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(57) **Abrégé/Abstract:**

The present invention relates to the identification, cloning and sequencing of nucleic acid molecules encoding an isoform of the enzyme glutamic acid decarboxylase and further relates to the use of these molecules and/or peptides and polypeptides encoded thereby in diagnostic tests for Insulin Dependent Diabetes Mellitus and other diseases in which glutamic acid decarboxylase is an autoantigen and in the treatment of patients suffering from these diseases.





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(54) Title: A METHOD FOR THE DIAGNOSIS AND TREATMENT OF GLUTAMIC ACID DECARBOXYLASE AUTO-ANTIGEN ASSOCIATED DISEASES		
(57) Abstract The present invention relates to the identification, cloning and sequencing of nucleic acid molecules encoding an isoform of the enzyme glutamic acid decarboxylase and further relates to the use of these molecules and/or peptides and polypeptides encoded thereby in diagnostic tests for Insulin Dependent Diabetes Mellitus and other diseases in which glutamic acid decarboxylase is an autoantigen and in the treatment of patients suffering from these diseases.		

**A METHOD FOR THE DIAGNOSIS AND TREATMENT OF GLUTAMIC
ACID DECARBOXYLASE AUTOANTIGEN ASSOCIATED DISEASES**

The present invention relates to the identification, cloning and sequencing of
5 nucleic acid molecules encoding an isoform of the enzyme glutamic acid
decarboxylase and further relates to the use of these molecules and/or
peptides and polypeptides encoded thereby in diagnostic tests for Insulin
Dependent Diabetes Mellitus and other diseases in which glutamic acid
decarboxylase is an autoantigen and in the treatment of patients suffering from
10 these diseases.

The enzyme glutamic acid decarboxylase (hereinafter referred to as "GAD")
catalyses the conversion of L-glutamic acid to the inhibitory neurotransmitter
 γ -amino butyric acid (hereinafter referred to as "GABA"). GAD is expressed
15 both in the GABA secretory neurons of the central nervous system (1-3), in
the β -cells of the pancreas (4,5), and in spermatozoa (6). Analysis of
immunoaffinity-purified, enzymatically active brain GAD has identified several
isomeric forms of GAD with M_r 54-67,000 (7,8). Using antisera raised to
purified brain GAD to screen brain cDNA expression libraries, cDNAs
20 encoding full length rat (9) and feline (10) GAD sequences have been isolated
and sequenced. Comparisons of the deduced amino acid sequences of rat and
feline GAD show that both proteins are 95% identical and, therefore, highly
conserved during evolution.

25 Autoantibodies reactive with GAD in GABA-ergic neurons are present in the
majority of sera from patients with the rare neurological disease Stiff Man
Syndrome (hereinafter referred to "SMS"; 11,12). Patients positive for GAD
autoantibodies have an increased frequency of polyendocrine autoimmunity
especially Insulin Dependent Diabetes Mellitus (hereinafter referred to as
30 "IDDM"). During the pre-clinical stage of IDDM and in patients with recent
onset clinical IDDM, autoantibodies are frequently detected against an islet
cell M_r 64,000 protein designated "64K" (13). In a recent report, the 64K

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autoantigen was presumptively identified as GAD (14). However, Genovese (15) has suggested that GAD is co-precipitated with a separate 64K protein, the latter distinguished by tryptic products of M_r 37,000/40,000 that are distinct from a M_r 50,000 product of GAD. GAD comprises at least two isoforms encoded by separate genes (16, 17, 18). The predicted molecular weights of the known isoforms are approximately 67,000 and 65,000 (referred to as the "67K" and "65K" isoforms, respectively). The distribution of GAD isoforms in different tissues is still not well defined, but it is likely that the 65K isoform accounts for the GAD component of the 64K autoantigen (17).

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In work leading up to the present invention, the inventors sought to clone the 67K isoform of GAD from human and other species for potential diagnostic and/or therapeutic use. In accordance with the present invention, human brain (HB), human pancreatic islet (HI) and mouse brain (MB) GAD (hereinafter referred to as "HBGAD", "HIGAD" and "MBGAD", respectively) have been cloned and sequenced. In further accordance with the present invention, recombinant GAD proteins corresponding to the 67K isoform and their fragments and derivatives were used as an antigen to detect antibodies and T-cells reactive with GAD thereby forming a basis for a new range of diagnostics and therapeutics for diseases of the type including preclinical and clinical IDDM and SMS and other diseases in which GAD is an autoantigen.

Accordingly, a first aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence which encodes or is complementary to a sequence which encodes human or mouse glutamic acid decarboxylase (GAD) corresponding to a 67K isoform of the enzyme or antigenically active fragments or derivatives thereof.

By the "67K isoform" is meant the form of GAD having approximately M_r 67,000 and/or any fragments, derivatives, homologues and/or immunological relatives thereof and which are distinguishable and/or otherwise distinct from the M_r 65,000 form of GAD and which reacts preferentially to T-cells and/or

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autoantibodies from individuals with clinical or preclinical IDDM, SMS and/or other similar diseases.

Preferably, the GAD is human pancreatic islet GAD (HIGAD), human brain
5 cell GAD (HBGAD) and/or mouse brain cell GAD (MBGAD). Preferably,
the nucleic acid molecule is DNA, at least a part of which has a nucleotide
sequence substantially corresponding to the sequence shown in Figure 1, 3, 7
and/or 11 or a fragment, derivative, homologue or immunological relative
thereof or one or more sequences complementary thereto. The present
10 invention, however also extends to any single or multiple nucleotide
substitutions, deletions and/or additions to the sequence shown in Figure 1, 3,
7 and/or 11 and which still encode a GAD or fragment or derivative thereof
having the requisite antigenic profile and reactive with autoantibodies or T-
cells. Furthermore, when the nucleic acid molecule is RNA, the
15 ribonucleotide sequence will, in a preferred embodiment, be substantially
complementary to one or more of the sequences shown in Figures 1, 3, 7
and/or 11 or a fragment, derivative, or homologue thereof.

The present invention also provides a recombinant nucleic acid (e.g. DNA)
20 molecule comprising a nucleotide sequence as described above operably linked
to an expression control sequence. Such a recombinant molecule may, for
example, comprise an expression vector. The present invention further extends
to a host cell such as a bacterium, yeast, mammalian or insect cell transformed
with such a recombinant molecule. A preferred mammalian cell line is the
25 Chinese Hamster Ovary (CHO) cell line.

Another aspect of this invention is directed to a synthetic (e.g. recombinant)
peptide or polypeptide displaying the antigenicity of all or a portion of an
isoform of GAD which is reactive with autoantibodies and/or T-cells.

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- Such a synthetic peptide or polypeptide may, for example, be prepared by recombinant means such as by the expression of a host cell transformed with the recombinant molecules described above. The peptide or polypeptide may be fused to another peptide or polypeptide. Alternatively, it may be prepared by chemical synthesis, such as by the well-known Merrifield solid-phase synthesis procedure. The synthetic (eg. recombinant) peptide or polypeptide may or may not retain GAD enzymatic activity. Furthermore, although synthetic GAD or fragments thereof represent a preferred embodiment, the present invention also extends to biologically pure preparations of the naturally occurring enzyme or its fragments. By "biologically pure" is meant a preparation of at least 60%, preferably at least 70%, more preferably at least 80% and still more preferably at least 90% by weight enzyme.
- 15 In a most preferred embodiment, the present invention extends to naturally occurring or synthetic peptide or polypeptides corresponding to MBGAD, HIGAD and/or HBGAD and to nucleotide sequences coding for same as well as to fragments, derivatives, homologues or immunological relatives thereof. By way of example, such fragments are shown in Figures 2, 4, 5, 6, 8, 9 and 10.
- 20 By "derivatives" is meant to include any single or multiple amino acid substitution, deletion and/or addition relative to the naturally occurring sequence or to the sequence as shown in Figures 1, 3, 7 and/or 11 and including any single or multiple substitution, deletion and/or addition to other molecules associated with the peptide or polypeptide including carbohydrate
- 25 lipid and/or other proteinaceous moieties. Such derivatives, therefore, include glycosylated or non-glycosylated forms or molecules with altered glycosylation patterns.

