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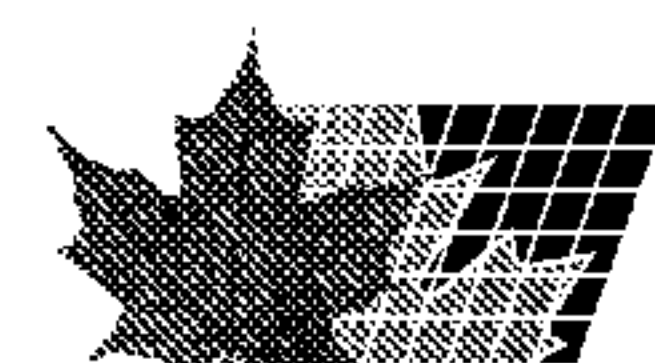
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(54) Title: CLONED GLUTAMIC ACID DECARBOXYLASE

(57) **Abrégé/Abstract:**

cDNA molecules coding for GAD₆₅ polypeptide. The invention provides cDNA molecules comprising a part of the cDNA sequence of GAD₆₅ which encode at least one epitope for autoantibodies to GAD₆₅. The invention also provides cloning vehicles capable of replication and expression comprising cDNA molecules coding for GAD₆₅. The invention further provides for hosts transformed with a vehicle having a cDNA molecule coding for GAD₆₅. In another embodiment, the invention provides for the detection of autoantibodies to GAD₆₅ using the GAD₆₅ polypeptides coded for by the cDNA molecules of the invention.



ABSTRACT OF THE DISCLOSURE

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cDNA molecules coding for GAD₆₅ polypeptide. The invention provides cDNA molecules comprising a part of the cDNA sequence of GAD₆₅ which encode at least one epitope for autoantibodies to GAD₆₅. The invention also provides cloning vehicles capable of replication and expression comprising cDNA molecules coding for GAD₆₅. The invention further provides for hosts transformed with a vehicle having a cDNA molecule coding for GAD₆₅. In another embodiment, the invention provides for the detection of autoantibodies to GAD₆₅ using the GAD₆₅ polypeptides coded for by the cDNA molecules of the invention.

CLONED GLUTAMIC ACID DECARBOXYLASE**BACKGROUND OF THE INVENTION**

5 The present invention was supported by Grant NS22256 from the National Institutes of Health. The United States Government has certain rights in this invention.

1. FIELD OF THE INVENTION

10 The present invention relates to the use of recombinant DNA technology for the transformation of a host organism with glutamic acid decarboxylase₆₅ (GAD₆₅) for the expression of GAD₆₅ polypeptides. Also encompassed are methods of using GAD₆₅ polypeptides diagnostically and therapeutically in autoimmune disease.

2. DESCRIPTION OF THE BACKGROUND ART

15 Insulin-dependent diabetes mellitus (IDDM; type I diabetes) is one of the most common metabolic disorders. In the United States, IDDM affects approximately one in 300 to 400 people, and epidemiological studies suggest that its incidence is increasing. The disease results from the autoimmune destruction of the insulin-producing β -cells of the pancreas. More specifically, the preonset stage is characterized by "insulinitis", in which lymphocytes infiltrate the pancreatic islets and selectively destroy the β -cells. The typical IDDM presentation of hyperglycemia appears only after at least 80% of the insulin-producing β -cells are lost. The
20 remaining β -cells are destroyed during the next few years.

25 Although insulin therapy allows most IDDM patients to lead normal lives, this replacement is imperfect and does not completely restore metabolic homeostasis. Thus, severe complications which result in dysfunctions of the eye, kidney, heart, and other organs are common in IDDM patients undergoing insulin therapy. Because of this, it is highly desirable to extend the latency period (e.g., through

administration of immunosuppressant drugs) between the start of β -cell destruction and the actual requirement of insulin replacement (i.e., when 80% of the β -cells are destroyed). Therefore, a diagnostic test which determines the beginning of β -cell destruction would allow the clinician to administer immunosuppressant drugs (Silverstein, et al., *New England Journal of Medicine*, 319:599-604, 1988) to extend this latency period and thus significantly delay the onset of insulin replacement side effects.

Many IDDM patients have sera which contain antibodies to a 64kD molecule (Baekkeskov, et al., *J.Clin.Invest.*, 79:926-934, 1987; Atkinson, et al., *Lancet*, 335:1357-1360, 1990), to islet cell cytoplasmic (ICA) molecules or islet cell surface (ICSA) molecules (Bottazzo, et al, *Lancet*, 1:668-672, 1980), or to insulin (Palmer, et al., *Science*, 222:1137-1139, 1983; Atkinson, et al., *Diabetes*, 35:894-898, 1986). Atkinson and coworkers (Atkinson, et al., *Lancet*, 335:1357-1360, 1990) have demonstrated that the presence of antibodies to the 64kD molecule in human sera appears to be the earliest and most reliable indicator that onset of IDDM symptoms will eventually occur.

