Example 3

Genetic Study of FIX

PCR amplification and direct sequencing of the exons and splice sites of the Proband FIX gene were carried out using standardized techniques and primers as reported in the literature (From: Methods in Molecular Medicine, Vol 31: Hemostasis and Thrombosis Protocols. Edited by D. J. Perry and K. J. Pasi. Humana Press Inc. Totowa, N.J. Chapter 16: Hemophilia B mutational analysis. By Peter Green). Briefly, amplification was carried out by using intron primer pairs flanking each of the eight exons of the FIX gene. The sequencing was undertaken with an ABI PRISM 310 sequencer (Perkin Elmer, Foster City, Calif.) using the ABI PRISM Big Dye Terminator kit for cycle sequencing reactions. The sequence data were analyzed using the Sequencing Analysis 3.0 programme (Perkin Elmer, Calif.). The sequence obtained was compared with the FIX sequence reported on the GenBank database (accession number: K02402).

Analysis of the nucleotide sequence of the Proband FIX gene has documented a single mutation in exon VIII of the FIX gene compared to the normal sequence. The patient was found to be a carrier for a mutation from G to T at position 34099 of the FIX gene (normal sequence of the FIX gene, Gene bank accession number: K02402) (or in the corresponding position 31134 according to the numbering given in the Database of mutations of Hemophilia B (Giannelli et al., Hemophilia B: Database of point mutations and short additions and deletions. Nucleic Acids Research 1990; 18:4053-9) able to change codon 338 from Arginine to Leucine. Therefore the FIX molecule present in the Proband's plasma (mutated FIX) differs from the normal FIX molecule only by the presence of the amino acid substitution in position 338 where there is a Leucine instead of Arginine.

Example 4

In Vitro Mutagenesis, Expression and Purification of Recombinant FIX Containing the Leu 338 Mutation

Site-specific mutagenesis was carried out according to standard techniques described by Kunkel (Kunkel T A. Rapid and efficient site-specific mutagenesis without phenotypic selection; Proc Natl Acad Sci, USA 1985, 82:488-492). Sequencing of the cDNA was carried out for assurance that the mutation was correct and that any new mutations had not been introduced. Expression of the recombinant FIX was obtained using "human embryonic kidney cell line 293" and the methods already reported in the literature (Chang J L, Jin J P, Lollar P, et al. Changing residue 338 in human factor IX from arginine to alanine causes an increase in catalytic activity. J. Biol. Chem. 1998; 273:12089-12094). The recombinant FIX was isolated from the supernatant (culture medium) by means of an immunoaffinity column, as aforedescribed. Briefly, the supernatant of the cell culture was collected every 24 hours for 10 days and conserved at −20° C. For the purification the supernatant was thawed out and benzamidine and EDTA were added to a final concentration of 5 milliMoles and 4 milliMoles respectively. After filtration through a Millipore filter, the supernatant was incubated with fast-flow Sepharose Q resin for 12 hours at 4° C. The resin was then re-equilibrated in Tris, NaCl and benzamidine buffer and loaded onto the column. Elution was undertaken with a 0-60 nM calcium gradient. The eluate was then dialyzed in a Tris-NaCl buffer. The preparation was then applied to the immunoaffinity column following the method described in example 2 (in the "in vitro" expression of the recombinant protein). Starting from the culture medium, the procedure was the same as for the plasma, except for the precipitation procedure using BaCl. The culture medium was centrifuged at 4000 g for 20 minutes then subjected to dialysis in Tris-NaCl and loaded onto the immunoaffinity column at a rate of 0.5 ml/min. The remaining steps were the same as those taken for the plasma.

The FIX with the G34099T gene mutation resulting in the 338Leu amino acid substitution, was obtained by in vitro mutagenesis and expression techniques. The level of expression in cell culture was found to be similar to that obtainable

with non-mutated recombinant FIX (normal molecule). Specifically, the expression level of the non-mutated recombinant FIX was between 750 and 880 ng/ml while for the recombinant factor IX with the gene mutation G34099T resulting in the 338Leu amino acid substitution, the level was between 590 and 629 ng/ml.

