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(54) **Titre : TRAITEMENT DE MALADIES A DEPOT D'IgA1**  
(54) **Title: TREATMENT OF IgA1 DEPOSITION DISEASES**

(57) **Abrégé/Abstract:**

The present invention discloses the use of bacterial IgA1 proteases to treat IgA1 deposition in tissue and organs. Bacterial IgA1 proteases specifically cleave IgA1 molecules and thus provide a means to specifically cleave and remove IgA1 depositions. Accordingly, therapeutic agents for the treatment of diseases characterized by IgA deposition are provided. In particular, therapeutic agents to treat IgA Nephropathy, Dermatitis herpetiformis (DH), and Henoch-Schoenlein purpura (HS) are disclosed.



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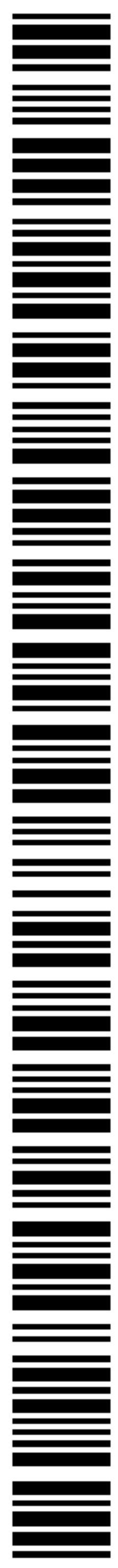
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(54) Title: TREATMENT OF IGAI DEPOSITION DISEASES

(57) Abstract: The present invention discloses the use of bacterial IgAl proteases to treat IgAl deposition in tissue and organs. Bacterial IgAl proteases specifically cleave IgAl molecules and thus provide a means to specifically cleave and remove IgAl depositions. Accordingly, therapeutic agents for the treatment of diseases characterized by IgA deposition are provided. In particular, therapeutic agents to treat IgA Nephropathy, Dermatitis herpetiformis (DH), and Henoch-Schoenlein purpura (HS) are disclosed.



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# **JUMBO APPLICATIONS / PATENTS**

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE  
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## TREATMENT OF IgA1 DEPOSITION DISEASES

### GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by a grant NIH RO1 DE 09677 from NIH, NIDCR. The U.S. Government has certain rights in the invention.

### BACKGROUND

Immunoglobulin A1 (IgA1) deposition in human tissues and organs is a characteristic of many human diseases including IgA nephropathy, dermatitis herpetiformis (DH), and Henoch-Schoenlein purpura (HS). IgA1 deposition is responsible for a variety of clinical manifestations such as renal failure, skin blistering, rash, arthritis, gastrointestinal bleeding and abdominal pain.

There are several available treatment options for patients that present with abnormal IgA1 deposition. These include administration of corticosteroids that have immunosuppressive and anti-inflammatory properties, dietary fish oil supplements that reduce renal inflammation, and angiotensin converting enzyme inhibitors that reduce the risk of progressive renal disease and renal failure. Such treatments do not directly act on IgA1 deposits in tissue or organs.

To address this issue of IgA1 deposit removal, exogenous proteolytic enzymes have been tested in IgA1 deposition animal models (Gesualdo L. et al, (1990) J. Clin. Invest. 86: 715-722 and Nakazawa M. et al. (1986) J. Exp. Med 164: 1973-1987). The proteases, chymopapain and subtilisin, act by proteolytic cleavage of IgA1 deposits in the kidney but are not specific for IgA1 molecules and will digest a variety of other proteins.

Thus, despite advances in the field, there is a need in the art for therapeutic agents that can be used to treat IgA1 deposition diseases.

### SUMMARY OF THE INVENTION

The present invention discloses the use of bacterial IgA1 proteases to treat IgA1 deposition in tissue and organs. Bacterial IgA1 proteases specifically cleave IgA1 molecules and

thus provide a means to specifically cleave and remove IgA1 depositions. Accordingly, therapeutic agents for the treatment of diseases characterized by IgA deposition are provided. In particular, therapeutic agents to treat IgA Nephropathy, Dermatitis herpetiformis (DH), and Henoch-Schoenlein purpura (HS) are disclosed.

Disclosed herein is a nucleic acid molecule encoding an IgA1 protease that is fused to an amino acid tag located upstream of an IgA1 protease auto-catalytic cleavage site.

In one embodiment, the tag, which is fused to the IgA1 protease, is a tag that specifically binds to a protein ligand, such as an antibody or peptide. The tag can be c-Myc, HA, VSV-G, HSV, FLAG, V5, or HIS.

In one aspect, a pharmaceutical composition for the treatment of IgA1 deposition is provided that comprises an IgA1 protease complexed with an antibody, such as an anti-IgA1 protease antibody.

In another aspect, a pharmaceutical composition for the treatment of IgA1 deposition is provided that comprises a tagged IgA1 protease that is complexed with a ligand of the tag. The tag fused to the IgA1 protease can be c-Myc, HA, VSV-G, HSV, FLAG, V5, or HIS. Accordingly, the ligand can be an anti-tag antibody such as anti-FLAG, anti-MYC, anti-VSV, anti-HA, or anti-V5. Alternatively, the ligand can be a peptide or non-peptide ligand, such as a chelating molecule.

In another aspect, a method for treatment of a disease characterized by IgA1 deposition is provided. The method involves administering to a patient a therapeutically effective amount of an IgA1 protease.

In one embodiment, the method for treatment uses an IgA1 protease fused to a tag complexed with a ligand of the tag, such as an anti-tag antibody. The tag fused to the IgA1 protease can be c-Myc, Flag, HA, VSV-G, HSV, FLAG, V5, or HIS. Accordingly, the anti-tag antibody can be anti-FLAG, anti-MYC, anti-VSV, anti-HA, or anti-V5.

In another embodiment, the disease characterized by IgA1 deposition is IgA Nephropathy, Dermatitis herpetiformis, or Henoch-Schoenlein purpura.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the hinge region of IgA1 and the cleavage sites for several IgA1 proteases within the hinge region (SEQ ID NO:1).

FIG. 2a illustrates the IgA1 protease precursor that undergoes auto-catalytic cleavage and releases a soluble mature IgA1 protease by auto-catalytic cleavage.

FIG. 2b shows an IgA1 protease wherein a His tag has been fused to the IgA1 protease such that the His tag is located near the carboxyl terminus of the mature IgA1 protease. The soluble IgA1 protease can then be complexed with an anti-His antibody for therapeutic purposes.

FIG. 3 shows a schematic of the *Haemophilus influenzae* Rd IgA1 protease precursor protein and shows an amino acid sequence that is upstream from the auto-catalytic cleavage site (site a), original sequence (SEQ ID NO:2). The mutated sequence (SEQ ID NO:3) shows where a His tag has been fused in frame to an IgA1 protease, 2 amino acids upstream from the proteolytic cleavage site. The corresponding nucleic acid sequences of the original sequence (SEQ ID NO:25) and mutated sequence (SEQ ID NO:26) are also shown.

FIG. 4 shows the PCR site directed mutagenesis fragments that were generated for insertion of a HIS tag into *H. influenzae* Rd IgA1 protease by conventional ligation techniques.

FIG. 5 shows the protein sequence of *Haemophilus influenzae* Rd (SEQ ID NO:4).

FIG. 6 shows the nucleotide sequence of *Haemophilus influenzae* Rd (SEQ ID NO:5).

#### DETAILED DESCRIPTION

The present invention relates to the use of bacterial Immunoglobulin A1 proteases (IgA1 proteases) to treat diseases that are characterized by IgA1 deposition.

## Definitions

As used herein, the term “IgA1 protease” refers to a bacterial enzyme that specifically cleaves human IgA1 molecules. By “specifically cleaves” is meant that the protease cleaves in the hinge region of human IgA1 molecules and does not cleave human IgA2 molecules. IgA1 proteases are expressed in gram negative and gram positive bacteria as a single-chain precursor that traverses the bacterial membrane. IgA1 proteases of gram negative bacteria undergo auto-catalytic cleavage releasing an N-terminal soluble IgA1 mature protease.

As used herein, the term “located upstream” refers to the spatial parameter of a tag wherein the amino acid tag sequence is located at least 2 amino acids, or 1, or none, amino-terminal to, and up to 50 amino acids amino-terminal to, the IgA1 protease site of auto-catalytic cleavage such that the tag is located 2, or 1, or none, to 50 amino acids upstream from the carboxyl terminus of the soluble, secreted, IgA1 protease.

As used herein, a “tag” refers to a polypeptide sequence 3 to 40 amino acids in length. A tag can possess a specific binding affinity for a peptide, protein ligand, or a non-peptide ligand. The specific binding affinity permits the IgA1 protease to which it is fused to be complexed with a ligand in order that the IgA1 protease can be detected, isolated, enlarged into a complex form, or used for therapeutic purposes. Herein, a tag also encompasses a fluorescent tag, a luminescent tag, or a chromogenic tag. Non-limiting examples of tags include c-Myc, HA, and VSV-G, HSV, FLAG, V5, and HIS.

By “complexed with a ligand” is meant that the IgA1 protease specifically binds to a binding partner, such as an antibody, or chelating molecule. The specific binding partner can be attached to a matrix, such as a bead. The term “specifically binds” refers to the interaction of two molecules, e.g., an antibody or a protein or peptide or a chelating agent, wherein the interaction is dependent upon the presence of particular structures on the respective molecules. For example, when the two molecules are protein molecules, a structure on the first molecule

recognizes and binds to a structure on the second molecule, rather than to proteins in general.

“Specific binding”, as the term is used herein, means that a molecule binds its specific binding partner with at least 2-fold greater affinity, and preferably at least 10-fold, 20-fold, 50-fold, 100-fold or higher affinity than it binds a non-specific molecule.

By “detected” is meant a manner of determining the presence or absence of the tag, such as “detection” by western blot with anti-tag monoclonal antibody, detection by immunofluorescence, or detection because the tag itself fluoresces. Non-limiting examples of suitable tags according to the invention include c-Myc, Flag, HA, and VSV-G, HSV, FLAG, V5, and HIS.

By “isolated” is meant that the IgA1 protease is separated from bacterial cell materials, such as cell membrane and any protein or nucleic acid present in bacterial growth media. Examples of non-limiting methods of isolation include the isolation of an IgA1 protease that has a poly-Histidine tag using a metal-chelate resin or beads, immunoprecipitation, and affinity column purification using anti-tag antibodies.

As used herein, the term “antibody” refers to an immunoglobulin molecule, or fragment thereof, that is capable of binding antigen, such as a tag or IgA1 protease. The term “antibody” is intended to include whole antibodies, e.g., of any isotype (IgG, IgA, IgM, IgE, etc), and includes fragments thereof which are also specifically reactive with a vertebrate, e.g., mammalian, protein. Antibodies can be fragmented using conventional techniques. Thus, the term includes segments of proteolytically-cleaved or recombinantly-prepared portions of an antibody molecule that are capable of selectively reacting with a certain protein. Non limiting examples of such proteolytic and/or recombinant fragments include Fab, F(ab')<sub>2</sub>, Fab', Fv, dAbs and single chain antibodies (scFv) containing a V<sub>L</sub> and V<sub>H</sub> domain joined by a peptide linker. The scFv's may be covalently or non-covalently linked to form antibodies having two or more binding sites. Thus, antibodies include polyclonal, monoclonal, or other purified

preparations of antibodies and recombinant antibodies. Herein, the term “anti-tag antibody” refers to an antibody that specifically binds to a tag.