Recently, Baekkeskov and coworkers established that the 64kD molecule and glutamic acid decarboxylase (GAD) have several antigenic epitopes in common and thus they may be identical or very similar molecules. Although this identification is an important finding, the use of this information as a diagnostic tool for predicting IDDM is quite cumbersome and limited unless knowledge of the molecular biology of GAD is known. Consequently, the cloning and subsequent production of large quantities of the 64kD molecule, or a GAD molecule which is antigenically substantially identical to the 64kD molecule, will allow the development of a diagnostic kit designed to predict IDDM. The present invention provides a means for accomplishing this result.

SUMMARY OF THE INVENTION

The present invention arose out of the discovery that recombinant DNA technology
5 could be used to produce eukaryotic GAD₆₅ polypeptide and that GAD₆₅ polypeptide
could be used in the diagnosis and therapy of patients with autoimmune disease.
Particularly relevant is the use of cloned eukaryotic GAD₆₅ polypeptide in the
diagnosis of patients having, or at risk of having, insulin-dependent diabetes mellitus
(IDDM).

10

A major advantage of the present invention is that it provides the art with a ready
source of eukaryotic GAD₆₅ polypeptide corresponding to that purified from natural
sources, while avoiding the problems associated with the isolation of naturally
occurring eukaryotic GAD₆₅ polypeptide when separating it from other eukaryotic
15 non-GAD₆₅ polypeptides. This absence of other eukaryotic non-GAD₆₅ polypeptides is
significant in that it allows the development of test systems which will only detect
antibodies specifically reactive with GAD₆₅ polypeptides.

Another advantage of providing eukaryotic GAD₆₅ polypeptide in host cells is that by
20 so doing, it is possible to obtain much larger quantities of the polypeptide than are
currently practicably available from natural sources. As a consequence, not only is it
possible to use the polypeptide of the invention to more accurately classify patients
with such autoimmune diseases as IDDM, but it is also now possible to provide
commercially useful quantities of GAD₆₅ polypeptide for use in diagnostic systems.

25

According to one aspect of the invention, there is provided an isolated cDNA
sequence consisting essentially of the cDNA sequence of Figure 2 or Figure 3.

According to another aspect of the invention, there is provided an isolated cDNA
30 sequence consisting essentially of a portion of the cDNA sequence of Figure 2 or
Figure 3 which encodes at least one epitope for autoantibodies to GAD₆₅.

3a

According to a further aspect of the invention, there is provided a method of providing GAD₆₅ polypeptide having at least one epitope for autoantibodies to GAD₆₅ comprising:

- 5 (a) providing a host cell which replicates and expresses an intron-free DNA sequence consisting essentially of a portion of the cDNA sequence of Figure 2 or Figure 3 which encodes the at least one epitope;
- (b) growing the host cell; and
- 10 (c) recovering the GAD₆₅ polypeptide.

According to another aspect of the invention, there is provided a method of detecting autoantibodies to GAD₆₅ which comprises:

- (a) contacting a sample with a recombinantly produced GAD₆₅
- 15 polypeptide wherein the polypeptide is substantially free of non-GAD eukaryotic polypeptides and is encoded by a portion of the cDNA sequence of Figure 2 or Figure 3;
- (b) incubating the components of step (a) for a period of time and under conditions sufficient for the autoantibodies to bind to the polypeptide;
- 20 (c) separating the autoantibodies bound to the polypeptide from the sample; and
- (d) detecting the presence of the autoantibodies bound to the polypeptide.

According to a further aspect of the invention, there is provided the use of a composition for ameliorating an autoimmune response in a patient; the composition comprising:

- (1) a therapeutically effective amount of a recombinant GAD₆₅ polypeptide, wherein the GAD₆₅ polypeptide binds with a receptor, is substantially free of non-GAD eukaryotic polypeptides, and is encoded by a portion of the cDNA
- 30 sequence of Figure 2 or Figure 3; and
- (2) a pharmaceutically acceptable carrier.

3b

According to another aspect of the invention, there is provided a kit useful for the detection of autoantibodies to GAD₆₅, comprising a compartmentalized carrier to receive in close confinement therein one or more containers wherein:

- 5 (a) a first container contains recombinantly produced GAD₆₅ encoded by at least a portion of the cDNA sequence of Figure 2 or Figure 3; and
- (b) a second container contains a detectably-labeled second antibody, wherein the second antibody binds an epitopic determinant present on the autoantibody.

10

According to a further aspect of the invention, there is provided an isolated cDNA sequence consisting essentially of the complement of any DNA sequence which hybridizes to the cDNA sequence of Figure 2 or Figure 3 under stringent conditions.

15

According to another aspect of the invention, there is provided a cDNA sequence comprising a cDNA sequence of Figure 2 or the complement to any DNA sequence which hybridizes to the sequence of Figure 2 under stringent conditions.

20

According to a further aspect of the invention, there is provided a cDNA sequence comprising a cDNA sequence of Figure 3 or the complement to any DNA sequence which hybridizes to sequence of Figure 3 under stringent conditions.

25

According to another aspect of the invention, there is provided a cDNA sequence consisting essentially of a cDNA sequence encoding a polypeptide having an amino acid sequence possessing at least one epitope for autoantibodies of GAD₆₅; the cDNA sequence being selected from a sequence of Figure 2 or Figure 3 or the complement to any DNA sequence which hybridizes to the sequence of Figure 2 or Figure 3 under stringent conditions.

