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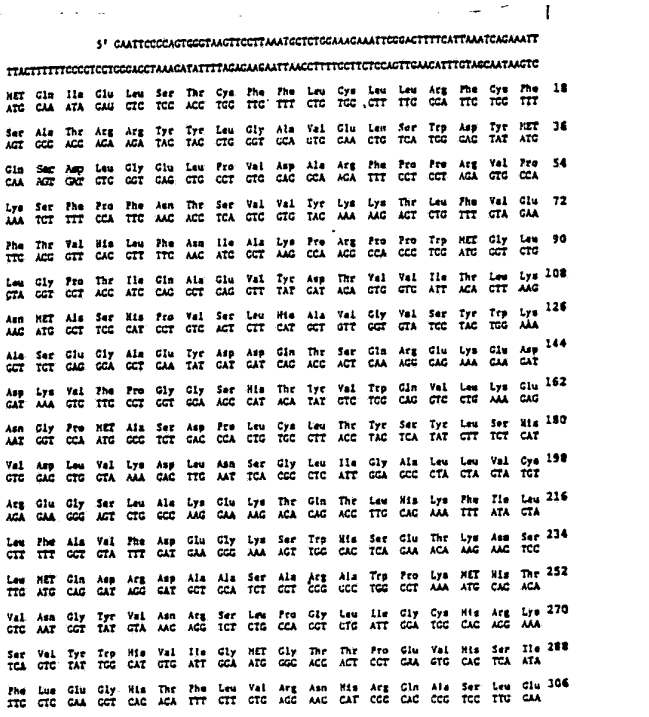
(57) Abstract

Preparations of recombinant DNA which code for cellular production of human and porcine factor VIII:C and methods of obtaining such DNA and expression thereof in bacteria and eucarryotic cells. New compounds, including deoxyribonucleotides and ribonucleotides which are utilized in obtaining clones which express factor VIII:C are also disclosed.



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PRODUCTION OF FACTOR VIII AND RELATED PRODUCTS

Prior Application

This application is a continuation-in-part application of U.S. Application Serial No. 546,650 filed on October 28, 1983.

Background of the Invention

This invention relates to the preparation of recombinant deoxyribonucleic acid (DNA) which codes for cellular production of human factor VIII:C, and of DNA which codes for porcine factor VIII:C, to methods of obtaining DNA molecules which code for factor VIII:C, and to expression of human and porcine factor VIII:C utilizing such DNA, as well as to novel compounds, including deoxyribonucleotides and ribonucleotides utilized in obtaining such clones and in achieving expression of human factor VIII:C. This invention also relates to human AHF and its production by recombinant DNA techniques.

Factor VIII:C is a blood plasma protein that is defective or absent in Hemophilia A disease. This disease is a hereditary bleeding disorder affecting approximately one in 20,000 males. Factor VIII:C has also been known or referred to as factor VIII, the antihemophilic factor (AHF), antihemophilic globulin (AHG), hemophilic factor A, platelet cofactor, thromboplastinogen, and thrombocytolysin. It is referred to as "Factor VIII:C", to indicate that it is the compound which affects clotting activity. As used herein, "factor VIII:C" and "AHF" are synonymous.

Although the isolation of AHF from blood plasma has been described in the literature, the precise structure of AHF has not previously been identified, due in part to the unavailability of sufficient quantities of pure material, and the proteolytic nature of many contaminants and purification agents. While some quantities of impure AHF have been available as a concentrated preparation processed from fresh-frozen human plasma, the extremely low concentration of AHF in human plasma and the high cost of obtaining and processing human plasma make the cost of

this material prohibitive for any extensive treatment of hemophilia.

The present invention makes it possible to produce human AHF using recombinant DNA techniques.

AHF, like other proteins, is comprised of some twenty different amino acids arranged in a specific array. By using gene manipulation techniques, a method has been developed which enables production of AHF by identifying and cloning the gene which codes for the human AHF protein, cloning that gene, incorporating that gene into a recombinant DNA vector, transforming a suitable host with the vector which includes that gene, expressing the human AHF gene in such host, and recovering the human AHF produced thereby. Similarly, the present invention makes it possible to produce porcine AHF by recombinant DNA techniques, as well as providing products and methods related to such porcine AHF production.

Recently developed techniques have made it possible to employ microorganisms, capable of rapid and abundant growth, for the synthesis of commercially useful proteins and peptides, regardless of their source in nature. These techniques make it possible to genetically endow a suitable microorganism with the ability to synthesize a protein or peptide normally made by another organism. The technique makes use of fundamental relationships which exist in all living organisms between the genetic material, usually DNA, and the proteins synthesized by the organism. This relationship is such that production of the amino acid sequence of the protein is coded for by a series of three nucleotide sequences of the DNA. There are one or more trinucleotide sequence groups (called codons) which specifically code for the production of each of the twenty amino acids most commonly occurring in proteins. The specific relationship between each given trinucleotide sequence and the corresponding amino acid for which it codes constitutes the genetic code. As a consequence, the amino acid sequence of every protein or peptide is reflected by a corresponding nucleotide sequence, according to

a well understood relationship. Furthermore, this sequence of nucleotides can, in principle, be translated by any living organism. For a discussion of the genetic code, see J. D. Watson, Molecular Biology of the Gene, (W. A. Benjamin, Inc., 1977), the disclosure of which is incorporated herein by reference, particularly at 347-77; C. F. Norton, Microbiology (Addison, Wesley 1981), and U. S. Patent No. 4,363,877, the disclosure of which is incorporated herein by reference.

The twenty amino acids from which proteins are made, are phenylalanine (hereinafter sometimes referred to as "Phe" or "F"), leucine ("Leu", "L"), isoleucine ("Ile", "I"), methionine ("Met", "M"), valine ("Val", "V"), serine ("Ser", "S"), proline ("Pro", "P"), threonine ("Thr", "T"), alanine ("Ala", "A"), tyrosine ("Tyr", "Y"), histidine ("His", "H"), glutamine ("Gln", "Q"), asparagine ("Asp", "N"), glutamic acid ("Glu", "E"), cysteine ("Cys", "C"), tryptophane ("Trp", "W"), arginine ("Arg", "R") and glycine ("Gly", "G"). The amino acids coded for by the various combinations of trinucleotides which may be contained in a given codon may be seen in Table 1:

TABLE 1

The Genetic Code

First Position	Second Position T	C	A	G	Third Position
T	Phe	Ser	Tyr	Cys	T
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Stop*	Stop*	A
	Leu	Ser	Stop*	Trp	G
C	Leu	Pro	His	Arg	T
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asp	Ser	T
	Ile	Thr	Asp	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	T
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

*The "Stop" or termination codon terminates the expression of the protein.

Knowing the deoxyribonucleotide sequence of the gene or DNA sequence which codes for a particular protein allows the exact description of that protein's amino acid sequence. However, the converse is not true; while methionine is coded for by only one codon, the other amino acids can be coded for by up to six codons (e.g. serine), as is apparent from Table 1. Thus there is considerable ambiguity in predicting the nucleotide sequence from the amino acid sequence.

In sum, prior to the present invention, very little was known about the structure of AHF, and, despite substantial work over many years, those skilled in this art were unable to determine the structure of AHF, or of its gene, or provide any procedure by which AHF could be produced in substantially pure form in substantial quantities.

The method described herein by which the gene for human AHF is cloned and expressed includes the following steps:

(1) Purification of porcine AHF;

(2) Determination of the amino acid sequence of porcine AHF;

(3) Formation of oligonucleotide probes, and use of those probes to identify and/or isolate at least a fragment of the gene which codes for porcine AHF;

(4) Use of the porcine AHF gene fragment to identify and isolate human genetic material which codes for human AHF;

(5) Using the previously described AHF DNA fragments to determine the site of synthesis of AHF from among the various mammalian tissues;

(6) Producing cDNA segments which code for human and porcine AHF, using messenger RNA obtained from the tissue identification in step 5;

(7) Constructing full length human and porcine cDNA clones from the cDNA segments produced in step 6, e.g. by ligating together cDNA segments which were cut by the same restriction enzymes;

(8) Forming DNA expression vectors which are capable of directing the synthesis of AHF;

(9) Transforming a suitable host with the expression vectors bearing the full length cDNA for human or porcine AHF;

(10) Expressing human or porcine AHF in the host; and

(11) Recovering the expressed AHF.

In the course of this work, a new technique of screening a genomic DNA library has been developed utilizing oligonucleotide probes based on the amino acid sequences contained in the AHF molecule.

The invention includes the above methods, along with the various nucleotides, vectors, and other products made in connection therewith.

Brief Summary of The Drawings

Figure 1 is a depiction of the amino acid sequence (A) determined for the amino terminal sequence of the 69,000 dalton thrombin cleavage product described in Example 1. The first residue was not identifiable. This sequence is compared with sequence (B) deduced from the nucleotide sequence of the porcine AHF exon described in Example 3, and with the human AHF exon sequence(C) described in Example 4.

Figure 2 illustrates the amino acid sequence, shown in single letter code, of bovine thrombin digestion fragments of (A) the amino terminus and (B) a 40 Kd thrombin cleavage product of the 166 Kd porcine AHF fragment isolated by Fass et al., infra.

Figure 3 illustrates the design of an oligonucleotide

probe for the identification and isolation of at least a portion of the porcine gene which codes for AHF.

Figure 4 illustrates the DNA sequence for a Sma I DNA fragment (34-S1) which contains a porcine AHF exon, derived from bacteriophage PB34 described in Example 3.

Figure 5 is a representation of the DNA sequence of the Hae III insert 34-H1 bearing the exon for porcine AHF, as described in Example 3. This sequence is included within the longer sequence shown in Figure 4 and corresponds to nucleotides 250-615 of the sequence in Figure 4.

Figure 6 is a representation of the DNA sequence along with the deduced amino acid sequence for nucleotides 34-84 for a portion of the Sau 3AI insert of clone 25-S1, showing a portion of the exon for human AHF, as described in Example 4.

Figure 7 illustrates the DNA nucleotide sequence (shown in one strand only) which contains the entire sequence coding for human AHF, along with the deduced amino acid sequence for human AHF.

Detailed Description of the invention

The following definitions are supplied in order to facilitate the understanding of this case. To the extent that the definitions vary from meanings circulating within the art, the definitions below are to control.

Amplification means the process by which cells produce gene repeats within their chromosomal DNA.

Cotransformation means the process of transforming a cell with more than one exogenous gene foreign to the cell, one of which confers a selectable phenotype on the cell.

Downstream means the direction going towards the 3' end of a nucleotide sequence.

An enhancer is a nucleotide sequence that can potentiate the transcription of genes independent of the identity of the gene, the position of the sequence in relation to the gene, or

the orientation of the sequence.

A gene is a deoxyribonucleotide sequence coding for a given mature protein. For the purposes herein, a gene shall not include untranslated flanking regions such as RNA transcription initiation signals, polyadenylation addition sites, promoters or enhancers.

A selection gene is a gene that confers a phenotype on cells which express the gene as a detectable protein.

A selection agent is a condition or substance that enables one to detect the expression of a selection gene.

Phenotype means the observable properties of a cell as expressed by the cellular genotype.

Genotype means the genetic information contained within a cell as opposed to its expression, which is observed as the phenotype.

Ligation is the process of forming a phosphodiester bond between the 5' and 3' ends of two DNA strands. This may be accomplished by several well known enzymatic techniques, including blunt end ligation by T4 ligase.

Orientation refers to the order of nucleotides in a DNA sequence. An inverted orientation of a DNA sequence is one in which the 5' to 3' order of the sequence in relation to another sequence is reversed when compared to a point of reference in the DNA from which the sequence was obtained. Such points of reference can include the direction of transcription of other specified DNA sequences in the source DNA or the origin of replication of replicable vectors containing the sequence.

Transcription means the synthesis of RNA from a DNA template.

Transformation means changing a cell's genotype by the cellular uptake of exogenous DNA. Transformation may be detected in some cases by an alteration in cell phenotype. Transformed cells are called transformants. Pre-transformation cells are referred to as parental cells.

Translation means the synthesis of a polypeptide from messenger RNA (mRNA).

The present invention permits, for the first time, the identification and isolation of porcine Factor VIII:C gene by recombinant DNA techniques. It also permits, for the first time, the isolation and identification of the gene which encodes human factor VIII:C by recombinant DNA techniques, by taking advantage of the homology between porcine AHF DNA and human AHF DNA in order to locate and isolate the human gene for AHF. This route to cDNA clones for producing AHF via homology with the porcine AHF gene avoids the tedious time consuming and expensive need to purify human AHF, which is highly expensive and essentially unavailable.

Porcine AHF is first highly purified, most preferably by a monoclonal antibody purification technique such as that disclosed by David Fass et al, "Monoclonal Antibodies to Porcine AHF Coagulant and Their Use in the Isolation of Active Coagulent Protein", Blood 59: 594-600 (1982), and Knutsen et al., "Porcine Factor VIII:C prepared by affinity Interaction with Von Willebrand Factor and Heterologous Antibodies," Blood, 59: 615-24 (1982), the disclosures of both of which are incorporated herein by reference. Porcine factor VIII:C polypeptides are bound to anti-VIII:C monoclonal antibodies which are immobilized on a suitable affinity chromatography column. Two large molecular weight polypeptides, having molecular sizes of about 166 and 130 Kd, are eluted with ethylenediamine tetraacetic acid. Another protein segment having a molecular weight of about 76 Kd, is then eluted from the column, utilizing about 50% ethylene glycol. The partial amino acid sequences of these polypeptides, and/or of fragments of these polypeptides obtained in a known manner, e.g. from enzymatic digestion of the proteins, using bovine thrombin or other suitable agents to break up the proteins, are then determined by known methods of analysis. Based on the amino acid sequence of these materials, oligonucleotide probes are

synthesized, at least some of which will hybridize with DNA segments which code for the corresponding segment of AHF. These oligonucleotides are then used to screen for segments of the gene which code for porcine AHF.

Once a portion of the porcine gene for AHF is obtained, that recombinant material is used to screen a human genomic DNA library to locate and isolate the gene which codes for human AHF. In this procedure, it is established that there are substantial similarities between human AHF and porcine AHF, and advantage is taken of those similarities to isolate and identify the human factor VIII:C gene or gene segments.

The similarities in the porcine and human proteins are attributable to corresponding similarities in the DNA sequences which code for the amino acid sequences. The genetic materials coding for the AHF proteins in humans and pigs are identical at a high percentage of positions, and thus exhibit hybridization when subjected to the procedure of, for example, Benton and Davis, "Screening gt Recombinant Clones by Hybridization to Single Plaques In Situ," Science, 196:180 (1977), the disclosure of which is incorporated herein by reference.

The cloned segments of the gene for human AHF are then used to identify a source for AHF mRNA from among various human tissues. Similarly, one or more of the cloned segments for porcine AHF are used to screen potential tissue sources for porcine mRNA. This step is accomplished by conventional procedures involving RNA extraction, gel electrophoresis, transfer to nitrocellulose sheets, and hybridization to radiolabelled cloned DNA as described for example in Maniatis, et. al., Molecular Cloning, A Laboratory Manual, (Cold Spring Harbor Laboratory, 1982). Once this tissue source is identified, cDNA libraries are prepared (in either plasmid or preferably bacteriophage lambda vectors, both of which are generally available) and screened for AHF cDNA clones as described below. The process of cDNA library screening is repeated until a set of

cDNA clones is obtained which, by DNA sequence determintion, is shown to comprise the entire DNA gene sequence encoding AHF.

Once the full length human or porcine AHF cDNA clone is obtained, known and appropriate means are utilized to express the AHF protein, e.g. insertion into an appropriate vector, and transfection into an appropriate host, selection of transformed cells (transformants), and culture these transformants, to express AHF activity.

Host-vector systems for the expression of AHF may be procaryotic, but the complexity of AHF makes the preferred expression system eucaryotic, preferably (at least for biologically-active AHF having clotting activity) a mammalian one. This is easily accomplished by eucaryotic (usually mammalian or vertebrate cells) transformation with a suitable AHF vector. Eucaryotic transformation is in general a well-known process, and may be accomplished by a variety of standard methods. These include the use of protoplast fusion, DNA microinjection, chromosome transfection, lytic and nonlytic viral vectors (For example, Mulligan et al., "Nature" (London) 277:108-114 (1979), cell-cell fusion (Fournier et al., "Proc. Nat. Acad. Sci." 74:319-323 (1977), lipid structures (U.S. patent 4,394,448) and cellular endocytosis of DNA precipitates (Bachetti et al., "Proc. Nat. Acad. Sci. 74:1590-1594 (1977). Other eucaryotic cells, such as yeasts or insect cells, may also be used to advantage.

Transformation which is mediated by lytic viral vectors is efficient but is disadvantageous for a number of reasons: The maximum size of transfected DNA is limited by the geometry of viral capsid packing, the exogenous genes are frequently deleted during viral replication, there is a requirement for helper virus or specialized hosts, host cells must be permissive, and the hosts are killed in the course of the viral infection.

Nonlytic transformations are based on the transcription and translation of virus vectors which have been incorporated

into a cell line as a stable episome. These systems generally require unique cell lines and suffer from a number of disadvantages. See "Trends in Biochemical Sciences", June 1983, pp. 209-212.

On the other hand, other transformation methods in which extrachromosomal DNA is taken up into the chromosomes of host cells have been characterized by low frequencies of transformation and poor expression levels. These initial difficulties were ameliorated by transformation with genes which inheritably confer selectable phenotypes on the small subpopulation of cells that are in fact transformed (selection genes). The entire population of transformed cells can be grown under conditions favoring cells having acquired the phenotype, thus making it possible to locate transformed cells conveniently. Thereafter, transformants can be screened for the capability to more intensely express the phenotype. This is accomplished by changing a selection agent in such a way as to detect higher expression.

Selection genes fall into three categories: Detectably amplified selection genes, dominant selection genes, and detectably amplified dominant selection genes.