As used herein, the term “IgA1 deposition” refers to the accumulation of IgA1 immunoglobulin in aggregated or non-aggregated form in human tissue or organs.

Herein, a “disease characterized by IgA1 deposition” refers to any disease in which IgA1 deposition occurs, such as, but not limited to IgA Nephropathy, Dermatitis herpetiformis, and Henoch-Schoenlein purpura.

As used herein, “IgA Nephropathy” refers to a kidney disease characterized by IgA1 deposits within the kidney.

As used herein, “Dermatitis herpetiformis” refers to a chronic blistering disease associated with deposits of IgA1 in skin and other tissues.

As used herein, “Henoch-Schoenlein purpura” refers to a skin and kidney disease characterized by deposition of IgA1 in skin tissue and kidney tissue.

As used herein, a “pharmaceutical composition” comprises a pharmacologically effective amount of an active agent and a pharmaceutically acceptable carrier. As used herein, “pharmacologically effective amount,” or simply “effective amount” refers to that amount of an agent effective to produce the intended pharmacological, therapeutic or preventive result. For example, if a given clinical treatment is considered effective when there is at least a 25% reduction in a measurable parameter associated with a disease or disorder.

#### I. IgA1 proteases

Herein, IgA1 proteases are used to treat diseases characterized by IgA1 deposition. IgA1 proteases are bacterial enzymes that specifically cleave human IgA1 molecules. Human IgA2 is resistant to nearly all known IgA1 proteases because IgA2 molecules lack a hinge region that is present in all IgA1 molecules. The hinge region of IgA1 molecules consist of a string of amino acids, that contain cleavage sites for a variety of IgA1 proteases, as illustrated in FIG. 1. IgA1

proteases are expressed in gram-negative bacteria as a single-chain precursor that traverses the inner membrane of bacterium. The precursor protein then inserts itself into the outer bacterial membrane and undergoes auto-catalytic cleavage, releasing a mature soluble IgA1 protease (FIG. 2a). IgA proteases of gram-positive bacteria are also useful in this invention, although they do not have an autocatalytic secretion mechanism. For such proteases, an epitope tag is added into the enzyme protein.

In one embodiment of the present invention a tag sequence is fused in frame to an IgA1 protease, such that the tag sequence is located near the carboxyl terminus of the secreted IgA1 protease (FIG. 2b). FIG. 3 shows a schematic of the *Haemophilus influenzae* Rd IgA1 protease precursor protein illustrating that a tag sequence (e.g. His tag) is fused in frame to an IgA1 protease upstream of the auto-catalytic cleavage sites a, b and c.

A variety of bacteria produce IgA1 proteases and are useful in the present invention. These include, but are not limited to *Haemophilus influenzae* type 1 and 2, *Neisseria meningitidis* type 1 and 2, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, *Streptococcus sanguis*, *Clostridium ramosum*, *Prevotella melaninogenica*, and *Ureaplasma urealyticum*.

The IgA1 protease nucleotide sequences of the present invention can be obtained from any bacteria where an IgA1 protease is expressed, as long as the IgA1 protease is capable of cleaving human IgA1 molecules. Nucleotide sequences encoding IgA1 proteases from numerous bacterial strains have already been identified and include: *Clostridium ramosum* (Genebank Accession, AY028440); *Ureaplasma urealyticum* (Genebank Accession, NC\_002162); *Haemophilus influenzae* (Genebank Accession, X59800) and bacterial strains Rd (Genebank Accession, NC-000907), 7768 (Genebank Accession, AF274862), 6338 (Genebank Accession, AF27486), 2509 (Genebank Accession, AF274859), *aegyptius* (Genebank Accession, AF369907), 8625 (Genebank Accession, AJ001741), HK284 (Genebank Accession, X82487), Da66 (Genebank Accession, X82467), HK635 (Genebank Accession, X82488), and other

deposited sequences from unidentified strains (Genebank Accession numbers, X59800, X82488, X64357, M87492, M87491, M87490, and M87489); *Neisseria meningitidis* (Genebank Accession number AF235032) and bacterial strains, Z2491 (Genebank Accession, NC-03316), B40 (Genebank Accession, AF012211), Z4099 (Genebank Accession, AF012210), Z4018 (Genebank Accession, AF012209), Z4400 (Genebank Accession, AF012208), Z3524 (Genebank Accession, AF012207) , Z4024 (Genebank Accession, AF012206), Z3910 (Genebank Accession, AF012205), Z3906 (Genebank Accession, AF012204), Z2491 (Genebank Accession, AF012203), IHN341 (Genebank Accession, AJ001740), NL3327 (Genebank Accession, AJ001739), NL823 (Genebank Accession, AJ001737), NL3293 (Genebank Accession, AJ001738), HK284 (Genebank Accession, X82487), ETH2 (Genbank Accession, X82469), NGO93 (Genbank Accession, X82482), NCG80 (Genbank Accession, X82479), NG117 (Genbank Accession, X82483), HF96 (Genbank Accession, X82475), HF54 (Genbank Accession, X82473), HF48 (Genbank Accession, X82480), HF13 (Genbank Accession, X82474), NGC65 (Genbank Accession, X82484), NCG16 (Genbank Accession, X82485), SM1894 (Genbank Accession, X82476), EN3771 (Genbank Accession, X82468), NG44/76 (Genbank Accession, X82481), SM1166 (Genbank Accession, X82486), HF159 (Genbank Accession, X82471), 81139 (Genbank Accession, X82477), HF117 (Genbank Accession, X82470), SM1027 (Genbank Accession, X82472) and Genebank Accession number, AF235032; *Nissseria gonorrhoeae* (Genebank Accession number, A12416) and bacterial strain, MS11 (Genebank Accession, S75490); *Streptococcus pneumoniae* (Genebank Accession number, X94909) and bacterial strains MGAS315 (Genebank Accession, NC-004070), R6 (Genebank Accession, NC-003098); and *Streptococcus Sanguis* (Genebank Accession, NC-003098) and bacterial strains SK85 (Genebank Accession, Y13461), SK49 (Genebank Accession, Y13460), SK4 (Genebank Accession, Y13459), SK162 (Genebank Accession, Y13458), SK161

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(Genebank Accession, Y13457), SK115 (Genebank Accession, Y13456, and Sk112 (Genebank Accession, Y13455).

### *Vector Construction*

In the present invention, sequences encoding IgA1 proteases are cloned into vectors suitable for expression of the protein, such that soluble IgA1 protease can be produced and isolated. The vectors can be constructed using standard methods (Sambrook et al., Molecular Biology: A laboratory Approach, Cold Spring Harbor, N.Y. 1989; Ausubel, et al., Current protocols in Molecular Biology, Greene Publishing, 1995), guided by the principles discussed below. In brief, conventional ligation techniques are used to insert DNA sequences encoding IgA1 protease into a bacterial cloning and/or expression vectors.

To prepare nucleic acids encoding IgA1 protease, a source of genes encoding for IgA1 proteases is required. The genes can be obtained from natural or synthetic sources. Methods for cloning novel IgA1 protease genes from bacterial strains are described in Lomholt H., et al., Mol. Microbiol. (1995) 15(3), 495-508; Fishman, Y. et al., (1985), p. 164-168 in G. K. Schoolnik (ed.), The Pathogenic Neisseria, Am. Soc. Microbiol., Washington DC; Koomey, J. et al., Proc. Natl. Acad. Sci. USA, (1982) 79: 7881-7885; Halter, R, et al., EMBO J., (1984) 3: 1595-1601; Bricker, J. et. al., Proc, Natl. Acad. Sci. USA, (1983), 80:2681-2685; Koomey, J. M. and Falkow, S., supra; Grundy, J. F. et al., J. Bacteriol, (1987) 169:4442-4450; and Gilbert, J.V. et al., Infect. Immun., (1988) 56:1961-1966.

Alternatively, DNA encoding a known IgA1 protease can be isolated from bacterial genomic DNA by polymerase chain reaction (PCR) amplification using primers specific for the IgA1 protease gene of interest. Briefly, bacterial genomic DNA is isolated using methods well known in the art, for example using bacterial genomic DNA isolation kits provided by QIAGEN or standard methods described in Sambrook et al., Molecular Biology: A laboratory Approach,

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Cold Spring Harbor, N.Y. (1989) and Ausubel, et al., Current protocols in Molecular Biology, Greene Publishing, (1995).

PCR is well known in the art (Mullis and Faloona, Methods Enzymol., (1987), 155:135).

In general, oligonucleotide primers useful according to the invention are single-stranded DNA or RNA molecules that hybridize selectively to a nucleic acid template that encodes IgA1 protease to prime enzymatic synthesis of a second nucleic acid strand. The primer is complementary to a portion of a target molecule present in a pool of nucleic acid molecules from the bacterial genome. It is contemplated that primers are prepared by synthetic methods, either chemical or enzymatic. Alternatively, such a molecule or a fragment thereof is naturally occurring, and is isolated from its natural source or purchased from a commercial supplier. Mutagenic oligonucleotide primers are 15 to 100 nucleotides in length, ideally from 20 to 40 nucleotides, although oligonucleotides of different length are of use. Preferably, the primers also comprise a unique restriction enzyme sequence.

Typically, selective hybridization occurs when two nucleic acid sequences are substantially complementary (at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary). See Kanehisa, Nucleic Acids Res., (1984), 12: 203

As a result, it is expected that a certain degree of mismatch at the priming site is tolerated. Such mismatch may be small, such as a mono-, di- or tri-nucleotide. Alternatively, it may comprise nucleotide loops, which we define as regions in which mismatch encompasses an uninterrupted series of four or more nucleotides.

Overall, five factors influence the efficiency and selectivity of hybridization of the primer to a second nucleic acid molecule. These factors, which are (i) primer length, (ii) the nucleotide sequence and/or composition, (iii) hybridization temperature, (iv) buffer chemistry and (v) the

potential for steric hindrance in the region to which the primer is required to hybridize, are important considerations when non-random priming sequences are designed.