Example 5

Functional Assay of FIX

The functional assay of FIX was carried out on the Proband's plasma with a coagulometric test using Actin (Dade Behring, Marburg, Germany) and FIX deficient plasma (Dade Behring, Marburg, Germany). Briefly for the coagulometric test a variant of the partial thromboplastin time (PTT) was used in a system containing FIX deficient plasma. After adding the calcium chloride the clotting time was measured in seconds. This clotting time was compared to those of a calibration curve obtained by serial dilutions of a pool of normal plasma as reference containing FIX at a quantity of 5 μg/ml (i.e. 100%), and the FIX percentage present in the sample being calculated on 100% of the normal plasma pool (according to common standardized methods).

The normal range for the test had been previously obtained by analyzing, using the same method, 100 healthy individuals of both sexes, aged between 20 and 70 years.

The activity levels of FIX in the Proband were found to be equal to 776% (normal range in 100 healthy individuals, 80-120%).

Example 6

Assay of FIX Antigen

The FIX antigen was determined with the ELISA test using a first anti-FIX monoclonal antibody (Affinity Biologicals, Ontario, Canada) coated (bound) onto the plate for the capture and a second monoclonal antibody labelled with Horseradish peroxidase (HRP) (Affinity Biologicals, Canada) for the detection of FIX. The reference curve was constructed by diluting a pool of normal plasma from 1:100 to 1:3200 in a buffer for the samples, according to standardized procedures. Briefly, the first antibody was bound to the plate after dilution in sodium bicarbonate buffer at basic pH (pH=9.0) at a final concentration of 4 μg/ml. After extensive washing of the plate with Tris-NaCl-Tween20 buffer, the samples, diluted 1:100 and 1:200 in the same buffer, were loaded into the wells and incubated at ambient temperature for 2 hours. After removal of the samples from the wells and extensive washing with the buffer, 100 μl of a solution containing the second antibody conjugated with HRP were added to each of the wells and incubated at ambient temperature for two hours. After further washes, 100 μl of a solution containing tetramethylbenzidine (TMB) were added and the developed color was measured by spectrophotometer with a 450 nanometer filter. The level of FIX antigen was calculated using the reference curve and expressed as a percentage of the pool of normal plasma. The normal test range was previously obtained using the same method by analyzing 100 healthy individuals of both sexes, aged between 20 and 70 years.

FIX antigen levels were found to be equal to 92% (normal range 80-120%). This result (combined with that obtained in example 5) was compatible with the presence of normal quantities of a synthesized circulating FIX, but with its procoagulant function being around 8-9 times greater than the normal FIX molecule.

Example 7

Activity and Antigen Levels of FIX After Reconstitution of a FIX Deficient Plasma with an FIX Extracted from the Proband's Plasma and with Recombinant FIX

After isolating FIX from the Proband's plasma, this FIX was used for reconstituting a FIX deficient plasma (Dade-Behring, Milan, Italy) with a final FIX concentration of 5 μg/ml (equal to 100% of normal). The measurements of FIX activity and antigen in the thus reconstituted plasma were 740% and 95% respectively, these being hence comparable with those of the Proband's plasma.

For assaying the activity of the recombinant FIX obtained in accordance with example 4, the same system was used after recomposition of a FIX deficient plasma with a quantity of mutated recombinant FIX (rFIX 338Leu) such as to restore the normal FIX concentration in normal human plasma, i.e. 5 μg/ml (corresponding to 100% of normal) (μg=micrograms). The measurements of recombinant factor IX activity and antigens were 780% and 90% respectively, these being hence comparable with those of the Proband's plasma. This indicates that the recombinant protein thus obtained, containing the amino acid substitution also present in factor IX of the Proband, has a biological activity at least 8-9 times greater than normal factor IX.

Example 8

SDS-PAGE and Immunoblotting of FIX

The SDS-PAGE and immunoblotting (Western blot) of the FIX was carried out on a 5-15% linear gradient gel according to standard procedures. Briefly, the samples containing normal FIX or recombinant FIX were loaded into the polyacrylamide gel wells and subjected to electrophoresis.