Detectably amplified selection genes are those in which amplification can be detected by exposing host cells to changes in the selection agent. Detectably amplified genes which are not dominant acting generally require a parental cell line which is genotypically deficient in the selection gene. Examples include the genes for hydroxymetholglutanyl CoA reductase (Sinensky, "Biochem. Biophys. Res. Commun" 78:863 (1977), ribonucleotide reductase (Meuth et al. "Cell:3:367 (1943), aspartate transcarbamylase; (Kemp et al. "Cell" 9:541 (1976), adenylate deaminase (DeBatisse et al. "Mol and Cell Biol." 2(11):1346-1353 (1982) mouse dihydrofolate reductase (DHFR) and, with a defective promoter, mouse thymidine kinase (TK).

Dominant selection genes are those which are expressed in

transformants regardless of the genotype of the parental cell. Most dominant selection genes are not detectably amplified because the phenotype is so highly effective in dealing with the selection agent that it is difficult to discriminate among cell lines that have or have not amplified the gene. Examples of dominant selection genes of this type include the genes for procaryotic enzymes such as xanthine-guanine phosphoribosyltransferase (Mulligan et al. "Proc. Nat. Acad. Sci." 78[4]:2072-2076 (1981) and aminoglycoside 3' - phosphotransferase (Colbere-Garapin et al., "J. Mol. Biol.", 150:1-14 (1981).

Some dominant selection genes also are detectably amplified. Suitable examples include the mutant DHFR gene described by Haber et al., "Somatic Cell Genet." 4:499-508 (1982), cell surface markers such as HLA antigens and genes coding for enzymes such as specific esterases that produce fluorescent or colored products from fluorogenic or chromogenic substrates as is known in the art.

Detectably-amplified, dominant selection genes are preferred for use herein. It should be understood that a dominant selection gene in some cases can be converted to a detectably amplified gene by suitable mutations in the gene.

Selection genes at first were of limited commercial utility. While they enabled one to select transformants having the propensity to amplify uptaken DNA, most selection genes produced products of no commercial value. On the other hand, genes for products which were commercially valuable generally did not confer readily selectable (or even detectable) phenotypes on their transformants. This would be the case, for example, with enzymes or hormones which do not provide transformed cells with unique nutrient metabolic or detoxification capabilities. Most proteins of commercial interest fall into this group, e.g. hormones, proteins participating in blood coagulation and fibrinolytic enzymes.

Subsequently it was found that eucaryotic cells having the propensity to be transformed with and amplify the selection gene would do the same in the case of the product gene. By following the selection gene one could identify a subpopulation of transformant cells which coexpress and coamplify the product gene along with the selection gene. It has been the practice to culture the transformants in the presence of the selection agent and to conclude that transformants having increased expression of the selection gene will also show increased expression of the product gene. This is not always the case, as will be more fully explored below. Axel et al. (U.S. patent 4,399,216) use the term cotransformation to describe the process of transforming a cell with more than one different gene, whether by vector systems containing covalently linked or unlinked genes, and in the latter case whether the genes are introduced into host cells sequentially or simultaneously. Cotransformation should "allow the introduction and stable integration of virtually any defined gene into cultured cells" (Wigler et al. "Cell", 16:777-785, (1975), and "by use of the cotransformation process it is possible to produce eucaryotic cells which synthesize desired proteinaceous and other materials" (U.S. patent 4,399,216, column 3, lines 37-42).

Transformation Vectors

Vectors used in AHF cotransformation will contain a selection gene and the AHF gene. In addition there usually will be present in the transformation or cotransformation vectors other elements such as enhancers, promoters, introns, accessory DNA, polyadenylation sites and 3' noncoding regions as will be described below.

Suitable selection genes are described above. It is preferred that the selection agent be one that prevents cell growth in the absence of the selection gene. That way, revertant cells in large scale culture that lose the selection gene (and presumably the AHF gene as well) will not over-grow the

fermentation. However, it would be desirable in the commercial production of AHF to avoid the use of cell toxins, thereby simplifying the product purification steps. Thus, a desirable selection gene would be one that enables transformants to use a nutrient critical for growth that they otherwise would not be able to use. The TK gene described above is an example.

Two classes of vectors have been employed in cotransformation. The first class are the unlinked vectors. Here the selection gene and the AHF gene are not covalently bound. This vector class is preferred because the step of ligating or otherwise bonding the two genes is not required. This simplifies the transformation process because the selection and product genes usually are obtained from separate sources and are not ligated in their wild-type environment. In addition, the molar ratio of the AHF and selection genes employed during cotransformation can be adjusted to increase cotransformation efficiency.

The second class of cotransformation vectors are linked vectors. These vectors are distinguished from unlinked vectors in that the selection and AHF genes are covalently bound, preferably by ligation.

The vectors herein may also include enhancers. Enhancers are functionally distinct from promoters, but appear to operate in concert with promoters. Their function on the cellular level is not well understood, but their unique characteristic is the ability to activate or potentiate transcription without being position or orientation dependent. Promoters need to be upstream of the gene, while enhancers may be present upstream or 5' from the promoter, within the gene as an intron, or downstream from the gene between the gene and a polyadenylation site or 3' from the polyadenylation site. Inverted promoters are not functional, but inverted enhancers are. Enhancers are cis-acting, i.e., they have an effect on promoters only if they are present on the same DNA strand. For a general discussion of enhancers see Khoury et

al., "Cell" 33:313-314 (1983).

Preferred enhancers are obtained from animal viruses such as simian virus 40, polyoma virus, bovine papilloma virus, retrovirus or adenovirus. Viral enhancers may be obtained readily from publically available viruses. The enhancer regions for several viruses, e.g., Rous sarcoma virus and simian virus 40, are well known. See Luciw et al., "Cell" 33:705-716 (1983). It would be a matter of routine chemistry to excise these regions on the basis of published restriction maps for the virus in question and, if necessary, modify the sites to enable splicing the enhancer into the vector as desired. For example, see Kaufman et al, "J. Mol. Biol.", 159:601-621 (1982) and "Mol. Cell Biol." 2(11):1304-1319 (1982) the disclosures of both of which are incorporated herein by reference. Alternatively, the enhancer may be synthesized from sequence data; the sizes of viral enhancers (generally less than about 150 bp) are sufficiently small that this could be accomplished practically.

Another element which should be present in the vector assembly is a polyadenylation splicing (or addition) site. This is a DNA sequence located downstream from the translated regions of a gene, shortly downstream from which in turn transcription stops and adenine ribonucleotides are added to form a polyadenine nucleotide tail at the 3' end of the messenger RNA. Polyadenylation is important in stabilizing the messenger RNA against degradation in the cell, an event that reduces the level of messenger RNA and hence the level of product protein.

Eucaryotic polyadenylation sites are well known. A concensus sequence exists among eucaryotic genes: The hexanucleotide 5'-AAUAAA-3' is found 11-30 nucleotides from the point at which polyadenylation starts. DNA sequences containing polyadenylation sites may be obtained from viruses in accord with published reports. Exemplary polyadenylation sequences can be obtained from mouse beta-globulin, and simian virus 40 late or early region genes, but viral polyadenylation sites are

preferred. Since these sequences are known, they may be synthesized in vitro and ligated to the vectors in conventional fashion.

A polyadenylation region must be located downstream from either the AHF and/or selection gene, but may be ligated to either gene. It may be ligated to the selection gene only, and not the product gene, and this will be the case whether the vectors are linked or unlinked. The sequence which separates the polyadenylation site from the translational stop codon is preferably an untranslated DNA oligonucleootide such as an unpromoted eucaryotic gene. Since such oligonucleotides and genes are not endowed with a promoter they will not be expressed. The oligonucleotide should extend for a considerable distance, on the order of up to about 1,000 bases, from the stop codon to the polyadenylation site. This 3' untranslated oligonucleotide generally results in an increase in product yields. The vector may terminate from about 10 to about 30 bp downstream from the concensus sequence, but it is preferable to retain the 3' sequences found downstream from the polyadenylation site in its wild-type environment. These sequences typically extend about from 200 to 600 base pairs downstream from the polyadenylation site.

The vectors described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors such selection genes, enhancers, promoters, and the like may be obtained from natural sources or synthesized as described above. Basically, if the components are found in DNA available in large quantity, e.g. components such as viral functions, or if they may be synthesized, e.g. polyadenylation sites, then with appropriate use of restriction enzymes large quantities of vector may be obtained by simply culturing the source organism, digesting its DNA with an appropriate endonuclease, separating the DNA fragments, identifying the DNA containing the element of interest and

recovering same. Ordinarily, a transformation vector will be assembled in small quantity and then ligated to a suitable autonomously replicating synthesis vector such as a procaryotic plasmid or phage. The pBR322 plasmid may be used in most cases. See Kaufman et al., op. cit.

The synthesis vectors are used to clone the ligated transformation vectors in conventional fashion, e.g. by transfection of a permissive procaryotic organsim, replication of the synthesis vector to high copy number and recovery of the synthesis vector by cell lysis and separation of the synthesis vector from cell debris.

The resulting harvest of synthesis vector may be directly transfected into eucaryotic cells, or the transformation vector may be rescued from the synthesis vector by appropriate endonuclease digestion, separation by molecular weight and recovery of the transformation vector. Transformation vector rescue is not necessary so long as the remainder of the synthesis vector does not adversely affect eucaryotic gene amplification, transcription or translation. For example, the preferred synthesis vector herein is a mutant of the E. coli plasmid pBR322 in which sequences have been deleted that are deleterious to eucaryotic cells. See Kaufman et al., op. cit. Use of this mutant obviates any need to delete the plasmid residue prior to cotransformation.

Cotransformation, Selection and Detection of Amplification

The cells to be transformed may be any eucaryotic cell, including yeast protoplasts, but ordinarily a nonfungal cell. Primary explants (including relatively undifferentiated cells such as stem cells), and immortal and/or transformed cell lines are suitable. Candidate cells need not be genotypically deficient in the selection gene so long as the selection gene is dominant acting.

The cells preferably will be stable mammalian cell lines

as is discussed above. Cell lines that are known to stably integrate selection genes into their chromosomal DNA are best, for example Chinese hamster ovary (CHO) cell lines. Also useable are HeLa, COS monkey cells, melanoma cell lines such as the Bowes cell line, mouse L cells, mouse fibroblasts and mouse NIH 3T3 cells.

Cotransformation with unlinked vectors may be accomplished serially or simultaneously, (see U.S. patent 4,399,216). Methods for facilitating cellular uptake of DNA are described above. Microinjection of the vector into the cell nucleus will yield the highest transformation efficiencies, but exposing parental cells to DNA in the form of a calcium phosphate precipitate is most convenient. Considerably better cotransformation efficiencies result from cotransformation with a molar excess of product to selection gene, on the order of 100:1.

The population of cells that has been exposed to transforming conditions is then processed to identify the transformants. Only a small subpopulation of any culture which has been treated for cotransformation will exhibit the phenotype of the selection gene. The cells in the culture are screened for the phenotype. This can be accomplished by assaying the cells individually with a cell sorting device where the phenotype is one that will produce a signal, e.g. fluorescence upon cleavage of a fluorogenic substrate by an enzyme produced by the selection gene. Preferably, however, the phenotype enables only transformants to grow or survive in specialized growth media as is further discussed above.

Selection transformants then will be screened for ligation of the product gene into their chromosomes or for expression of the product itself. The former can be accomplished using Southern blot analysis, the latter by standard immunological or enzymatic assays.

Once the tranformants have been identified, steps are taken to amplify expression of the product gene by further

cloning in the presence of a selection agent such as MTX. See U.S. Patent 4,399,216.

Cotransformants which may be produced in accordance with the processes described herein are suitable for in vivo transfections of higher organisms in accordance with known techniques. Primary explants or stable cell lines from a potential host animal are cotransformed and inoculated into the host or a substantially otherwise syngeneic host which is genotypically deficient in the product protein.

The invention will be further understood with reference to the following illustrative embodiments, which are purely exemplary, and should not be taken as limitive of the true scope of the present inevntion, as described in the claims.

Unless otherwise noted, restriction endonucleases are utilized under the conditions and in the manner recommended by their commercial suppliers. Ligation reactions are carried on as described by Maniatis et al., Molecular Cloning, A Laboratory Manual, (Cold Spring Harbor Laboratory 1982) at 245-6, the disclosure of which is incorporated herein by reference, using the buffer described at page 246 thereof and using a DNA concentration of 1-100 ug/ml, at a temperature of 23°C for blunt ended DNA and 16°C for "sticky ended" DNA. "Phosphatasing" as described herein, refers to dephosphorylation of DNA, and is carried out in the manner described by Maniatis et al., supra, e.g. at page 133 et seq. "Kinasing" refers to phosphorylation of DNA. Electrophoresis is done in 0.5-1.5% Agarose gels containing 90 mM Tris-borate, 10 mM EDTA. "Nick-translating" refers to the method for labeling DNA with ^{32}P as described by Rigby et al., J. Mol. Biol., 113:237 (1977). All radiolabeled DNA is labeled with ^{32}P, whatever labeling technique was used.

By "rapid prep" is meant a rapid, small scale production of bacteriophage or plasmid DNA, e.g., as described by Maniatis et al., supra, at p. 365-373.

In accordance with another aspect of this invention,

there is provided a pharmaceutical preparation of AHF. A pharmaceutical preparation of human AHF produced in accordance with this invention may be prepared for parenteral administration in accordance with procedures well known in the art.

The pharmaceutical preparation for human use comprises sterilized AHF recovered from transformed cells. In addition to the AHF polypeptide or sufficient portion thereof which produces AHF activity, there may be included one or more acceptable carriers therefor and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with other ingredients of the preparation and not deleterious to the recipient thereof. The preparation may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy.

The pharmaceutical preparation of this invention, suitable for parenteral administration, may conveniently comprise a sterile lyophilized preparation of the AHF polypeptide which may be reconstituted by addition of sterilized solution to produce solutions preferably isotonic with the blood of the recipient. The preparation may be presented in unit or multi-dose containers, for example sealed ampoules or vials.

It should be understood that in addition to the sterile AHF or solution thereof, the pharmaceutical preparation of this invention may include one or more additional ingredients such as diluents, buffers, binders, surface active agents, thickeners, lubricants, preservatives (including anti-oxidants) and the like. Gelatine, lactose, starch, magnesium sterate, micronized silica gel, cocoa butter, talc, vegetabilic and animalic fats and oils, vegetabilic rubber and polyalkylene glycol and other known carriers for pharmaceuticals are all suitable for manufacturing the pharmaceutical preparations of the present invention. Preparations for parenteral use include an ampoule of a sterile solution or suspension with water or other pharmaceutically acceptable liquid as the carrier therefor, or an ampoule of

sterile solid AHF for dilution with a pharmaceutically acceptable liquid.

The pharmaceutical preparation of the present invention is useful in the treatment of Hemophilia A. These preparations also provide an important tool in the in vivo as well as the in vitro study of the processes involved in clotting and in the study of the immunulogical and biological characteristics of the AHF molecule.

Example 1. Protein Sequence Analysis

Porcine factor VIII:C was purified by Dr. David Fass according to published procedures, Knutsen and Fass (1982); Fass et al. (1982), supra. Amino acid sequence analysis is performed on a bovine thrombin digest product of the 76,000 dalton protein as described below.

Porcine AHF polypeptides bound to the anti-VIII:C monoclonal antibody column, can be sucessively eluted in two steps (Fass et.al. 1982). The two larger molecular weight species, 166,000 and 130,000 daltons, can be eluted with EDTA. The remaining polypeptide, having a molecular weight of about 76,000 daltons, can then be eluted with 50% ethylene glycol.

The 76,000 dalton protein is digested with thrombin after extensive dialysis in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl. Bovine thrombin digests are performed at room temperature for 60 minutes using 1 unit/ml of bovine thrombin, followed by the addition of another 1 unit/ml thrombin and incubation for an additional 60 minutes. The thrombin digestions are terminated by heating for 10 minutes at 90°C in 0.01% SDS. The major thrombin digest product of the 76,000 dalton protein is a polypeptide, 69,000 daltons (69 Kd).

Less than 1 µg of the 69 Kd polypeptide species described above is iodinated to serve as a radioactive marker after SDS gel electrophoresis, in accordance with the procedure of U. K. Laemmli, Nature, 227:680 (1970), the disclosure of which is

incorporated herein by reference. The polypeptide, dissolved in TAS buffer (50 mM Tris-acetate (pH 7.8), 0.1% SDS) is added to 100 µl of the same buffer containing 5 mCi of carrier-free iodine-125. 50 µl of 2.5 mg/ml chloramine T (Baker) in TAS buffer are added and the solution agitated for 1 minute. The reactions are stopped by adding 50 µl 2.5 mg/ml sodium metabisulfate in TAS buffer followed by 1 minute agitation. Labeled protein is separated from unincorporated I^{125} by chromatography using a small volume Sephadex G-25 M column (PD-10, Pharmacia). The column is pre-equilibrated with several column volumes of TAS buffer containing 2.5 mg/ml sodium iodide. The void volume is collected and protein integrity analyzed by SDS gel electrophoresis in accordance with the procedures of Laemmli, (1970), supra.

The radioactively labeled 69 Kd protein is added to its unlabeled counterpart for subsequent monitoring. The protein is then individually electrophoretically concentrated. The protein solutions are adjusted to 0.1% SDS, 10 mM dithiothreitol and Coomassie brilliant blue (Serva) are added to make the solution a very pale blue.

The solutions are then dialyzed briefly (1-2 hours) in TAS buffer using Spectrapore dialysis tubing (made by Spectrum Medical Industries, Inc., with molecular weight cut off at about 14,000). The dialyzed protein samples are then placed in an electrophoretic concentrator, of the design suggested by Hunkapiller, et al Meth. Enzymol., Enzyme Structures, Part I, 91:227 (1983), and electrophoretically concentrated in TAS buffer for 24 hours at 50 volts.