There is a positive correlation between primer length and both the efficiency and accuracy with which a primer will anneal to a target sequence: longer sequences have a higher melting temperature ( $T_M$ ) than do shorter ones, and are less likely to be repeated within a given target sequence, thereby minimizing promiscuous hybridization. Primer sequences with a high G-C content or that comprise palindromic sequences tend to self-hybridise, as do their intended target sites, since unimolecular, rather than bimolecular, hybridization kinetics are generally favored in solution: at the same time, it is important to design a primer containing sufficient numbers of G-C nucleotide pairings to bind the target sequence tightly, since each such pair is bound by three hydrogen bonds, rather than the two that are found when A and T bases pair. Hybridization temperature varies inversely with primer annealing efficiency, as does the concentration of organic solvents, e.g. formamide, that might be included in a hybridization mixture, while increases in salt concentration facilitate binding. Under stringent hybridization conditions, longer probes hybridize more efficiently than do shorter ones, which are sufficient under more permissive conditions. Stringent hybridization conditions typically include salt concentrations of less than about 1M, more usually less than about 500 mM and preferably less than about 200 mM. Hybridization temperatures range from as low as 0°C to greater than 22°C, greater than about 30°C, and (most often) in excess of about 37°C. Longer fragments may require higher hybridization temperatures for specific hybridization. As several factors affect the stringency of hybridization, the combination of parameters is more important than the absolute measure of any one alone.

Primers preferably are designed using computer programs that assist in the generation and optimization of primer sequences. Examples of such programs are "PrimerSelect" of the DNASTar<sup>TM</sup> software package (DNASTar, Inc.; Madison, WI) and OLIGO 4.0 (National

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Biosciences. Inc.). Once designed, suitable oligonucleotides are prepared by a suitable method, e.g. the phosphoramidite method described by Beaucage and Carruthers (1981) Tetrahedron Lett., 22: 1859) or the triester method according to Matteucci and Caruthers (1981) J. Am. Chem. Soc., 103: 3185, or by other chemical methods using either a commercial automated oligonucleotide synthesizer or VLSIPS™ technology.

PCR is performed using template bacterial DNA (at least 1 fg: more usefully, 1-1000 ng) and at least 25 pmol of oligonucleotide primers; it may be advantageous to use a larger amount of primer when the primer pool is heavily heterogeneous, as each sequence is represented by only a small fraction of the molecules of the pool, and amounts become limiting in the later amplification cycles. A typical reaction mixture includes: 2 µl of DNA, 25 pmol of oligonucleotide primer, 2.5 µl of 10X PCR buffer 1 (Perkin-Elmer, Foster City, CA), 0.4 µl of 1.25 mM dNTP, 0.15 µl (or 2.5 units) of Taq DNA polymerase (Perkin Elmer, Foster City, CA) and deionized water to a total volume of 25 µl. Mineral oil is overlaid and the PCR is performed using a programmable thermal cycler.

The length and temperature of each step of a PCR cycle, as well as the number of cycles, is adjusted in accordance to the stringency requirements in effect. Annealing temperature and timing are determined both by the efficiency with which a primer is expected to anneal to a template and the degree of mismatch that is to be tolerated; obviously, when nucleic acid molecules are simultaneously amplified and mutagenised, mismatch is required, at least in the first round of synthesis. An annealing temperature of between 30°C and 72°C is used. Initial denaturation of the template molecules normally occurs at between 92°C and 99°C for 4 minutes, followed by 20-40 cycles consisting of denaturation (94-99°C for 15 seconds to 1 minute), annealing (temperature determined as discussed above: 1-2 minutes), and extension (72°C for 1-5 minutes, depending on the length of the amplified product). Final extension is generally for 4 minutes at 72°C, and may be followed by an indefinite (0-24 hour) step at 4°C.

Subsequent to PCR amplification, the DNA can be isolated by standard means, such as gel electrophoresis, or column purification. The DNA encoding the bacterial IgA1 protease can then be digested with appropriate restriction enzymes and ligated into a suitable cloning and/or expression vector.

#### *Vectors and host cells*

Any vector can be used in the present invention. As used herein, vector refers to a discrete element that is used to introduce heterologous DNA into bacterial cells for the expression and/or replication thereof. Numerous vectors suitable for the present invention are publicly available, including bacterial plasmids and bacteriophage. Each vector contains various functional components, which generally include a cloning (or "polylinker") site, an origin of replication and at least one selectable marker gene. If given vector is an expression vector, it additionally possesses one or more of the following: enhancer element, promoter, transcription termination and signal sequences, each positioned in the vicinity of the cloning site, such that they are operatively linked to the gene encoding an IgA1 protease according to the invention.

Both cloning and expression vectors generally contain nucleic acid sequences that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria. For example, the origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria.

Advantageously, a cloning or expression vector may contain a selection gene also referred to as a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will therefore not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other

toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available in the growth media.

Since the replication of vectors according to the present invention is most conveniently performed in *E. coli*, an *E. coli*-selectable marker, for example, the  $\beta$ -lactamase gene that confers resistance to the antibiotic ampicillin, is of use. These can be obtained from *E. coli* plasmids, such as pBR322 or a pUC plasmid such as pUC18 or pUC19.

Expression vectors usually contain a promoter that is recognized by the host organism and is operably linked to the coding sequence of interest. Such a promoter may be inducible or constitutive. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Promoters suitable for use with prokaryotic hosts include, for example, the  $\beta$ -lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (*trp*) promoter system and hybrid promoters such as the *tac* promoter. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the coding sequence. A preferred promoters of the present invention are the isopropylthiogalactoside (IPTG)-regulatable promoters.

Any bacterial strain is considered a suitable host cell for expression of and cloning of the IgA1 proteases of the present invention. An exemplary host is *E.coli*.

#### *Introduction of vectors to host cells.*

Vectors can be introduced to selected host cells by any of a number of suitable methods known to those skilled in the art. For example, vector constructs may be introduced to appropriate bacterial cells by infection using bacteriophage vector particles such as lambda or M13, or by any of a number of transformation methods for plasmid vectors or for bacteriophage

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DNA. For example, standard calcium-chloride-mediated bacterial transformation is still commonly used to introduce naked DNA to bacteria (Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), but electroporation may also be used (Ausubel et al., Current Protocols in Molecular Biology, (1988), (John Wiley & Sons, Inc., NY, NY)).

*Purification of soluble IgA1 protease*

After introduction of an expression vector encoding IgA1 protease into a suitable bacterial host cell, the bacteria are propagated for the overproduction of soluble IgA1 protease by standard means (Sambrook et al., Molecular Biology: A laboratory Approach, Cold Spring Harbor, N.Y. (1989) and Ausubel, et al., Current protocols in Molecular Biology, Greene Publishing, (1995).

Briefly, bacteria, such as *E. Coli*, which harbor an expression vector that encodes IgA1 protease, or bacteria that have DNA encoding IgA1 protease integrated into the bacterial genome, are grown in bacterial growth media at 37°C. When the bacterial cultures reach log phase, soluble IgA1 protease is purified from the growth media by means well known in the art.

For example, *H. influenzae* Rd bacteria that express 6x His-IgA1 protease are cultured as 20L (two 10L) in a fermentor charged with brain-heart infusion broth supplemented with NAD and hemin. The cells are grown at 37°C until they reach stationary phase, 16-20h. The bacterial mass is then removed with a Pellicon system, and each 10 L of culture supernatant containing the active enzyme is concentrated to 400 ml. The buffers are adjusted to have the protein in 25 mM Tris/HCl buffer, pH 7.5, with 0.05% NaN<sub>3</sub>. To remove unwanted protein, 80 ml batches of this concentrate is applied to a 40 ml bed-volume DE-52 anion-exchange column equilibrated in 25 mM Tris buffer. IgA protease does not bind to this column, and is collected as flow through using 500 ml Tris buffer. Yield of recovery is typically 85-90% based on assay using human IgA substrate. Ammonium sulfate is then used to precipitate the enzyme (60% saturation

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ammonium sulfate; 390 gm per L). The precipitate is dissolved with the following buffer: 50 mM sodium phosphate, 12.5 mM Tris/HCl, 0.3 M NaCl and 0.025% sodium azide, adjusted to pH 7.5, and the enzyme is then dialyzed against this buffer for several days. The final volume of enzyme solution is approximately 200 ml for each 10L of starting culture.

For affinity purification, 40 ml aliquots of the enzyme solution is applied to Ni-NTA-agarose in a column with bed volume of 40 ml. The bound enzyme is washed three times with volumes of 500 ml of buffers containing 50 mM sodium phosphate, 12.5 mM Tris/HCl, 0.3 M NaCl and 0.025% sodium azide. pH of these buffer washes is reduced in stepwise fashion, beginning with pH 7.5, then 6.6, then 6.0, intended to remove weakly adherent, non-enzyme proteins from the nickel ligand. The final wash again uses buffer at pH 7.5, now 200 ml. The 6xHis-IgA protease is then eluted from Ni-NTA agarose using 50 ml 0.1 M imidazole in 50 mM Tris/HCl, pH 7.5. The recovered enzyme is concentrated by positive pressure filtration using a 100 kDa cut-off Centricon™ membrane, washed three times with 25mM Hepes, pH 7.15, and then stored in Hepes buffer.

#### *Assay for IgA1 protease activity*

The IgA1 protease is tested for enzyme activity by standard means as described in Plaut, AG and Bachovchin WW, IgA-specific prolyl endopeptidases: serine type. Methods Enzymol. 1994;244:137- 51.

The assay can be performed with purified protease or IgA1 protease present in bacterial growth media. An IgA1 protease has sufficient activity to be useful according to the invention if it has one Unit activity, with Unit equal to one microg human IgA1 cleaved per minute at 37°C.

#### II. Tagged IgA1 protease

In one embodiment, the IgA1 protease is fused to a tag. Fusing a tag to the IgA1 proteases of the present invention aids in purification and detection of the protease, as well as

provides a means in which the IgA1 protease can form a complex with a ligand, such as an anti-tag antibody, for therapeutic purposes.

To generate an IgA protease comprising a tag, a sequence encoding a tag can be ligated in frame to a sequence encoding an IgA1 protease using conventional molecular biology techniques. The tag sequence is ligated upstream of the DNA sequence encoding the IgA1 protease auto-catalytic cleavage site such that, upon cleavage of the IgA1 protease precursor protein, a soluble IgA1 protease comprising a tag is secreted into bacterial growth media.

Alternatively, an IgA1 protease comprising a tag is generated by PCR-based site directed mutagenesis. There are a number of site-directed mutagenesis methods known in the art which allow one to mutate specific regions within a protein. These methods are embodied in a number of kits available commercially for the performance of site-directed mutagenesis, including both conventional and PCR-based methods. Examples include the EXSITE™ PCR-based site-directed mutagenesis kit available from Stratagene (Catalog No. 200502; PCR based) and the QUIKCHANGE™ site-directed mutagenesis kit from Stratagene (Catalog No. 200518; PCR based), and the CHAMELEON® double-stranded site-directed mutagenesis kit, also from Stratagene (Catalog No. 200509). Briefly, a tag sequence is introduced into a PCR fragment by inclusion of a sequence encoding the tag near the 5' or 3' end of one of the PCR primers. The PCR fragment is generated in a manner to provide appropriate restriction sites such that the fragment can be digested then ligated into parental vector for replacement of specific amino acid codons.