The FIX was then subjected to transblotting on a polyvinylidene fluoride (PVDF) membrane using a semidry apparatus (Novablot, GE-Healthcare, Milan, Italy).

The FIX was detected on the PVDF membrane after transblotting using an anti-FIX monoclonal antibody conjugated to HRP (Affinity Biologicals, Ontario, Canada).

SDS-PAGE and immunoblot of a normal FIX polypeptide, a modified FIX polypeptide according to the present invention, a recombinant modified FIX polypeptide according to the present disclosure shows that the FIX isolated from the Proband, the 338Leu recombinant FIX and the normal FIX exhibit the same electrophoretic mobility and the same immunoblot pattern.

Therefore no significant differences (neither quantitative nor qualitative) between normal human FIX, 338 Leu natural mutant human FIX and 338Leu recombinant FIX were found using this technique.

From the aforedescribed, it is clear that the presence of a leucine in a position corresponding to position 338 surprisingly increases the activity of FIX polypeptide by almost eight times.

The present invention proves to be a particular improvement on the state of the art as it provides a modified FIX polypeptide which in vivo in man does not cause any side effects other than an increased coagulation activity.

Example 9

In Vitro Mutagenesis, Expression and Purification of the Recombinant FIX Containing the 338 Asp Mutation (338 Aspartic Acid, 338D)

The site-specific mutagenesis was carried out according to standard techniques described by Kunkel (Kunkel T A. Rapid and efficient site-specific mutagenesis without phenotypic selection; Proc Natl Acad Sci USA 1985, 82: 488-492) by inserting a guanine in place of cytosine in position 34098, and an alanine in place of guanine in position 34099 and a thymine in place of alanine in position 34100 (the mutagenesis was also repeated by inserting a guanine in place of cytosine in position 34098, an adenine in place of guanine in position 34099 and a guanine in place of adenine in position 34100).

Sequencing of the cDNA was carried out for assurance that the mutation was correct and that any new mutations had not been introduced. Expression of the recombinant FIX was obtained using "human embryonic kidney cell line 293" and the methods already reported in the literature (Chang J L, Jin J P, Lollar P, et al. Changing residue 338 in human factor IX from arginine to alanine causes an increase in catalytic activity. J. Biol. Chem. 1998; 273:12089-12094). The recombinant FIX was isolated from the supernatant (culture medium) by means of an immunoaffinity column, as aforedescribed. Briefly, the supernatant of the cell culture was collected every 24 hours for 10 days and conserved at −20° C. For the purification the supernatant was thawed out and benzamidine and EDTA were added to a final concentration of 5 milliMoles and 4 milliMoles respectively. After filtration through a Millipore filter, the supernatant was incubated with fast-flow Sepharose Q resin for 12 hours at 4° C. The resin was then re-equilibrated in Tris, NaCl and benzamidine buffer and loaded onto the column. Elution was undertaken with a 0-60 nM calcium gradient. The eluate was then dialyzed in a Tris-NaCl buffer. The preparation was applied to the immunoaffinity column following the method described in example 2 (in the "in vitro" expression of the recombinant protein). Starting from the culture medium, the procedure was the same as for the plasma, except for the precipitation procedure using BaCl. The culture medium was centrifuged at 4000 g for 20 minutes then subjected to dialysis in Tris-NaCl and loaded onto the immunoaffinity column at a rate of 0.5 ml/min. The remaining steps were the same as those taken for the plasma.

The FIX with the amino acid substitution 338Asp was obtained by in vitro mutagenesis and expression techniques. The level of expression in cell culture was found to be similar to that obtainable with non-mutated recombinant FIX (normal molecule). Specifically, the expression level of the non-mutated recombinant FIX was between 750 and 880 ng/ml while for the recombinant factor IX with the 338Asp amino acid substitution, the level was between 650 and 740 ng/ml.

Example 10

Activity and Antigen Levels of FIX after Reconstitution of a FIX Deficient Plasma with Recombinant FIX with 338Asp Mutation

For the assay of the activity of recombinant FIX obtained in accordance with example 9, the same system was used after recomposition of a FIX deficient plasma with a quantity of mutated recombinant FIX (rFIX 338Asp) such as to restore the normal concentration of FIX in normal human plasma, i.e. 5 μg/ml (corresponding to 100% of normal) (μg=micrograms). Measurements of recombinant factor IX activity and antigens were 460% and 98% respectively. This indicates that the recombinant protein thus obtained (FIX 338 Asp), has a biological activity at least 5 times greater than normal factor IX.