30

According to a further aspect of the invention, there is provided a method of providing GAD₆₅ polypeptide having at least one epitope for autoantibodies to GAD₆₅ comprising:

- (a) providing a host cell which replicates and expresses an intron-free DNA sequence consisting essentially of a portion of the complement to

3c

any DNA sequence which hybridizes to the sequence of Figure 2
which encodes at least one epitope.

- (b) growing the host cell; and
- 5 (c) recovering the GAD₆₅ polypeptide.

According to another aspect of the invention, there is provided a method of providing
GAD₆₅ polypeptide having at least one epitope for autoantibodies to GAD₆₅
comprising:

- 10 (a) providing a host cell which replicates and expresses an intron-free
DNA sequence consisting essentially of a portion of the complement to
any DNA sequence which hybridizes to the sequence of Figure 3
which encodes at least one epitope.
- (b) growing the host cell; and
- 15 (c) recovering the GAD₆₅ polypeptide.

DESCRIPTION OF THE DRAWINGS

FIGURE 1 Cloning strategy for obtaining GAD₆₅ and GAD₆₇ specific cDNA probes.

FIGURE 2 DNA sequence and corresponding amino acid sequence for rat GAD₆₅.

FIGURE 3 DNA sequence and corresponding amino acid sequence for human GAD₆₅.

FIGURE 4 Comparison of rat GAD₆₅ and human GAD₆₅ amino acid sequences.

FIGURE 5 GAD₆₅ and GAD₆₇ cDNAs hybridize to different size RNAs.

FIGURE 6 Southern blots hybridized with cDNA probes specific for GAD₆₅ and GAD₆₇.

FIGURE 7 Immunological identification of GAD₆₅ and GAD₆₇.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the manipulation of genetic materials by recombinant procedures which make possible the production of polypeptides possessing part or all of the primary structural conformation for one or more of the epitopes for binding autoantibodies to glutamic acid decarboxylase₆₅ (GAD₆₅). These polypeptides are highly useful for the immunological detection of autoantibodies reactive with them, since such autoantibodies are indicative of autoimmune diseases such as insulin dependent diabetes mellitus and "stiff man" syndrome. These polypeptides can also be used for purposes of screening

drugs, such as those that alter GAD function, and for generation of polyclonal and monoclonal antibodies which, in turn, can be used diagnostically to detect GAD₆₅.

5 The development of specific DNA sequences encoding eukaryotic GAD₆₅ polypeptide for splicing into DNA vectors can be accomplished using a variety of techniques. For example, alternative methods which can be employed include (1) the isolation of a double stranded DNA sequence from the genomic DNA of the eukaryote; (2) the chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and (3) the *in vitro* synthesis of
10 a double stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA.

15 The manufacture of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct manufacture of DNA sequences is not possible and the method of choice is the formation of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation
20 of plasmid-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double stranded DNA or RNA probe sequences
25 duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single stranded form (Jay, et al., *Nucleic Acid Research*, 11:2325, 1983).

Hybridization procedures are useful for the screening of recombinant clones by using labeled mixed synthetic oligonucleotide probes wherein each is potentially the complete complement of a specific DNA sequence in the hybridization sample which includes a heterogeneous mixture of denatured double stranded DNA. For such screening, hybridization is preferably performed on either single stranded DNA or denatured double stranded DNA. These procedures are particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed toward avoidance of non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, et al., *Nucleic Acid Research*, 9:879, 1981).

In addition, a GAD cDNA library can be screened by injecting the various cDNAs into oocytes, allowing sufficient time for expression of the cDNA gene products to occur, and testing for the presence of the desired cDNA expression product, for example, by using antibody specific for GAD₆₅ polypeptide or by using functional assays for GAD₆₅ enzymatic activity.

Alternatively, a cDNA library can be screened indirectly for GAD₆₅ peptides having at least one epitope using antibodies to GAD₆₅ (Chang and Gottlieb, *J. Neurosci.*, 8:2123, 1988). Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of GAD₆₅ cDNA. Preferred are antibodies directed to an epitope found in the first 100 amino acids of the N-terminal portion of GAD₆₅.

Of the three above-noted methods for developing specific DNA sequences for use in recombinant procedures, the use of genomic DNA isolates, is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides because of the presence of introns.

The present invention provides novel polypeptides of GAD₆₅ which have part or all of the primary structural conformation, that is, a continuous sequence of amino acid residues, having at least one epitope for antibodies to GAD₆₅.

5 It is possible to use the polypeptide fragments of the invention rather than intact GAD₆₅ to detect autoantibodies to GAD₆₅. The term "polypeptide," as applied to GAD₆₅ polypeptide, denotes any sequence of amino acids having an epitope for autoantibodies to GAD₆₅, wherein the sequence of amino acids is encoded by all or part of the cDNA sequences of the invention.

10 The polypeptides resulting from microbial expression of the DNA sequences of the invention can be further characterized by their freedom from association with other eukaryotic polypeptides or other contaminants which might otherwise be associated with GAD₆₅ in its natural cellular environment or in such extracellular fluids as plasma or urine.