In one embodiment, the tag of the present invention has a specific binding affinity for an antibody, so that the protease forms an immuno-complex upon binding ligand. For example, the tag may comprise a unique epitope for which antibodies are readily available. Alternatively, the tag can comprise metal-chelating amino acids (e.g. His) so that the IgA proteases can complex with a metal-chelating resin or bead, for example nickle-NTA beads.

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In another embodiment, the tag comprises a detectable marker, such as an enzyme, or comprises an amino acid that can be labeled with a detectable marker. Detectable markers include, for example, radioisotopes, fluorescent molecules, chromogenic molecules, luminescent molecules, and enzymes. Useful detectable markers in the present invention include biotin for staining with labeled streptavidin conjugate, fluorescent dyes (e.g., fluorescein, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g.,  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^{32}\text{P}$ ), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold. Patents teaching the use of such detectable markers include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,336,241.

Non-limiting examples of suitable tags according to the invention include c-Myc, HA, and VSV-G, HSV, FLAG, V5, and HIS. The amino acid and nucleic acid sequence for each tag is shown below.

Tag                      Peptide and Nucleic Acid Sequences

HIS

Protein: HHHHHH (SEQ ID NO:6)

DNA: CAC CAT CAC CAT CAC CAT (SEQ ID NO:7)

c-Myc

Protein: EQKLISEEDL (SEQ ID NO:8)

DNA: GAG CAA AAG CTC ATT TCT GAA GAG GAC TTG (SEQ ID NO:9)

HA

Protein: YPYDVPDYA (SEQ ID NO:10)

DNA: TAC CCT TAT GAT GTG CCA GAT TAT GCC (SEQ ID NO:11)

VSV-G

Protein: YTDIEMNRLGK (SEQ ID NO:12)

DNA: TAT ACA GAC ATA GAG ATG AAC CGA CTT GGA AAG (SEQ ID NO:13)

HSV

Protein: QPELAPEDPED (SEQ ID NO:14)

DNA: CAG CCA GAA CTC GCC CCG GAA GAC CCC GAG GAT (SEQ ID NO:15)

V5

Protein: GKPIPNNLLGLDST (SEQ ID NO:16)

DNA: GGC AAA CCA ATC CCA AAC CCA CTG CTG GGC CTG GAT AGT ACT (SEQ ID NO:17)

FLAG

Protein: DYKDDDDDKG (SEQ ID NO:18)

DNA: GAT TAC AAA GAC GAT GAC GAT AAA GGA (SEQ ID NO:19)

Placing a tag on an IgA1 protease has the benefit of enabling easy detection of the IgA1 protease both *in vivo* and *in vitro*. A tag that comprises an epitope for an antibody can be detected either using anti-tag antibodies or antibodies that are conjugated to a detectable marker. The detectable marker can be a naturally occurring or non-naturally occurring amino acid that bears, for example, radioisotopes (e.g.,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ), fluorescent or luminescent groups, biotin, haptens, antigens and enzymes. There are many commercially available Abs to tags, such as c-myc, HA, VSV-G, HSV, V5, His, and FLAG. In addition, antibodies to tags used in the invention can be produced using standard methods to produce antibodies, for example, by monoclonal antibody production (Campbell, A.M., *Monoclonal Antibodies Technology: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, the Netherlands (1984); St. Groth et al., *J. Immunology*, (1990) 35: 1-21; and Kozbor et al., *Immunology Today* (1983) 4:72). The anti-tag antibodies can then be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, etc) using methods well known in

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the art, such as described in international application WO 00/70023 and (Harlour and Lane (1989) *Antibodies*, Cold Spring Harbor Laboratory, pp. 1- 726.

Assays for detecting tags include, but are not limited to, Western Blot analysis, Immunohistochemistry, Elisa, FACS analysis, enzymatic assays, and autoradiography. Means for performing these assays are well known to those of skill in the art. For example, radiolabels may be detected using photographic film or scintillation counters and fluorescent markers may be detected using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label.

The tag can be further used to isolate the IgA1 protease away from other cellular material. For example, by immunoprecipitation, or by using anti-tag antibody affinity columns or anti-tag antibody conjugated beads. When a HIS tag is used, isolation can be performed using a metal-chelate column (See Hochuli in *Genetic Engineering: Principles and Methods* ed. JK Setlow, Plenum Press, NY, chp 18, pp 87-96). Means for performing these types of purification are well known in the art.

In a preferred embodiment, an anti-tag antibody is used to generate an IgA1 protease immuno-complex such that the IgA1 protease retains enzymatic activity once complexed. Such an immuno-complex can be used in pharmaceutical preparations for the treatment of IgA1 deposition diseases. For example, an IgA1 immuno-complex, when administered to a patient, is believed to become trapped in the glomerulus of the kidney, a site of IgA1 deposition in IgA Nephropathy and Henoch-Schoenlein purpura disease.

### III. Treatment of IgA1 deposition diseases

Herein, IgA1 proteases are used as therapeutic agents to treat IgA1 deposition diseases. The abnormal deposition of IgA1 molecules is known to cause renal failure, skin blistering, rash, arthritis, gastrointestinal bleeding and abdominal pain.

#### *IgA nephropathy*

In one embodiment, the invention provides a method for treating IgA Nephropathy by administering to a patient in need of such treatment an IgA1 protease. IgA nephropathy is a disease of the kidney. The disease is considered to be an immune-complex-mediated glomerulonephritis, which is characterized by granular deposition of IgA1 in the glomerular mesangial areas. Nephropathy results and is defined by proliferative changes in the glomerular mesangial cells.

IgA nephropathy is one of the most common types of chronic glomerulonephritis and a frequent cause of end-stage renal disease.

#### *Dermatitis herpetiformis*

The invention further provides a method for treating Dermatitis herpetiformis (DH) by administering to a patient in need of such treatment an IgA1 protease. Dermatitis herpetiformis is a chronic blistering skin disease associated with deposits of IgA1 at the dermal-epidermal junction (Hall, RP & T.J. Lawley, J. Immunol. (1985) 135(3): 1760-5). DH patients have granular IgA1 deposits and have an associated gluten-sensitive enteropathy (GSE).

#### *Henoch-Schoenlein purpura*

In another embodiment, the invention provides a method for treating Henoch-Schoenlein purpura (HS) by administering to a patient in need of such treatment an IgA1 protease. Henoch-Schoenlein purpura is a skin and kidney disease. HSP is characterized by deposition of IgA1-containing immune complexes in tissue. The disease is diagnosed by observing evidence of

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IgA1 deposition in the skin tissue or kidney via immunofluorescence microscopy. The clinical manifestations typically include rash; arthralgias; abdominal pain; and renal disease.

#### *Animal models*

The therapeutic effect of IgA proteases of the present invention can be tested in any suitable animal model known to those skilled in the art. Some exemplary animal models are described below.

##### 1. IgA Nephropathy

A number of rat and mice animal models of IgA Nephropathy are available and are useful in the present invention. These models are described in Emancipator, S. N. et al., (1987) Animal models of IgA Nephropathy *In* IgA Nephropathy. A. R. Clarkson, editor. Martinus Nijhoff publishing, Boston. 188-203.

An exemplary model is described in Gesualdo L. et al, (1990) J. Clin. Invest. 86: 715-722.

Briefly, an IgA antibody/dextran sulfate complex is injected into mice. The immuno-complex lodges in the kidney and the mice present with glomerulonephritis that resembles typical cases of human IgA Nephropathy. How the model is made and used for testing therapeutic agents is described in more detail below.

Soluble immune complexes of dextran sulfate (500 kD, Sigma Chemical Co., St. Louis, MO) and monoclonal IgA anti- $\beta$ 1-6 glycoside (J558: Litton Bionetics, Kensington, MD) are prepared at threefold excess (26.5  $\mu$ g dextran/mg J558 (Nephropathy model); 22.0  $\mu$ g dextran/mg MOPC 104 E (normal control)). Complexes containing 3 mg antibody are injected into Swiss-Webster mice via tail vein injection. After 1 hour, the point of maximal deposition of IgA complexes in the kidney, mice are injected intraperitoneally with multiple doses of either saline or therapeutic agent at given intervals, such as 10 minute intervals. The mice are killed 1 hour after the last injection.

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Kidneys are then isolated from each mouse to look at IgA1 deposition and morphology by light, immunofluorescence, and electron microscopy.

Briefly, to monitor IgA1 deposition, snap-frozen samples of renal cortex, cryostat sectioned at 4um, are stained with fluoresceinated IgG fractions of goat antisera specific for mouse IgA (US Biochemical Corp) by direct immunofluorescence to semiquantitatively score for IgA1 deposits (Nakazawa, M. et al., (1986) Lab. Invest. 55:551-556, and Nakazawa, M. et al., (1986) J. Exp. Med. 164:1973-1987). A therapeutic agent is regarded as an effective agent when the number of IgA1 deposits scored is reduced towards the number of IgA1 deposits observed in a normal kidney.

Morphological changes, such as expansion of mesangial matrix and mesangial hypercellularity, is scored by staining sections of renal cortex with PAS reagent (Gesualdo, L. et al, (1990) J. Clin. Invest. 86: 715-722). Briefly, renal cortex is fixed in 10% formalin, embedded in paraffin and stained. Expansion of mesangial matrix and mesangial hypercellularity is scored semiquantitatively according to the methods described in Nakazawa, M. et al. (1986) Lab. Invest. 55:551-556, and Nakazawa, M. et al. (1986) J. Exp. Med. 164:1973- 1987.

Normal mesangial matrix is scored as 0. Expansion of mesangial matrix is scored as +1 when widened mesangial stalks are observed, +2 when matrix encroachment on capillary lumens is observed, and +3 when conspicuous widening of mesangial stalk is observed along with a decrease in capillary lumen. A therapeutic agent is regarded as effective agent when the expansion of mesangial matrix is reduced towards the morphology of the matrix observed in a normal kidney, for example to a score of +2, or +1, or 0.

Normal mesangial cellularity is scored as 0 and is defined as 3 or fewer cell nuclei per mesangial area. Hypercellularity is scored as +1 when 4 to 6 cell nuclei per mesangial area are observed, as +2 when 4 to 6 cell nuclei per mesangial area are observed in most areas but some

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areas have 7 or more nuclei, and as +3 when 7 or more cell nuclei per mesangial area are observed in most areas. A therapeutic agent is regarded as effective agent when the mesangial hypercellularity is reduced towards that observed in a normal kidney, for example to a score of +2, or +1, or 0.

Total glomerular area, matrix area, and glomerular cellularity are also quantified in randomly selected glomeruli from each mouse by computer morphometry (Cue image analysis system, Olympus Corp., Columbia, MD.) (Gesualdo L. et al, (1990) J. Clin. Invest. 86: 715-722). Briefly, cubes of cortex are fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, post fixed in 1% OsO<sub>4</sub>, and embedded in Spurr's epoxy (Polysciences, Inc. Warrington, PA). 50-70 nm sections are stained with uranyl acetate and lead hydroxide. Coded grids are examined in a JEOL JEM 100 EX microscope and matrix, cellularity, and immune deposits are semiquantified as described in Nakazawa, M. et al., (1986) J. Exp. Med. 164:1973- 1987.