The present invention also contemplates a method for the detection of autoantibodies associated with IDDM which method comprises contacting a

30 peptide or polypeptide corresponding to all, or an antigenic portion of, GAD, which GAD corresponds to the 67K isoform of the enzyme, or a fragment or

derivative thereof with a biological sample from a patient to be tested for a time and under conditions sufficient for a complex to form between the peptide or polypeptide and an antibody reactive to GAD and then detecting the complex. Preferably, the biological sample is serum. Even more
5 preferably, the peptide or polypeptide is immobilised onto a solid support before, during or after contact with the serum. Methods of detection are well known and include colorimetric, fluorometric and radioactive procedures. Other detection means can also be used such as involving agglutination. This assay can be varied in any number of ways without departing from the scope of
10 the present invention.

The present invention also extends to the use of a peptide or polypeptide corresponding to the 67K isoform of GAD, or antigenic fragments thereof, as an antigen in a diagnostic test for diseases of the type including IDDM and
15 SMS, or for screening asymptomatic individuals by detection or determination of the titre of antibodies in a patient's serum, for example using ELISA or RIA technology or an agglutination assay using antigen-coated beads or the like.

20 This aspect of the present invention may conveniently be carried out by the detection and/or determination of the titre of autoantibodies in a biological sample (e.g. serum) from a human subject, said method comprising contacting said sample with a peptide or polypeptide corresponding to an antigenic portion of the 67K isoform of GAD or a fragment or derivative thereof for a
25 time and under conditions sufficient for a complex to form between the peptide or polypeptide and an antibody reactive to GAD and then detecting the complex and/or amount of peptide or polypeptide which has been bound in the complex. Preferably, the peptide or polypeptide is immobilised onto a solid support before, during or after contact with the sample and the peptide
30 or polypeptide is as hereinbefore defined.

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- Alternatively, such diseases may be detected or at least a negative result re-confirmed or otherwise by screening for GAD associated immune complexes. It is possible, for example, that a negative autoantibody result could have been
- 5 caused by autoantibodies forming complexes with GAD thereby not being available for binding in the aforementioned assay. To conveniently detect GAD immune complexes, serum or other biological fluid is contacted with an anti-GAD antibody (e.g. a monoclonal antibody) for a time and under conditions sufficient for a GAD-autoantibody immune complex to bind.
- 10 Preferably, the anti-GAD antibody is first immobilised onto a solid support. An anti-immunoglobulin antibody, generally with a label or other reporter molecule attached, is then used to screen for the antibody component of the GAD complex.
- 15 One skilled in the art will immediately recognise that the assays as contemplated herein may be modified without departing from the scope of the present invention. All such modifications and variations of these assays are encompassed by the present invention.
- 20 The invention also extends to use of the peptides and/or polypeptides, or fragments, or derivatives of the present invention in the treatment of patients. In this later aspect, such methods of treatment include their use as an adsorbent to remove autoantibodies or autoreactive cells from a patient, their use in direct administration to a patient as a means of desensitising or inducing
- 25 tolerance to eliminate or diminish reactivity of autoreactive T-cells or autoantibodies to the IDDM autoantigen or to generate T-cell lines or clones to be used for or as therapeutic agents.
- As contemplated herein, the method of treatment includes but is not limited to
- 30 the following examples of treatment. A first example of treatment is desensitisation or tolerance induction using an effective amount of GAD peptide or polypeptide or fragments thereof to alter T-cell recognition of GAD

and induce T-cell suppression. This may be achieved by using the known effect of certain ultraviolet wavelengths, especially UV-B, to modify antigen presentation through the skin (see 19). Effective amounts of GAD peptide or polypeptide or fragments thereof would be applied epicutaneously to the skin of subjects exhibiting peripheral blood T-cell reactivity to GAD, after exposure of skin to UV-B radiation. Treatment would be repeated until such time that T-cell reactivity to GAD was suppressed. A second treatment involves application of GAD to the skin together with one or more cytokines such as but not limited to $\text{TNF}\alpha$ or β . A third treatment involves T-cell immunisation whereby T-cell lines are generated to GAD peptide or polypeptide or fragments thereof by standard procedures, cells attenuated by fixation with agents such as glutaraldehyde or paraformaldehyde, washed under sterile conditions and re-injected to patients for a time and under conditions causing suppression of the endogenous T-cell response to GAD. These approaches of treatment are applicable to the prevention of clinical IDDM in asymptomatic subjects with preclinical IDDM or subjects with recent onset clinical IDDM, as well as to the recurrence of IDDM in subjects who have received pancreas, islet cell or insulin-producing cell transplants. These approaches are also applicable to SMS and other diseases where GAD is an autoantigen. In accordance with the present invention the effective amount of GAD peptide or polypeptide is 0.1 μg to 10 mg per dose and preferably 1.0 μg to 1 mg per dose. A dose may comprise a single administration or an administration protocol. Administration may be by any convenient means such as, but not limited to, intravenous, subcutaneous, epicutaneous, infusion, oral, topical, intranasal, suppository or intraperitoneal administration. The GAD peptide or polypeptide may be administered alone or in combination with one or more other active molecules, molecules which facilitate the GAD peptide or polypeptide activity such as cytokines, and in particular, $\text{TNF-}\alpha$ and/or $\text{TNF-}\beta$.

In yet a further embodiment, the present invention contemplates the use of a peptide or polypeptide corresponding to the 67K isoform of GAD, or antigenic fragments or derivatives thereof, to measure reactivity of a patient's cells to the

10 IDDM autoantigen. The peptide or polypeptide, or fragments or derivatives thereof, may be added, in solution or bound to a solid support together with cells from a patient derived from peripheral blood or from tissue biopsies either unfractionated, fractionated or derived as a continuous cell line. Reactivity to the autoantigen may then be measured by standard proliferation assays such as incorporation of tritiated thymidine, standard cytotoxic assays such as release of marker radioactivity from target cells, measurements of expressed or secreted molecules such as cytokines or other standard assays of cellular reactivity which are well known in the art.

20 In one embodiment of this aspect of this invention there is provided a diagnostic kit for assaying patient T-cells. Standard 96 well plates, as used in ELISA assays, are pre-coated with a monoclonal antibody (MAb) to a T-cell cytokine such as γ -interferon (γ -IFN) with or without antigen. Alternatively, antigen is added in soluble form together with aliquots of peripheral blood mononuclear cells or T-cells. Incubation is allowed to proceed for two or more days, the cells are washed off, wells washed again and plates developed with a labelled second MAb to the cytokine such as anti- γ -IFN conjugated with alkaline phosphatase or horseradish peroxidase. Colorimetric reaction and read-out systems can then be utilised. Alternatively, it is possible to visualise microscopically individual spots on bottoms of wells representing cytokine produced at the single T-cell level,

thereby enabling the precursor frequency of antigen-reactive T-cells to be determined.

The present invention encompasses other forms of kits and diagnostic assays including a kit comprising a container adapted to contain a synthetic peptide or polypeptide corresponding to the 67K isoform of GAD or its fragments, derivatives, homologues and/or immunological relatives. The kit may contain a second container adapted to contain or receive a sample to be tested. A third container
10 may be presently adapted to contain reagents for detecting GAD-antibody complexes. Alternatively, where the kit is to detect GAD immune complexes, the kit may comprise one or more containers (e.g. wells)

adapted to contain a GAD specific antibody (e.g. a monoclonal antibody). Additional containers with the kit may then contain receptacles for receiving fluid samples and a labelled antibody.

- 5 In further accordance with the present invention, expression of the cDNA insert encoding the GAD's described herein or fragments thereof, may be achieved in a number of different ways.

As an example, successful expression of the autoantigen as a fusion protein can
10 be achieved using the pGEX vectors which give expression of glutathione S-transferase fusion proteins, using *E. coli* as the host cells. Expression could also be achieved, by way of example, using the well-known pEV vectors or the polyhistidine expression vectors (23) again using *E. coli* as the host cells. Alternatively, GAD may be expressed as a non-fused polypeptide, by using
15 appropriate vector and host cell combinations. Other vector and host cell combinations which can be used in accordance with the present invention including a number of well described yeast shuttle vectors for use in yeast cells, or eukaryotic vectors useful in continuous cell lines, (eg CHO cells) or transgenic animals.