The 69 Kd AHF polypeptide, concentrated by the above procedure, is electrophoresed through an SDS-polyacrylamide gel in accordance with Laemmli, supra. The purified protein, identified by autoradiography of the radioactively labeled tracer polypeptide, is excised from the gel and electroeluted and concentrated as descibed by Hunkapiller et al., supra. The

concentrated sample is suitable for direct amino acid sequence analysis using the gas phase sequenator as described in Hewick et al., J. Biol. Chem. 256:7990 (1981).

The amino terminal sequence extending from the 2-42 residue of the 69,000 dalton thrombin cleavage product is as depicted in Figure 1. The first residue "X" was not identifiable. The amino acid sequences of (A) the amino terminus and (B) a 40 kd bovine thrombin digestion product from the 166 kd AHF fragment noted by Fass et al., supra, are shown in Figure 2. The amino acid sequence of the amino terminus of the 76 Kd polypeptide is: X-Ile Ser Leu Pro Thr Phe Gln Pro Glu Glu Asp Lys Met Asp Tyr Asp Asp Ile Phe.

Example 2

Chemical Synthesis of Oligonucleotide Probes for a Porcine AHF Gene

(a) Pentapeptide Probe Pool

The partial amino acid sequence of a fragment of porcine AHF having been determined allows porcine AHF oligonucleotide probes to be designed and synthesized. From the genetic code (Table 1) it is possible to predict the gene sequence that codes for this sequence of amino acids. Because the genetic code is degenerate, there are more than one possible DNA coding sequences for each amino acid sequence. Accordingly, a plurality or pool of complementary oligonucleotide probe sequences are provided for a region of the AHF molecule which required only a reasonable number of oligonucleotides to ensure the correct DNA sequence. Such regions are selected by searching for tracks of five to eight contiguous amino acids which have the lowest degeneracy. After the region is selected, a pool of oligonucleotides is synthesized, which would include all possible DNA sequences which could code for the five to eight amino acids in the selected region.

In the 69,000 dalton thrombin-cleavage fragment of porcine AHF there is a pentapeptide sequence running from the

18th through the 22nd amino acid from the amino terminus, which could be coded for by up to 16 different DNA sequences, each having five codons, for a length of 15 nucleotides.

	18		20		22
	Trp	Asp	Tyr	Gly	Met
Possible mRNA Sequences	UGG	GAU or GAC	UAU or UAC	GGU or GGC or GGA or GGG	AUG

The probes are made by synthesizing a limited number of mixtures of oligonucleotides with two to eight or more oligonucleotides per mixture. These mixtures are referred to as pools. Enough pools are made to encompass all possible coding sequences.

These oligonucleotides can be synthesized manually, e.g., by the phospho-tri-ester method, as disclosed, for example in R. L. Letsinger, et al., J. Am. Chem. Soc. 98:3655 (1967), the disclosure of which is incorporated by reference. Other methods are well known in the art. See also Matteucci and Caruthers, J. Am. Chem. Soc. 103:3185 (1981), the disclosure of which is incorporated by reference.

Preferably, however, the synthetic oligonucleotide probes for the desired polypeptide sequences are prepared by identical chemistry with the assistance of the completely automatic Applied Biosystems DNA synthesizer, Model 380A, as indicated above.

The oligonucleotides thus prepared can then be purified on a reverse HPLC column, as described by H. Fritz, et al., Biochemistry, 17:1257 (1978), the disclosure of which is incorporated herein by reference. After detritylation with 80% HOAc, the resulting oligonucleotide is normally pure and can be

used directly as a probe. If there are any contaminants, the synthetic DNA can be further purified on the same HPLC column, preferably using a slightly different gradient system.

Oligonucleotides are labelled, e.g. by using [-32P]ATP and T4 polynucleotide kinase, and their sequence checked either by two-dimensional homochromatography as described by Sanger et al., PNAS U.S.A. 70:1209 (1973) or by the Maxam-Gilbert method, Meth. Enzymology, 65:499 (1977), the disclosures of both of which are incorporated herein by reference.

(b) Forty Five Nucleotide Probes

A unique aspect of the present invention has been the use of oligonucleotide probes to screen a genomic DNA library for the AHF gene or fragments thereof. While oligonucleotides have been used for screening of cDNA libraries, see, e.g. M. Jaye, et al, Nucleic Acids Research 11:2325 (1982), the disclosure of which is incorporated herein by reference, genomic libraries have previously been screened successfully only with cDNA probes, i.e. probes which were generated only after the tissue source of the mRNA for a described protein had been identified and utilized to generate a cDNA clone which precisely matched the DNA sequence of the gene sought by the genomic search.

In the present case, it has been shown possible to use oligonucleotides to identify gene segments in a genomic library which code for the amino acid sequence of the proteins of interest, and identification of such gene segments provides an exact probe for use in mRNA, cDNA or further genomic screening techniques.

Preferably, as here, oligonucleotides corresponding to at least two segments of the amino acid sequence of the protein of interest are utilized. Preferably at least one of the oligonucleotide probes is used in the form of one or more pools of oligonucleotides which in the aggregate include every possible DNA sequence which could code for the amino acid sequences selected. Preferably one relatively short probe, e.g. 11 to 25

nucleotides, preferably 15 to 20 nucleotides is utilized in conjunction with a relatively long probe, e.g. 30 to 200 nucleotides, preferably 40 to 50 nucleotides. The second probe can be used for confirmation, and is not always necessary for identification of the DNA segment. Preferably at least one of the probes, and more preferably the longer of the probes is designed in accordance with the Rules 1 to 4 described below.

Rule 1. Codon Preference In the absence of other considerations, the nucleotide sequence was chosen which matched prevailing or similar sequences in similar mammalian genes. See Mechanisms of Ageing Dev., 18:(1982).

Rule 2. Advantageous GT Pairing The nucleotide G, in addition to bonding to its compliment C, can also form weak bonds with the nucleotide T. See K. L. Agarwal et al., J. Biol. Chem. 256:1023 (1981). Thus, faced with a choice of G or A for the third portion of an ambigious codon, it is preferable to choose G, since if the resulting hybridization would occur even if the actual nucleotide in the position is a T, rather than a C, the hybridization would still be stable. If an A were chosen incorrectly, the corresponding A:C incompatability could be enough to destroy the ability of the probe to hybridize with the genomic DNA.

Rule 3. Avoidance of 5'CG Sequences

When selecting from among the possible ambiguities, select those nucleotides which will not contain the 5'CpG sequence, either intra codon or inter codon.

Rule 4. Mismatch Position In choosing the codon sequences to use, consideration was given to the postulation that mismatches near the ends of the molecule do not adversely affect the stability of the hybridization as do mismatches near the center of the molecule. Thus, for example, where substantial doubt occurred concerning the sequence of a particular codon, and that codon was close to the center of the probe, the tendency was to test a pool of possible nucleotide sequences for that codon,

whereas codon positions near the ends of the probe were more likely to be subject to determinations on the basis of codon preference.

The chosen sequence for the 45-mer probes, as well as the amino acid sequences, the possible DNA sequences, and the "Actual Probe Sequence", i.e. the complement of actual coding strand determined for the AHF exon, as shown in Figure 3.

Thus, out of nucleotide positions involving choices, three were covered by using pools containing both possible nucleotide alternatives, five were predicted correctly, one was predicted in a way to maintain neutrality though incorrect, and four others were in error.

Despite the approximately 11% mismatch (5/45) the pool of 45-mer oligonucleotides are adequate to strongly identify a porcine AHF gene fragment, as described below.

Example 3. Screening of Porcine Genomic Library

A porcine genomic library is constructed using the bacteriophage vector Lambda J1. Lambda J1 is derived from L47.1 (Loenen et al., Gene 20:249 (1980)) by replacement of the 1.37 kb and 2.83 kb Eco RI-Bam HI fragments with a 95 bp Eco Rl-Hind III-Xba I-Bgl II-Bam HI polylinker. The 6.6 kb Bam HI fragment is then present as a direct repeat in reverse orientation relative to L47.1. The cloning capacity for Bam HI fragments is 8.6 - 23.8 kb. Bam HI cleaved porcine DNA (prepared as described by Piccini et al., Cell, 30:205 (1982)) is phenol extracted, ethanol precipitated and concentrated by centrifugation in a Microfuge. 0.67 ug of Bam HI porcine DNA is ligated to 2 ug of Lambda J1 Bam HI "arms", prepared as described in Maniatis, et al., supra, pp 275-279, in a volume of 10 ul ligation buffer with 10 units T4 DNA ligase (Maniatis et al., supra, p 474). The ligated DNA is packaged and plated as described in Maniatis et al., supra, p 291.

Approximately 4 x 10^5 pfu are plated on E. coli stain C600 on 15 cm plastic petri plates containing NZCYM agarose, at

8,000 pfu/plate. These recombinant phage are screened by the method of Woo (1979) using the 45-mer probe described above, radioactively labeled with ^{32}P as described above, as a probe. Filters are then hybridized in 5xSSC, 5x Denhardt's, 0.1% SDS, and 5 x 10^6 cpm/ml probe at 45° C for 16 hours, washed in 5 x SSC, 0.1% SDS at 50° C and subjected to autoradiography using intensifying screens (DuPont Lightning-Plus). Autoradiography reveals numerous phage which hybridized, to varying degrees, with the 45-mer. The filters are then denatured in 0.5M NaOH, neutralized in 1.0M Tris pH7.5, 1.5M NaCl and hybridized to the 15-mer as described for the 45-mer except the hybridization and washing temperature is 37° C. One phage which hybridized to both probes is picked from the original plate and 100 pfu plated and the plaques screened as described above using the 15-mer as probe.

A positive phage, named PB34, is picked as a plug and used to make a plate lysate as described in Maniatis et al, supra, pp 65-66. A small-scale isolation of PB34 DNA is achieved using the procedure described in Maniatis et al., supra, pp 371-372. 10µl of this DNA was cut with the restriction enzyme Hae III and then phosphatased using calf alkaline phosphatase (Boehringer-Mannheim). After phenol extraction, 20 ng of Sma I cut M13mp8 DNA is added, the solution is made 0.2M NaCl and nucleic acid precipitated by the addition of 2 volumes ethanol. Precipitated DNA is pelleted by centrifugation and redissolved in 2 µl of a ligation mixture and the DNA is ligated for 30 minutes at 23° C, diluted to 50 µl with ligase buffer and ligated an additional 3 hours. 5 µl of this reaction is used to transform E. coli strain JM101/TG1.

Cells are made competent for transformation by growing to an O.D.600 of 0.5 at 37° C in 50 ml SOBM media (SOBM is 2% tryptone, 0.5% yeast extract, 0.1M NaCl, 0.11g KOH per liter, 20mM $MgSo_4$). Cells are pelleted by centrifugation at 2500 rpm for 10 minutes at 4° C. The cells are resuspended in 3.5 ml

100mM RbCl, 45mM $MnCl_2$, 50mM $CaCl_2$, 10mM potassium MES pH 6.4 (MES = methylethane sulfonic acid). 200µl of competent cells are transformed with the DNA contained in 5 ml of the ligation reaction at 0° C for 30 minutes. The cells are then heat-shocked at 42° C for 90 seconds after which 4 ml of 0.8% agarose/SOBM containing 100 µl stationary JM101/TG1 cells are added and plated on 10 cm SOBM agar petri plates.

A subclone, containing a Hae III fragment from PB34, hybridizing to the 15-mer is identified by screening using the procedure of Benton and Davis, Supra. This clone is isolated and prepared for use as a template. The DNA sequence of the Hae III fragment present in this clone, designated as 34-H1, is shown in Figure 5. M13 template DNA is prepared by growing 1.5 ml of infected cells 5 hours at 37° C. Cells are pelleted by centrifugation for 10 minutes in a Beckman microfuge. 1.0 ml of supernatant (containing virus) is removed and 200 µl of 20% polyethylene glycol, 2.5 M NaCl is added. This sample is then incubated at room temperature for 15 minutes followed by centrifugation for 5 minutes in a Beckman microfuge. The pellet is dissolved in 100 µl TE, 7.5 µl of 4M NaCHCOO pH 4.5 is added and the sample extracted twice with a 1:1 mixture of phenol-chloroform and once with chloroform. The single-stranded phage DNA is then precipitated by the addition of 2 volumes of ethanol. Precipitated DNA is pelleted by centrifugation in a Beckman microfuge and dissolved in 30 µl 1mM Tris pH 8.0, 0.1 mM EDTA. The DNA sequencing is performed by the dideoxy chain termination technique, see, e.g. Sanger et al., PNAS U.S.A., 74:5463 (1977), utilizing the 15-mer as a primer. The sequence observed, which is included in the sequences represented in Figs. 4 and 5, confirmed the subclone as containing a porcine AHF exon as it includes the identical 14 amino acids encompassed by the region $phenylalanine_2$ to $glutamine_{15}$ in the amino terminal sequence of the 69K fragment represented in Figure 1. Further confirmation came from sequencing from the 5' end of the Hae III

insert in the 34-H1 vector, by priming with the "Universal primer" (Bethesda Research Labs) at a point adjacent the polylinker in that vector. Also, the insert from this clone, named 34-H1, was recloned into M13mp9 by restricting the DNA with Eco RI and Hind III, phosphatasing with calf alkaline phosphatase, and ligating to Eco RI, Hind III cleaved M13mp9 DNA. This clone, which contains an inversion of the Hae III segment relative to the universal primer, was also sequenced as described above. The resulting sequence data for all of insert 34-H1 is shown in Figure 5. This sequence confirms that this subclone contains an exon of the porcine AHF gene that could encode, from nucleotides 169 to 267, at least the thirty amino acids from the $phenylanine_2$ through $arginine_{31}$ of the 69K fragment (Fig. 1).

It appears likely that $arginine_{31}$ borders an intron because termination codons can be found downstream from nucleotide 267 (Fig. 5) in all three reading frames, and a sequence similar to the consensus 5' splice site sequence is also found in that region between nucleotides 266-267. Further, the amino acid sequence which would be encoded by the downstream DNA differs completely from that observed in the 69 Kd fragment of porcine AHF.

PB34 DNA was cut with Bam HI and electrophoresed through an agarose gel and the bands visualized by ultraviolet light after staining the gel in 5 µg/ml ethidium bromide. Three inserts of approximately 6.6 kb, 6.0 kb, and 1.8 kb were observed. The DNA in the gel was transferred to nitrocellulose as described in Maniatis et al., supra, pp 383-386. Hybridization of the filter to the 15-mer and autoradiography were performed as described above. Autoradiography revealed that the 6.0 kb band contained an AHF gene fragment which hybridized to the 15-mer probe.

Thus, for the first time, a section of the gene coding for porcine AHF had been isolated and identified. The bacteriophage lambda recombinant clone PB 34 is on deposit at the

American Type Culture Collection under Accession Number ATCC 40087.

Example 4. Location of Human AHF Gene

The human genomic library described by Maniatis et al., Cell, 15:687 (1978) is screened for the human AHF gene by infecting E. coli strain LE392 (publicly available) with 6x10^5 pfu and plating on 15cm NZCYM agar plates at a density of 20,000 pfu/plate. These phage are screened using the procedure of Benton and Davis, supra with the 6.0 kb porcine AHF fragment described in Example 3, labeled with ^{32}P by nick translation, as the probe. A phage exhibiting a strong hybridization signal is picked and plated at about 100 pfu/10cm plate and screened in duplicate as described above using the radioactively labeled 45-mer as one probe and the 6.0 kb Bam HI fragment of PB34 as the other. A phage, named HH25, which hybridizes to both probes is identified, a plate stock made and rapid prep DNA prepared as described above. The phage DNA is cut with Sau3A I, phosphatased with calf alkaline phosphatase phenol extracted, and co-precipitated with 20 ng of Bam HI cut M13 mp8 DNA. Precipitated DNA is pelleted by centrifugation and redissolved in 2 µl ligase buffer containing T4 DNA ligase. Ligation is performed for 2 minutes at 16° C, diluted to 50 µl in ligase buffer containing T4 DNA ligase and incubated an additional 3 hours at 16° C. 5 µl of this reaction mixture is used to transform E. coli strain JM101/TG1 as described in Example 3 above.

The plaques are screened using the Benton and Davis procedure and probing with radioactively labeled 15-mer. A phage plaque, named 25-S1, exhibiting hybridization is isolated and single-stranded phage DNA prepared for use as a DNA sequencing template as described above. Sequencing is performed using the dideoxy chain termination technique described by Sanger et al., supra, utilizing the 15-mer as primer, and gives the information

strand DNA sequence shown in Figure 6. 84 nucleotides sequenced in this manner demonstrated 85% homology with the homologous region of porcine AHF. There is also only one amino acid difference between the porcine AHF 69K region 2-16 as shown in Figure 1 and the corresponding region which was deduced from the human nucleotide sequence of Figure 6. This high degree of homology shows that the DNA of the recombinant phage HH-25 emanates from the AHF gene.

Thus, for the first time, an exon for the human AHF gene has been isolated and identified. A bacteriophage lambda recombinant HH 25 is on deposit at the American Type Culture Collection under the Accession Number ATCC 40086.

Example 5 Identifying Cells Actively Transcribing AHF

The porcine and human AHF exons described above are useful for a variety of functions, one of which is as a screening agent which permits identification of the tissue which is the site of synthesis of AHF in vivo. A number of screening methods are available, based on the use of the exon as an exact complement to the mRNA which is produced during the course of natural expression of AHF. In the screening procedures, tissue from various parts of the body is treated to liberate the mRNA contained therein, which is then hybridized to a DNA segment containing the exon for AHF, and if a molecule of mRNA does hybridize to that exon, the tissue which is the source of that mRNA is the source of AHF.

1. Screening Procedure

Porcine or human tissue from various organs, including kidneys, liver, pancreas, spleen, bone marrow, lymph nodes, etc., is prepared by guanidine hydrochloride extraction as described by Cox Methods Enzymol., 12B:120 (1968), the disclosure of which is incorporated herein by reference, with some modifications. Briefly, tissue is explanted into 8 M guanidine hydrochloride (or 4M guanidine isothiocyanate as proposed by Chirgwin, et al.,

Biochemistry 18: 5294 (1979), the disclosure of which is incorporated herein by reference, see also Maniatis, et al., supra, at 189 et seq.), 50 mM Tris (pH 7.5), 10 mM EDTA and homogenized in an Omnimixer (Sorvall) at top speed for 1 minute. The homogenate is clarified at 5000 rpm for 5 minutes in a Sorval HB-4 rotor and the RNA is precipitated by the addition of 0.5 volumes of ethanol. The RNA is dissolved and precipitated 3 more times from 6 M guanidine hydrochloride before being dissolved in H_2O.