15 Studies by the present inventors unequivocally establish that GAD₆₅ and GAD₆₇ are encoded by distinct genes and are not produced, for example, by post-transcriptional or post-translational modification of a common genomic sequence. Evidence proving that GAD₆₅ and GAD₆₇ are encoded by different genes include: (a) the largest contiguous sequence of exact identity between GAD₆₅ and GAD₆₇ cDNAs is only 17 nucleotides in length, (b) cDNAs from GAD₆₅ and GAD₆₇ do not cross hybridize with each other's or with each other's mRNA under low stringency conditions (2.0 x SSC, 0.01% SDS, 23°C), and (c) GAD₆₅ and GAD₆₇ cDNAs do not cross hybridize with isolated genomic clones encoding GAD₆₇ and GAD₆₅, respectively.

25 The term "host" is meant to include not only prokaryotes, but also such eukaryotes as yeast, filamentous fungi, as well as plant and animal cells which can replicate and express an intron-free DNA sequence of eukaryotic GAD₆₅. However, prokaryotes are preferred as the host organism.

The term "prokaryotes" is meant to include all bacteria which can be transformed or transfected with the gene for the expression of GAD₆₅. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, *E. coli*, *S. typhimurium*, *Serratia marcescens* and *Bacillus subtilis*.

5 A recombinant DNA molecule coding for the GAD₆₅ polypeptides can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Especially preferred is the use of a plasmid or a virus containing the GAD₆₅ coding sequence for purposes of prokaryotic transformation or transfection, respectively.

10 Methods for preparing fused, operably linked genes and expressing them in bacteria are well-known in the art (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression of GAD₆₅ in prokaryotic hosts.

15 In general, expression vectors containing promotor sequences which facilitate the efficient transcription of the inserted eukaryotic genetic sequence are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of the transformed cells. The
20 transformed prokaryotic hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. The polypeptides of the invention can then be isolated from the grown medium, cellular lysates, or cellular membrane fractions.

25 The isolation and purification of the microbially expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibody.

By having provided the sequence of amino acid residues of GAD₆₅, the present invention provides for the manufacture of DNA sequences which code for the host expression of polypeptide analogs or derivatives of GAD₆₅ which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues and which share some or all of the epitopes of naturally-occurring polypeptide forms.

The novel DNA sequences of the invention include all sequences useful in providing the expression in prokaryotic or eukaryotic host cells of polypeptides which have at least a part of the primary structural conformation for one or more epitopes capable of reacting with autoantibodies to GAD₆₅ which are comprehended by: (a) the DNA sequence as set forth in Figures 2 or 3 or their complementary strands; (b) DNA sequences which hybridize to DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to DNA sequences defined in (a) and (b) above. Specifically comprehended in (b) are genomic DNA sequences which encode allelic variant forms of GAD₆₅. Part (c) specifically comprehends the manufacture of DNA sequences which encode GAD₆₅, GAD₆₅ fragments, and GAD₆₅ analogs wherein the DNA sequences thereof may incorporate codons which facilitate translation of mRNA in non-vertebrate hosts.

Since the cDNA sequence of the invention encodes essentially the entire human or rat GAD₆₅ molecule, it is now a matter of routine to prepare, subclone, and express smaller polypeptide fragments of cDNA from this or a corresponding cDNA sequence which would encode as few as one epitope for autoantibodies to human or rat GAD₆₅. The presence of such an epitope on a cloned polypeptide can then be confirmed using, for example, sera from a patient with autoantibodies to GAD₆₅. An example of such a smaller peptide is the first approximately 100 amino acids from the N-terminus of GAD₆₅ (shown in Figure 3). This amino acid sequence is essentially absent from GAD₆₇.

The GAD₆₅ of the invention is particularly suited for use in immunoassays in which it can be utilized in liquid phase or bound to a solid phase carrier. In addition, GAD₆₅ used in these assays can be detectably labeled in various ways.

5 Examples of immunoassays which can utilize the GAD₆₅ of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA), the sandwich (immunometric assay) and the Western blot assay. Detection of
10 antibodies which bind to the GAD₆₅ of the invention can be done utilizing immunoassays which run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. The concentration of GAD₆₅ which is used will vary depending on the type of immunoassay and nature of the detectable label which is used. However, regardless of the type of immunoassay which is used, the concentration of GAD₆₅ utilized can be readily determined by one of ordinary skill in the art using routine
15 experimentation.

The GAD₆₅ of the invention can be bound to many different carriers and used to detect the presence of antibody specifically reactive with the polypeptide. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and
20 modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding GAD₆₅, or will be able to ascertain such, using routine experimentation.

25 There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds.

Alternatively, the polypeptide of the invention can be used to detect antibodies to GAD₆₅ by measuring GAD enzymatic activity. For example, GAD₆₅ and a specimen suspected of having antibodies to GAD₆₅ can be incubated for a period of time and under conditions sufficient to allow binding to occur between GAD₆₅ and the antibodies. The reaction product is precipitated and then tested for GAD enzymatic activity.