20

The present invention will now be further described with reference to the following non-limiting Figures and Examples.

In the Figures:

25

Figure 1 shows a comparison of the 540 nucleotide DNA sequences corresponding to human brain GAD (HBGAD) and human islet GAD (HIGAD) excluding oligonucleotide sequences.

- 30 Figure 2 shows the deduced amino acid sequences of HBGAD and HIGAD and their alignment with the equivalent region in the feline GAD (amino acids 218-398).

Figure 3 shows the nucleotide sequence and deduced amino acid sequence corresponding to the full length mouse brain GAD (MBGAD).

Figure 4 shows the nucleotide sequence and deduced amino acid sequence corresponding to the N-terminal fragment of MBGAD designated MBGAD12 that encodes amino acids 1-204 of the published feline GAD sequence (10) .

Figure 5 shows the nucleotide sequence and deduced amino acid sequence corresponding to the mid-region fragment of MBGAD, designated MBGAD34 corresponding to amino acids 198-404 of the published feline GAD sequence.

Figure 6 shows the nucleotide sequence and deduced amino acid sequence corresponding to the C-terminal fragment of MBGAD, designated MBGAD56 corresponding to amino acids 392-593 of the published feline GAD sequence.

Figure 7 shows the full length nucleotide sequence and deduced amino acid sequence corresponding to human brain GAD (HBGAD-FL).

Figure 8 shows the nucleotide sequence and deduced amino acid sequence corresponding to the N-terminal fragment of HBGAD, designated HBGAD17 corresponding to amino acids 1-250 of the published feline GAD sequence .

Figure 9 shows the nucleotide sequence and deduced amino acid sequence corresponding to the mid region fragment of HBGAD or HIGAD, designated HBGAD14 or HIGAD14 corresponding to amino acids 208-404 of the published feline GAD sequence.

Figure 10 shows the nucleotide sequence and deduced amino acid sequence corresponding to the C-terminal region fragment of HBGAD, designated HBGAD65, corresponding to amino acid 392-594 of the published feline GAD sequence .

Figure 11 shows the full length nucleotide sequence and deduced amino acid sequence corresponding to human islet GAD (HIGAD-FL).

EXAMPLE 1

5

MATERIALS AND METHODS

Mouse RNA. Mouse RNA was obtained from brains of BALB/C mice.

Human RNA. RNA was obtained from human adult brain and pancreatic islets. Islets were isolated from a donor pancreas by an intraductal collagenase
10 distension procedure. Individually hand-picked islets were lysed in 5 M guanidinium isothiocyanate, 10 mM Tris pH 7.6, 10mM EDTA and RNA purified by centrifugation through a 5.7M CsCl cushion. Total RNA from human brain was a gift of Claude Bernard from Latrobe University School of Behavioural Science, Australia.

15

Human cDNA libraries. Two λ gt-11 based human cDNA expression libraries were used as a source of GAD cDNA. A brain-stem cDNA library was purchased from Clontech and the islet-cell library was a gift of Alan Permutt from the Washington School of Medicine, St. Louis. cDNA was prepared from
20 phage stocks by a plate lysis method (20).

Polymerase chain reaction (PCR). Based on the published rat (9) and feline (10) GAD cDNA sequences, oligonucleotide primers were designed from conserved regions. The primers used to isolate the various clones are shown in
25 Table 1. First strand synthesis of total RNA (1 μ g) was performed in 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 100 μ M dNTPs (PCR buffer) containing 2 pmole of complimentary primer, 40 U of RNasin and 5 U of MoMLV reverse transcriptase at 37°C for 30 min in a 50ul reaction volume. λ gt-11 cDNA (100ng) or 10 μ l of the first strand reaction was amplified in PCR
30 buffer containing 20 pmole of each primer and 2.5 U of TaqI polymerase by 30 thermal cycles (one cycle: 1.5 min at 95°C; 2.0 min at 37-45°C; 2.0 min at 72°C). Reactions were analysed on low melting agarose gels and products of

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the expected size purified by phenol extraction (20).

5 Cloning and DNA sequencing. PCR amplified DNA fragments were cloned into the plasmid expression vector pGEX 1-3(21) and also into the histidine expression vector pDS56,(-1) and (-2) (23). Nucleotide sequence was determined by the dideoxy chain termination method (22) using the M13 universal primer and specific primers designed from internal GAD sequence, as described in Table 1.

TABLE 1 : ORIGIN OF GAD cDNA CLONES

GAD CLONE	AA REGION EQUIVALENT TO FELINE GAD	PCR SOURCE	OLIGONUCLEOTIDES 5' - 3'
MBGAD 12	1 - 204	BRAIN RNA	RGAD1 - ATTGGATCCACCGAGCTGATGGCGTCTTC RGAD2 - CCGAATTCGCCATTCGCCAGCTAAACC
MBGAD34	198 - 404	BRAIN RNA	RGAD3 - ATTGGATCCGGTTTAGCTGGCGGAATGGC RGAD4 - CCGAATTCGTGTGAGGGTTCAGGTGAC
MBGAD56	392 - 593	BRAIN RNA	RGAD5 - ATTGGATCCGTCACCTGGAACCCCTCACA RGAD6 - CCGAATTCATTACAGATCCTGGCCCCA
HBGAD	208 - 404	BRAIN cDNA LIBRARY	GAD1 - ACTGCCAATACCAATATGTTACATATGA RGAD4 - CCGAATTCGTGTGAGGGTTCAGGTGAC
HIGAD	208 - 404	ISLET cDNA LIBRARY	GAD1 - ACTGCCAATACCAATATGTTACATATGA RGAD4 - CCGAATTCGTGTGAGGGTTCAGGTGAC
HBGAD17	1 - 250	BRAIN RNA	RGAD1 - ATTGGATCCACCGAGCTGATGGCGTCTTC GAD7 - GGAGAAAATATCCCATCACC
HBGAD14	208 - 404	BRAIN RNA	GAD1 - ACTGCCAATACCAATATGTTACATATGA RGAD4 - CCGAATTCGTGTGAGGGTTCAGGTGAC
HBGAD65	392 - 594	BRAIN RNA	GAD6 - ATTGGATCCGGCATAGAAAGGGCCAA GAD5 - CCCATAAATCATGTTCTTG
HBGAD-FL HIGAD-FL	1 - 594 1 - 594	BRAIN RNA PANCREAS RNA	RGAD1 - ATTGGATCCACCGAGCTGATGGCGTCTTC GAD5 - CCCATAAATCATGTTCTTG
HIGAD14	208 - 404	ISLET RNA	GAD1 - ACTGCCAATACCAATATGTTACATATGA RGAD4 - CCGAATTCGTGTGAGGGTTCAGGTGAC

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EXAMPLE 2

Cloning of Human GAD

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5 To clone human GAD cDNA, oligonucleotide pairs overlapping nucleotide stretches conserved between rat and feline sequences were synthesised and used in PCR reactions to amplify cDNA extracted from brain and islet λ gt-11 expression libraries as well as from RNA extracted from human brain or human islets. In extensive PCR reactions using various combinations of
10 oligonucleotide primers and temperatures of annealing, a product of 600 nucleotides was obtained from both brain and islet cDNA templates with the oligonucleotides primers:

5' ACTGCCAATACCAATATGTTTCACATATGA 3' and

5' CCGAATTCTGTAGAGGGTTCCAGGTGAC 3' (complementary,
15 contains an Eco RI site) which would correspond to nucleotide positions 739-768 and 1312-1330 of the published feline cDNA (10), respectively, representing the middle portion of the GAD open reading frame. The two 600 nucleotide PCR products were digested with EcoRI and SmaI ligated with pGEX-3X DNA cleaved with EcoRI and SmaI and transformed into E. coli.
20 Restriction analysis of plasmid DNA from transformants identified a human brain GAD clone (HBGAD) and an islet GAD clone (HIGAD).