Messenger RNA from this pool is enriched by chromatography on oligo (dT) cellulose (Collaborative Research).

This mRNA is then subjected to electrophoresis through an agarose gel containing formaldehyde, as described by Maniatis et al., supra, at 202-3. The mRNA in the gel is then transferred to a nitrocellulose filter (Maniatis et al., supra, at 203-4).

The thus obtained mRNA is hybridized with the radiolabeled porcine or human exon DNA obtained as described above, and the existence of hybrids detected by autoradiography. A radioactive signal indicates that the tissue source of the mRNA is a source of synthesis of AHF in the body.

Alternatively, mRNA can be screened using the S_1 protection screening method.

S_1 nuclease is an enzyme which hydrolyzes single stranded DNA, but does not hydrolyze base paired nucleotides, such as hybridized mRNA/DNA. Thus the existence of a radioactive band after acrylamide gel electrophoresis and autoradiography shows that the single stranded DNA corresponding to the AHF exon has been protected by the complementary mRNA, i.e. the mRNA for AHF. Thus the tissue which was the source of that mRNA is a site of synthesis of AHF in vivo. That tissue can be used as a source for AHF mRNA. This method of screening can be somewhat more sensitive than screening method described above.

A probe consisting of single stranded radioactively labeled DNA complementary to the mRNA is synthesized using the

universal primer of M13 to prime DNA synthesis of the porcine genomic subclone 34-H1. The reaction is performed in a 100 µl solution of 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 50 mM NaCl, 40 µM dGTP, dTTP, dCTP, 60 µCi of 32p-dATP (400Ci/mmole), 10 ng universal primer, 200-400 ng of 34-53 template DNA, and the Klenow fragment of DNA polymerase I (E. coli). The reaction is incubated at 23° C for 60 minutes, 10 minutes at 70° C, 50 units of PstI added and incubated an additional 60 minutes.

The reaction is terminated by phenol/chloroform extraction, NaCl added to 0.2M and then precipitated with two volumes 100% ethanol. The precipitated DNA is pelleted by centrifugation, redissolved in 20% sucrose, 50mM NaOH, 0.1% cresol green and then electrophoresed through 2% agarose in 50 mM NaOH, 10 mM EDTA. The resulting single stranded fragment is localized by autoradiography, the band excised and DNA isolated by electroelution in dialysis tubing.

Sample mRNA is prepared from liver, spleen, etc. tissue by the guanadine hydrochloride method described above.

The probe is then hybridized to sample mRNA (obtained from the oligo (dT) chromatography enriching step) in 50% formamide, 0.4M NaCl, 40 mM PIPES [piperazine-N,N' bis(2-ethanesulfonic acid)] pH 6.5, 1 mM EDTA, 5-50 µg mRNA, 2 µg labeled DNA in a volume of 15 µl. The hybridization is terminated by the addition of 200 µl cold S1 nuclease buffer (0.25 M NaCl, 0.3 M $NaCH_3COO$[pH 4.5], 1 mM $ZnSO_4$, 5% glycerol, and 1000 units S1 nuclease. The reaction is incubated at 45° C for 30 minutes. Samples are phenol extracted, ethanol precipitated with 10 µg yeast tRNA carrier and subjected to analysis by electrophoresis through 5% polyacrylamide sequencing gels as described in Maxam-Gilbert, PNAS USA, 74:560 (1977).

Example 6 Use of AHF Exon DNA to Obtain AHF mRNA From Tissue

Once a tissue which is a source of mRNA is identified,

AHF mRNA from that tissue is extracted and used to construct a cDNA library. The cDNA.library is then utilized for identifying and constructing a full length cDNA clone which encodes the amino acid sequence for AHF, without the introns contained in the genomic clone,as described below. Thereafter, the cDNA which encodes the AHF protein is inserted into an appropriate expression vector, in an appropriate host, for expression of AHF.

Since human AHF is in great demand for treatment of hemophilia and other uses, the preparation of human AHF cDNA is described.

1. Obtaining mRNA for Human AHF

mRNA from the human tissue responsible for AHF synthesis is prepared by the guanidine hydrochloride extraction method as described by Cox and modified by Chirgwin, et al. as disclosed above.

Further fractionation of mRNA obtained from the oligo (dT) cellulose chromatography column is obtained by sedimenting on 5-20% sucrose gradients containing 10 mM Tris-HCl (pH 7.4), 1mM EDTA and 0.2% SDS by centrifugation for 24 hours at 22,000 rpm in a Beckman SW28 rotor. Fractions (1.0 ml) are collected, sodium acetate was added to 0.2M, and the fractions are ethanol precipitated twice before dissolving in water. The size distribution of the fractionated RNA is determined by electrophoresis through 1.4% agarose gels containing 2.2 M formaldehyde.

mRNA sedimenting with an S (Svedberg) value greater than 28 is pooled for the synthesis of double stranded cDNA. 10 µg of this RNA is denatured at room temperature in 10 µl of 10 mM methylmercuryhydroxide. 140 mM 2-mercaptoethanol is added to inactivate the methylmercuryhydroxide. The RNA is then diluted to 50 µl containing 140 mM KCl, 100 mM Tris-HCl (pH 8.3 at 42°C), 1 mM of each deoxynucleotide triphosphate, 200 µg/ml oligo(dT)12-18, 10 mM $MgCl_2$, and 0.1 µCi ^{32}P-dCTP/ml. These reactions are performed at 42°C for 1 hour after the addition of

3 µl of 17 units/µl AMV reverse transcriptase (Life Sciences). The reaction is terminated by the addition of 0.25 M EDTA (pH 8.0) to 20 mM. The resulting mixture is extracted once with an equal volume of phenol/chloroform (1:1) followed by one chloroform extraction. The sample is then chromatographed on a 5 ml Sepharose CL-4B column (Pharmacia) equilibrated in 10 mM Tris-HCl (pH 8.00, 100 mM NaCl, 1 mM EDTA. The void volume is collected and the nucleic acids (including any RNA/cDNA hybrids) are precipitated by the addition of 2.5 volumes of ethanol.

Preferably in conjunction with the above procedures an AHF exon oligonucleotide segment is also used in place of oligo dTr to prime the reverse transcription, as described in Ullrich et al., Nature, 303:821 (1983).

The RNA-cDNA hybrids are dissolved in 35 µl of deionized H_2O, made 100 mM potassium cacodylate (pH 6.8), 100 µM dCTP, 1 mM 2-mercaptoethanol, 1 mM cobalt chloride and enzymatically "tailed" by the addition of 10 units of deoxytidyl terminal transferase (pH Biochemicals) and incubating the reaction for 30 seconds at 37°C. The reaction is terminated by adding 0.25 M EDTA to 10 mM. Tris-HCl (pH 8.0) is added to 300 mM and the sample extracted once with an equal volume of phenol-chloroform (1:1) and then with an equal volume of chloroform. Nucleic acids are precipitated from this product by the addition of 2.5 volumes of ethanol.

The dC tailed hybrid molecules are then annealed with 170 µg/ml oligo(dG)14-18 cellulose in 10 mM KCl, 1 mM EDTA for 10 minutes at 43°C and then an additional 10 minutes at 23°C. This reaction product is then diluted to 100 µl containing 100 mM ammonium sulfate, 1 mM 2-mercaptoethanol, 100 mM $MgCl_2$, 100 µg/ml bovine serum albumin (Sigma, Cohn fraction V), and 100 µM nicotinamide adenine dinucleotide. Second strand cDNA synthesis is initiated by the addition of 1 unit RNase H (P-L Biochemicals), 1 unit of E. coli DNA ligase, and 10 units of DNA polymerase I and incubated at 16°C for 12 hours.

The sample is then chromatographed over Sepharose CL-4B as described above. Double stranded DNA is ethanol precipitated and dC tailed as described for the RNA-cDNA hybrid tailing.

2. Screening for Human AHF DNA

dC tailed double stranded cDNA obtained as described above is annealed with an equimolar amount of dG tailed pBR322 (New England Nuclear) in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl at 37° for 2 hours. The annealed chimeric molecules are then frozen at -20°C until use in bacterial transformation.

Bacterial transformation is done using the MC1061 strain of E. coli(source). Cells (50 ml) are grown to an optical density of 0.25 at 600 nm. Cells are concentrated by centrifugation at 2,500 rpm for 12 minutes, washed in 10 ml of sterile 100 mM $CaCl_2$, and again pelleted by centrifugation as described above. Cells are resuspended in 2 ml of sterile 100 mM $CaCl_2$ and kept at 4° for 12 hours. The annealed chimeric molecules are incubated at a ratio of 5 ng of double stranded cDNA per 200 µl competent cells at 4°C for 30 minutes. The bacteria are then subjected to a two minute heat pulse of 42°C. 1.0 ml of L-broth is then added and the cells incubated for 1 hour at 37°. Cells are then plated onto LB-agar plates containing 5 µg/ml tetracycline.

Human AHF clones are identified using the colony hybridization procedure of Grunstein and Hogness PNAS U.S.A. 72:3961 (1975), the disclosure of which is incorporated herein by reference. The cDNA library is plated onto a nitrocellulose filter (Schleicher and Schuell) overlaying L-broth/ 5 µg/ml tetracycline agar plates. Colonies are grown overnight at 37°C and then the filter placed on sterile Whatman 3 M paper. A pre-moistened nitrocellulose filter is then pressed against the master filter and the filters keyed using an 18 gauge needle. The replica filter is then grown on LB-tetracycline plates at 37°C until the colonies reached a diameter of 1-2 mm. The filters are then transferred to LB plates containing 150 µg/ml

chloramphenicol and incubated at 37°C for 16-24 hours.

Filters are then removed and placed onto Whatman 3 M paper saturated with 0.5 M NaOH for 5 minutes at room temperature. Filters are then neutralized by placing onto Whatman 3 M saturated with 1 M Tris-HCl (pH 7.5), 1.5 M NaCl, and then Whatman 3 M saturated with 2x standard saline citrate (SSC). SSC (1x) is 0.15 M NaCl, .015 M sodium citrate.

Filters are air dried and baked in vacuo at 80°C for 2 hours. Prehybridization of filters is done at 65°C for 30 minutes in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, .1% SDS followed by 30 minutes in 7x SSC, 5x Denhardt's (1x Denhardt's is 0.02% polyvinylpyrollidine, 0.02% ficoll, 0.02% bovine serum albumin), 100 ug/ml denatured salmon sperm DNA, and 0.1% SDS. ^{32}P-labelled human exon DNA, prepared as described above, are added to 10^6 cpm/ml and the hybridization performed 12-16 hours at 37°C. Filters are then washed in several changes of 7x SSC, .1% SDS for 1-2 hours at 37°C. Filters are then air-dried and subjected to autoradiography with Kodak XAR film and a Dupont Lightning Plus intensifying screen.

Those colonies showing a hybridization signal above background are grown in L-broth containing 50 ug/ml tetracycline for rapid prep purification of plasmid DNA. Plasmid DNA is purified by the method of Holmes et al., Anal. Biochem., 114:193 (1981), the disclosure of which is incorporated herein by reference. An aliquot of this DNA is cleaved with restriction endonuclease Pst 1 and the fragments electrophoresed through 1% agarose/TBE gels and blotted according to the procedure of E. Southern, J. Mol. Biol. 98:503 (1975), Methods Enzymol. 69:152 (1980), the disclosure of which is incorporated herein by reference. The nitrocellulose filters are hybridized with radiolabeled human AHF exon DNA as described for colony hybridization. Those plasmids which contained Pst I inserts hybridizing to the AHF exon DNA are used for DNA sequencing analysis.

For sequencing, plasmid DNA, (purified from 0.75 ml of culture by the procedure of Holmes, et al., supra), is digested to completion with the restriction endonuclease Sau 3aI. The resulting DNA segment, identified as 34-S1, has a DNA sequence which is depicted in Figure 4. The DNA is ethanol precipitated after extraction with phenol-chloroform and redissolved in 10 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). 5 µl of the DNA solution is ligated with 20 ng of Bam HI cleaved M13 mp 9 replicative form DNA in 100 ul of 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and an excess of T4 DNA ligase. Ligations are done at 15°C for 2-4 hours.

5 µl of the ligation reactions are used to transform 200 ul of E. coli strain JM101/TG1 as described above. Recombinants are identified as white plaques when grown on LB-agar plates containing X-gal as an indication for beta-galactosidase activity as described by Davies, et al., J. Mol. Biol. 36:413 (1968).

Recombinant phage harboring sequences hybridizing to the human exon are identified by the procedure of Benton, et al., Science 196:180 (1977), the disclosure of which is incorporated herein by reference, using radioactively labelled human AHF exon as a probe. Plaques showing a hybridization signal are picked and grown in 1.5 ml cultures of L-broth. Single-stranded phage DNA prepared from these cultures is used as template in oligonucleotide-primed DNA synthesis reactions. Sequencing is done using the dideoxy chain-termination procedure, see, e.g., Sanger et al., PNAS U.S.A. 74:5463 (1977).

Human AHF recombinants are identified by comparing their nucleotide sequence with that which is known from the human exon sequence of human AHF.

3. Porcine AHF mRNA

Human AHF recombinants are used to screen a porcine tissue library constructed exactly as described for the human tissue cDNA library. Prospective porcine AHF recombinant DNA clones are identified by the Grunstein-Hogness procedure using

the porcine ^{32}P labeled exon fragment as a probe as described above. The probe is the porcine AHF exon segment labelled with 32P by nick-translation as described by Rigby et al., J. Mol. Biol., 113:237 (1977), the disclosure of which is incorporated herein by reference.

Colonies exhibiting hybridization signals are grown for rapid prep plasmid purification purposes as described above. Plasmid DNA is cleaved with the restriction endonuclease Pst 1, electrophoresed through 1% agarose/TBE gels, and blotted according to the procedure of E. Southern (1975). The blots are hybridized with nick-translated porcine AHF recombinant DNA labelled with ^{32}P.

Full-length clones may be constructed in a conventional manner, such as by ligation of DNA fragments from overlapping clones at restriction enzyme sites common to both clones as is well known in the art ("gene walking").

4. Identifying full-length clones from steps 2 or 3

The distance between the 5' end of an existing clone and the 5' end of the mRNA can be analyzed by the primer-extension technique described in Agarwal et al., "J.B.C." 256(2):1023-1028 (1981) utilizing an oligonucleotide primer whose sequence comes from the 5' (amino-terminal) region of the existing AHF clone. If gels developed using this procedure show more than one transcript one should consider the most intense band as representing the full mRNA transcript.

There are, however, many mRNAs which contain a long region of 5' untranslated sequence. Thus, expression of human AHN DNA may not be contingent upon the acquisition of a complete cDNA clone. For example, a clone may be obtained which by DNA sequencing analysis demonstrates the existence of a methionine codon followed by a sequence which is analogous to or identical to a known eucaryotic protein secretion signal and which is in frame with the remaining codons. Transformation and expression should be conducted for a clone which contains the met-secretion

signal sequence, which is of the expected size and which contains a poly (T) 3' terminus.

5. Alternative Procedure

As an alternative to the methods in Sections 1-3 described above, it is preferred that the cDNA clones for porcine or human AHF be identified using a bacteriophage vector in the following manner.

mRNA from human fetal liver tissue responsible for AHF synthesis was prepared by the guanidine hydrochloride method as described by Cox and modified by Chirgwin, et al. as set forth above in Example 5, Section 1. First strand cDNA was synthesized from 10 µg of polyA+ fetal liver RNA by the procedure described in Example 6, Section 1, supra. Specifically, 10 µg of this RNA was denatured at room temperature in 10 µl of 10 mM methylmercuryhydroxide. 140 mM 2-mercaptoethanol was added to inactivate the methylmercuryhydroxide. The RNA was then diluted to 50 µl containg 140 mM KCl, 100 mM Tris-HCl (pH 8.3 at 42 °C), 1 mM of each deoxynucleotide triphosphate, 200 µg/ml olio(dT)12-18, 10 mM $MgCl_2$, and 0.1 µCi 32p-dCTP/ml. These reactions were performed at 42°C for 1 hour after the addition of 3 µl of 17 units/µl AMV reverse transcriptase (Life Sciences).

For the first strand synthesis of a primer-extended library, 200 picomoles of a unique complimentary 38mer was included in the CH_3HgOH denaturation step, and after 10 minutes at 23°C, the reaction was made 140mM beta-mercaptoethanol, 0.7M KCl, 1mM EDTA, 20mM Tris-HCl (pH 8.3 at 42°), 1 unit/ul of RNasin (Biotec) and incubated at 50°C for 2 minutes and at 42°C for 2 minutes. This was then diluted to 50 µl containing 140 mM KCl, 100 mM Tris-HCl (pH 8.3 at 42°C), 1 mM of each deoxynucleotide triphosphate, 10 mM $MgCl_2$, and 0.1 µCi 32p-dCTP/µl. The reaction was incubated at 42 °C for 1 hour after the addition of 3 µl of 17µ/µl AMV reverse transcriptase.