For purposes of the invention, the antibody which binds to GAD₆₅ of the invention may be present in various biological fluids and tissues. Any sample containing a detectable amount of antibodies to GAD₆₅ can be used. Normally, a sample is a liquid such as urine, saliva, cerebrospinal fluid, blood, serum and the like, or a solid or semi-solid such as tissue, feces and the like.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes and the like, each of the container means comprising one of the separate elements to be used in the method. For example, one of the container means may comprise GAD₆₅ bound to a carrier. A second container may comprise soluble, detectably-labeled second antibody, in lyophilized form or in solution.

In addition, the carrier means may also contain a plurality of containers each of which comprises different, predetermined amounts of GAD₆₅. These latter containers can then be used to prepare a standard curve into which can be interpolated the results obtained from the sample containing the unknown amount of autoantibodies to GAD₆₅.

In using the kit all the user has to do is add, to a container, a premeasured amount of a sample containing a measurable, yet unknown amount of autoantibodies to GAD₆₅ to be detected, a premeasured amount of carrier-bound GAD₆₅ present in the first container, and a premeasured amount of the detectably

5 labeled second antibody present in the second container. Alternatively, the non-detectably labeled GAD₆₅ can be provided attached to the container to which the sample and the detectably labeled second antibody are added. After an appropriate time for incubation, an immune complex is formed and is separated from the supernatant fluid, and the immune complex or the supernatant fluid are detected, as by radioactive counting or addition of an enzyme substrate, and color development.

10 The term "ameliorate" denotes a lessening of the detrimental effect of the autoimmune response in the patient receiving therapy. The term "therapeutically effective" means that the amount of GAD₆₅ polypeptide used is of sufficient quantity to ameliorate the cause of disease due to the autoimmune response.

15 The recombinant GAD₆₅ polypeptides of the invention can also be used therapeutically in patients having an autoimmune response to GAD₆₅. Such therapy can be accomplished by, for example, the administration of recombinant GAD₆₅ polypeptide. Such administration can utilize unlabeled as well as labeled GAD₆₅ polypeptide. When unlabeled GAD₆₅ polypeptide is utilized advantageously, it would be in a form wherein, for example, the GAD₆₅ polypeptides are in fragments which are too small to stimulate an immune response, but large enough to bind, or block, the continuance of the autoimmune response. For example, GAD₆₅ could be digested enzymatically into epitope-sized peptides (typically 5-12 amino acids in length) and thereby bind to Fab binding portions present in the body fluids, or on the surface of immune cells, of the patient with autoimmune disease.

25 Alternatively, the recombinant GAD₆₅ polypeptides of the invention could be administered labeled with a therapeutic agent. These agents can be coupled either directly or indirectly to the GAD₆₅ polypeptides of the invention. One example of indirect coupling is by use of a spacer moiety. These spacer moieties, in turn, can be either insoluble or soluble (Diener, *et al.*, *Science*, 231:148, 1986) and can be selected to enable drug release from the GAD₆₅ polypeptide at the

target site. Examples of therapeutic agents which can be coupled to the GAD₆₅ polypeptides of the invention for immunotherapy are drugs, radioisotopes, lectins, and toxins.

5 The drugs with which can be conjugated to the GAD₆₅ polypeptides of the invention include compounds which are classically referred to as drugs such as mitomycin C, daunorubicin, and vinblastine.

10 In using radioisotopically conjugated GAD₆₅ polypeptides of the invention for immunotherapy, certain isotopes may be more preferable than others depending on such factors as leukocyte distribution as well as stability and emission. Depending on the autoimmune response, some emitters may be preferable to others. In general, α and β particle-emitting radioisotopes are preferred in immunotherapy. Preferred are short range, high energy α emitters such as ²¹²Bi. Examples of radioisotopes which can be bound to the GAD₆₅ polypeptides of the invention for therapeutic purposes are ¹²⁵I, ¹³¹I, ⁹⁰Y, ⁶⁷Cu, ²¹²Bi, ²¹¹At, ²¹²Pb, ⁴⁷Sc, ¹⁰⁹Pd and ¹⁸⁸Re.

15 Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. However, ricin is a toxic lectin which has been used immunotherapeutically. This is accomplished by binding the α -peptide chain of ricin, which is responsible for toxicity, to the antibody molecule to enable site specific delivery of the toxic effect.

20 Toxins are poisonous substances produced by plants, animals, or microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin is a substance produced by *Corynebacterium diphtheria* which can be used therapeutically. This toxin consists of an α and β subunit which under proper conditions can be separated. The toxic A component can be bound to GAD₆₅ polypeptide and used for site specific delivery to a leukocyte expressing a receptor for GAD₆₅ polypeptide.

Other therapeutic agents which can be coupled to the GAD₆₅ polypeptides of the invention, as well as *ex vivo* and *in vivo* therapeutic protocols, are known, or can be easily ascertained, by those of ordinary skill in the art.

5 The dosage ranges for the administration of the GAD₆₅ polypeptides of the invention are those large enough to produce the desired effect in which the symptoms or cellular destruction of the autoimmune response are ameliorated. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the
10 patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary from about 0.1mg/m² to about 2000mg/m², preferably about 0.1mg/m² to about 500mg/m²/dose, in one or more dose administrations daily, for one or several days.

15 The GAD₆₅ polypeptides of the invention can be administered parenterally by injection or by gradual perfusion over time. The GAD₆₅ polypeptides of the invention can be administered intravenously, intraperitoneally, intra-muscularly, subcutaneously, intracavity, or transdermally.