The 540 nucleotide DNA sequences determined for both HBGAD and HIGAD, excluding the oligonucleotide sequences, are shown in Fig. 1. These
25 two sequences display 90% similarity with the feline GAD sequence and therefore, confirm the identity of the human clones. Alignment of the HBGAD sequence with the HIGAD sequence showed that they were identical except for four nucleotide changes at position 88 (T-A), 91(T-C) 128(C-T) and 366(C-T).

Figure 2 shows the deduced amino acid sequences of HBGAD and HIGAD and their alignment with the corresponding region in the feline GAD protein (aa 218-393). The four nucleotide differences between HBGAD and HIGAD would result in three conservative amino acid changes at residues 247 (leucine → isoleucine) and 260 (threonine → isoleucine) and 248 (phenylalanine → leucine); residue 339 (leucine) remains unchanged because the nucleotide difference at position 366 is silent. These amino acid differences between the middle one-third of the brain and islet GAD proteins provide evidence for the existence of isomeric forms of GAD in human tissue.

Infiltration of the pancreatic islets with mononuclear cells culminates in the destruction of insulin-producing β cells and clinical IDDM (20). The enzyme GAD has recently been identified as a putative islet autoantigen in IDDM based on the ability of several IDDM sera to co-precipitate the 64K islet cell protein and GAD (14) and it has been shown that peripheral blood T cells from subjects with pre-clinical and clinical IDDM can be activated by islet membrane preparations containing the 64K autoantigen and GAD (24, 25). The finding of sequence differences between brain and islet GAD may now provide a genetic basis for selective autoimmune destruction of pancreatic islets.

EXAMPLE 3

Construction of a Full Length Human Brain and Islet GAD cDNA

Normal brain RNA was reverse-transcribed with either GAD 5 (5' CCCATAAACTCATGTTCTTG 3') or GAD 7 (5' GGAGAAAATATCCCATCACC 3') oligonucleotides. As shown in Table 1, amplification of the GAD7 and GAD5 first strand products by PCR using GAD specific oligonucleotides generated a cDNA encoding aa 1-250 HBGAD17 and an overlapping cDNA that encodes aa 208-594. One hundred nanograms of each fragment was denatured at 95 °C in PCR buffer and hybrid

molecules extended and amplified using RGAD 1 and GAD 5 oligonucleotides that anneal at the end of the hybridised molecules (Table 1) to generate a full length human GAD clone that encodes the 594aa GAD open reading frame to generate a full length HBGAD and HIGAD (Figures 7 and 11).

EXAMPLE 4

Cloning of Mouse Brain GAD

Mouse Brain GAD was cloned as described above for HBGAD and HIGAD except that primers RGAD1 and RGAD6 (Table 1) were used.

10

EXAMPLE 5

T-Cell Responses to Recombinant Proteins

67 subjects were tested for their T-cell response to HBGAD and HIGAD.

Subject backgrounds were as follows:

- 15 Recent onset clinical Diabetics (less than 3 months after onset of symptoms)
- 44 Pre-clinical Diabetics (asymptomatic first degree relatives of a person with IDDM who are positive for islet cell antibodies that react with islets in frozen sections of human pancreas)
- 8 Controls (normal healthy young adults)

20

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll Hypaque* density gradient centrifugation, and washed twice. The cells were then resuspended

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(2×10^6 /ml) in complete culture medium (RPMI 1640 with Hepes buffer 20 mM, penicillin 100 units/ml, streptomycin 100 μ l/ml, 10^{-5} M 2-mercaptoethanol and 5% autologous serum) and seeded (200 μ l/well) into 96 well round-bottomed microtitre plates. The recombinant GAD fusion proteins HBGAD are HIGAD which contain the 196 amino acid middle portions of human brain and human islet GAD respectively, as described in Table 1 were

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added to final concentrations of 10, 1.0 and 0.1 $\mu\text{g/ml}$, together with and glutathione-S-transferase (GST) to which the recombinant GAD antigen is fused. Sonicated fetal pig islets, which the present inventors have shown to contain GAD (24), as well as fetal pig liver, thyroid and kidney were also used
5 as sources of antigen(s).

The cultures were incubated for 5 days in a humidified 5% CO_2 atmosphere with the addition of ^3H -thymidine (1 $\mu\text{Ci/well}$) for the last 17 hours. The cells were then harvested for scintillation counting. Median counts per minute
10 (cpm) of each quadruplicate were used to derive stimulation indices, ie. cpm with antigen/cpm without antigen. A positive result was defined as a stimulation index greater than that obtained with GST (recombinant GAD proteins or greater than 2.0 (fetal tissues).

15 TABLE 2

Reactivity of Peripheral Blood T-cells

	Antigens		
	H islet GAD14	H brain GAD14	Fetal pig proislets
20 Subject group			
Recent onset clinical diabetes	10/15	8/14	5/12
25 Preclinical diabetes	25/44	18/36	16/34
Controls	3/8	3/8	1/8

30

The results shown in Table 2 indicate that overall, 35/59 (59%) recent onset or preclinical subjects have circulating T-cells capable of proliferating in response to human islet GAD and (26/50) 52% to human brain GAD.

5

EXAMPLE 6

Antibody Responses to Recombinant Proteins

Sera samples from subjects were tested for an antibody response to the N-terminal fragment of recombinant murine brain GAD, MBGAD12 as well as against the full length recombinant human brain GAD.

Protein Used As Antigen

15 Recombinant mouse brain GAD12 was cloned and expressed as a fusion protein with glutathione-S-transferase (GST) in the pGEX system. MBGAD12 was cleaved with thrombin and the GAD portion affinity purified from GST using glutathione agarose beads. MBGAD34, MBGAD56, HBGAD17 and HBGAD65 were cloned and expressed as fusion proteins with six histidine
20 residues at the N-terminus using the polyhistidine expression system.

ELISAS

In all ELISA assays, the recombinant GAD proteins, were coated at 1 µg/mL on plastic wells of a 96-well plate, wells were exposed to blocking buffer, washed and incubated with doubling dilutions of test sera, washed and exposed to alkaline phosphatase - conjugated second antibody, washed, developed with n-nitrophenol chromogen and read at 405nm. An OD > mean +2 SD with control sera was taken as positive.

30

Subject Patients were as follows:-

The results of ELISA using MBGAD12, MBGAD34 and MBGAD56, and
5 HBGAD17 and HBGAD65 are shown in Tables 3 and 4, respectively:

TABLE 3

	MBGAD12	MBGAD34	MBGAD56
10			
Preclinical IDDM	5/9	5/9	4/9
Recent onset Clinical IDDM	2/13	4/13	3/13
Controls	0/22	0/20	0/20

15

Seven of nine (78%) preclinical IDDM and six of 13 (46%) recent-onset IDDM sera reacted with at least one of the MBGAD peptides. Only three of nine (33%) and one of 13 (8%) preclinical and recent onset IDDM sera, respectively, reacted with all three MBGAD fragments. None of the three
20 GAD peptides was recognised preferentially by either sera group. These findings indicate that patterns of sera reactivity with recombinant MBGAD are heterogenous and that at least three major epitopes exist in the GAD67 isoform.