Hematuria (the presence of red blood cells in urine) and proteinuria (the presence of protein in urine) are also a suitable measure of IgA Nephropathy. Briefly, mice are placed in metabolic cages and urine is collected for 24 hours. The urine is then centrifuged and assayed for protein by turbidimetry in 3% sulfalicylic acid and hematuria by microscopy, as described in Nakazawa, M. et al., (1986) J. Exp. Med. 164:1973- 1987.

Typically, a normal mouse without IgA Nephropathy will have less than three red blood cells per high power field (40X), while mice with IgA Nephropathy will have greater than 10 red blood cells per high power field. A reduction in the number of red blood cells per high power field is indicative that the therapeutic agent is effective for IgA Nephropathy. Mice are tested for hematuria and proteinuria before treatment to determine the reference value indicative of disease. A reduction in the reference value, as compared to the value for hematuria and proteinuria obtained before treatment, of 5%, 10%, 30%, 40% preferably 50%, and more

preferably greater than 50% after treatment with the therapeutic agent is indicative that the agent is effective for treatment of IgA1 Nephropathy.

#### IV Dosage, Formulation and Administration

Herein, bacterial IgA proteases are used to treat IgA deposition diseases. The IgA1 protease of the present invention can be used in a composition that is combined with a pharmaceutically acceptable carrier. Such a composition may also contain diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. In one aspect, the IgA1 protease is complexed with an antibody to form a therapeutic immuno-complex. Such a therapeutic immuno-complex is particularly useful for treatment of diseases characterized by IgA1 deposition in the kidney since the large immuno-complex is believed to lodge in the renal glomerulus upon administration.

The term “pharmaceutically acceptable” means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. For drugs administered orally, pharmaceutically acceptable carriers include, but are not limited to pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, tablets may be coated with a material such as glyceryl monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract.

Pharmaceutically acceptable salts can be formed with inorganic acids such as acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate heptanoate, hexanoate, hydrochloride hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, thiocyanate, tosylate and undecanoate. Base salts include ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salt with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with amino acids such as arginine, lysine, and so forth. Also, the basic nitrogen-containing groups can be quarternized with such agents as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chloride, bromides and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl and diamyl sulfates, long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides, aralkyl halides like benzyl and phenethyl bromides and others. Water or oil-soluble or dispersible products are thereby obtained.

The composition may also contain other agents, which either enhance the activity of the composition, or compliment its activity or use in treatment, or maintain the activity of the therapeutic agent in storage. Such additional factors and/or agents may be included in the composition to produce a synergistic effect or to minimize side effects. Additionally, administration of the composition of the present invention may be administered concurrently with other therapies.

Administration of the therapeutic agent of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, topical application or cutaneous, subcutaneous, intraperitoneal, parenteral or intravenous injection.

The compositions containing the therapeutic agent of the present invention can be administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent, *i.e.*, carrier or vehicle.

Modes of administration of the therapeutic agent of the present invention include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intra-arterial injection and infusion. Pharmaceutical compositions for parenteral injection comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (*e.g.*, glycerol, propylene glycol, polyethylene glycol and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (*e.g.*, olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity may be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents, and/or compounds to shield the immunogenic determinant of the therapeutic agent. Prevention of the action of microorganisms may be improved by the inclusion of various antibacterial and antifungal agents such as paraben, chlorobutanol, phenol sorbic acid and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride and the like. Prolonged absorption of an injectable pharmaceutical form may be brought about by the inclusion of agents, such as aluminum monostearate and gelatin, which delay absorption. Injectable depot forms are made by forming microencapsule matrices of the therapeutic agent in biodegradable polymers

such as polylactide-polyglycolide, poly(orthoesters) and poly(anhydrides). Depending upon the ratio of therapeutic agent to polymer and the nature of the particular polymer employed, the rate of therapeutic agent release can be controlled. Depot injectable formulations are also prepared by entrapping the therapeutic agent in liposomes or microemulsions which are compatible with body tissues. The injectable formulations may be sterilized, for example, by filtration through a bacterial-retaining filter or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable media just prior to use.

The formulations include those suitable for oral, rectal, ophthalmic (including intravitreal or intracameral), nasal, topical (including buccal and sublingual), intrauterine, vaginal or parenteral (including subcutaneous, intraperitoneal, intramuscular, intravenous, intradermal, intracranial, intratracheal, and epidural) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

As used herein, a “therapeutically effective amount” means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, i.e., treatment, healing, prevention or amelioration of the relevant medical condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. Generally, a composition will be administered in a single dose in the range of 100 µg – 10 mg/kg body weight, preferably in the range of 1 µg – 100 µg/kg body weight. This dosage may be repeated daily, weekly, monthly, yearly, or as considered appropriate by the treating physician.

When a therapeutically effective amount of the therapeutic agent of the present invention is administered orally, the composition of the present invention can be in the form of a liquid, the composition contains from about 0.5 to 90% by weight of protein of the present invention, and preferably from about 1 to 50% protein of the present invention.

When a therapeutically effective amount of the therapeutic agent of the present invention is administered by intravenous, cutaneous or subcutaneous injection, the protein will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to protein of the present invention, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

Topical administration, in which the composition is brought in contact with tissue(s), may be suitable for Dermatitis herpetiformis. By "contacting" is meant not only topical application, but also those modes of delivery that introduce the composition into the tissues, or into the cells of the tissues.

Use of timed release or sustained release delivery systems are also included in the invention. Such systems are highly desirable in situations where surgery is difficult or impossible, *e.g.*, patients debilitated by age or the disease course itself, or where the risk-benefit analysis dictates control over cure.

A sustained-release matrix, as used herein, is a matrix made of materials, usually polymers, which are degradable by enzymatic or acid/base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. The sustained-release matrix desirably is chosen from biocompatible materials such as liposomes, polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (co-polymers of lactic acid and glycolic acid) polyanhydrides, poly(ortho)esters, polyproteins, hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene, polyvinylpyrrolidone and silicone. A preferred biodegradable matrix is a matrix of one of either polylactide, polyglycolide, or polylactide co-glycolide (co-polymers of lactic acid and glycolic acid).

The amount of the therapeutic agent of the present invention in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments, which the patient has undergone.

Ultimately, the attending physician will decide the amount of the therapeutic agent of the present invention with which to treat each individual patient. Initially, the attending physician will administer low doses of the therapeutic agent of the present invention and observe the patient's

response. Larger doses of may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the therapeutic agent of the present invention will be in the range of 12 to 72 hours of continuous intravenous administration, at a rate of approximately 30 mg/hour. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

## EXAMPLES

### Example 1. Construction of tagged IgA1 protease

A His tag has been fused in frame into *Haemophilus influenzae* IgA1 protease by PCR-based site directed mutagenesis using plasmid pFG26 that contains the DNA sequence encoding *Haemophilus influenzae* IgA1 protease. Two PCR fragments were generated from pFG26 as illustrated in FIG. 4. The first fragment, Xba I and pml I fragment, containing the newly inserted HIS tag and pml I site was generated using oligonucleotide primers “HFD6His1”(primer 1) and “HFD6His2” (primer 2) shown below. The second fragment, pml I and Acc I fragment, was generated using primer 3 and primer 4 also shown below.

Primer 1: HFD-5 XbaI: 5' GATCCGCTTACCAATTATGC 3' (SEQ ID NO:20)

Primer 2: HFD6His1:  
5'CTTGGTACGCTAGGCACGTGATGATGATGATGAGGTGTTGTGATATTT  
GTCG-3' (SEQ ID NO:21)

Primer 3: HFD6His2: 5'-CCTAATAATATTCAAGCTCACGTGCCTAGCGTACC-3'  
(SEQ ID NO:22)

Primer 4: HFD-F-ACCI: 5'-TTCAGCAGAAGTCTCTTGC-3' (SEQ ID NO:23)

After amplification of the two fragments by PCR, the fragments were digested with either Xba I and Pml I or Pml I and Acc I and ligated into the Xba I and Acc I sites of the parental

pFG26 plasmid using conventional techniques. The mutation was confirmed by DNA sequence and the new plasmid designated pJQ/Rd6His. The fragments were designed such that DNA codons for six Histidines replaced the original codons at position 1007-1012 of the IgA1 protease; asn-asn-ile-gln-ala-asn (SEQ ID NO:24).

Example 2. Generation of a Bacterial strain that expresses Tagged IgA1 protease.

A *Haemophilus influenzae* bacterial strain that expresses only a tagged IgA1 protease that is enzymatically active was generated by standard recombination techniques. Briefly, the plasmid pJQ/Rd6His that was generated in Example 1 was cut with restriction enzymes Cla I and Nde I. The gene was isolated and transformed into a *Haemophilus influenzae* bacterial Rd strain (Rd 3-13) that produces an IgA1 protease without enzyme activity (Plaut AG, Qiu, J, Grundy, F. and Wright, A. J Infect Dis. (1992) Jul;1 66(1):43-52) to allow for insertion of the His tagged IgA1 protease into the bacterial genome by recombination. The bacteria were then screened for restoration of enzyme activity by testing bacterial growth media of selected colonies for the presence of active protease using human IgA1 as a substrate.

Introduction of the 6-His mutation into the active enzyme was confirmed by verifying the presence of a Pml I site using PCR fragment of the genomic DNA. This strain was designated Rd 6His.

The Rd 6His strain had identical growth rate and colony morphology as did wild type strain Rd. IgA protease activity yield and the size of the enzyme were indistinguishable from wild type. Although the 6 His mutation was introduced just two amino acids away from the auto-proteolytic a site, there was no detectable problem with either the enzyme secretion from the bacterial cell, nor its self-processing.

A monoclonal anti-5His antibody (Qiagen, Inc) bound to the protease as determined by Western blot analysis. When combined with the monoclonal antibody in solution, Rd 6His IgA protease retained full activity.

## Example 3.

The therapeutic effect of IgA1 protease for the treatment of IgA Nephropathy can be tested in a mouse model for IgA Nephropathy.

Multiple Swiss-Webster mice (Charles River Laboratories) are used per experiment and divided into groups (typically, 10 mice/group). Mice are first placed in a metabolic chamber, and urine collected for 24 hours to determine the amount of hematuria (the presence of red blood cells in urine) and proteinuria (the presence of protein present in urine) in urine. This provides a base value for hematuria and proteinuria in normal healthy mice. IgA Nephropathy is then induced in the mice as described in Gesualdo L. et al, (1990) J. Clin. Invest. 86: 715-722.

After induction of Nephropathy, mice are injected intraperitoneally with multiple doses of either saline (control), inactive IgA1 protease (control), active IgA1 protease, and IgA1 protease complexed with immunoglobulin (e.g. complexed with anti-tagged antibody, or anti-IgA1 protease antibody). An example dose regime includes injection of 0.1-0.5 mg of protease intraperitoneally, twice a day for 5 days. The dosage and dose regime can be varied according to therapeutic effect observed in pilot experiments, for example on a single mouse. Typically, maximal doses are chosen which do not elicit histological or clinical toxicological effects. When the IgA1 protease is complexed with a ligand, varied concentrations of ligand are used to determine the most effective Protease/ligand ratio. The mode of administration can also be varied, for example administration can be performed by intravenous injection.