20 Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's
25 dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the GAD₆₅ polypeptides of the invention, the medicament being used for therapy of autoimmune response to GAD₆₅.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLE 1

CLONING AND EXPRESSION OF GAD₆₅

A. RECOMBINANT DNA PROCEDURES

In order to obtain cDNA probes specific for GAD₆₅ and GAD₆₇, total RNA was extracted from adult rat brain by guanidine isothiocyanate-caesium gradient using the method of Chirgwin, *et al.* (*Biochemistry*, 18:5294, 1979). Poly (A) RNA was purified on oligo dT cellulose, using the protocol by Bethesda Research Laboratories (BRL). First strand synthesis was performed by using MMLV-reverse transcriptase (BRL), with conditions suggested, except that poly d(N₆)-mers (Pharmacia) were used as primers. This cDNA-RNA mixture was heat inactivated at 65°C for 15 min and stored at -20°C. For PCR, 1/50 of the sample was added to the 100 μ l reaction. Degenerate oligonucleotides were synthesized (Applied Biosystems) to encode the underlined common amino acid sequences of feline (from cDNA) (Kobayashi, *et al.*, *J.Neurosci.*, 7:2768, 1987) and rat (from peptides) (Chang and Gottlieb, *J.Neurosci.*, 8:2123, 1988) GAD (Figure 1). The 5'-end sequence of each degenerate oligonucleotide contained one strand of the DNA sequence recognized by either SstI and HindIII (5' oligo) or SstI and SstII (3'-end oligo). These primers were used for selective amplification by polymerase chain

5 reaction of the generated cDNA template as described by Gould, et al. (*Proc.Natl.Acad.Sci.,USA*, 86:1934, 1989). PCR products were subcloned into HindIII/SstI double digested Bluescript SK vector (Stratagene), transformed into DH5 (BRL), and plated by standard methods (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

10 Colony hybridization was done with an 5'-³²P end labeled oligonucleotide specific to feline GAD₆₇ (Kobayashi, et al., *J.Neurosci.*, 7:2768, 1987). End labeling of oligonucleotide, hybridization conditions, and washing conditions were done as described (Wallace, et al., in *Guide to Molecular Cloning Techniques*; Berger, et al., Eds. in *Methods of Enzymology*; Abelson, et al., Eds. *Academic Press, Inc.*, San Diego, 432-442, 1987), except that the nitrocellulose filters were washed at 50°C for 15 min. Colonies which were positive and negative in the hybridization were individually picked and grown overnight in Terrific Broth (Tartof, et al., *Focus*, 9:12, 1987). DNA was isolated using a boiling method (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989) and templates were denatured by 0.2N NaOH and purified by Sephacryl S400 spun columns (Pharmacia). Sequencing of denatured double stranded template was by the chain-termination method (Sanger, et al., *Proc.Natl.Acad.Sci.,USA*, 74:5463, 1977) using the T7-sequencing kit (Pharmacia).

25 As shown in Figure 1, PCR-generated rat GAD₆₅ and GAD₆₇ cDNAs were used as probes to screen a lambda ZAP (Stratagene) rat hippocampus library provided by S. Heinemann (Salk Institute) by standard techniques (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). A 2400 nucleotide GAD₆₅ cDNA (the largest clone) was isolated and subcloned by "zapping" as described by Stratagene. When a rat GAD₆₇ cDNA was obtained which was smaller than a 3.2kb rat GAD₆₇ cDNA clone already on hand, the larger cDNA was sequenced. Exo III deletions (Henikoff, *Gene*, 28:351, 1984) were made in both directions for GAD₆₅ and GAD₆₇ and templates were

5 prepared and sequenced as described above. Anchored PCR (Frohman, et al., *Proc.Natl.Acad.Sci.,USA*, 85:8998, 1988) was done to clone the remaining 5'-ends of GAD₆₅ and GAD₆₇ mRNAs which were not represented in the original cDNA clones isolated in the library screening. Sequencing of these clones revealed that neither GAD₆₅ nor GAD₆₇ mRNAs contained any further initiation codons (AUGs) in frame with the previously designated initiation codons of the original cDNA clones.

EXAMPLE 2

CHARACTERIZATION OF CLONED GAD₆₅

10 A. NORTHERN BLOT HYBRIDIZATION

15 Two PCR-derived cDNA probes were hybridized to Northern blots containing rat brain RNA in order to determine whether the GAD₆₇ and GAD₆₅ cDNAs were derived from two different mRNAs. RNA was extracted as described in Example 1. Poly (A) RNA was separated by electrophoresis in formaldehyde and transferred onto Biotrans (ICN) membranes, and hybridization was performed as described by Well, et al. (*J.Neurosci.*, 16:311, 1986), except that 100 μ l/ml of poly (A) was added. Probes were labeled to approximately 10⁹ dpm/ μ g by the oligolabeling procedure of Feinberg and Vogelstein (*Anal.Biochem.*, 132:6, 1983). Identical results were subsequently obtained with full-length clones of GAD₆₅ and GAD₆₇ cDNAs.