TABLE 4

SUBJECTS	HBGAD 17	HBGAD65
Pre-clinical IDDM	7/9	3/9
Recent onset IDDM	3/7	3/7
Controls	0/16	0/16

10

The results using the two human brain GAD fragments HBGAD17 and HBGAD65 in an ELISA format are comparable with those obtained using the equivalent mouse brain GAD peptides MBGAD12 and MBGAD56.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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	10	20	30	40	50
HBGAD	ATTGCACCCGTGTTTGTTCATGGAACAGATTACTCTTAAGAAGATGAG				
HIGAD	ATTGCACCCGTGTTTGTTCATGGAACAGATTACTCTTAAGAAGATGAG				

	60	70	80	90	100
HBGAD	AAAGATCGTTGGATGGTCAAATAAAGATGGTGATGGGTTATTTTCTCCTG				
HIGAD	AAAGATCGTTGGATGGTCAAATAAAGATGGTGATGGGTTACTTTTCTCCTG				

	110	120	130	140	150
HBGAD	GGGGAGCCATATCCAATATGTACAGCACCATGGCTGCTCGTTACAAGTAC				
HIGAD	GGGGAGCCATATCCAATATGTACAGCATCATGGCTGCTCGTTACAAGTAC				

	160	170	180	190	200
HBGAD	TTCCCAGAAGTGAAGACAAAAGGCATGGCGGCTGTGCCCCAAACTGGTCCT				
HIGAD	TTCCCAGAAGTGAAGACAAAAGGCATGGCGGCTGTGCCCCAAACTGGTCCT				

	210	220	230	240	250
HBGAD	CTTCACCTCAGAACACAGTCACTATTCCATAAAGAAAGCCGGGGCTGCGC				
HIGAD	CTTCACCTCAGAACACAGTCACTATTCCATAAAGAAAGCCGGGGCTGCGC				

	260	270	280	290	300
HBGAD	TTGGCTTTGGAACCGACAAATGTGATTTTGATAAAGTGCAATGAAGGGGG				
HIGAD	TTGGCTTTGGAACCGACAAATGTGATTTTGATAAAGTGCAATGAAGGGGG				

Fig.1.

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Fig.1.
(cont.)

	310	320	330	340	350
HBGAD	AAGATAATTCCGGCTGATTTAGAGGCCAAAAATTCTTGATGCCAAACAAAA				
HIGAD	AAGATAATTCCGGCTGATTTAGAGGCCAAAAATTCTTGATGCCAAACAAAA				
	360	370	380	390	400
HBGAD	GGGCTATGTTCCCTCTATGTCAATGCAACCGCAGGCACGACTGTTTACG				
HIGAD	GGGCTATGTTCCCTTTATGTCAATGCAACCGCAGGCACGACTGTTTACG				
	410	420	430	440	450
HBGAD	GAGCATTGATCCCAATCCAGGAAATTGCGGACATATGTGAGAAATACAAAC				
HIGAD	GAGCATTGATCCCAATCCAGGAAATTGCGGACATATGTGAGAAATACAAAC				
	460	470	480	490	500
HBGAD	CTTTGGCTGCATGTGGATGCTGCCCTGGGGTGGTGGACTGCTCATGTCCCCG				
HIGAD	CTTTGGCTGCATGTGGATGCTGCCCTGGGGTGGTGGACTGCTCATGTCCCCG				
	510	520	530	540	550
HBGAD	GAAGCACCGCCACAAACTCAGCGGCATAGAAAGGGCCCAATTCA				
HIGAD	GAAGCACCGCCACAAACTCAGCGGCATAGAAAGGGCCCAATTCA				

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HBGAD IAPVFLMEQITLKKMRKIVGWSNKDGDGLFSPGG^{*}AINMYSTMAARYKY^{*}
HIGAD IAPVFLMEQITLKKMRKIVGWSNKDGDGILSPGGAINMYSIMAARYKY
FBGAD IAPVFLMEQITLKKMREIVGWSSKDGDGIFSPGGAINMYSIMAARYKF

HBGAD FPEVKTGMAAVPKLVLTSEHSHYSIKKAGAA LGFGTDNVILIKCNERG
HIGAD FPEVKTGMAAVPKLVLTSEHSHYSIKKAGAA LGFGTDNVILIKCNERG
FBGAD FPEVKTGMAAVPKLVLTSEHSHYSIKKAGAA LGFGTDNVILIKCNERG

HBGAD KIIIPADLEAKILDAKQKGYVPLYVNATAGTTVYGAFDPIQEIADICEKYN
HIGAD KIIIPADLEAKILDAKQKGYVPLYVNATAGTTVYGAFDPIQEIADICEKYN
FBGAD KIIIPADLEAKILEAKQKGYVPLYVNATAGTTVYGAFDPIQEIADICEKYN

HBGAD LWLHVDAAWGGGLMSRKHRHKLSGIERANS
HIGAD LWLHVDAAWGGGLMSRKHRHKLSGIERANS
FBGAD LWLHVDAAWGGGLMSRKHRHKLSGIERANS

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Fig.2.

• aa differences between HBGAD and HIGAD

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M A S S T P S P A T S S N A G A D P N T
ATGGCGTCTTCCACTCCTTGGCCTGCAACCTCCTCGAACGCGGAGCGGATCCTAATACT 60
T N L R P T T Y D T W C G V A H G C T R
ACCAACCTGCGCCCTACAACTATGATACCTTGGTGGCGTAGCCCATGGATGCACCAGA 120
K L G L K I C G F L Q R T N S L E E K S
AACTGGGCCCTGAAGATCTGTGGCTTCTTACAAGGACC AATAGCCTGGAAGAGAAGAGT 180
R L V S A F R E R Q S S K N L L S C E N
CGTCTTGTGAGCGCCTTCAGGGAGAGGCGAGTCTCCAGAACCCTGCTTTCCTGTGAAAC 240
S D Q G A R F R R T E T D F S N L F A Q
AGTGACCAGGTGCCCCGCTTCCGGCGCACAGAGACCGACTTCTCCAACCCTGTTTGCTCAA 300
D L L P A K N G E E Q T A Q F L L E V V
GATCTGCTTCCAGCTAAGAACGGGAGGAGCAAACTGCGCAGTCTTGTGCTGGAAGTGGTA 360
D I L L N Y V R K T F D R S T K V L D F
GACATACTCCTCAACTATGTCCGCAAGACATTGTGATCGCTCCACCAAGGTTCTGGATTTC 420
H H P H Q L L E G M E G F N L E L S D H
CACCACCCACACAGTTGCTGGAAGGCATGGAAGGCTTTAATTGGAGCTGTCTGACCAC 480
P E S L E Q I L V D C R D T L K Y G V R
CCCGAGTCTCTGGAGCAGATCCTGGTTGACTGTAGAGACACCCTGAAGTACGGGGTTCGC 540
T G H P R F F N Q L S T G L D I I G L A
ACAGGTCACCCCTCGATTTTTCAACCAGCTCTCTACTGGTTGGATATCATTTGGTTAGCT 600
G E W L T S T A N T N M F T Y E I A P V
GGCGAATGGCTGACATCGACTGCCAATACCAATATGTTCAATATGAAATTGCACCCGTG 660
F V L M E Q I T L K K M R K I V G W S N
TTTGTCTCATGGAACAGATTACTCTTAAGAAGATGAGAAAGATCGTTGGATGGTCAAAT 720

Fig.3.

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K D G D G I F S P G G A I S N M Y S I M	780
AAAGATGGTGGGATATTTTCTCTGGGGAGCCATATCCAATATGTACAGCATCATG	
A A R Y K Y F P E V K T K G M A A V P K	
GCTGCTCGTTACAAGTACTTCCAGAGTGAAGACAAAAGGCATGGCGGCTGTGCCCCAAA	840
L V L F T S E H S H Y S I K K A G A A L	
CTGGTCCTCTCACCTCAGAACACAGTCACTATTCCATAAAGAAAGCCGGGGCTGCGCTT	900
G F G T D N V I L I K C N E R G K I I P	
GGCTTTGGAACCGACAATGTGATTTTGATAAAGTGCAATGAAGGGGAAGATAATTCCG	960
A D L E A K I L D A K Q K G Y V P L Y V	
GCTGATTTAGAGGCAAAAATTCTTGATGCCAAACAAAGGGCTATGTTCCTTTATGTC	1020
N A T A G T T V Y G A F D P I Q E I A D	
AATGCAACCGCAGGCACGACTGTTTACGGAGCATTCGATCCCAATCCAGGAAATTGCGGAC	1080
I C E K Y N L W L H V D A A W G G L L	
ATATGTGAGAAATACAACTTTTGGCTGCTGATGTGGATGCTGCTGGGTGGTGGACTGCTC	1140
M S R K H R H K L S G I E R A N S V T W	
ATGTCCCGGAAGCACCGCCACAACTCAGCGGCATAGAAAGGCCCAATTCAGTCACCTGG	1200
N P H K M M G V L L Q C S A I L V K E K	
AACCTCACAAGATGATGGGCGTGCTGCTCCAGTGCTCTGCCATTCTGGTCAAGGAAAAG	1260
G I L Q G C N Q M C A G Y L F Q P D K Q	
GGTACTCCAAGGATGCAACCAGATGTGTGCAGGCTACCTCTTCCAGCCAGACAAGCAG	1320
Y D V S Y D T G D K A I Q C G R H V D I	
TATGACGTCTCCTATGACACCGGGGACAAGGCGATTTCAGTGTGGCCCATGTGGACATC	1380
F K F W L M W K A K G T V G F E N Q I N	
TTCAAGTTCTGGCTGATGTGGAAAGCAAGGGCACCGTGGGATTTGAAACCAGATCAAC	1440

Fig.3.
(cont.)