Example 11

SDS-PAGE and Immunoblotting of FIX

The SDS-PAGE and immunoblotting (Western blot) of the FIX was carried out on a 5-15% linear gradient gel according to standard procedures. Briefly, the samples containing normal FIX or recombinant FIX were loaded into the polyacrylamide gel wells and subjected to electrophoresis.

The FIX was then subjected to transblotting on a polyvinylidene fluoride (PVDF) membrane using a semidry apparatus (Novablot, GE-Healthcare, Milan, Italy).

The FIX was detected on the PVDF membrane after transblotting using an anti-FIX monoclonal antibody conjugated to HRP (Affinity Biologicals, Ontario, Canada).

The 338Asp recombinant FIX and the normal FIX exhibit the same electrophoretic mobility and the same immunoblot pattern. Therefore no significant differences (neither quantitative nor qualitative) between normal human FIX and 338Asp recombinant FIX were found using this technique.

From the aforedescribed, it is clear that the presence of an Aspartic acid in a position corresponding to position 338 surprisingly increases the activity of FIX polypeptide by almost eight times.

The present invention proves to be a particular improvement on the state of the art as it provides a modified FIX polypeptide which in vivo in man does not cause any side effects other than an increased coagulation activity.

Example 12

In Vitro Mutagenesis, Expression and Purification of Recombinant FIX Containing the 338Gln Mutation (338 Glutamine, 338Q)

Site-specific mutagenesis was carried out according to standard techniques described by Kunkel (Kunkel T A. Rapid and efficient site-specific mutagenesis without phenotypic selection; Proc Natl Acad Sci USA 1985, 82: 488-492) by inserting an adenine in place of guanine in position 34099 (the mutagenesis was also repeated by inserting an adenine in place of guanine in position 34099, and a guanine in place of adenine in position 34100). Sequencing of the cDNA was carried out for assurance that the mutation was correct and that any new mutations had not been introduced. Expression of the recombinant FIX was obtained using "human embryonic kidney cell line 293" and the methods already reported in the literature (Chang J L, Jin J P, Lollar P, et al. Changing residue 338 in human factor IX from arginine to alanine causes an increase in catalytic activity. J. Biol. Chem. 1998; 273:12089-12094). The recombinant FIX was isolated from the supernatant (culture medium) by means of an immunoaffinity column, as aforedescribed. Briefly, the supernatant of the cell culture was collected every 24 hours for 10 days and conserved at −20° C. For the purification the supernatant was thawed out and benzamidine and EDTA were added to a final concentration of 5 milliMoles and 4 milliMoles respectively. After filtration through a Millipore filter, the supernatant was incubated

with fast-flow Sepharose Q resin for 12 hours at 4° C. The resin was then re-equilibrated in Tris, NaCl and benzamidine buffer and loaded onto the column. Elution was undertaken with a 0-60 nM calcium gradient. The eluate was then dialyzed in a Tris-NaCl buffer. The preparation was then applied to the immunoaffinity column following the method described in example 2 (in the "in vitro" expression of the recombinant protein). Starting from the culture medium, the procedure was the same as for the plasma, except for the precipitation procedure using BaCl. The culture medium was centrifuged at 4000 g for 20 minutes then subjected to dialysis in Tris-NaCl and loaded onto the immunoaffinity column at a rate of 0.5 ml/min. The remaining steps were the same as those taken for the plasma.

The FIX with the amino acid substitution 33801n was obtained by in vitro mutagenesis and expression techniques. The level of expression in cell culture was found to be similar to that obtainable with non-mutated recombinant FIX (normal molecule). Specifically, the expression level of the non-mutated recombinant FIX was between 750 and 880 ng/ml while for the recombinant factor IX with the 338Gln amino acid substitution, the level was between 600 and 720 ng/ml.