After first strand synthesis the reactions were diluted to 150ul containing 10mM $MgCl_2$, 50mM Tris pH 7.4, 5mM

2-mercaptoethanol, 7.5mM NH_4SO_4, 250 uM of each deoxynucletide triphosphate, and second strand synthesis initiated by the addition of 1 unit of RNase H (E. coli) and 45 units of DNA polymerse I. Reactions were incubated at 16°C for 8 hours and then terminated by the addition of EDTA to 20mM and brought to a final volume of 200ul with H_2O. EcoRl methylation was then performed by addition of S-adenosyl methionine to 50uM and 40 units of EcoRl methylase. These reactions were incubated for 1 hour at 37°C, terminated by phenol-chloroform extraction, and chromatographed using Sephadex G50 equilibrated in 10mM Tris-HCl (ph 8.0), 1mM EDTA, .1M NaCl. The void volume was pooled and precipitated by ethanol addition.

cDNA molecules were blunt-ended in 200ul containing 50mM Tris pH 8.3, 10mM $MgCl_2$, 10mM 2-mercaptoethanol, 50mM NaCl, 50uM of each deoxynucleotide triphosphate, 100ug/ml ovalbumin and 5 units of T4 polymerase. The reaction was incubated at 37° for 30 minutes and then terminated by phenol-chloroform extraction. Nucleic acids were then precipitated by ethanol addition.

EcoRl "linkers" (Collabortive Research) were then ligated to the blunted cDNA molecules under standard conditions, Manitis, et al., supra, at 243, in a total volume of 45ul. The reactions were terminated by the addition of EDTA to 15mM and extracted with phenol-chloroform. Nucleic acids were precipitated by ethanol and pelleted by centrifugation. The linkered cDNA was redissolved in 200ul of 100uM Tris-HCl (pH 7.2), 5mM $MgCl_2$, 50mM NaCl and digested with 300 units of EcoRl for 2 hours at 37°C. The reaction was then extracted with phenol-chloroform and chromatographed over Sepharose CL-4B equilibrated with 10mM Tris (pH 8.0), 1mM EDTA, 0.1M NaCl. cDNA in the void volume was collected, precipitated by ethanol and pelleted by centrifugation.

cDNA was redissolved in 10mM Tris-HCl (pH 8.0), 1mM EDTA and ligated to EcoRl cleaved, phosphatased lambda Charon 21A DNA at various ratios of cDNA to vector DNA using standard ligation

conditions. Ligated DNA was packaged and titered using established procedures, Manitis, et al., supra, at 64, 256. The library was plated and screened using 32p-labelled human exon DNA under conditions described in Benton and Davis, supra. Overlapping clones which spanned approximately 10,000 base pairs were obtained and a substantial portion thereof was sequenced to reveal one long open reading frame encoding human AHF. The recombinant DNA nucleotide sequence obtained therefrom coding for human AHF is shown in Fig. 7 along with the deduced amino acid sequence for human AHF. The overlapping clones were assembled into vector pSP64 (Promega Biotec) using conventional techniques well known in the art, i.e. by ligation of DNA fragments from overlaping clones at resttriction enzyme sites common to both clones. A pSP64 recombinant clone containing the nucleotide sequence depicted in Figure 7, designated as pSP64-VIII, is on deposit at the American Type Culture Collection under Accession Number ATCC _____.

EXAMPLE 7

Expression of human or porcine AHF

This example contemplates expression of AHF using the full length clone obtained by the method of Example 6 in a cotransformation system.

1. Preparation of Transformation Vector

A direct method for obtaining the AHF transformation vector is described below. The pCVSVL plasmid is partially digested with Pst I, the digest separated on gel electrophoresis. After visualization, the linear DNA fragment band corresponding to full length plasmid is isolated as pCVSVL-B1.

The cDNA for AHF is rescued from the Example 5 cloning vectors by partial digest with Pst I. The partial digest is separated on gel electrophoresis, and the band corresponding to the molecular weight of the full length cDNA is isolated. pCVSVL-B1 is annealed with this DNA fragment, ligated with T4

ligase at 15°C, transfected into E. coli strain HB101 and transformants selected for tetracycline resistance. The selected E. coli transformants are grown in the presence of tetracycline. Plasmid pCVSVL-Bla is recovered in conventional fashion. Proper orientation of the cDNA in the plasmid may be determined in conventional fashion by assymetric digestion with an appropriate endonuclease(s).

2. Cotransformation: Selection and Amplification

Plasmid pCVSVL-Ala or pCVSVL-Bla and pAdD26SVpA#3 (Kaufman et al., op. cit.) are mixed together (50 µg HP and .5 µg pAdD265VpA #3) and precipitated by the addition of NaOAc pH 4.5 to .3M and 2.5 vols. of ethanol. Precipitated DNA is allowed to air dry, is resuspended in 2X HEBSS (.5ml) and mixed vigorously with .25 M $CaCl_2$ (.5ml) as described (Kaufman et al., op. cit). The calcium-phosphate-DNA precipitate is allowed to sit 30' at room temperature and applied to CHO DUKX-B1 cells (Chasin and Urlaub, 1980, available from Columbia University). The growth and maintenance of these cells has been described (Kaufman et al., op. cit, Chasin and Urlaub 1980).

The DUKX-B1 cells are subcultured at $5x10^5$/10cm dish 24 hr. prior to transfection. After 30 minutes incubation at room temperature, 5ml of a media with 10% fetal calf serum is applied and the cells are incubated at 37° for 4.5 hr. The media is then removed from the monolayer, 2ml of a-media with 10% fetal calf serum, 10µg/ml of thymidine, adenosine, and deoxyadenosine, and penicillin and streptomycin. Two days later the cells are subcultured 1:15 into a-media with 10% dialyzed fetal calf serum, and penicillin and streptomycin, but lacking nucleosides. Cells are then fed again with the same media after 4-5 days.

Colonies appear 10-12 days after subculturing into selective media. Methotrexate (MTX) selection and detection of AHF gene and selection gene amplification are conducted in accordance with Axel et a., U.s. Patent 4,399,216, or Kaufman et al., op. cit.

AHF yields may be improved by cotransforming the permanent cell line EA.hy 926 (Edgell et al., "Proc. Natl. Acad. Sci" 80:3734-3737 (1983) in place of DUKX-B1 cells. In this case the cotransforming selection gene should be the dominant DHFR gene disclosed by Haber et al., "Somatic Cell Genetics" 4:499-508 (1982). Otherwise, the cotransformation and culture will be substantially as set forth elsewhere herein.

3. Production of AHF

AHF-producing CHO transformants selected in Section 2 are maintained in seed culture using standard techniques. The culture is scaled up to 10 liters by cell culture in conventional media. This medium need not contain MTX and will not contain nucleosides so as to exclude selection gene revertants.

The culture supernatant is monitored for clotting activity following standard assays for use with blood plasma samples (reduction in clotting time of Factor VIII-deficient plasma). The supernatant may be purified by conventional techniques such as polyethylene glycol and glycine precipitations (as are known for use in purifying AHF from blood plasma) in order to increase the sensitivity of the FACTOR VIII assays.

When FACTOR VIII activity has reached a peak the cells are separated from the culture medium by centrifugation. The Factor VIII is then recovered and purified by polyethylene glycol and glycine precipitations. Clotting activity is demonstrated in Factor VIII deficient plasma.

4. Alternative Procedure for Production of AHF

A full length cDNA containing the entire AHF coding region was constructed from the exon in HH-25 described above, two cDNA clones which overlapped the extreme 5' and 3' regions of that exon, and a third clone which overlapped the 3' cDNA clone and continued past the termination codon. This was constructed such that synthetic Sal I sites were placed just 5' to the initiator methionine and at a position 3' to the translational stop signal at the end of the AHF coding sequences. This allowed

the placement into and excision from the polylinker Sal I site of pSP64. The Sal I fragment from this clone (pSP64-VIII) was purified and ligated to pCVSVL2. pCVSVL2 is a plasmid which is identical to the expression vector pCVSVL (Kaufman, et al., Mol. Cell Biol., 2:1304 (1982) the disclosure of which in incorporated herein by reference), except that it has deleted the Pst I site located 3' of the SV40 polyadenylation sequence which is accomplished by conventional procedures and that it contains a duplication of the SV40 Ava II "D" fragment upstream from the adenovirus major late promoter (MLP). pCVSVL2 was derived from pAdD26SVpA(1) (See Kaufman et al., supra) by adding XhoI linkers at each end of two SV40 Ava II "D" fragments, and inserting them into the XhoI site in pAdD26SVpA(1). The Ava II "D" fragments are both inserted such that the SV40 late promoter is in the same orientation as the Ad2 major late promoter. For plasmids pCVSVL and pAdD26SVpA(3), see Kaufman et. al., supra. To insert the Sal I fragment of pSP64-VIII into pCVSVL2, the unique Pst I site in pCVSVL2 was converted to a Sal I site by the procedure described in Rothstein et al., Methods Enzym., 69:98 (1980), and the Sal I fragment excised from pSP64-VIII was inserted to yield pCVSVL2-VIII. pCVSVL2-VIII contains the correct 5' to 3' orientation which allows transcription of the AHF coding sequence from the adenovirus MLP of the vector. pCVSVL2-VIII is on deposit at the American Type Culture Collection under Accession Number _____.

The pCVSVL2-VIII was introduced into monkey COS-7 cells (Mellon et al., Cell 27:279 (1981) using the DEAE dextran transfection protocol described by Sampayrac and Danna, PNAS U.S.A. 78:7575 (1981). The cells were cultured in serum-free medium which was assayed for AHF activity 3 days after transfection.

Factor VIII:C activity was determined by the chromogenic substrate assay, described by Didisheim, Science 129:389 (1959), which confirmed that human Factor VIII:C expression had been

achieved at a level of about 0.05 units/ml.

CLAIMS:

1. A DNA sequence coding for human factor VIII:C substantially free of other human genes.

2. A cloned human factor VIII:C gene.

3. A transformed host containing a gene for human factor VIII:C, said host being selected from bacteria, yeasts, and mammalian cells.

4. Isolated DNA coding for human factor VIII:C, comprising a polydeoxyribonucleotide having the sequence:

5'CGC AGC TTT CAG AAG AAA ACA CGA CAC
TAT TTT ATT GCT GCA GTG GAG AGG 3'

5. The DNA of claim 4, excised from human genomic DNA.

6. The DNA of claim 4, linked directly or indirectly to DNA from a non-human source.

7. A cloning vector comprising the DNA segment of claim 4.

8. An expression vector for human factor VIII:C, comprising the DNA segment of claim 4.

9. A transformed microorganism containing the expression vector of claim 7.

10. A transformed cell line containing the expression vector of claim 7.

11. A screening agent for identifying deoxyribonucleotide sequences and ribonucleotide sequences which encode for at least a portion of the human gene factor VIII:C, comprising a deoxyribonucleotide having at least a ten nucleotide portion of the sequence or its inverse complement:

5'TTT CAG AAG AGA ACC CGA CAC TAT TTC
ATT GCT GCG GTG GAG CAG CTC TGG GAT
TAC GGC ATG AGC GAA TCC CCC CGG GCG
CTA AGA AAC AGG 3'

12. The DNA of claim 11, excised from porcine genomic DNA.

13. The DNA of claim 11, linked to DNA from a non-porcine source.

14. A cloning vector comprising the DNA segment of claim 11.

15. A DNA sequence coding for porcine factor VIII:C substantially free of other porcine genes.

16. A cloned porcine factor VIII:C gene.

17. A transformed host containing a gene for porcine factor VIII:C, said host being selected from bacteria, yeasts and mammalian cells.

18. Isolated DNA coding for porcine factor VIII:C, comprising a polydeoxyribonucleotide having the sequence:

5' TTT CAG AAG AGA ACC CGA CAC TAT TTC
ATT GCT GCG GTG GAG CAG CTC TGG GAT
TAC GGC ATG AGC GAA TCC CCC CGG GCG
CTA AGA AAC AGG 3'

19. An expression vector for porcine factor VIII:C, comprising the DNA segment of claim 18.

20. A transformed microorganism containing the expression vector of claim 19

21. A method of isolating a gene fragment which encodes for at least a portion of a protein, comprising forming a genomic DNA library of an organism that produces the protein, forming at least one oligonucleotide probe whose sequence was selected solely based on the amino acid sequence contained in the protein, contacting the genomic library with the oligonucleotide under conditions favoring DNA/DNA hybridization, identifying the genomic DNA which hybridizes to the oligonucleotide, and isolating a segment of the genomic DNA which contains the oligonucleotide-hybridizing DNA.

22. A method of in accordance with claim 21, wherein at least one oligonucleotide probe comprises a plurality of oligonucleotides which contain differing DNA sequences, each of which corresponds in DNA sequence to the amino acid sequence.

23. The method according to claim 22, wherein at least one oligonucleotide probe contains a plurality of oligonucleotides, one of which contains each possible DNA sequence which corresponds with a selected amino acid sequence in the protein.

24. The method of claim 22, wherein the oligonucleotide probe has a length of from about 10 to 200 nucleotides.

25. The method of claim 22, wherein the oligonucleotides have a length of from 10 to 20 nucleotides.

26. The method of claim 22, wherein the oligonucleotides have a chain length of from about 30 to 200 nucleotides.

27. The method of claim 22, wherein the oligonucleotides have a chain length of about 40 to 50 nucleotides.

28. A method in accordance with claim 22, wherein at least two oligonucleotide probes are formed corresponding in DNA sequence to amino acid sequences in the protein, and each oligonucleotide probe is contacted with the genomic DNA under hybridizing conditions.

29. The method of claim 28, wherein one oligonucleotide probe comprises a plurality of oligonucleotides having a length of 11 to 20 nucleotides, and another oligonucleotide probe conprises at least one oligonucleotide having a length of 40 to 200 nucleotides.

30. The method of claim 28, wherein one nucleotide probe comprises at least one oligonucleotide having a length of 40 to 90 nucleotides.

31. A method of isolating a gene which encodes for human factor VIII:C, comprising screening a cRNA library with a probe which corresponds to at least a ten nucleotide sequence of the following sequence or its inverse complement:

5'CGC AGC TTT CAA AAG AAA ACA CGA CAC
TAT TTT ATT GCT GCA GTG GAG AGG 3'

constructing a cDNA library from the mRNA which hybridizes with the probe, and forming a gene which encodes for human factor VIII:C by ligating AHF cDNA segments from the cDNA library.

32. A method for producing AHF, comprising

(a) preparing one or more oligonucleotide probes which

hybridize with human DNA which encodes AHF, or which hybridize with the complement of such DNA;

(b) using at least one such probe to identify cells containing mRNA transcripts having nucleotide sequences corresponding to AHF;

(c) preparing cDNA from the step (b) mRNA;

(d) assembling the step (c) cDNA into a replicable cDNA sequence encoding at least the amino acid sequence of mature human AHF;

(e) cloning the step (d) sequence;

(f) transforming a parental cell which does not express AHF with the step (e) sequence;

(g) culturing the step (f) transformed cell; and

(h) recovering AHF from the culture.

33. The method of claim 32 wherein the cell is a mammalian cell.

34. The method of claim 33 wherein the cell is cotransformed with a selection gene.

35. Substantially pure human factor VIII:C, made by the process of claim 32.

36. The DNA sequence of claim 1, wherein said sequence comprises one or more DNA sequences selected from the DNA nucleotide sequence depicted in Figure 7 or one or more DNA sequences which hybridize to the DNA nucleotide sequence depicted in Figure 7 and which on expression codes for a polypeptide which exhibits factor VIII:C activity.

37. The DNA sequence of claim 1, wherein said sequence comprises the DNA sequence shown in Figure 7.

38. The DNA sequence of claim 1, wherein said sequence codes for a polypeptide having the amino sequence shown in Figure 7.

39. A recombinant DNA sequence which codes for a polypeptide which produces factor VIII:C activity.

40. The DNA sequence of claim 39, wherein the polypeptide comprises at least a major portion of the amino acid sequence of Figure 7.

41. A polypeptide expressed by DNA which exhibits human factor VIII:C activity comprising amino acid sequences selected from the amino acid sequence depicted in Figure 7 or amino acid sequences expressed by DNA sequences which hybridize to the DNA nucleotide sequence depicted in Figure 7.

42. The polypeptide of claim 41, wherein said polypeptide comprises amino acids corresponding to a segment of the amino acid sequence of Figure 7 sufficient to provide factor VIII:C activity, substantially free of human fibrinogen and fibronectin.

43. The polypeptide of claim 41, wherein said polypeptide is substantially free from other human polypeptides.

44. The polypeptide of claim 43, wherein sid other human polypeptides are human factor VIII:vWF, fibrinogen and fibronectin.

45. A vector comprising a DNA sequence coding for a number of amino acid groups in the sequence depicted in Figure 7 sufficient to produce factor VIII:C activity.

46. A transformed host containing exogenous DNA coding

for a number of amino acid groups in the sequence depicted in Figure 7 sufficient to produce factor VIII:C activity.

47. The transformed host of claim 46, wherein the host is selected from bacteria, yeast, insect or mammalian cells.

48. A method of making human factor VIII:C, comprising transforming a host with a DNA sequence which codes for a polypeptide having a number of amino acid groups depicted in Figure 7 sufficient to produce factor VIII:C activity, and expressing polypeptides from that sequence.

49. The method of claim 48, wherein the polypeptide has the amino acid sequence depicted in Figure 7.

50. The method of claim 48, wherein the DNA segment has the sequence depicted in Figure 7.

51. The method of claim 48, further comprising recovering human factor VIII:C from said transformant.

52. A glycosylated polypeptide consisting essentially of a number of amino acids in the sequence shown in Figure 7 sufficient to provide factor VIII:C activity.

53. A pharmaceutical preparation useful for therapeutic treatment of Hemophilia A comprising a sterile preparation of the polypeptide of claim 41.