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K C L E L A D Y L Y A K I K N R E E F E	
AAATGCCCTGGAGCTGGCTGATTACCTCTACGCCAAGATTAAACACAGAGAAGAGTTTGAG	1500
M V F D G E P E H T N V C F W Y I P Q S	
ATGGTTTTCGATGGTGAGCCTGAGCACACAATGTCTGTTTCTGGTACATTCCACAAAGC	1560
L R G V P D S P E R E K L H R V A P K	
CTTAGAGGGGTTCCAGATAGCCCTGAGCGACGAGAAAGCTACACAGGGTGGCTCCCAAG	1620
I K A L M M E S G T T M V G Y Q P Q G D	
ATCAAAGCTCTGATGATGGAGTCAGGAACAACCATGGTCGGCTACCAGCCTCAAGGGGAC	1680
K A N F F R M V I S N P A A T Q S D I D	
AAGGCCAACTTCTTCGGATGGTTCATCTCTAACCCAGCCGCCACCCAGTCTGACATCGAT	1740
F L I E E I E R L G Q D L *	
TTCCTCATTGAGGAGATAGAGAGGTTGGGCCAGGATCTGTAA	1782

Fig.3.

(cont.)

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M A S S T P S P A T S S N A G A D P N T
ATGGCGTCTTCCACTCCTTCGCCCTGCAACCTCCTCGAACGCGGAGCGGATCCTAATACT 60
T N L R P T T Y D T W C G V A H G C T R
ACCAACCTGCGCCCTACAACGTATGATACTTGGTGGCGTAGCCCATGGATGCCACAGA 120
K L G L K I C G F L Q R T N S L E E K S
AAACTGGGCCCTGAAGATCTGTGGCTTCTTACAAGGACCAATAGCCTGGAAGAGAAGAGT 180
R L V S A F R E R Q S S K N L L S C E N
CGTCTTGTGAGCGCCTTCAGGGAGAGGCAGTCCTCCAAGAACCTGCTTTCCTGTGAAAAC 240
S D Q G A R F R R T E T D F S N L F A Q
AGTGACCAGGGTGCCCGCTTCCGGCGCACAGAGACCGACTTCTCCAACCTGTTTGCTCAA 300
D L L P A K N G E E Q T A Q F L L E V V
GATCTGCTTCCAGCTAAGAACGGGAGGAGCAAACTGCGCAGTTCTTGCTGGAAGTGTA 360
D I L L N Y V R K T F D R S T K V L D F
GACATACTCCTCAACTATGTCCGCAAGACATTTGATCGCTCCACCAAGGTTCTGGATTTC 420
H H P H Q L L E G M E G F N L E L S D H
CACCACCCACACCAGTTGCTGGAAGGCATGGAAGGCTTTAATTGGAGCTGTCTGACCAC 480
P E S L E Q I L V D C R D T L K Y G V R
CCCGAGTCTCTGGAGCAGATCCTGGTTGACTGTAGAGACACCCTGAAGTACGGGGTTCGC 540
T G H P R F F N Q L S T G L D I I G L A
ACAGGTCACCCCTCGATTTTTCAACCAGCTCTCTACTGGTTTGGATATCATTTGTTAGCT 600
G E W
GGCGAATGG 609

Fig.4.

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G L A G E W L T S T A N T N M F T Y E I 651
GGTTTAGCTGGCGAATGGCTGACATCGACTGCCAATACCAATATGTTACATATGAAATT
A P V F V L M E Q I T L K K M R K I V G
GCACCCGTGTTTCTCATGGAACAGATTACTCTTAAGAAGATGAGAAAGATCGTTGGA 711
W S N K D G D G I F S P G G A I S N M Y
TGGTCAAATAAAGATGGTGATGGGATATTTCTCTGGGGAGCCATATCCAATATGTAC 771
S I M A A R Y K Y F P E V K T K G M A A
AGCATCATGGCTGCTCGTTACAAGTACTTCCAGAAAGTGAAGACAAAAGGCATGGCGGCT 831
V P K L V L F T S E H S H Y S I K K A G
GTGCCAAACTGGTCCTCTTCACCTCAGAACACAGTCACTATTCCATAAGAAAGCCGGG 891
A A L G F G T D N V I L I K C N E R G K
GCTGCGCTTGGCTTTGGAAACCGACAATGTGATTTTGATAAAGTGCAATGAAAGGGGAAG 951
I I P A D L E A K I L D A K Q K G Y V P
ATAATTCCGGCTGATTTAGAGGCCAAAATTTCTTGATGCCAAACAAAGGGCTATGTTCCT 1011
L Y V N A T A G T T V Y G A F D P I Q E
CTTTATGTCAATGCAACCGCAGGCACGACTGTTTACGGAGCATTCGATCCCAATCCAGGAA 1071
I A D I C E K Y N L W L H V D A A W G G
ATTGCGGACATATGTGAGAAATACAACCTTTGGCTGCATGTGGATGCTGCTGGGTGGT 1131
G L L M S R K H R K L S G I E R A N S
GGACTGCTCATGTCCCGGAAGCACCGCCACAACTCAGCGGCATAGAAAGGCCCAATTCA 1191
V T W N P H
GTCACCTGGAACCTCAC 1209

Fig.5.

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G I E R A N S V T W N P H K M M G V L L 1230
GGCATAGAAAGGGCCAATTCACTGGAACCTCACAAAGATGATGGCGTGCTGCTC 1230
Q C S A I L V K E K G I L Q G C N Q M C
CAGTGCTCTGCCATTCTGGTCAAGGAAAGGGTATACTCCAAGGATGCAACCAGATGTGT 1290
A G Y L F Q P D K Q Y D V S Y D T G D K
GCAGGCTACCTCTTCCAGCCAGACAAGCAGTATGACGTCTCCTATGACACCGGGGACAAG 1350
A I Q C G R H V D I F K F W L M W K A K
GCGATTCAGTGTGGCCGCCATGTGGACATCTTCAAGTTCTGGCTGATGTGGAAGCAAAG 1410
G T V G F E N Q I N K C L E L A D Y L Y
GGCACCGTGGGATTTGAAACCAAGATCAACAAATGCCTGGAGCTGGCTGATTACCTCTAC 1470
A K I K N R E E F E M V F D G E P E H T
GCCAAGATTAAACAGAGAAGAGTTTGAGATGGTTCGATGGTGAGCCTGAGCACACA 1530
N V C F W Y I P Q S L R G V P D S P E R
AATGTCTGTTTCTGGTACATTCCACAAAGCCTTAGAGGGGTTCCAGATAGCCCTGAGCGA 1590
R E K L H R V A P K I K A L M M E S G T
CGAGAAAGCTACACAGGGTGGCTCCCAGATCAAAGCTCTGATGATGGAGTCAGGAACA 1650
T M V G Y Q P Q G D K A N F F R M V I S
ACCATGGTCGGCTACCGCCTCAAGGGGACAAGGCCAACTTCTCCGGATGGTCATCTCT 1710
N P A A T Q S D I D F L I E E I E R L G
AACCAGCCGCCACCCAGTCTGACATCGATTTCCTCATTTGAGGAGATAGAGAGGTTGGGC 1770
Q D L *
CAGGATCTGTAA 1782

Fig.6.