Example 13

Levels of Activity and Antigen of the FIX After Reconstitution of a FIX Deficient Plasma with Recombinant FIX with 338Gln Mutation

For the assay of the activity of recombinant FIX obtained in accordance with example 12, the same system was used after recomposition of a FIX deficient plasma with a quantity of mutated recombinant FIX (rFIX 338Gln) such as to restore the normal concentration of FIX in normal human plasma, i.e. 5 μg/ml (corresponding to 100% of normal) (μg=micrograms). Measurements of recombinant factor IX activity and antigens were 1360% and 99% respectively. This indicates that the recombinant protein thus obtained (FIX 338 Gln) has a biological activity at least 13 times greater than normal factor IX.

Example 14

SDS-PAGE and Immunoblotting of FIX

The SDS-PAGE and immunoblotting (Western blot) of the FIX was carried out on a 5-15% linear gradient gel according to standard procedures. Briefly, the samples containing normal FIX or recombinant FIX were loaded into polyacrylamide gel wells and subjected to electrophoresis.

The FIX was then subjected to transblotting on a polyvinylidene fluoride (PVDF) membrane using a semidry apparatus (Novablot, GE-Healthcare, Milan, Italy).

The FIX was detected on the PVDF membrane after transblotting using an anti-FIX monoclonal antibody conjugated to HRP (Affinity Biologicals, Ontario, Canada).

The 338Gln recombinant FIX and the normal FIX exhibited the same electrophoretic mobility and the same immunoblot pattern. Therefore no significant differences (neither quantitative nor qualitative) between normal human FIX and 338Gln recombinant FIX were found using this technique.

From the aforedescribed, it is clear that the presence of a glutamine in a position corresponding to position 338 surprisingly increases the activity of FIX polypeptide by almost thirteen times.

The present invention proves to be a particular improvement on the state of the art as it provides a modified FIX polypeptide which in vivo in humans does not cause any side effects other than an increased coagulation activity.

Therefore evidence is provided that:

1) it has been discovered a naturally occurring FIX mutant (arginine 338 leucine) with a 8-9 fold increased functional activity as compared to FIX wild-type;

2) recombinant modified FIX polypeptides (not known before) with 5 folds (FIX arginine 338 aspartic acid), 8 to 9 folds (FIX arginine 338 leucine), 13 folds (FIX arginine 338 glutamine) increased functional (procoagulant) activity, respectively, as compared to FIX wild-type can be generated.

The use of the mutants of the invention, which show such a specific functional activity of 5 folds or above, and in particular 8 to 9 folds as compared to FIX wild type, for medical use and in particular for the prophylaxis and treatment of Hemophilia B patients; said use of the mutants of the invention has never been considered before and is part of the present invention.

The use of the mutants of the invention, which show such a specific functional activity of 5 folds or above, and in particular 8 to 9 folds as compared to FIX wild type, for gene therapy of Hemophilia B patients has never been considered before and is part of the present invention.

The use of the mutants of the invention, which show a specific functional activity of 5 folds or above, and in particular 8 to 9 folds as compared to FIX wild type, for the prophylaxis and treatment of hemorrhagic coagulopathies other than Hemophilia B or for gene therapy of such diseases has never been considered before and is part of the present invention.

It has to be noted that the use of the mutants of the invention, which show a specific functional activity of 5 folds or above, and in particular of FIX arginine 338 leucine which shows 8 to 9 folds increased functional activity as compared to FIX wild type, is considered optimal for the treatment of patients with hemophilia B because of the presence of an identical naturally occurring mutant in humans (never described before, and is part of the present invention) which does not generate neutralizing antibodies. In addition, the FIX functional activity levels express by FIX arginine 338 leucine, is possibly the best option being higher than that of FIX arginine 338 alanine (previously known and described in WO 99/03496, with a modest increase in activity of 2 to 3 folds that of FIX wild-type) and not too high to cause thrombotic complications in hemophilia B patients or patients with other hemorrhagic coagulopathies.

The invention of FIX arginine 338 leucine, is also the best choice for the use of FIX mutants in gene therapy by using viral vectors, given the actual efficiency and yield of the method for the treatment (partial correction) of Hemophilia B.