20 As shown in Figure 5, lanes 1 and 2 contain 1 μ g of poly (A) selected RNA extracted from rat cerebellum. Lane 1 was hybridized to a cDNA probe for the rat cognate of feline GAD₆₇ (Kobayashi, et al., *J.Neurosci.*, 7:2768, 1987) and lane 2 with a cDNA probe for the rat peptide sequence (which corresponds to GAD₆₅).

The cDNA probe for the rat peptide sequence hybridized to a 5.7kb RNA, while the cDNA probe for the rat cognate of our feline cDNA, hybridized to a 3.7kb RNA. This demonstrates that GAD₆₅ and GAD₆₇ are not derived from the same mRNA.

5 **B. GENOMIC HYBRIDIZATION OF GAD₆₇ AND GAD₆₅**

In order to investigate the possibility that GAD₆₇ and GAD₆₅ arise from separate genes, cDNAs of both GAD₆₇ and GAD₆₅ were hybridized to DNA blots containing genomic DNA.

10 For Southern blots, genomic DNA was extracted from rat liver as described (Kaiser, et al., in *DNA Cloning*, vol.I, A Practical Approach, D.M. Glover ed., IRL Press, Oxford, 38-40, 1985). DNA (10μg/sample) was digested to completion with EcoRI and HindIII using conditions recommended by the suppliers (BRL, Gaithersburg, MD). DNA fragments were separated by electrophoresis at 1.5v/cm for 16 hrs in 0.8% agarose. The DNA was then transferred to Zeta-Probe *
15 membranes (Bio-Rad), hybridized, and washed, as described by Gatti, et al. (*Biotechniques*, 2:148, 1984), except that 5μg/ml Carnation dried milk was substituted for Denhardt's solution. Probes for Southern blots were labeled as described in Example 1, above.

20 As shown in Figure 6, genomic DNA digested with HindIII and EcoRI are in lanes 1 and 3 and lanes 2 and 4, respectively. GAD₆₇ cDNA was hybridized to lanes 1 and 2, whereas GAD₆₅ cDNA was hybridized to lanes 3 and 4. Numbers along the side of the gel are the DNA fragment sizes in kilobases.

25 This data shows that the two cDNAs hybridize to genomic fragments of different sizes. In addition, the greatest contiguous stretch of identical nucleotide sequence of GAD₆₅ and GAD₆₇ cDNAs is only 17 nucleotide bases in length. Thus, GAD₆₇ and GAD₆₅ are encoded by two distinct genes.

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C. ENZYMATIC COMPARISON OF GAD₆₇ AND GAD₆₅

Studies were done comparing the effect of PLP on the activity of GAD₆₇ and GAD₆₅. In so doing, both cDNAs were subcloned into vectors that allowed their expression in bacteria (Studier, et al., *J.Mol.Biol.*, 189:113, 1986). Overexpression of "fusionless" GAD₆₅ and GAD₆₇ was accomplished by subcloning GAD₆₅ cDNA into the NcoI site of pET-8c and GAD₆₇ cDNA into the NheI site of pET-5c vectors (Studier, et al., *J.Mol.Biol.*, 189:113, 1986).

To obtain compatible sticky ends for correct in-frame subcloning of both cDNAs, selective amplification was performed by PCR using conditions suggested by United States Biochemical (USB), with 200 μ M dNTPs and 1.5mM MgCl₂ in the mixture and annealing at 55°C with 20 cycles to decrease infidelity of AmpliTaq (USB). Primers specific for GAD₆₅ and GAD₆₇ contained one DNA strand of the NcoI and SpeI recognition sites, respectively. Since there is a NheI restriction site within the coding region of GAD₆₇, SpeI (which is compatible with NheI) was used.

PCR products were subcloned into their respective pET vectors, transformed into DH5 and plated as described (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). Colonies were picked and grown overnight in LB broth with 50 μ g/ml ampicillin. Subclones with correct orientation were transformed into BL21(DE3) strain (Studier, et al., *J.Mol.Biol.*, 189:113, 1986) for overexpression. As a negative control, the pET-8C vector with no insert was transformed and subsequently induced. Single colonies were picked, grown, induced by 1mM isopropyl-B-D-thiogalactopyranoside (IPTG), and analyzed on SDS-PAGE gels as described (Sambrook, et al., *Molecular Cloning a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 17.15-17.16, 1989).

5 To measure GAD activity, we induced 10ml cultures of bacteria at OD₆₀₀-0.5 with 1mM IPTG. Two hours after induction, bacteria was spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM 2-aminoethylisothiuronium bromide (AET), and 60mM potassium phosphate, pH 7.1). After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford, *Anal.Biochem.*, 72:248, 1986) in the supernatant (supernatant was stored in aliquots at -70°C). Brain homogenates were prepared as described (Legay, et al., *J.Neurochem.*, 46:1478, 1986). GAD activity was measured as described (Krieger, et al., *J.Neurochem.*, 33:299, 1984) with 0.2mM PLP present or absent and 20μl of brain homogenate or bacterial lysate in the incubation mixture. Production of ¹⁴CO₂ in bacterial lysates was linear with respect to time of incubation and protein concentration.