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M A S S T P S S A T S S N A G A D P N
ATGGCGTCTTCGACCCCATCTTCGTCCGCAACCTCCTCGAACGCGGAGCGGACCCCAAT 60
T T N L R P T T Y D T W C G V A H G C T
ACCACTAACCTGCGCCCCACAACGTACGATACCTGGTGGCGGTGCCCATGGATGCACC 120
R K L G L K I C G F L Q R T N S L E E K
AGAAACTGGGGCTCAAGATCTGCGGCTTCTTGCAAGGACCAACAGCCTGGAAGAGAAG 180
S R L V S A F K E R Q S S K N L L S C E
AGTCGCCCTTGTGAGTGCCCTTCAAGGAGGCAATCCTCCAAGAACCTGCTTTCCTGTGAA 240
N S D R D A R F R R T E T D F S N L F A
AACAGCGACCGGATGCCCGCTTCCGGCGACAGAGACTGACTTCTCTAATCTGTTGCT 300
R D L L P A K N G E E Q T V Q F L L E V
AGAGATCTGCTTCCGGCTAAGAACGGTGAGGAGCAACCGTGCAATTCTCTCTGGAAGTG 360
V D I L L N Y V R K T F D R S T K V L D
GTGGACATACTCCTCAACTATGTCCGCAAGACATTTGATCGCTCCACCAAGGTGCTGGAC 420
F H H P H Q L L E G M E G F N L E L S D
TTTCATCACCCACACCAGTTGCTGGAAGGCATGGAGGCTTCAACTTGGAGCTCTCTGAC 480
H P E S L E Q I L V D C R D T L K Y G V
CACCCCGAGTCCCTGGAGCAGATCCTGGTCGACTGCAGAGACACCTTGAAGTATGGGTT 540
R T G H P R F F N Q L S T G L D I I G L
CGCACAGGTCACTCGATTTTTC AACCCAGCTCTCCACTGGATTGGATATTATTGGCCTA 600
A G E W L T S T A N T N M F T Y E I A P
GCTGGAGAATGGCTGACATCAACGGCCAATACCAACATGTTACATATGAAATTGCACCA 660
V F V L M E Q I T L L K K M R E I V G W S
GTGTTGTCTCATGGAAACAAATAACACTTAAGAAGATGAGAGAGATAGTTGGATGGTCA 720

Fig.7

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Fig. 7.

(cont.)

S	K	D	G	D	G	I	F	S	P	G	G	A	I	S	N	M	Y	S	I
AGTAAAGATGGTGATGGGATAATTTCTCCTGGGGCGCCATATCCAACATGTACAGCATC 780																			
M	A	A	R	Y	K	Y	F	P	E	V	K	T	K	G	M	A	A	V	P
ATGGCTGCTCGCTACAAGTACTTCCC GG AAGTTAAGACAAAGGCCATGGGGCTGTGCCT 840																			
K	L	V	L	F	T	S	E	Q	S	H	Y	S	I	K	K	A	G	A	A
AAACTGGTCCTCTTCACCTCAGAACAGAGTCACTATTCCATAAAGAAAGCTGGGGCTGCA 900																			
L	G	F	G	T	D	N	V	I	L	I	K	C	N	E	R	G	K	I	I
CTTGGCTTTGGAACTGACAAATGTGATTTTGATAAAGTGCAATGAAGGGGAAAATAATT 960																			
P	A	D	F	E	A	K	I	L	E	A	K	Q	K	G	Y	V	P	F	Y
CCAGCTGATTTTGAGGCCAAAAATTCTTGAAGCCAAACAGAAGGATATGTTCCCTTTTAT 1020																			
V	N	A	T	A	G	T	T	V	Y	G	A	F	D	P	I	Q	E	I	A
GTCAATGCAACTGCTGGCACGACTGTTTTATGGAGCTTTTGTATCCGATACAAGAGATTGCA 1080																			
D	I	C	E	K	Y	N	L	W	L	H	V	D	A	A	W	G	G	G	L
GATATATGTGAGAAATATAACCCTTTGGTTGTCATGTCTGCTGCTGGGAGGTGGGCTG 1140																			
L	M	S	R	K	H	R	H	K	L	N	G	I	E	R	A	N	S	V	T
CTCATGTCCAGGAAGCACCGCCATAAACTCAACGGCATAGAAAGGCCAACTCAGTCACC 1200																			
W	N	P	H	K	M	M	G	V	L	L	Q	C	S	A	I	L	V	K	E
TGGAACCC TCACAAGATGATGGGCGTGCTGTTGTCAGTGTCTTGCCATTCTCGTCAA GGAA 1260																			
K	G	I	L	Q	G	C	N	Q	M	C	A	G	Y	L	F	Q	P	D	K
AAGGGTATACTCC AAGGATGC AAC CAGATGTGTGCAGGATACCTCTTCCAGCCAGACAAG 1320																			
Q	Y	D	V	S	Y	D	T	G	D	K	A	I	Q	C	G	R	H	V	D
CAGTATGATGTCTCTACGACACCGGGGACAAGGCAATTCAGTGTGGCCGCCACGTGGAT 1380																			
I	F	K	F	W	L	M	W	K	A	K	G	T	V	G	F	E	N	Q	I
ATCTTCAAGTTCTGGCTGATGTGGAAAGCAAGGCACAGTGGGATTTGAA AAC CAGATC 1440																			

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N K C L E L A E Y L Y A K I K N R E E F
AACAAATGCCCTGGAACTGGCTGAATACCTCTATGCTCAAGATTAAAAACAGAGAAGAAATTT 1500
E M V F N G E P E H T N V C F W Y I P Q
GAGATGGTTTCAATGGCGAGCCTGAGCACACAACGCTCTGTTTGTGATATTCACCAA 1560
S L R G V P D S P Q R R E K L H K V A P
AGCCTCAGGGGTGTGCCAGACAGCCCTCAACGACGGGAAAGCTACACAAGGTGGCTCCA 1620
K I K A L M M E S G T T M V G Y Q P Q G
AAAATCAAAGCCCTGATGATGGAGTCAGGTACGACCATGGTTGGCTACCAAGCCCCAAGGG 1680
D K A N F F R M V I S N P A A T Q S D I
GACAAGGCCAACTTCTTCCGGATGGTTCATCTCCAACCCAGCCGCTACCCAGTCTGACATT 1740
D F L I E E I E R L G Q D L *
GACTTCTCATTGAGGAGATAGAAAGACTGGGCCAGGATCTGTAA 1785

Fig.7.
(cont.)

M A S S T P S S A T S S N A G A D P N
ATGGCGTCTTCGACCCCATCTTCGTCCGCAACCTCCTCGAACGGGAGCGGACCCCAAT 60
T T N L R P T T Y D T W C G V A H G C T
ACCACTAACCTGCGCCCCACAACTGACGATACCTGGTGGCGGTGCCCATGGATGCACC 120
R K L G L K I C G F L Q R T N S L E E K
AGAAACTGGGGCTCAAGATCTGCGGCTTCTTGCAAGGACCAACAGCCTGGAAGAGAAG 180
S R L V S A F K E R Q S S K N L L S C E
AGTCGCCCTTGAGTGCCCTTCAAGGAGAGGCAATCCTCCAAGAACCTGCTTCTCTGTGAA 240
N S D R D A R F R T E T D F S N L F A
AACAGCGACCGGATGCCCGCTTCCGGCGCACAGAGACTGACTTCTCTAATCTGTTGCT 300
R D L L P A K N G E E Q T V Q F L L E V
AGAGATCTGCTTCCGGCTAAGAACGGTGAGGAGCAACCGTGCAATTCCTCCTGGAAGTG 360
V D I L L N Y V R K T F D R S T K V L D
GTGGACATACTCCTCAACTATGTCCGCAAGACATTTGATCGCTCCACCAAGGTGCTGGAC 420
F H H P H Q L L E G M E G F N L E L S D
TTTCATCACCCACACCAGTTGCTGGAAGGCATGGAGGCTTCAACTTGGAGCTCTCTGAC 480
H P E S L E Q I L V D C R D T L K Y G V
CACCCCGAGTCCCTGGAGCAGATCCTGGTCGACTGCAGAGACACCTTGAAGTATGGGTT 540
R T G H P R F F N Q L S T G L D I I G L
CGCACAGGTCACTCGATTTTTCAACCAGCTCTCCACTGGATTGGATATTATTGGCCTA 600
A G E W L T S T A N T N M F T Y E I A P
GCTGGAGAAATGGCTGACATCAACGGCCAATACCAACATGTTACATATGAAATGCAACCA 660
V F V L M E Q I T L K K M R E I V G W S
GTGTTTGTCTCATGGAAACAAATAACACTTAAGAAGATGAGAGAGATAGTTGGATGGTCA 720
S K D G D G I F S P
AGTAAAGATGGTGATGGGATATTCTCTCCT 750

Fig.8.