54. A method of treating Hemophilia A comprising administering an effictive dose of the polypeptide of claim 41.

FIG. 1

A. Amino Acid Sequence of 69 Kd Porcine AKF Fragment

Phe	Gln	Lys	Arg	Thr	Arg	His	Tyr	Phe
Ile	Ala	Ala	Val	Glu	Gln	Leu	Trp	Asp
Tyr	Gly	Met	Ser	Glu	Ser	Pro	Arg	Ala
Leu	Arg	Asn	Arg	Ala	Gln	Asn	Gly	Glu
Val	Pro	Arg	Phe	Lys				

B. Amino Acid Sequence Encoded by Porcine AHF Exon (34-H1)

Arg	Ser	Phe	Gln	Lys	Arg	Thr	Arg	His	Tyr	Phe
		Ile	Ala	Ala	Val	Glu	Gln	Leu	Trp	Asp
		Tyr	Gly	Met	Ser	Glu	Ser	Pro	Arg	Ala

C. Amino Acid Sequence Encoded in Human AHF Exon (25-S1)

Arg	Ser	Phe	Gln	Lys	Lys	Thr	Arg	His	Tyr
	Phe	Ile	Ala	Ala	Val	Glu	Arg		

FIG. 2

A. Amines Terminus of 166Kd Polypeptide

X I R R Y Y L G A V E L S W D Y R Q S E L L R E L H V D T R F P A

B. Fragment of 166Kd Polypeptide

X V A K K H P K T W V H Y I S A E E E D W D Y A P A V P S P S D R S/T Y K S L

FIG. 3

Amino Acid No.	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Amino Acid Sequence	His	Tyr	Phe	Ile	Ala	Ala	Val	Glu	Gln	Leu	Trp	Asp	Tyr	Gly	Met
Possible mRNA Sequences	CAU CAC	UAU UAC	UUU UUC	AUU AUC AUA	GCU GCC GCA GCG	GCU GCC GCA GCG	GUU GUC GUA GUG	GAA GAG	CAA CAG	CUU CUC CUA CUG UUA UUG	UGG	GAU GAC	UAU UAC	GGU GGC GGA GBG	AUG
Guessed Probe Sequence	GTG	ATG	AAA AAG	TAA	CGA	CGA	CAC	CTT CTC	GTC	GAC	ACC	CTA CTG	ATA	CCG	TAC
Actual Probe Sequence	GTG	ATA	AAG	TAA	CGA	CGC	CAC	CTC	GTC	GAG	ACC	CTA	ATG	CCC	TAC

FIG. 4

AAA	ACA	CGG	GGC	ACC	TGT	TAA	CCT	27 GAA
CAA	AGT	AAA	TAG	ACC	TGG	AAG	GAC	54 TCC
CTC	CAA	GCT	TCT	GGG	TCC	CCC	GAT	81 GCC
CAA	AGA	GTG	GGA	ATC	CCT	AGA	GAA	108 GTC
ACC	AAA	AAG	CAA	AGC	TCT	CAG	GAC	135 GAA
AGA	CAT	CAT	CAG	TTT	ACC	CCT	GGA	162 CCG
TCA	CGA	AAG	CAA	TCA	TTC	AAT	AGC	189 AGC
AAA	AAA	TGA	AGG	ACA	AGC	CGA	GAC	216 CCA
AAG	AGA	AGC	CGC	CTG	GAC	GAA	GCA	243 GGG
AGG	GCC	TGG	AAG	GCT	GTG	CGC	TCC	270 AAA
GCC	TCC	GGT	CCT	GCG	ACG	GCA	TCA	297 GAG
GGA	CAT	AAG	CCT	TCC	TAC	TTT	TJA*	324 CCC
GGA	GGA	AGA	CAA	AAT	GGA	CTA	TGA	351 TGA
TAT	CTT	CTA	ACT	GAA	ACG	AAG	GGA	378 GAA
GAT	TTT	GAC	ATT	TAC	GGT	GAG	GAT	405 GAA
AAT	CAG	GAC	CCT	CGC	AGC	TTT	CAG	432 AAG
AGA	ACC	CGA	CAC	TAT	TTC	ATT	GCT	459 GCG
GTG	GAG	CAG	CTC	TGG	GAT	TAC	GGG	486 ATG
AGC	GAA	TCC	CCC	CGG	GCG	CTA	AGA	513 AAC
AGG	TAT	GGC	TAC	GTT	GGC	TAC	TCC	540 TCT
GTC	CTA	CCC	TGG	GGA	CCT	TTG	TCT	567 TGA
GCA	GGT	GCC	GAA	GCC	ATG	GGA	AAG	594 GCA
CAA	GCA	GTC	TGG	GGG	TGG	AGA	GGC	621 CAC
AGT	GGG	AGG	ATG	TGC	TTG	TTG	GGG	648 AGC
ACA	GCG	TGG	TCG	GGC	AGG	GAA	GAG	675 CAG
ACC	GAC	CTG	AGG	AGA	G			

* J Indicates an ambiguity, in accordance with the Stanford code.