According to certain aspects of the present invention there are provided polypeptides, nucleotide sequences, nucleic acids, vectors, methods and uses in accordance with the following points.

1. A modified FIX (factor IX) polypeptide comprising:

an amino acid chosen from the group consisting of: leucine, cysteine, aspartic acid, glutamic acid, histidine, lysine, asparagine, glutamine, tyrosine in a position corresponding to position 338.

2. A polypeptide according to claim **1** wherein the amino acid is chosen from the group consisting of: leucine, aspartic acid, glutamine.

3. A polypeptide according to claim 1 wherein the amino acid is chosen from the group consisting of: aspartic acid, glutamine.

4. A polypeptide according to claim 1 wherein the amino acid is aspartic acid.

5. A polypeptide according to claim 1 wherein the amino acid is glutamine.

6. A polypeptide according to claim 1 wherein the amino acid is leucine.

7. A polypeptide according to one of the previous points, and having a homology of at least 70% with a peptide sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

8. A polypeptide according to one of the previous points, and having a homology of at least 90% with a peptide sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

9. A polypeptide according to one of the previous points, and having a percentage identity of at least 70% with a peptide sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

10. A polypeptide according to one of the previous points, and having a percentage identity of at least 90% with a peptide sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

11. A polypeptide according to one of the previous points wherein the peptide sequence is SEQ ID NO: 2.

12. A nucleotide sequence encoding a FIX polypeptide according to one of the previous points.

13. A nucleotide sequence according to point 12 wherein the nucleotide sequence is a DNA sequence and consists of intron portions and exon portions, the exon portions having an overall sequence with at least 70% homology relative to an overall sequence of exon regions of a SEQ ID NO: 5 sequence.

14. A nucleotide sequence according to point 13 wherein the overall sequence of the exon portions has at least 90% homology with the overall sequence of the exon regions of the SEQ ID NO: 5 sequence.

15. A nucleotide sequence according to one of points 12 to 14 wherein the nucleotide sequence has at least 50% homology with the sequence having the accession number K02402 (GenBank).

16. A nucleotide sequence according to one of points 12 to 15, comprising in positions corresponding to 34098, 34099 and 34100 a triplet chosen from the group consisting of: TTA, UUA, TTG, UUG, CTT, CUU, CTC, CUC, CTA, CUA, CTG, CUG, GAT, GAU, GAC, CAA, CAG.

17. A nucleotide sequence according to point 16, comprising in positions corresponding to 34098, 34099 and 34100 a triplet chosen from the group consisting of: TTA, UUA, TTG, UUG, CTT, CUU, CTC, CUC, CTA, CUA, CTG, CUG, CAA, CAG.

18. A nucleotide sequence according to point 16, comprising in positions corresponding to 34098, 34099 and 34100 a triplet chosen from the group consisting of: TTA, UUA, TTG, UUG, CTT, CUU, CTC, CUC, CTA, CUA, CTG, CUG.

19. A nucleotide sequence according to point 16, comprising in positions corresponding to 34098, 34099 and 34100 a triplet chosen from the group consisting of: CAA, CAG.

20. A nucleotide sequence according to point 16, comprising in positions corresponding to 34098, 34099 and 34100 a triplet chosen from the group consisting of: GAT, GAU, GAC, CAA, CAG.

21. A nucleotide sequence according to point 16, comprising in positions corresponding to 34098, 34099 and 34100 a triplet chosen from the group consisting of: GAT, GAU, GAC.

22. A nucleotide sequence according to one of points 12 to 18, comprising a thymine in a position corresponding to position 34099.

23. A nucleic acid comprising a nucleotide sequence according to one of points 12 to 22.

24. A nucleic acid according to point 23, and comprising a promoter in operational linkage with said nucleotide sequence.

25. A vector comprising a nucleic acid according to point 23 or 24.

26. A method for producing a modified FIX polypeptide, whereby the modified FIX polypeptide is expressed by means of a nucleic acid according to point 23 or 24.

27. A method according to point 26, comprising the steps of: introducing a vector of point 25 into a cell; and

culturing the cell such that the FIX polypeptide is expressed.