1-24 h after administration of the last dose of therapeutic agent, mice are tested for relief of IgA Nephropathy by measuring hematuria and proteinuria in mice that did not receive therapeutic agent. A reduction in the amount of hematuria and proteinuria as compared to mice that did not receive therapeutic agent, towards those values observed in normal healthy mice is indicative of therapeutic success.

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PCT/US2004/006615

Morphological measures of IgA Nephropathy are also assessed. Mice are sacrificed after urine collection and the kidneys are removed to determine the amount of i) IgA1 deposition, ii) expansion of mesangial matrix and ii) mesangial hypercellularity as described. A reduction in parameters i-iii as compared to that observed in mice that did not receive therapeutic agent are indicative of therapeutic success.

Example 4.

The therapeutic effect of IgA1 protease for the treatment of Dermatitis herpetiformis can be tested in a mouse model for Dermatitis herpetiformis.

Example 5.

The therapeutic effect of IgA1 protease for the treatment of Henoch-Schoenlein purpura can be tested in a mouse model for Henoch-Schoenlein purpura.

# **DEMANDES OU BREVETS VOLUMINEUX**

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS  
COMPREND PLUS D'UN TOME.**

**CECI EST LE TOME \_\_1\_\_ DE \_\_2\_\_**

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

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# **JUMBO APPLICATIONS / PATENTS**

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE  
THAN ONE VOLUME.**

**THIS IS VOLUME \_\_1\_\_ OF \_\_2\_\_**

NOTE: For additional volumes please contact the Canadian Patent Office.

## Claims

1. An IgA1 protease for use in cleaving IgA1 deposits in a patient.
2. The IgA1 protease of claim 1, wherein the cleaving of IgA1 deposits results in a reduction of IgA1 deposits in the patient.
3. The IgA1 protease of claim 1 or claim 2, wherein said deposits are associated with IgA nephropathy.
4. The IgA1 protease of claim 1 or claim 2, wherein said deposits are associated with dermatitis herpetiformis.
5. The IgA1 protease of claim 1 or claim 2, wherein said deposits are associated with Henoch-Schoenlein purpura.
6. The IgA1 protease of any one of claims 1 to 5, wherein the IgA1 protease is fused to a tag.
7. The IgA1 protease of claim 6, wherein the tag is c-Myc, HA, VSV-G, HSV, FLAG, V5, or HIS.
8. The IgA1 protease of any one of claims 1 to 7, wherein the IgA1 protease is *Streptococcus pneumoniae* IgA1 protease, *Streptococcus sanguis* IgA1 protease, *Clostridium ramosum* IgA1 protease, *Haemophilus influenzae* IgA1 protease, *Neisseria meningitidis* IgA1 protease, or *Neisseria gonorrhoeae* IgA1 protease.
9. The IgA1 protease of claim 8, wherein the IgA1 protease is *Streptococcus sanguis* IgA1 protease, *Clostridium ramosum* IgA1 protease, *Haemophilus influenzae* IgA1 protease, or *Neisseria gonorrhoeae* IgA1 protease.
10. The IgA1 protease of claim 9, wherein the IgA1 protease is *Haemophilus influenzae* IgA1 protease.

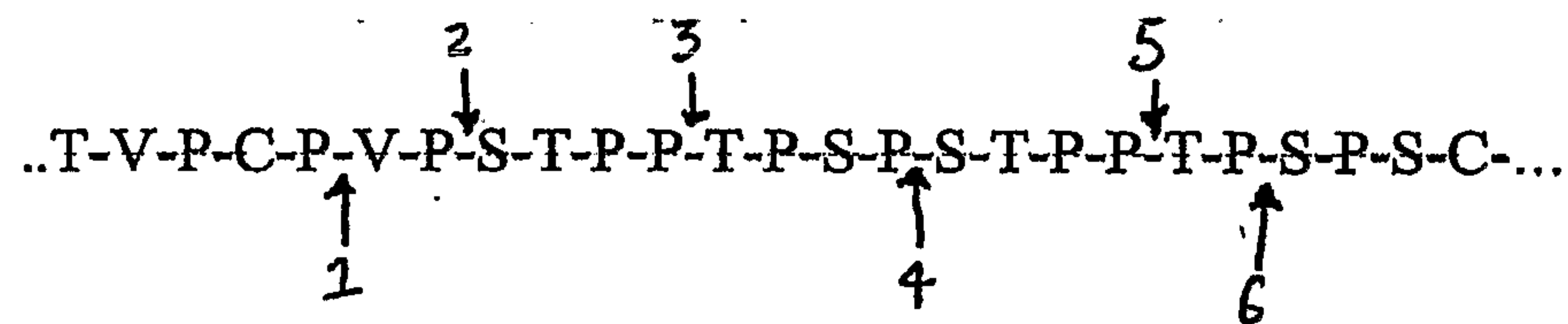
11. The IgA1 protease of claim 10, wherein the Haemophilus influenzae IgA1 protease is Haemophilus influenzae type 1 IgA1 protease or Haemophilus influenzae type 2 IgA1 protease.
12. The IgA1 protease of claim of 9, wherein the IgA1 protease is Neisseria meningitidis IgA1 protease.
13. The IgA1 protease of claim of 12, wherein the Neisseria meningitidis IgA1 protease is Neisseria meningitidis type 1 IgA1 protease and Neisseria meningitidis type 2 IgA1 protease.
14. The IgA1 protease of any one of claims 1 to 13, wherein the IgA1 protease cleaves at the hinge region of IgA1.
15. Use of an IgA1 protease for the preparation of a medicament for use in the treatment of IgA nephropathy, dermatitis herpetiformis or Henoch-Schoenlein purpura.
16. A pharmaceutical composition for the treatment of a disease that is IgA nephropathy, dermatitis herpetiformis or Henoch-Schenlein purpura, comprising IgA1 protease and a pharmaceutically acceptable carrier.
17. A pharmaceutical composition for the treatment of a disease that is renal failure, skin blistering, rash, arthritis, gastrointestinal bleeding or abdominal pain, comprising IgA1 protease and a pharmaceutically acceptable carrier.
18. The pharmaceutical composition of claim 16 or claim 17, wherein said IgA1 protease comprises a tag.
19. The pharmaceutical composition of claim 18, wherein said tag specifically binds to a ligand.
20. The pharmaceutical composition of claim 19, wherein said ligand is an antibody.
21. The pharmaceutical composition of claim 18, wherein said tag is: c-Myc, HA, VSV-G, HSV, FLAG, V5, or HIS.

22. The pharmaceutical composition of claim 19, wherein said antibody is an anti-tag antibody.
23. The pharmaceutical composition of claim 22, wherein said anti-tag antibody is: anti-HIS, anti-FLAG, anti-MYC, anti-VSV, anti-HA, and anti-V5.
24. The pharmaceutical composition of claim 16, wherein the disease is IgA nephropathy.
25. The pharmaceutical composition of claim 16, wherein the disease is **Dermatitis** herpetiformis.
26. The pharmaceutical composition of claim 16, wherein the disease is Henoch-Schoenlein purpura.
27. The pharmaceutical composition of any one of claims 16-26, which composition is formulated for administration in combination with an antibody.
28. The pharmaceutical composition of any one of claims 16-26, which composition is formulated for intravenous delivery.

FIGURE 1

IgA1 Hinge Region

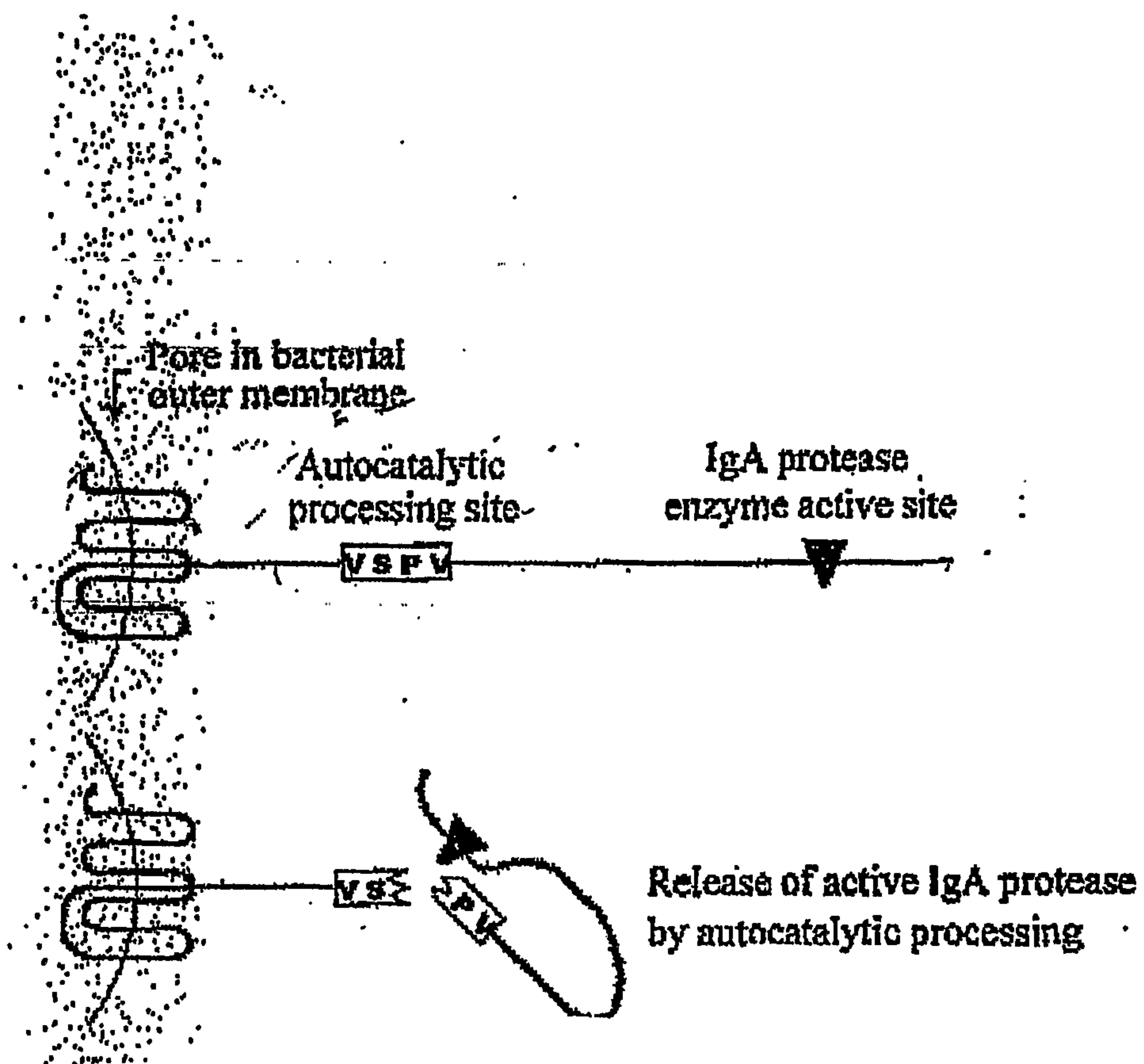
Amino acids 217-241



1. *Clostridium Rasmusum*
2. *Prevotella species*
3. *Streptococcus pnemoniae*  
*Streptococcus saguis*
4. *Haemophilus Influenzae* (1)  
*Haemophilus aegyptitus*
5. *Ureaplasma ureatyticum*  
*Neisseria meningitidis* (2)  
*Neisseria gonorrhoeae* (2)  
*Haemophilus Influenzae* (2)
6. *Neisseria meningitidis* (1)  
*Neisseria gonorrhoeae* (1)