FIG.5

```
(250)          9              18              27
5'   TGG  AAG  GCT  GTG  CGC  TCC  AAA  GCC  TCC  GGT  CCT

      36             45                             63
     GCG  ACG  GCA  TCA  GAG  GGA  CAT  AAG  CCT  TCC  TAC

          72             81             90             99
     TTT  TJA  CCC  GGA  GGA  AGA  CAA  AAT  GGA  CTA  TGA

                108            117            126
     TGA  TAT  CTT  CTA  ACT  GAA  ACG  AAG  GGA  GAA  GAT

       135            144            153            162
     TTT  GAC  ATT  TAC  GGT  GAG  GAT  GAA  AAT  CAG  GAC

            171            180            189            198
     CCT  CGC  AGC  TTT  CAG  AAG  AGA  ACC  CGA  CAC  TAT
           R    S    F    Q    K    R    T    R    H    Y

                207            216            225
     TTC  ATT  GCT  GCG  GTG  GAG  CAG  CTC [TGG  GAT  TAC
      F    I    A    A    V    E    Q    L    W    D    Y

       234            243            252            261
     GGG  ATG] AGC  GAA  TCC  CCC  CGG  GCG  CTA  AGA
      G    M    S    E    S    P    R    A    L    R

            267
     AAC  AGG  TAT  GGC  TAC  GTT  GGC  TAC  TCC  TCT
      N    R

     GTC  CTA  CCC  TGG  GGA  CCT  TTG  TCT  TGA  GCA

     GGT  GCC  GAA  GCC  ATG  GGA  AAG  GCA  CAA  GCA

     GTC  TGG  GGG  TGG  AGA  (3')
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FIG. 6

HUMAN SEQ. DATA (Complement of 15 mer desired sequence)

5'	GAC	ATT	TAT	GAT	GAG	GAT	GAA
	ATT	CAG	AGC	CCC	CGC	AGC	TTT
	CAG	AAG	AAA	ACA	CGA	CAC	TAT
	TTT	ATT	GCT	GCA	GTG	GAG	AGG

FIG. 7

5' GAATTCCGCAGTGGGTAAGTTCCTTAAATGCTCTGGAAAGAAATTCGGACTTTTCATTAAATCAGAAATT

TTACTTTTTTCCCCTCCTGCGAGCTAAAGATATTTTAGAGAAGAATTAACCTTTTGCTTCTCCAGTTGAACATTTGTAGCAATAAGTC

MET	Gln	Ile	Glu	Leu	Ser	Thr	Cys	Phe	Phe	Leu	Cys	Leu	Leu	Arg	Phe	Cys	Phe	18
ATG	CAA	ATA	GAG	CTC	TCC	ACC	TGC	TTC	TTT	CTG	TGC	CTT	TTG	CGA	TTC	TGC	TTT	
Ser	Ala	Thr	Arg	Arg	Tyr	Tyr	Leu	Gly	Ala	Val	Glu	Leu	Ser	Trp	Asp	Tyr	MET	36
AGT	GCC	ACC	AGA	AGA	TAC	TAC	CTG	GGT	GCA	GTG	GAA	CTG	TCA	TGG	GAC	TAT	ATG	
Gln	Ser	Asp	Leu	Gly	Glu	Leu	Pro	Val	Asp	Ala	Arg	Phe	Pro	Pro	Arg	Val	Pro	54
CAA	AGT	GAT	CTC	GGT	GAG	CTG	CCT	GTG	GAC	GCA	AGA	TTT	CCT	CCT	AGA	GTG	CCA	
Lys	Ser	Phe	Pro	Phe	Asn	Thr	Ser	Val	Val	Tyr	Lys	Lys	Thr	Leu	Phe	Val	Glu	72
AAA	TCT	TTT	CCA	TTC	AAC	ACC	TCA	GTC	GTG	TAC	AAA	AAG	ACT	CTG	TTT	GTA	GAA	
Phe	Thr	Val	His	Leu	Phe	Asn	Ile	Ala	Lys	Pro	Arg	Pro	Pro	Trp	MET	Gly	Leu	90
TTC	ACG	GTT	CAC	CTT	TTC	AAC	ATC	GCT	AAG	CCA	AGG	CCA	CCC	TGG	ATG	GCT	CTG	
Leu	Gly	Pro	Thr	Ile	Gln	Ala	Glu	Val	Tyr	Asp	Thr	Val	Val	Ile	Thr	Leu	Lys	108
CTA	GGT	CCT	ACC	ATC	CAG	GCT	GAG	GTT	TAT	GAT	ACA	GTG	GTC	ATT	ACA	CTT	AAG	
Asn	MET	Ala	Ser	His	Pro	Val	Ser	Leu	His	Ala	Val	Gly	Val	Ser	Tyr	Trp	Lys	126
AAC	ATG	GCT	TCC	CAT	CCT	GTC	AGT	CTT	CAT	GCT	GTT	GGT	GTA	TCC	TAC	TGG	AAA	
Ala	Ser	Glu	Gly	Ala	Glu	Tyr	Asp	Asp	Gln	Thr	Ser	Gln	Arg	Glu	Lys	Glu	Asp	144
GCT	TCT	GAG	GGA	GCT	GAA	TAT	GAT	GAT	CAG	ACC	AGT	CAA	AGG	GAG	AAA	GAA	GAT	
Asp	Lys	Val	Phe	Pro	Gly	Gly	Ser	His	Thr	Tyr	Val	Trp	Gln	Val	Leu	Lys	Glu	162
GAT	AAA	GTC	TTC	CCT	GGT	GGA	AGC	CAT	ACA	TAT	GTC	TGG	CAG	CTC	CTG	AAA	GAG	
Asn	Gly	Pro	MET	Ala	Ser	Asp	Pro	Leu	Cys	Leu	Thr	Tyr	Ser	Tyr	Leu	Ser	His	180
AAT	GGT	CCA	ATG	GCC	TCT	GAC	CCA	CTG	TGC	CTT	ACC	TAC	TCA	TAT	CTT	TCT	CAT	
Val	Asp	Leu	Val	Lys	Asp	Leu	Asn	Ser	Gly	Leu	Ile	Gly	Ala	Leu	Leu	Val	Cys	198
GTG	GAC	CTG	GTA	AAA	GAC	TTG	AAT	TCA	GGC	CTC	ATT	GGA	GCC	CTA	CTA	GTA	TGT	
Arg	Glu	Gly	Ser	Leu	Ala	Lys	Glu	Lys	Thr	Gln	Thr	Leu	His	Lys	Phe	Ile	Leu	216
AGA	GAA	GGG	AGT	CTG	GCC	AAG	GAA	AAG	ACA	CAG	ACC	TTG	CAC	AAA	TTT	ATA	CTA	
Leu	Phe	Ala	Val	Phe	Asp	Glu	Gly	Lys	Ser	Trp	His	Ser	Glu	Thr	Lys	Asn	Ser	234
CTT	TTT	GCT	GTA	TTT	GAT	GAA	GGG	AAA	AGT	TGG	CAC	TCA	GAA	ACA	AAG	AAC	TCC	
Leu	MET	Gln	Asp	Arg	Asp	Ala	Ala	Ser	Ala	Arg	Ala	Trp	Pro	Lys	MET	His	Thr	252
TTG	ATG	CAG	GAT	AGG	GAT	GCT	GCA	TCT	GCT	CGG	GCC	TGG	CCT	AAA	ATG	CAC	ACA	
Val	Asn	Gly	Tyr	Val	Asn	Arg	Ser	Leu	Pro	Gly	Leu	Ile	Gly	Cys	His	Arg	Lys	270
GTC	AAT	GGT	TAT	GTA	AAC	AGG	TCT	CTG	CCA	GGT	CTG	ATT	GGA	TGC	CAC	AGG	AAA	
Ser	Val	Tyr	Trp	His	Val	Ile	Gly	MET	Gly	Thr	Thr	Pro	Glu	Val	His	Ser	Ile	288
TCA	GTC	TAT	TGG	CAT	GTG	ATT	GGA	ATG	GGC	ACC	ACT	CCT	GAA	GTG	CAC	TCA	ATA	
Phe	Lue	Glu	Gly	His	Thr	Phe	Leu	Val	Arg	Asn	His	Arg	Gln	Ala	Ser	Leu	Glu	306
TTC	CTC	GAA	GGT	CAC	ACA	TTT	CTT	GTG	AGG	AAC	CAT	CGC	CAC	CCG	TCC	TTG	GAA	

Ile	Ser	Pro	Ile	Thr	Phe	Leu	Thr	Ala	Gln	Thr	Leu	Leu	MET	Asp	Leu	Gly	Gln	324
ATC	TCG	CCA	ATA	ACT	TTC	CTT	ACT	GCT	CAA	ACA	CTC	TTG	ATG	GAC	CTT	GGA	CAG	
Phe	Leu	Leu	Phe	Cys	His	Ile	Ser	Ser	His	Gln	His	Asp	Gly	MET	Glu	Ala	Tyr	342
TTT	CTA	CTG	TTT	TGT	CAT	ATC	TCT	TCC	CAC	CAA	CAT	GAT	GGC	ATG	GAA	GCT	TAT	
Val	Lys	Val	Asp	Ser	Cys	Pro	Glu	Glu	Pro	Gln	Leu	Arg	MET	Lys	Asn	Asn	Glu	360
GTC	AAA	GTA	GAC	AGC	TGT	CCA	GAG	GAA	CCC	CAA	CTA	CGA	ATG	AAA	AAT	AAT	GAA	
Glu	Ala	Glu	Asp	Tyr	Asp	Asp	Asp	Leu	Thr	Asp	Ser	Glu	MET	Asp	Val	Val	Arg	378
GAA	GCG	GAA	GAC	TAT	GAT	GAT	GAT	CTT	ACT	GAT	TCT	GAA	ATG	GAT	GTG	GTC	AGG	
Phe	Asp	Asp	Asp	Asn	Ser	Pro	Ser	Phe	Ile	Gln	Ile	Arg	Ser	Val	Ala	Lys	Lys	396
TTT	GAT	GAT	GAC	AAC	TCT	CCT	TCC	TTT	ATC	CAA	ATT	CGC	TCA	GTT	GCC	AAG	AAG	
His	Pro	Lys	Thr	Trp	Val	His	Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	414
CAT	CCT	AAA	ACT	TGG	GTA	CAT	TAC	ATT	GCT	GCT	GAA	GAG	GAG	GAC	TGG	GAC	TAT	
Ala	Pro	Leu	Val	Leu	Ala	Pro	Asp	Asp	Arg	Ser	Tyr	Lys	Ser	Gln	Tyr	Leu	Asn	432
GCT	CCC	TTA	GTC	CTC	GCC	CCC	GAT	GAC	AGA	AGT	TAT	AAA	AGT	CAA	TAT	TTG	AAC	
Asn	Gly	Pro	Gln	Arg	Ile	Gly	Arg	Lys	Tyr	Lys	Lys	Val	Arg	Phe	MET	Ala	Tyr	450
AAT	GGC	CCT	CAG	CGG	ATT	GGT	AGG	AAG	TAC	AAA	AAA	GTC	CGA	TTT	ATG	GCA	TAC	
Thr	Asp	Glu	Thr	Phe	Lys	Thr	Arg	Glu	Ala	Ile	Gln	His	Glu	Ser	Gly	Ile	Leu	468
ACA	GAT	GAA	ACC	TTT	AAG	ACT	CGT	GAA	GCT	ATT	CAG	CAT	GAA	TCA	GGA	ATC	TTG	
Gly	Pro	Leu	Leu	Tyr	Gly	Glu	Val	Gly	Asp	Thr	Leu	Leu	Ile	Ile	Phe	Lys	Asn	486
GGA	CCT	TTA	CTT	TAT	GGC	GAA	GTT	GGA	GAC	ACA	CTG	TTG	ATT	ATA	TTT	AAG	AAT	
Gln	Ala	Ser	Arg	Pro	Tyr	Asn	Ile	Tyr	Pro	His	Gly	Ile	Thr	Asp	Val	Arg	Pro	504
CAA	GCA	AGC	AGA	CCA	TAT	AAC	ATC	TAC	CCT	CAC	GGA	ATC	ACT	GAT	GTC	CGT	CCT	
Leu	Tyr	Ser	Arg	Arg	Leu	Pro	Lys	Gly	Val	Lys	His	Leu	Lys	Asp	Phe	Pro	Ile	522
TTG	TAT	TCA	AGG	AGA	TTA	CCA	AAA	GGT	GTA	AAA	CAT	TTG	AAG	GAT	TTT	CCA	ATT	
Leu	Pro	Gly	Glu	Ile	Phe	Lys	Tyr	Lys	Trp	Thr	Val	Thr	Val	Glu	Asp	Gly	Pro	540
CTG	CCA	GGA	GAA	ATA	TTC	AAA	TAT	AAA	TGG	ACA	GTG	ACT	GTA	GAA	GAT	GGG	CCA	
Thr	Lys	Ser	Asp	Pro	Arg	Cys	Leu	Thr	Arg	Tyr	Tyr	Ser	Ser	Phe	Val	Asn	MET	558
ACT	AAA	TCA	GAT	CCT	CGG	TGC	CTG	ACC	CGC	TAT	TAC	TCT	AGT	TTC	GTT	AAT	ATG	
Glu	Arg	Asp	Leu	Ala	Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Ile	Cys	Tyr	Lys	Glu	576
GAG	AGA	GAT	CTA	GCT	TCA	GGA	CTC	ATT	GGC	CCT	CTC	CTC	ATC	TGC	TAC	AAA	GAA	
Ser	Val	Asp	Gln	Arg	Gly	Asn	Gln	Ile	MET	Ser	Asp	Lys	Arg	Asn	Val	Ile	Leu	594
TCT	GTA	GAT	CAA	AGA	GGA	AAC	CAG	ATA	ATG	TCA	GAC	AAG	AGG	AAT	GTC	ATC	CTG	
Phe	Ser	Val	Phe	Asp	Gln	Asn	Arg	Ser	Trp	Tyr	Leu	Thr	Glu	Asn	Ile	Gln	Arg	612
TTT	TCT	GTA	TTT	GAT	CAG	AAC	CGA	AGC	TGG	TAC	CTC	ACA	GAG	AAT	ATA	CAA	CGC	
Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp	Pro	Glu	Phe	Gln	Ala	Ser	630
TTT	CTC	CCC	AAT	CCA	CCT	GGA	GTG	CAG	CTT	GAG	GAT	CCA	GAG	TTC	CAA	GCC	TCC	
Asn	Ile	MET	His	Ser	Ile	Asn	Gly	Tyr	Val	Phe	Asp	Ser	Leu	Gln	Leu	Ser	Val	648
AAC	ATC	ATG	CAC	AGC	ATC	AAT	GGC	TAT	GTT	TTT	GAT	AGT	TTG	CAG	TTG	TCA	GTT	

Cys	Leu	His	Glu	Val	Ala	Tyr	Trp	Tyr	Ile	Leu	Ser	Ile	Gly	Ala	Gln	Thr	Asp	666
TGT	TTG	CAT	CAG	GTG	GCA	TAC	TGG	TAC	ATT	CTA	AGC	ATT	GGA	GCA	CAG	ACT	GAC	
Phe	Leu	Ser	Val	Phe	Phe	Ser	Gly	Tyr	Thr	Phe	Lys	His	Lys	MET	Val	Tyr	Glu	684
TTC	CTT	TCT	GTC	TTC	TTC	TCT	GGA	TAT	ACC	TTC	AAA	CAC	AAA	ATG	GTC	TAT	GAA	
Asp	Thr	Leu	Thr	Leu	Phe	Pro	Phe	Ser	Gly	Glu	Thr	Val	Phe	MET	Ser	MET	Glu	702
GAC	ACA	CTC	ACC	CTA	TTC	CCA	TTC	TCA	GGA	GAA	ACT	GTC	TTC	ATG	TCG	ATG	GAA	
Asn	Pro	Gly	Leu	Trp	Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly	720
AAC	CCA	GGT	CTA	TGG	ATT	CTG	GGG	TGC	CAC	AAC	TCA	GAC	TTT	CGG	AAC	AGA	GGC	
MET	Thr	Ala	Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp	Tyr	Tyr	738
ATG	ACC	GCC	TTA	CTG	AAG	GTT	TCT	AGT	TGT	GAC	AAG	AAC	ACT	GGT	GAT	TAT	TAC	
Glu	Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys	Asn	Asn	Ala	Ile	756
GAG	GAC	AGT	TAT	GAA	GAT	ATT	TCA	GCA	TAC	TTG	CTG	AGT	AAA	AAC	AAT	GCC	ATT	
Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Ser	Arg	His	Pro	Ser	Thr	Arg	Gln	Lys	Gln	774
GAA	CCA	AGA	ACC	TTC	TCC	CAG	AAT	TCA	AGA	CAC	CCT	AGC	ACT	AGG	CAA	AAG	CAA	
Phe	Asn	Ala	Thr	Thr	Ile	Pro	Glu	Asn	Asp	Ile	Glu	Lys	Thr	Asp	Pro	Trp	Phe	792
TTT	AAT	GCC	ACC	ACA	ATT	CCA	GAA	AAT	GAC	ATA	GAG	AAG	ACT	GAC	CCT	TGG	TTT	
Ala	His	Arg	Thr	Pro	MET	Pro	Lys	Ile	Gln	Asn	Val	Ser	Ser	Ser	Asp	Leu	Leu	810
GCA	CAC	AGA	ACA	CCT	ATG	CCT	AAA	ATA	CAA	AAT	GTC	TCC	TCT	AGT	GAT	TTG	TTG	
MET	Leu	Leu	Arg	Gln	Ser	Pro	Thr	Pro	His	Gly	Leu	Ser	Leu	Ser	Asp	Leu	Gln	828
ATG	CTC	TTG	CGA	CAG	AGT	CCT	ACT	CCA	CAT	GGG	CTA	TCC	TTA	TCT	GAT	CTC	CAA	
Glu	Ala	Lys	Tyr	Glu	Thr	Phe	Ser	Asp	Asp	Pro	Ser	Pro	Gly	Ala	Ile	Asp	Ser	846
GAA	GCC	AAA	TAT	GAG	ACT	TTT	TCT	GAT	GAT	CCA	TCA	CCT	GGA	GCA	ATA	GAC	AGT	
Asn	Asn	Ser	Leu	Ser	Glu	MET	Thr	His	Phe	Arg	Pro	Gln	Leu	His	His	Ser	Gly	864
AAT	AAC	AGC	CTG	TCT	GAA	ATG	ACA	CAC	TTC	AGG	CCA	CAG	CTC	CAT	CAC	AGT	GGG	
Asp	MET	Val	Phe	Thr	Pro	Glu	Ser	Gly	Leu	Gln	Leu	Arg	Leu	Asn	Glu	Lys	Leu	882
GAC	ATG	GTA	TTT	ACC	CCT	GAG	TCA	GGC	CTC	CAA	TTA	AGA	TTA	AAT	GAG	AAA	CTG	
Gly	Thr	Thr	Ala	Ala	Thr	Glu	Leu	Lys	Lys	Leu	Asp	Phe	Lys	Val	Ser	Ser	Thr	900
GGG	ACA	ACT	GCA	GCA	ACA	GAG	TTG	AAG	AAA	CTT	GAT	TTC	AAA	GTT	TCT	AGT	ACA	
Ser	Asn	Asn	Leu	Ile	Ser	Thr	Ile	Pro	Ser	Asp	Asn	Leu	Ala	Ala	Gly	Thr	Asp	918
TCA	AAT	AAT	CTG	ATT	TCA	ACA	ATT	CCA	TCA	GAC	AAT	TTG	GCA	GCA	GGT	ACT	GAT	
Asn	Thr	Ser	Ser	Leu	Gly	Pro	Pro	Ser	MET	Pro	Val	His	Tyr	Asp	Ser	Gln	Leu	936
AAT	ACA	AGT	TCC	TTA	GGA	CCC	CCA	AGT	ATG	CCA	GTT	CAT	TAT	GAT	AGT	CAA	TTA	
Asp	Thr	Thr	Leu	Phe	Gly	Lys	Lys	Ser	Ser	Pro	Leu	Thr	Glu	Ser	Gly	Gly	Pro	954
GAT	ACC	ACT	CTA	TTT	GGC	AAA	AAG	TCA	TCT	CCC	CTT	ACT	GAG	TCT	GGT	GGA	CCT	
Leu	Ser	Leu	Ser	Glu	Glu	Asn	Asn	Asp	Ser	Lys	Leu	Leu	Glu	Ser	Gly	Leu	MET	972
CTG	AGC	TTG	AGT	GAA	GAA	AAT	AAT	GAT	TCA	AAG	TTG	TTA	GAA	TCA	GGT	TTA	ATG	
Asn	Ser	Gln	Glu	Ser	Ser	Trp	Gly	Lys	Asn	Val	Ser	Ser	Thr	Glu	Ser	Gly	Arg	990
AAT	AGC	CAA	GAA	AGT	TCA	TGG	GGA	AAA	AAT	GTA	TCC	TCA	ACA	GAG	AGT	GGT	AGG	

Leu	Phe	Lys	Gly	Lys	Arg	Ala	His	Gly	Pro	Ala	Leu	Leu	Thr	Lys	Asp	Asn	Ala	1,008
TTA	TTT	AAA	GGG	AAA	AGA	GCT	CAT	GGA	CCT	GCT	TTG	TTG	ACT	AAA	GAT	AAT	GCC	
Leu	Phe	Lys	Val	Ser	Ile	Ser	Leu	Leu	Lys	Thr	Asn	Lys	Thr	Ser	Asn	Asn	Ser	1,026
TTA	TTC	AAA	GTT	AGC	ATC	TCT	TTG	TTA	AAG	ACA	AAC	AAA	ACT	TCC	AAT	AAT	TCA	
Ala	Thr	Asn	Arg	Lys	Thr	His	Ile	Asp	Gly	Pro	Ser	Leu	Leu	Ile	Glu	Asn	Ser	1,044
GCA	ACT	AAT	AGA	AAG	ACT	CAC	ATT	GAT	GGC	CCA	TCA	TTA	TTA	ATT	GAG	AAT	AGT	
Pro	Ser	Val	Trp	Gln	Asn	Ile	Leu	Glu	Ser	Asp	Thr	Glu	Phe	Lys	Lys	Val	Thr	1,062
CCA	TCA	GTC	TGG	CAA	AAT	ATA	TTA	GAA	AGT	GAC	ACT	GAG	TTT	AAA	AAA	GTG	ACA	
Pro	Leu	Ile	His	Asp	Arg	MET	Leu	MET	Asp	Lys	Asn	Ala	Thr	Ala	Leu	Arg	Leu	1,080
CCT	TTG	ATT	CAT	GAC	AGA	ATG	CTT	ATG	GAC	AAA	AAT	GCT	ACA	GCT	TTG	AGG	CTA	
Asn	His	MET	Ser	Asn	Lys	Thr	Thr	Ser	Ser	Lys	Asn	MET	Glu	MET	Val	Gln	Gln	1,098
AAT	CAT	ATG	TCA	AAT	AAA	ACT	ACT	TCA	TCA	AAA	ACC	ATG	GAA	ATG	GTC	CAA	CAG	
Lys	Lys	Glu	Gly	Pro	Ile	Pro	Pro	Asp	Ala	Gln	Asn	Pro	Asp	MET	Ser	Phe	Phe	1,116
AAA	AAA	GAG	GGC	CCC	ATT	CCA	CCA	GAT	GCA	CAA	AAT	CCA	GAT	ATG	TCG	TTC	TTT	
Lys	MET	Leu	Phe	Leu	Pro	Glu	Ser	Ala	Arg	Trp	Ile	Gln	Arg	Thr	His	Gly	Lys	1,134
AAG	ATG	CTA	TTC	TTG	CCA	GAA	TCA	GCA	AGG	TGG	ATA	CAA	AGG	ACT	CAT	GGA	AAG	
Asn	Ser	Leu	Asn	Ser	Gly	Gln	Gly	Pro	Ser	Pro	Lys	Gln	Leu	Val	Ser	Leu	Gly	1,152
AAC	TCT	CTG	AAC	TCT	GGG	CAA	GGC	CCC	AGT	CCA	AAG	CAA	TTA	GTA	TCC	TTA	GGA	
Pro	Glu	Lys	Ser	Val	Glu	Gly	Gln	Asn	Phe	Leu	Ser	Glu	Lys	Asn	Lys	Val	Val	1,170
CCA	GAA	AAA	TCT	GTG	GAA	GGT	CAG	AAT	TTC	TTG	TCT	GAG	AAA	AAC	AAA	GTG	GTA	