28. A modified FIX polypeptide according to one of points 1 to 11 for use as a medicament.

29. A modified FIX polypeptide according to one of points 1 to 11 for the treatment of at least one coagulopathy.

30. Use of a modified FIX polypeptide according to one of points 1 to 11 for preparing a drug for the treatment of at least one coagulopathy in a mammal.

31. A nucleotide sequence according to one of points 12 to 22 for use as a medicament.

32. A method for detecting the nucleotide sequence of one of points 12 to 22.

33. A method for detecting the modified FIX polypeptide according to one of points 1 to 11.

34 A method according to point 32 comprising an step of amplification by PCR.

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KPGQFPWQ                                                          68

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LT                                                                 182

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CQGDSGGPHV TEVEGTSFLT GIISWGEECA MKGKYGIYTK VSRYVNWIKE KTKLT       415
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The invention claimed is:

[1. A pharmaceutical composition comprising:

a modified recombinant FIX (factor IX) polypeptide comprising at least 70% identity to SEQ ID NO: 2 and a leucine in position 338 of SEQ ID NO: 2; the modified recombinant FIX polypeptide in an amount suitable to provide a daily dosage comprised between 0.1 μg/kg and 400 μg/kg body weight, and

at least one pharmaceutically acceptable excipient.**]**

[2. The pharmaceutical composition according to claim **1**, wherein the polypetide comprises at least 90% identity to SEQ ID NO: 2.**]**

[3. The pharmaceutical composition according to claim **1** formulated for oral, parenteral or rectal administration or suited to administration by inhalation or insufflation.**]**

[4. A method of treatment, the method comprising administering to an individual in need thereof the pharmaceutical composition according to claim **1**.**]**

[5. The method according to claim **4** wherein the administering is performed to treat at least one coagulopathy in the individual.**]**

[6. A sequence of nucleotides encoding a modified FIX (Factor IX) polypeptide comprising at least 70% identity to SEQ ID NO: 2 and a leucine at position 338 of SEQ ID NO: 2.**]**

[7. The sequence of nucleotides according to claim **6**, wherein the nucleotide sequence has at least 90% homology with the sequence having accession number K02402 (GenBank).**]**

8. *A nucleotide sequence encoding a modified FIX (Factor IV) polypeptide comprising 100% identity to SEQ ID NO: 2 except for an alanine at position 148 and a leucine at position 338 of SEQ ID NO: 2 in combination with positions corresponding to positions 34098, 34099 and 34100 of a sequence having accession number K02402 (GenBank) having a triplet selected from the* group consisting of: **[**TTA, UUA, TTG, UUG, CTT, CUU,**]** CTC, CUC, **[**CTA, CUA,**]** CTG, *and* CUG**[**, GAT, GAU and GAC**]**.

[9. A nucleic acid comprising the nucleotide sequence according to claim **6**.**]**

[10. The nucleic acid according to claim **9**, the nucleic acid comprising a promoter in operational linkage with said nucleotide sequence.**]**

[11. A vector comprising the nucleic acid according to claim **9**.**]**

[12. A pharmaceutical composition comprising the sequence of nucleotide according to claim **6**.**]**

[13. A method for producing a modified FIX (Factor IX) polypeptide, the method comprising

expressing the modified FIX polypeptide by means of a nucleic acid according to claim **9**.**]**

[14. A method to perform gene therapy, the method comprising

administering to an individual in need thereof the nucleotide sequence according to claim **6** via a vector configured for gene therapy.**]**

[15. The method according to claim **14**, wherein the administering is performed to treat at least one coagulopathy in the individual.**]**

16. The nucleotide sequence of claim 8, wherein the triplet is selected from the group consisting of CTC and CTG.

17. The nucleotide sequence of claim 8, wherein the triplet is selected from the group consisting of CUC and CUG.

18. The nucleotide sequence of claim 8, wherein the triplet is CTC.

19. The nucleotide sequence of claim 8, wherein the triplet is CTG.

20. The nucleotide sequence of claim 8, wherein the triplet is CUC.

21. The nucleotide sequence of claim 8, wherein the triplet is CUG.

* * * * *