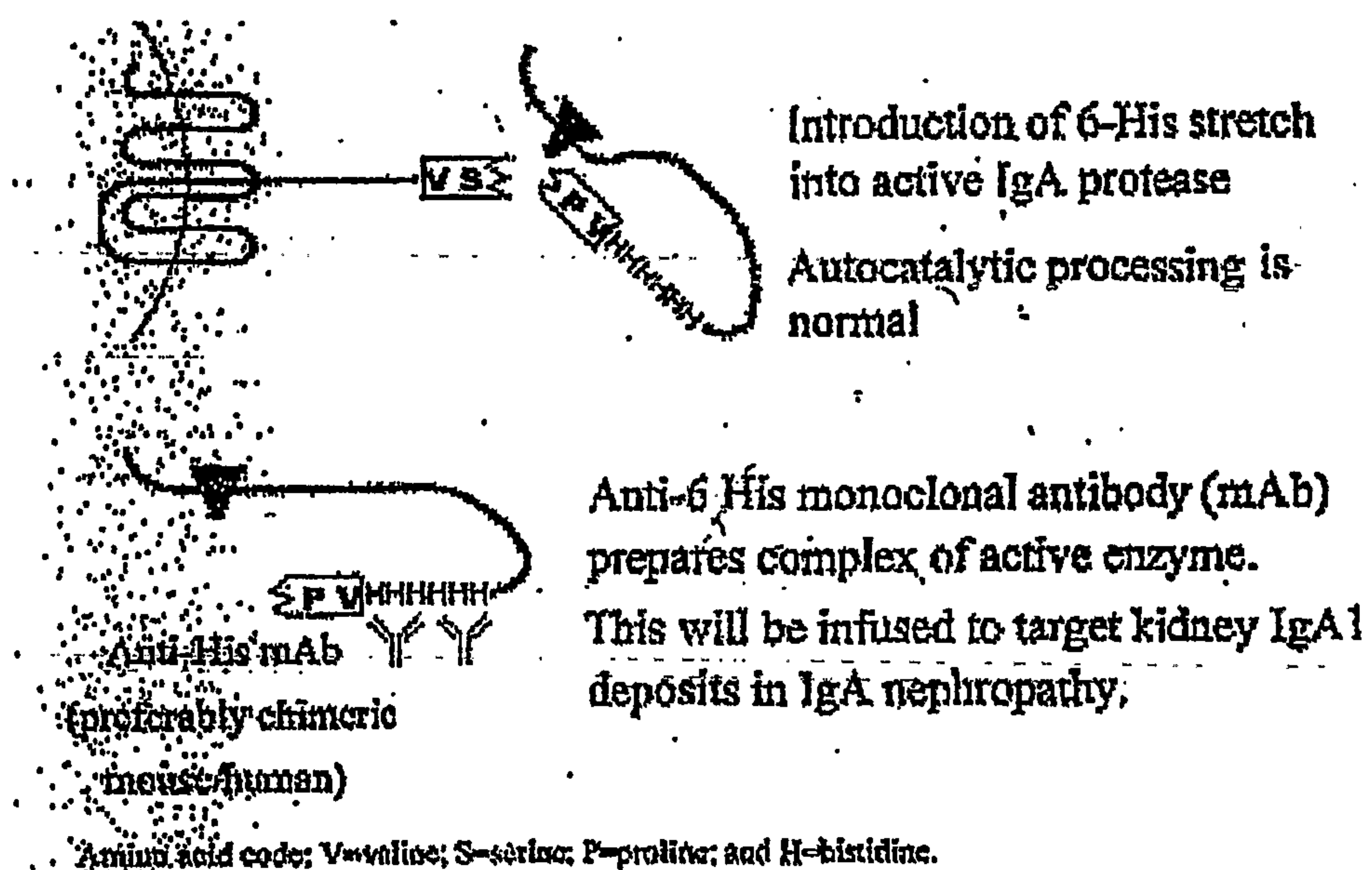
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FIGURE 2A



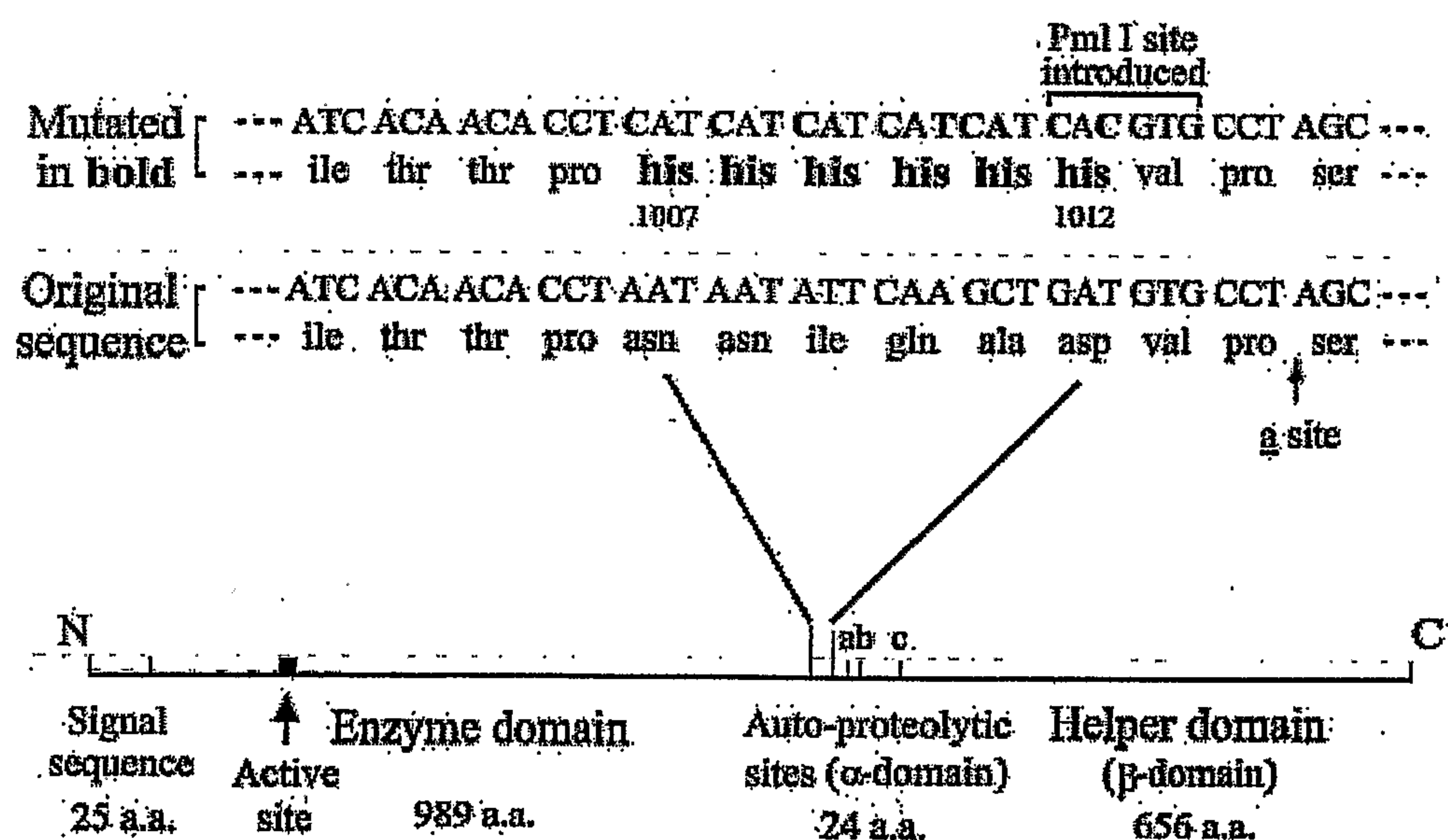
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FIGURE 2B



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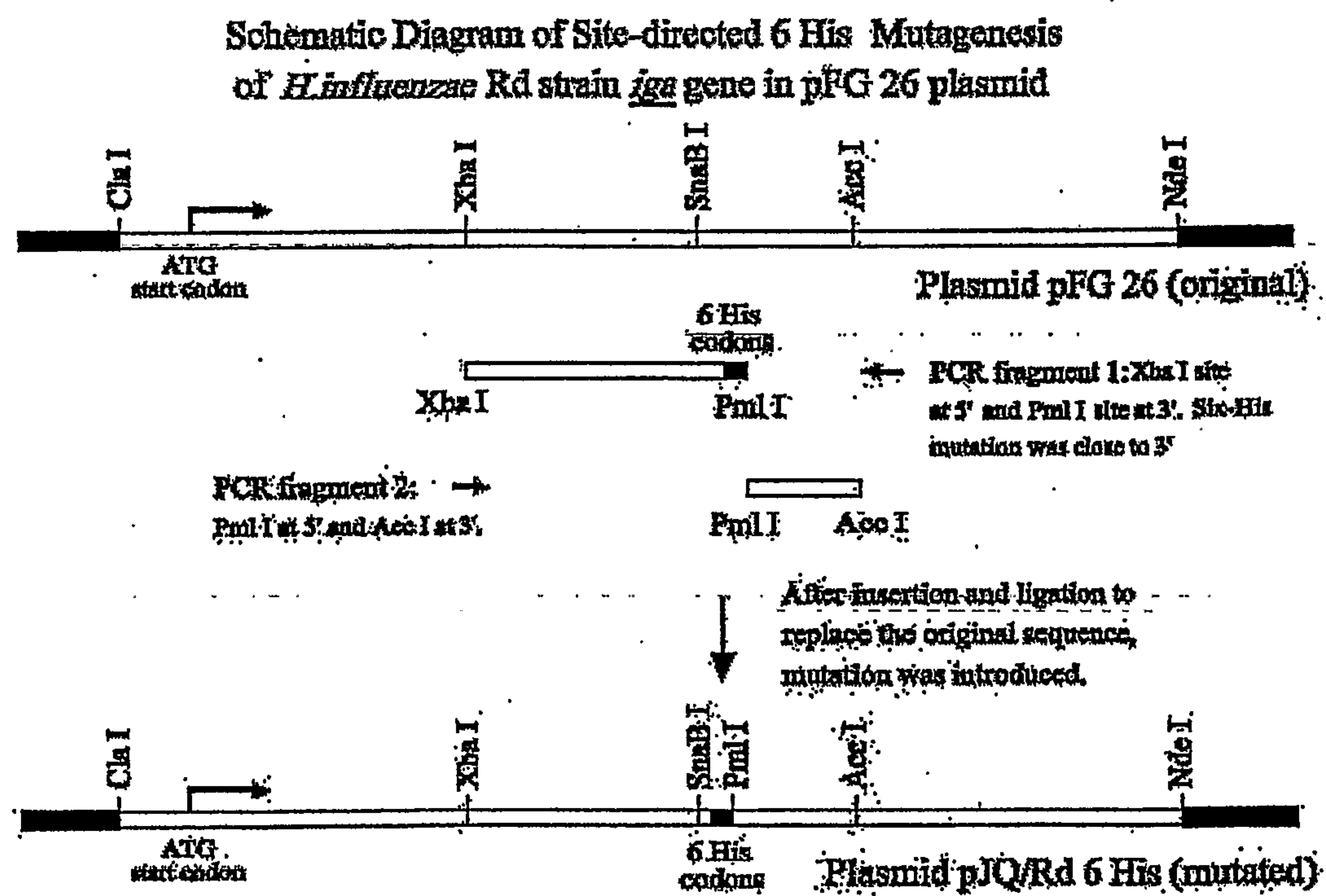
FIG. 3