Val	Gly	Lys	Gly	Glu	Phe	Thr	Lys	Asp	Val	Gly	Leu	Lys	Glu	MET	Val	Phe	Pro	1,188
GTA	GGA	AAG	GGT	GAA	TTT	ACA	AAG	GAC	GTA	GGA	CTC	AAA	GAG	ATG	GTT	TTT	CCA	
Ser	Ser	Arg	Asn	Leu	Phe	Leu	Thr	Asn	Leu	Asp	Asn	Leu	His	Glu	Asn	Asn	Thr	1,206
AGC	AGC	AGA	AAC	CTA	TTT	CTT	ACT	AAC	TTG	GAT	AAT	TTA	CAT	GAA	AAT	AAT	ACA	
His	Asn	Gln	Glu	Lys	Lys	Ile	Gln	Glu	Glu	Ile	Glu	Lys	Lys	Glu	Thr	Leu	Ile	1,224
CAC	AAT	CAA	GAA	AAA	AAA	ATT	CAG	GAA	GAA	ATA	GAA	AAG	AAG	GAA	ACA	TTA	ATC	
Gln	Glu	Asn	Val	Val	Leu	Pro	Gln	Ile	His	Thr	Val	Thr	Gly	Thr	Lys	Asn	Phe	1,242
CAA	GAG	AAT	GTA	GTT	TTG	CCT	CAG	ATA	CAT	ACA	GTG	ACT	GGC	ACT	AAG	AAT	TTC	
MET	Lys	Asn	Leu	Phe	Leu	Leu	Ser	Thr	Arg	Gln	Asn	Val	Glu	Gly	Ser	Tyr	Glu	1,260
ATG	AAG	AAC	CTT	TTC	TAA	CTG	AGC	ACT	AGG	CAA	AAT	GTA	GAA	GGT	TCA	TAT	GAG	
Gly	Ala	Tyr	Ala	Pro	Val	Leu	Gln	Asp	Phe	Arg	Ser	Leu	Asn	Asp	Ser	Thr	Asn	1,278
GGG	GCA	TAT	GCT	CCA	GTA	CTT	CAA	GAT	TTT	AGG	TCA	TTA	AAT	GAT	TCA	ACA	AAT	
Arg	Thr	Lys	Lys	His	Thr	Ala	His	Phe	Ser	Lys	Lys	Gly	Glu	Glu	Glu	Asn	Leu	1,296
AGA	ACA	AAG	AAA	CAC	ACA	GCT	CAT	TTC	TCA	AAA	AAA	GGG	GAG	GAA	GAA	AAC	TTG	
Glu	Gly	Leu	Gly	Asn	Gln	Thr	Lys	Gln	Ile	Val	Glu	Lys	Tyr	Ala	Cys	Thr	Thr	1,314
GAA	GGC	TTG	GGA	AAT	CAA	ACC	AAG	CAA	ATT	GTA	GAG	AAA	TAT	GCA	TGC	ACC	ACA	
Arg	Ile	Ser	Pro	Asn	Thr	Ser	Gln	Gln	Asn	Phe	Val	Thr	Gln	Arg	Ser	Lys	Arg	1,332
AGG	ATA	TCT	CCT	AAT	ACA	AGC	CAG	CAG	AAT	TTT	GTC	ACG	CAA	CGT	AGT	AAG	AGA	

Ala	Leu	Lys	Gln	Phe	Arg	Leu	Pro	Leu	Glu	Glu	Thr	Glu	Leu	Glu	Lys	Arg	Ile	1,350
GCT	TTG	AAA	CAA	TTC	AGA	CTC	CCA	CTA	GAA	GAA	ACA	GAA	CTT	GAA	AAA	AGG	ATA	
Ile	Val	Asp	Asp	Thr	Ser	Thr	Gln	Trp	Ser	Lys	Asn	MET	Lys	His	Leu	Thr	Pro	1,368
ATT	GTG	GAT	GAC	ACC	TCA	ACC	CAG	TGG	TCC	AAA	AAC	ATG	AAA	CAT	TTG	ACC	CCG	
Ser	Thr	Leu	Thr	Gln	Ile	Asp	Tyr	Asn	Glu	Lys	Glu	Lys	Gly	Ala	Ile	Thr	Gln	1,386
AGC	ACC	CTC	ACA	CAG	ATA	GAC	TAC	AAT	GAG	AAG	GAG	AAA	GGG	GCC	ATT	ACT	CAG	
Ser	Pro	Leu	Ser	Asp	Cys	Leu	Thr	Arg	Ser	His	Ser	Ile	Pro	Gln	Ala	Asn	Arg	1,404
TCT	CCC	TTA	TCA	GAT	TGC	CTT	ACG	AGG	AGT	CAT	AGC	ATC	CCT	CAA	GCA	AAT	AGA	
Ser	Pro	Leu	Pro	Ile	Ala	Lys	Val	Ser	Ser	Phe	Pro	Ser	Ile	Arg	Pro	Ile	Tyr	1,422
TCT	CCA	TTA	CCC	ATT	GCA	AAG	GTA	TCA	TCA	TTT	CCA	TCT	ATT	AGA	CCT	ATA	TAT	
Leu	Thr	Arg	Val	Leu	Phe	Gln	Asp	Asn	Ser	Ser	His	Leu	Pro	Ala	Ala	Ser	Tyr	1,440
CTG	ACC	AGG	GTC	CTA	TTC	CAA	GAC	AAC	TCT	TCT	CAT	CTT	CCA	GCA	GCA	TCT	TAT	
Arg	Lys	Lys	Asp	Ser	Gly	Val	Gln	Glu	Ser	Ser	His	Phe	Leu	Gln	Gly	Ala	Lys	1,458
AGA	AAG	AAA	GAT	TCT	GGG	GTC	CAA	GAA	AGC	AGT	CAT	TTC	TTA	CAA	GGA	GCC	AAA	
Lys	Asn	Asn	Leu	Ser	Leu	Ala	Ile	Leu	Thr	Leu	Glu	MET	Thr	Gly	Asp	Gln	Arg	1,476
AAA	AAT	AAC	CTT	TCT	TTA	GCC	ATT	CTA	ACC	TTG	GAG	ATG	ACT	GGT	GAT	CAA	AGA	
Glu	Val	Gly	Ser	Leu	Gly	Thr	Ser	Ala	Thr	Asn	Ser	Val	Thr	Tyr	Lys	Lys	Val	1,494
GAG	GTT	GGC	TCC	CTG	GGG	ACA	AGT	GCC	ACA	AAT	TCA	GTC	ACA	TAC	AAG	AAA	GTT	
Glu	Asn	Thr	Val	Leu	Pro	Lys	Pro	Asp	Leu	Pro	Lys	Thr	Ser	Gly	Lys	Val	Glu	1,512
GAG	AAC	ACT	GTT	CTC	CCG	AAA	CCA	GAC	TTG	CCC	AAA	ACA	TCT	GGC	AAA	GTT	GAA	
Leu	Leu	Pro	Lys	Val	His	Ile	Tyr	Gln	Lys	Asp	Leu	Phe	Pro	Thr	Glu	Thr	Ser	1,530
TTG	CTT	CCA	AAA	GTT	CAC	ATT	TAT	CAG	AAG	GAC	CTA	TTC	CCT	ACG	GAA	ACT	AGC	
Asn	Gly	Ser	Pro	Gly	His	Leu	Asp	Leu	Val	Glu	Gly	Ser	Leu	Leu	Gln	Gly	Thr	1,548
AAT	GGG	TCT	CCT	GGC	CAT	CTG	GAT	CTC	GTG	GAA	GGG	AGC	CTT	CTT	CAG	GGA	ACA	
Glu	Gly	Ala	Ile	Lys	Trp	Asn	Glu	Ala	Asn	Arg	Pro	Gly	Lys	Val	Pro	Phe	Leu	1,566
GAG	GGA	GCG	ATT	AAG	TGG	AAT	GAA	GCA	AAC	AGA	CCT	GGA	AAA	GTT	CCC	TTT	CTG	
Arg	Val	Ala	Thr	Glu	Ser	Ser	Ala	Lys	Thr	Pro	Ser	Lys	Leu	Leu	Asp	Pro	Leu	1,584
AGA	GTA	GCA	ACA	GAA	AGC	TCT	GCA	AAG	ACT	CCC	TCC	AAG	CTA	TTG	GAT	CCT	CTT	
Ala	Trp	Asp	Asn	His	Tyr	Gly	Thr	Gln	Ile	Pro	Lys	Glu	Glu	Trp	Lys	Ser	Gln	1,602
GCT	TGG	GAT	AAC	CAC	TAT	GGT	ACT	CAG	ATA	CCA	AAA	GAA	GAG	TGG	AAA	TCC	CAA	
Glu	Lys	Ser	Pro	Glu	Lys	Thr	Ala	Phe	Lys	Lys	Lys	Asp	Thr	Ile	Leu	Ser	Leu	1,520
GAG	AAG	TCA	CCA	GAA	AAA	ACA	GCT	TTT	AAG	AAA	AAG	GAT	ACC	ATT	TTG	TCC	CTG	
Asn	Ala	Cys	Glu	Ser	Asn	His	Ala	Ile	Ala	Ala	Ile	Asn	Glu	Gly	Gln	Asn	Lys	1,638
AAC	GCT	TGT	GAA	AGC	AAT	CAT	GCA	ATA	GCA	GCA	ATA	AAT	GAG	GGA	CAA	AAT	AAG	
Pro	Glu	Ile	Glu	Val	Thr	Trp	Ala	Lys	Gln	Gly	Arg	Thr	Glu	Arg	Leu	Cys	Ser	1,656
CCC	GAA	ATA	GAA	GTC	ACC	TGG	GCA	AAG	CAA	GGT	AGG	ACT	GAA	AGG	CTG	TGC	TCT	
Gln	Asn	Pro	Pro	Val	Leu	Lys	Arg	His	Gln	Arg	Glu	Ile	Thr	Arg	Thr	Thr	Leu	1,674
CAA	AAC	CCA	CCA	GTC	TTG	AAA	CGC	CAT	CAA	CGG	GAA	ATA	ACT	CGT	ACT	ACT	CTT	

Gln	Ser	Asp	Gln	Glu	Glu	Ile	Asp	Tyr	Asp	Asp	Thr	Ile	Ser	Val	Glu	MET	Lys	1,692
CAG	TCA	GAT	CAA	GAG	GAA	ATT	GAC	TAT	GAT	GAT	ACC	ATA	TCA	GTT	GAA	ATG	AAG	
Lys	Glu	Asp	Phe	Asp	Ile	Tyr	Asp	Glu	Asp	Glu	Asn	Gln	Ser	Pro	Arg	Ser	Phe	1,710
AAG	GAA	GAT	TTT	GAC	ATT	TAT	GAT	GAG	GAT	GAA	AAT	CAG	AGC	CCC	CGC	AGC	TTT	
Gln	Lys	Lys	Thr	Arg	His	Tyr	Phe	Ile	Ala	Ala	Val	Glu	Arg	Leu	Trp	Asp	Tyr	1,728
CAA	AAG	AAA	ACA	CGA	CAC	TAT	TTT	ATT	GCT	GCA	GTG	GAG	AGG	CTC	TGG	GAT	TAT	
Gly	MET	Ser	Ser	Ser	Pro	His	Val	Leu	Arg	Asn	Arg	Ala	Gln	Ser	Gly	Ser	Val	1,746
GGG	ATG	AGT	AGC	TCC	CCA	CAT	GTT	CTA	AGA	AAC	AGG	GCT	CAG	AGT	GGC	AGT	GTC	
Pro	Gln	Phe	Lys	Lys	Val	Val	Phe	Gln	Glu	Phe	Thr	Asp	Gly	Ser	Phe	Thr	Gln	1,764
CCT	CAG	TTC	AAG	AAA	GTT	GTT	TTC	CAG	GAA	TTT	ACT	GAT	GGC	TCC	TTT	ACT	CAG	
Pro	Leu	Tyr	Arg	Gly	Glu	Leu	Asn	Glu	His	Leu	Gly	Leu	Leu	Gly	Pro	Tyr	Ile	1,782
CCC	TTA	TAC	CGT	GGA	GAA	CTA	AAT	GAA	CAT	TTG	GGA	CTC	CTG	GGG	CCA	TAT	ATA	
Arg	Ala	Glu	Val	Glu	Asp	Asn	Ile	MET	Val	Thr	Phe	Arg	Asn	Gln	Ala	Ser	Arg	1,800
AGA	GCA	GAA	GTT	GAA	GAT	AAT	ATC	ATG	GTA	ACT	TTC	AGA	AAT	CAG	GCC	TCT	CGT	
Pro	Tyr	Ser	Phe	Tyr	Ser	Ser	Leu	Ile	Ser	Tyr	Glu	Glu	Asp	Gln	Arg	Gln	Gly	1,818
CCC	TAT	TCC	TTC	TAT	TCT	AGC	CTT	ATT	TCT	TAT	GAG	GAA	GAT	CAG	AGG	CAA	GGA	
Ala	Glu	Pro	Arg	Lys	Asn	Phe	Val	Lys	Pro	Asn	Glu	Thr	Lys	Thr	Tyr	Phe	Trp	1,836
GCA	GAA	CCT	AGA	AAA	AAC	TTT	GTC	AAG	CCT	AAT	GAA	ACC	AAA	ACT	TAC	TTT	TGG	
Lys	Val	Gln	His	His	MET	Ala	Pro	Thr	Lys	Asp	Glu	Phe	Asp	cys	Lys	Ala	Trp	1,854
AAA	GTG	CAA	CAT	CAT	ATG	GCA	CCC	ACT	AAA	GAT	GAG	TTT	GAC	TGC	AAA	GCC	TGG	
Ala	Tyr	Phe	Ser	Asp	Val	Asp	Leu	Glu	Lys	Asp	Val	His	Ser	Gly	Leu	Ile	Gly	1,872
GCT	TAT	TTC	TCT	GAT	GTT	GAC	CTG	GAA	AAA	GAT	GTG	CAC	TCA	GGC	CTG	ATT	GGA	
Pro	Leu	Leu	Val	Cys	His	Thr	Asn	Thr	Leu	Asn	Pro	Ala	His	Gly	Arg	Gln	Val	1,890
CCC	CTT	CTG	CTC	TGC	CAC	ACT	AAC	ACA	CTG	AAC	CCT	GCT	CAT	GGG	AGA	CAA	GTG	
Thr	Val	Gln	Glu	Phe	Ala	Leu	Phe	Phe	Thr	Ile	Phe	Asp	Glu	Thr	Lys	Ser	Trp	1,908
ACA	GTA	CAG	GAA	TTT	GCT	CTG	TTT	TTC	ACC	ATC	TTT	GAT	GAG	ACC	AAA	AGC	TGG	
Thy	Phe	Thr	Glu	Asn	MET	Glu	Arg	Asn	Cys	Arg	Ala	Pro	Cys	Asn	Ile	Gln	MET	1,926
TAC	TTC	ACT	GAA	AAT	ATG	GAA	AGA	AAC	TGC	AGG	GCT	CCC	TGC	AAT	ATC	CAG	ATG	
Glu	Asp	Pro	Thr	Phe	Lys	Glu	Asn	Thr	Arg	Phe	His	Ala	Ile	Asn	Gly	Tyr	Ile	1,944
GAA	GAT	CCC	ACT	TTT	AAA	GAG	AAT	TAT	CGC	TTC	CAT	GCA	ATC	AAT	GGC	TAC	ATA	
MET	Asp	Thr	Leu	Pro	Gly	Leu	Val	MET	Ala	Gln	Asp	Gln	Arg	Ile	Arg	Trp	Tyr	1,962
ATG	GAT	ACA	CTA	CCT	GGC	TTA	GTA	ATG	GCT	CAG	GAT	CAA	AGG	ATT	CGA	TGG	TAT	
Leu	Leu	Ser	MET	Gly	Ser	Asn	Glu	Asn	Ile	His	Ser	Ile	His	Phe	Ser	Gly	His	1,980
CTG	CTC	AGC	ATG	GGC	AGC	AAT	GAA	AAC	ATC	CAT	TCT	ATT	CAT	TTC	AGT	GGA	CAT	
Val	Phe	Thr	Val	Arg	Lys	Lys	Glu	Glu	Tyr	Lys	MET	Ala	Leu	Tyr	Asn	Leu	Tyr	1,998
GTG	TTC	ACT	GTA	CGA	AAA	AAA	GAG	GAG	TAT	AAA	ATG	GCA	CTG	TAC	AAT	CTC	TAT	
Pro	Gly	Val	Phe	Glu	Thr	Val	Glu	MET	Leu	Pro	Ser	Lys	Ala	Gly	Ile	Trp	Arg	2,016
CCA	GGT	GTT	TTT	GAG	ACA	GTG	GAA	ATG	TTA	CCA	TCC	AAA	GCT	GGA	ATT	TGG	CGG	

Val	Glu	Cys	Leu	Ile	Gly	Glu	His	Leu	His	Ala	Gly	MET	Ser	Thr	Leu	Phe	Leu	2,034
GTG	GAA	TGC	CTT	ATT	GGC	GAG	CAT	CTA	CAT	GCT	GGG	ATG	AGC	ACA	CTT	TTT	CTG	
Val	Tyr	Ser	Asn	Lys	Cys	Gln	Thr	Pro	Leu	Gly	MET	Ala	Ser	Gly	His	Ile	Arg	2,052
GTG	TAC	AGC	AAT	AAG	TGT	CAG	ACT	CCC	CTG	GGA	ATG	GCT	TCT	GGA	CAC	ATT	AGA	
Asp	Phe	Gln	Ile	Thr	Ala	Ser	Gly	Gln	Tyr	Gly	Gln	Trp	Ala	Pro	Lys	Leu	Ala	2,070
GAT	TTT	CAG	ATT	ACA	GCT	TCA	GGA	CAA	TAT	GGA	CAG	TGG	GCC	CCA	AAG	CTG	GCC	
Arg	Leu	His	Tyr	Ser	Gly	Ser	Ile	Asn	Ala	Trp	Ser	Thr	Lys	Glu	Pro	Phe	Ser	2,088
AGA	CTT	CAT	TAT	TCC	GGA	TCA	ATC	AAT	GCC	TGG	AGC	ACC	AAG	GAG	CCC	TTT	TCT	
Trp	Ile	Lys	Val	Asp	Leu	Leu	Ala	Pro	MET	Ile	Ile	His	Gly	Ile	Lys	Thr	Gln	2,106
TGG	ATC	AAG	GTG	GAT	CTG	TTG	GCA	CCA	ATG	ATT	ATT	CAC	GGC	ATC	AAG	ACC	CAG	
Gly	Ala	Arg	Gln	Lys	Phe	Ser	Ser	Leu	Tyr	Ile	Ser	Gln	Phe	Ile	Ile	MET	Tyr	2,124
GGT	GCC	CGT	CAG	AAG	TTC	TCC	AGC	CTC	TAC	ATC	TCT	CAG	TTT	ATC	ATC	ATG	TAT	
Ser	Leu	Asp	Gly	Lys	Lys	Trp	Gln	Thr	Tyr	Arg	Gly	Asn	Ser	Thr	Gly	Thr	Leu	2,142
AGT	CTT	GAT	GGG	AAG	AAG	TGG	CAG	ACT	TAT	CGA	GGA	AAT	TCC	ACT	GGA	ACC	TTA	
MET	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser	Gly	Ile	Lys	His	Asn	Ile	Phe	Asn	2,160
ATG	GTC	TTC	TTT	GGC	AAT	GTG	GAT	TCA	TCT	GGG	ATA	AAA	CAC	AAT	ATT	TTT	AAC	
Pro	Pro	Ile	Ile	Ala	Arg	Tyr	Ile	Arg	Leu	His	Pro	Thr	His	Tyr	Ser	Ile	Arg	2,178
CCT	CCA	ATT	ATT	GCT	CGA	TAC	ATC	CGT	TTG	CAC	CCA	ACT	CAT	TAT	AGC	ATT	CGC	
Ser	Thr	Leu	Arg	MET	Glu	Leu	MET	Gly	Cys	Asp	Leu	Asn	Ser	Cys	Ser	MET	Pro	2,196
AGC	ACT	CTT	CGC	ATG	GAG	TTG	ATG	GGC	TGT	GAT	TTA	AAT	AGT	TGC	AGC	ATG	CCA	
Leu	Gly	MET	Glu	Ser	Lys	Ala	Ile	Ser	Asp	Ala	Gln	Ile	Thr	Ala	Ser	Ser	Tyr	2,214
TTG	GGA	ATG	GAG	AGT	AAA	GCA	ATA	TCA	GAT	GCA	CAG	ATT	ACT	GCT	TCA	TCC	TAC	
Phe	Thr	Asn	MET	Phe	Ala	Thr	Trp	Ser	Pro	Ser	Lys	Ala	Arg	Leu	His	Leu	Gln	2,232
TTT	ACG	AAT	ATG	TTT	GCC	ACC	TGG	TCT	CCT	TCA	AAA	GCT	CGA	CTT	CAC	CTC	CAA	
Gly	Arg	Ser	Asn	Ala	Trp	Arg	Pro	Gln	Val	Asn	Asn	Pro	Lys	Glu	Trp	Leu	Gln	2,250
GGG	AGG	AGT	AAT	GCC	TGG	AGA	CCT	CAG	GTG	AAT	AAT	CCA	AAA	GAG	TGG	CTG	CAA	
Val	Asp	Phe	Gln	Lys	Thr	MET	Lys	Val	Thr	Gly	Val	Thr	Thr	Gln	Gly	Val	Lys	2,268
GTG	GAC	TTC	CAG	AAG	ACA	ATG	AAA	GTC	ACA	GGA	GTA	ACT	ACT	CAG	GGA	GTA	AAA	
Ser	Leu	Leu	Thr	Ser	MET	Tyr	Val	Lys	Glu	Phe	Leu	Ile	Ser	Ser	Ser	Gln	Asp	2,286
TCT	CTG	CTT	ACC	AGC	ATG	TAT	GTG	AAG	GAG	TTC	CTC	ATC	TCC	AGC	AGT	CAA	GAT	
Gly	His	Gln	Trp	Thr	Leu	Phe	Phe	Gln	Asn	Gly	Lys	Val	Lys	Val	Phe	Gln	Gly	2,304
GGC	CAT	CAG	TGG	ACT	CTC	TTT	TTT	CAG	AAT	GGC	AAA	GTA	AAG	GTT	TTT	CAG	GGA	
Asn	Gln	Asp	Ser	Phe	Thr	Pro	Val	Val	Asn	Ser	Leu	Asp	Pro	Pro	Leu	Leu	Thr	2,322
AAT	CAA	GAC	TCC	TTC	ACA	CCT	GTG	GTG	AAC	TCT	CTA	GAC	CCA	CCG	TTA	CTG	ACT	
Arg	Tyr	Leu	Arg	Ile	His	Pro	Gln	Ser	Trp	Val	His	Gln	Ile	Ala	Leu	Arg	MET	2,340
CGC	TAC	CTT	CGA	ATT	CAC	CCC	CAG	AGT	TGG	GTG	CAC	CAG	ATT	GCC	CTG	AGG	ATG	

2,352

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr End
GAG GTT CTG GGC TGC GAG GCA CAG GAC CTC TAC TGA GGGTGGCCACTGCATGCCACCTGCCAGTG

CCGTCACCTCTCCCTCCTCAGCTCCAGGGCATGTGTCCCTCCCTGGCTTGCTTCTACCTTTGTGCTAAATCCTAGCAGACACTGCCTTG

AAGCCTCCTGAATTAACTATCATCAGTCCTGCATTTCTTTGGTGGGGGGCCCAGGAGGGTGCATCCATTTTAACTTAACTCTTACCTATT

TTCTGCAGCTGCTCCCAGA

INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/01641

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[3] C12Q 1/68; C12P 21/00,21/02; C12N 15/00, 5/00, 1/20, 1/16, 1/18, 1/00; C07H 15/12; C07G 7/00; A61K 37/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/6, 68, 70, 172.3, 240, 253, 255, 256, 317; 536/27; 260/112R; 424/177

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer Data Bases: Biosis Files 5, 55 and 255, Chemical Abstracts

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	N, Choo et al., Nature, Vol. 299, 178-180, September 1982	1-54
Y	N, Kurachi et al. Proc. Natl. Acad. Sci. USA, Vol.79, pp. 6461-6464, November 1982	1-54
Y	N, Jaye et al., Nucleic Acids Research, Vol. 11, No. 8, pp. 2325-2335, 1983	1-54
Y	N, Knutson et al., Blood, Vol. 59, pp. 615-624, March 1982	1-54
Y	N, Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, pp. 224-228, 1982	1-54
Y	N, Tuddenham et al., J. Lab. Clin. Med. Vol. 93, No. 1, pp. 40-53, January 1979	1-34, 36-41 45-51, 53
X	N, Tuddenham et al., J. Lab. Clin. Med. Vol. 93, No. 1, pp. 40-53, January 1979	35, 42-44, 52, 54

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
18 December 1984	10 JAN 1985
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Alvin E. Tanenholtz