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Fig.9.

T A N T N M F T Y E I A P V F V L M E Q 681
ACGGCCAATACCAACATGTTACATATGAATTCACAGTGTTCCTCATGGAACAA
I T L K K M R E I V G W S S K D G D G I
ATAACACTTAAGAAGATGAGAGAGATAGTTGGATGGTCAAGTAAAGATGGTGATGGGATA 741
F S P G G A I S N M Y S I M A A R Y K Y
TTTTCTCCTGGGGGCCCATATCCAACATGTACAGCATCATGGCTGCTCGCTACAAGTAC 801
F P E V K T K G M A A V P K L V L F T S
TTCCCGGAAGTTAAGACAAAGGGCATGGCGCTGTGCTTAAACTGGTCTCTTCACCTCA 861
E Q S H Y S I K A G A A L G F G T D N
GAACAGAGTCACTATTCCATAAAGAAAGCTGGGGCTGCACTTGGCTTTGGAACTGACAAT 921
V I L I K C N E R G K I I P A D F E A K
GTGATTTTGATAAAGTGCAATGAAGGGGAAATAATTCAGCTGATTTTGAGGCAAAA 981
I L E A K Q K G Y V P F Y V N A T A G T
ATTCTTGAAGCCAAACAGAGGGGATATGTTCCCTTTTATGTCAATGCAACTGCTGGCAG 1041
T V Y G A F D P I Q E I A D I C E K Y N
ACTGTTTATGGAGCTTTTGTATCCGATACAAGAGATTGCAGATATATGTGAGAAATATAAC 1101
L W L H V D A A W G G L L M S R K H R
CTTTGGTTGTCATGTCGATGCTGCCCTGGGGAGGTGGGCTGCTCATGTCCAGGAAGCACCGC 1161
H K L N G I E R A N S V T W N P H
CATAAACTCAACGGCATAGAAAGGCCAACTCAGTCACCTGGAACCTTCAC 1212

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G I E R A N S V T W N P H K M M G V L L
GGCATAGAAAGGCCAACTCAGTCACCTGGAAACCCTCACAAGATGATGGGCGTGCTGTG 1233

Q C S A I L V K E K G I L Q G C N Q M C
CAGTGCTCTGCCATTCTCGTCAAGGAAAGGGTATACTCCAAGGATGCCAACAGATGTGT 1293

A G Y L F Q P D K Q Y D V S Y D T G D K
GCAGGATACCTCTTCCAGCCAGACAAGCAGTATGATGTCTCTACGACACCGGGGACAAG 1353

A I Q C G R H V D I F K F W L M W K A K
GCAATTCAGTGTGGCCGCCACGTGGATATCTTCAAGTTCTGGCTGATGTGGAAAGCAAAG 1413

G T V G F E N Q I N K C L E L A E Y L Y
GGCACAGTGGGATTTGAAACACAGATCAACAAATGCCCTGGAACCTGGCTGAATACCTCTAT 1473

A K I K N R E E F E M V F N G E P E H T
GCCAAGATTAAACAGAGAAGAAATTTGAGATGGTTTCAATGGCGAGCCTGAGCACACA 1533

N V C F W Y I P Q S L R G V P D S P Q R
AACGTCTGTTTTTGGTATATTCCACAAGCCTCAGGGGTGTGCCAGACAGCCCTCAACGA 1593

R E K L H K V A P K I K A L M M E S G T
CGGGAAAGCTACACAAGGTGGCTCCAAAAATCAAAGCCCTGATGATGGAGTCAGGTACG 1653

T M V G Y Q P Q G D K A N F F R M V I S
ACCATGGTTGGCTACCAGCCCCCAAGGGACAAAGCCAACTTCTCCGGATGGTCTCTCC 1713

N P A A T Q S D I D F L I E E I E R L G
AACCAGCCGCTACCCAGTCTGACATTGACTTCTCATTTGAGGAGATAGAAAGACTGGGC 1773

Q D L *
CAGGATCTGTAA 1785

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Fig.10.

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M A S S T P S S A T S S N A G A D P N
ATGGCGTCTTCGACCCCATCTTCGTCGCAACCTCCTCGAACGGGAGCGGACCCCAAT 60
T T N L R P T T Y D T W C G V A H G C T
ACCACTAACCTGCGCCCCACAACTGATACCTGGTGCGGCGTGCCACGGATGCACC 120
R K L G L K I C G F L Q R T N S L E E K
AGAAACTGGGGCTCAAGATCTGCGGCTTCTTGCAAGGACCAACAGCCTGGAAGAGAAG 180
S R L V S A F K E R Q S S K N L L S C E
AGTCGCCCTTGAGTGCCCTTCAAGGAGAGGCAATCCTCCAAGAACCTGCTTTCCTGTGAA 240
N S D R D A R F R R T E T D F S N L F A
AACAGCGACCGGATGCCCGCTTCCGGCGCACAGAGACTGACTTCTCTAATCTGTTGCT 300
R D L L P A K N G E E Q T V Q F L L E V
AGAGATCTGCTTCCGGCTAAGAACGGTGAGGAGCAACCGTGCAATTCCTCCTGGAAGTG 360
V D I L L N Y V R K T F D R S T K V L D
GTGGACATACTCCTCAACTATGTCCGCAAGACATTTGATCGCTCCACCAAGGTGCTGGAC 420
F H H P H Q L L E G M E G F N L E L S D
TTTCATCACCCACACAGTTGCTGGAAGGCATGGAGGGCTTCAACTTGGAGCTCTCTGAC 480
H P E S L E Q I L V D C R D T L K Y G V
CACCCCGAGTCCCCTGGAGCAGATCCTGGTCGACTGCAGAGACACCTTGAAGTATGGGTT 540
R T G H P R F F N Q L S T G L D I I G L
CGCACAGGTCATCCTCGATTTTTCAACCAGCTCTCCACTGGATTGGATATTATTGGCCTA 600
A G E W L T S T A N T N M F T Y E I A P
GCTGGAGAATGGCTGACATCAACGGCCAATACCAATGTTTCACATATGAAATTGCACCA 660
V F V L M E Q I T L K K M R E I V G W S
GTGTTGTCCTCATGGAAACAAATAACACTTAAGAAAGATGAGAGATAGTTGGATGGTCA 720

Fig.11.

**Fig.11.
(cont.)**

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N K C L E L A E Y L Y A K I K N R E E F
AACAAATGCCCTGGAACTGGCTGAATACCTCTATGCCCAAGATTAAACAGAGAAGAAATTT 1500

E M V F N G E P E H T S V C F W Y I P Q
GAGATGGTTTTCAATGGCGAGCCTGAGCACACAAAGCGTCTGTTTTTGGTATATTCACAA 1560

S L R G V P D S P Q R R E K L H K V A P
AGCCTCAGGGGTGTGCCAGACAGCCCTCAACGACGGGAAAGCTACACAAGGTGGCTCCA 1620

K I K A L M M E S G T T M V G Y Q P Q G
AAATCAAAGCCCTGATGATGGAGTCAAGTACGACCATGGTTGGCTACCAAGCCCAAGGG 1680

D K A N F F R M V I S N P A A T Q S D I
GACAAGGCCAACTTCTTCCGGATGGTCACTCTCCAACCCAGCCGCTACCCAGTCTGACATT 1740

D F L I E E I E R L G Q D L *
GACTTCCTCATTGAGGAGATAGAAAGACTGGGCCAGGATCTGTAA 1785
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Fig.11.
(cont.)