Schematic diagram showing *H. influenzae* Rd IgA protease precursor contains 4 sections: signal sequence, enzyme domain,  $\alpha$ -domain, and "helper" or  $\beta$ -domain. Active site of the protease is near N-terminus of the enzyme domain (arrow). Top of the diagram shows details of the protein sequence mutation. 6xHis epitope is 2 residues away from the main autocatalytic site, g site.

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FIGURE 4



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FIG. 5

***Haemophilus influenzae* Rd protein sequence**

MLNKKFKLNFIALTVAYALTPYTEAALVRDDVDYQIFRDFAE/NKGRFSVGATNVEVRD  
 KNNHSLGNVLPNGIPMIDFSVVDVDKRIATLINPQYVVGVKHVSNGVSELHFGNLNGN  
 MNNGNAKSHRDVSSEENRYFSVEKNEYPTKLNGKAVTTEDQTQKRREDYYMPRLDKF  
 VTEVAPIEASTASSDAGTYNDQNKYPAFVRLGSGSQFIYKKGDNYSLILNNHEVGGNNL  
 KLVGDAYTYGIAGTPYKVNHENNGLIGFGNSKEEHSDPKGILSQDPLTNYAVLGDSGSP  
 LFVYDREKKGKWLFLGSYDFWAGYNKKSWQEWNIYKPEFAKTVLDKDTAGSLTGSNTQ  
 YNWNPTGKTSVISNGSESLNVDLFDSSQDTSKKNNHGKSVTLRSGGTLTLNNNIDQGA  
 GGLFFEGDYEVKGTSDSTTWKGAGVSVADGKTVTWKVHNPKSDRLAKIGKGTLIVEGK  
 GENKGSLKVGDTVLKQQADANNKVKAFSQVGVSGRSTVVLNDDKQVDPNSIYFGF  
 RGGRLDANGNNLTFEHIRNIDDGARLVNHNTSKTSTVTITGESLITDPNTITPYNIDAPDE  
 DNPYAFRIKDGGQLYLNLENYTYIALRKGASTRSELPKNSGESNENWLYMGKTSDEA  
 KRNVMNHINNERMNGFNGYFGEEEGKNNGNLNVTFKGKSEQNRFLTGGTNLNGDLK  
 VEKGTFLFSGRPTPHARDIAGISSTKKDQHFENNEVVVEDDWINRNFKATNINVTNNA  
 TLYSGRNVANITSNITASDNAKVHIGYKAGDTV CVRSDYTGYVTCTTDKLSDKALNSFN  
 ATNVSGNVNLSGNANFVLGKANLFGTISGTGNSQVRLTENSHWHLTGDSNVNQLNLDK  
 GHIHLNAQNDANKVTTYNTLTVNSLSGNGSFYYLTDLSNKQGDKV VVTKSATGNFTLQ  
 VADKTGEPTKNELTFDASNATRNNLNVSLVGNTVDLGAWKYKL RNVNGRYDLYNPE  
 VEKRNQTVDTTNTITPNNIQADVPSVPSNNEEIARVETPVPPPAPATPSETTETVAENSKQ  
 ESKTVEKNEQDATETTAQNGEVAEEAKPSVKANTQTNEVAQSGSETEETQTTEIKETAK  
 VEKEEKAKVEKDEIQEAPQMASETSPKQAKPAPKEVSTD TKVEETQVQAQPQTQSTTVA  
 AAEATSPNSKPAEETQPSEKTNAEPVTPVVSKNQ TENTTDQPTEREKTAKVETEKTQEPP  
 QVASQASPKQE QSETVQPQAVLESENVPTVNNAEEVQAQLQTQTSATVSTKQPAPENSI  
 NTGSATAITETA EKSDKPQTETA ASTEDASQHKANTVADNSVANNSSESSDPKSRRRRSIS  
 QPQETSAEETTAASTDETTIADNSKRSKPNRRSRRSVRSEPTVTNGSDRSTVALRDLTST  
 NTNAVISDAMAKAQFVALNVGKA V SQHISQLEMNNEGQYNVWVSNTSMNENYSSSQY  
 RRFSSKSTQTQLGWDQTISNNVQLGGVFYTYVRNSNNFDKASSKNTLAQVNFYSKY YAD  
 NHWYLGIDLGYGKFQSNLKTNHNAKFARHTAQFGLTAGKAFNLGNFGITPIVGVRYSY  
 LSNANFALAKDRIKVNPISVKTAFAQVDLSYTYHLGEFSVTPILSARYDTNQSGSGKINVN  
 QYDFAYNVENQQQYNAGLKLKYHNVKLSLIGGLTKAKQAEKQKTAELKLSFSF"

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FIGURE 6

**Haemophilus influenzae Rd nucleotide sequence**

atgctaaata aaaaattcaa actcaatfff attgcgctta ctgtcgcta cgcattaacc  
 ccttatacag aagctgcgtt agtgagagac gatgtggatt atcaaatatt tctgtatttt  
 gcagaaaata aaggagatt ttctgttgt gcaacaaatg tggaagtga agataaaaat  
 aaccactctt taggcaatgt ttacctaatt ggcatccga tgattgatt tagtggtgtg  
 gatgtagata aacgcacgc cacattgata aatccacaat atgtagtagg tgtaaacac  
 gttagtaacg gcgtgagtga actacatttt gggaacttaa atggcaatat gaataatggc  
 aatgctaaat cgcaccgaga tgtatcttca gaagaaaata gatattttc cgttgagaaa  
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 cgccgtgaag actactatat gccacgtctt gataaatttg ttaccgaagt tgcaccaata  
 gaggcctcaa ctgcaagtag tgatgctggc acatataatg atcagaataa atatcctgct  
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 tatggtattg caggcacacc ttataaagta aaccacgaaa ataattggact aattggtttt  
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 gaatggaata ttataaacd tgaatttgca aaaactgttc tagataaaga tactgcaggt  
 tcttiaactg gttctaacac ccaatacaat tggaatccta ctggcaaac aagcgttatt  
 tctaattggt ctgaatctct aatgttgat ttattcgata gtagtcagga tactgactct  
 aagaagaaca atcacggaaa aagtgtgact cttagaggaa gtggaacgct taccttaaat  
 aataatatcg atcaaggcgc aggcggcttg ttcttgaag gagattatga agttaaggc

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FIGURE 6 (cont.)

acttctgata gtaccacttg gaaaggagct ggcgtttctg ttgctgatgg aaaaacagta  
 acgtggaaag tacataaccc gaaatctgat cgtttagcta aaatcggcaa aggaacatta  
 attgtagaag gaaagggaga aaataaaggt tcgctaaaag tgggcgatgg tactgttate  
 ttaaacaac aagctgatgc caataataaa gttaaagcct ttcacaagt aggtatagta  
 agtggctgct caactgttgt acttaatgat gataagcaag tagatccaaa ttccatttac  
 ttggctta gaggtggctg attagatgcc aatggcaata atctacttt tgaacatate  
 cgtaatatg atgatggcgc aagactagta aatcacaata ccagcaaac ctctactgta  
 acaattactg gggaaagtct aattacagat ccaatacaa ttactccata taatatagac  
 gcaccagatg aagataatcc ttatgccttt cgacggatta aagatggagg acagctctat  
 ttaaatttg aaaattacac ttattatgcg ttaagaaaag gtgcgagcac tcgttcagaa  
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 gtgacttgca ctactgcaa gttatccgat aaagccctta atagctttaa cgccaccaat  
 gtatctggca atgtaaatt atcaggtaat gcaaacttg tottaggcaa agctaacta  
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 catitaacag gcgatagcaa tgtaatcag ttaaatttag acaaggggca tattcattta  
 aatgcacaaa acgatgcaa taaagtaact acatataca cgctgactgt gaatagctta  
 tcaggtaacg gttcttcta ttatttaact gatctttcca ataaacaagg cgacaaagtt

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FIGURE 6 (cont.)

gttgtaacta aatccgccac aggtaacttt acattacaag tggcagataa aacaggcgag  
 cctacaaaaa atgaactcac gcttttgat gcgtaaatg ctacaagaaa taatttgat  
 gtgtcattag ttgggaatac cgttgattta ggtgcttgga aatataaatt acgtaatgt  
 aatggacgtt acgatttgta taaccagag gtggaaaaa gaaatcaaac tgtcgatag  
 acaaatatca caacacctaa taatattcaa gctgatgtgc ctagegtacc aagtaacaat  
 gaagaaatag cccgtgtiga aacaccagtt ccaccacctg cgctgtctac accatcagag  
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 agtagaagaa gtgttcgctc ggaaccaact gttacaaatg gcagcgatcg ttctacagta  
 gcattgcgag atctcacaag tacaacaca aatgcggtaa ttctgatgc aatggcaaaa

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FIGURE 6 (cont.)

gcacaattg ttgcattaaa tgtggggaaa gcagtttctc aacatattag ccagttagaa  
atgaataacg aggggcaata taacgtttgg gtatctaata cttcaatgaa cgaaaattat  
tctcaagtc aatatcgtcg ttttagttct aaaagtagcg aaactcaact tggttgggat  
caaacaatct caaacaatgt tcagttaggt ggcgtgttla cttatgttcg caatagtaac  
aacttigata aggcaagcag taaaaaactc ctagcacaag ttaatttcta ttctaaatat  
tatgcggata atcatiggtta ttggggcatt gatttaggct acggcaagtt ccaaagcaac  
ctaaaaacca atcataatgc gaaattigct cgccatactg cacaatttgg ttaaccgca  
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