10

TABLE 1

Source	GAD Specific Activity ^a		Fold Increase in Induction
	- PLP	+ PLP	
BL21(DE3) + pET-8c	12 ± 0.4	9 ± 1	--
5 BL21(DE3) + pET-GAD ₆₅	115 ± 3	773 ± 61	6.7
BL21(DE3) + pET-GAD ₆₇	160 ± 2	389 ± 8	2.4
Rat Brain	131 ± 5	216 ± 2	1.6

^a cpms of ¹⁴CO₂/μg protein/hr of triplicates ± S.E.M.

10 As shown in Table 1, bacterial lysates containing GAD₆₅ or GAD₆₇ catalyze the conversion of [1-¹⁴C]-glutamate to GABA and ¹⁴CO₂.

15 PLP stimulates the enzymatic activity of GAD₆₅ more than GAD₆₇. This greater stimulation probably reflects the faster cycling of GAD₆₅ through the inactivation cycle proposed by Martin and coworkers (Martin, *Cell.Mol.Neurobiol.*, 7:237, 1987). This faster cycling suggests that GAD₆₅ contributes more to the pool of apo-GAD that exists *in vivo* (Miller, *et al.*, *Brain Res.Bull.*, 5(Suppl.2):89, 1980). Thus, *in vivo*, PLP appears to regulate GAD₆₅ activity more than GAD₆₇ activity.

20 GAD₆₅ activity in bacterial lysates is similar to the five-fold PLP stimulation of GAD activity found in synaptosomes prepared from rat substantia nigra (Miller, *et al.*, *J.Neurochem.*, 33:533, 1979). Because both GADs are more dependent upon

added PLP in bacteria than is the GAD activity in crude rat brain homogenates, the endogenous PLP concentration of bacteria lysates may be less than rat brain homogenates.

D. IMMUNOLOGICAL IDENTIFICATION OF GAD₆₅ AND GAD₆₇

5 Rat brain homogenates and bacterial lysates were extracted as described above. Equal volumes of loading buffer were added to each sample as described (Harlow, et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). Proteins were separated by electrophoresis in a 10% acrylamide gel in SDS and electrophoretically transferred to nitrocellulose (Harlow, et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). The unreacted sites were blocked with a phosphate buffered saline (PBS) solution containing 2% bovine serum albumin (fraction V), 1% gelatin, and 1% Triton-X-100* at 42°C for one hr. After washing, the nitrocellulose filter was then cut into three sections and incubated with the following primary antibodies: lanes 1 to 4 with a 1/2000 dilution of the antiserum of Oertel, et al. (*Neuroscience*, 6:2689, 1981), which recognizes both GAD₆₇ and GAD₆₅; lanes 5-8 with a 1/2000 dilution of K-2 antiserum, which recognizes only GAD₆₇; lanes 9-12 with a 1/2000 dilution of GAD-6 monoclonal antibody, which is specific for GAD₆₅ (Chang, et al., *J.Neurosci.*, 8:2123, 1988). All filters were extensively washed and appropriate secondary antibodies were incubated and washed. Bound antibodies were detected with ¹²⁵I-labeled protein A and autoradiography. Each lane contained the following: lanes 1, 5, and 9 are BL21(DE3) + pET-GAD₆₇; lanes 2, 6, and 10 are BL21(DE3) + pET-GAD₆₅; lanes 3, 7, and 11 are rat brain homogenate; and lanes 4, 8, and 12 are BL21(DE3) + pET-8c.

The immunoblots of bacterially produced GAD₆₅ and GAD₆₇ demonstrated that GAD₆₅ indeed corresponds to the smaller GAD in brain extracts, and GAD₆₇ to the larger form (Figure 7). Previous work has demonstrated the correspondence of

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5 GAD₆₇ to the larger GAD for feline GAD₆₇, and for mouse GAD₆₇ (Katarova, et al., *Eur.J.Neurosci.*, 2:190, 1990; 235, 1987). The mobilities of bacterially produced GAD₆₅ and GAD₆₇ (as detected with the antiserum of Oertel, et al. (*Neuroscience*, 6:2689, 1981) are identical to the immunoreactive doublet seen in rat brain homogenate.

10 The smaller molecular weight and larger molecular weight forms of GAD in rat brain are thus identical in antigenicity and size to the products of GAD₆₅ and GAD₆₇ cDNAs, respectively. Consequently, the two GADs in rat brain are GAD₆₅ and GAD₆₇. From this data it can also be concluded that the molecular identity of the reported PLP-dependent and PLP-independent GADs by Tapia (Bayon, et al., *J.Neurochem.*, 29:519, 1977) are GAD₆₅ and GAD₆₇, respectively. Martin and coworkers (Spink, et al., *Brain Res.*, 421:235, 1987) have reported the existence of four kinetically different forms of rat brain GAD. However, immunoblotting experiments (with the antisera used here) of these forms have not been reported.

15 **E. DISTRIBUTION OF GAD₆₅ and GAD₆₇ IN RNAs IN BRAIN TISSUE**

Experiments were done to determine the distribution of GAD₆₅ and GAD₆₇ in RNAs in cerebellum using *in situ* hybridization.

20 Transcripts of, respectively, 3.2kb and 2.3kb from GAD₆₅ and GAD₆₇ cDNAs, were radiolabeled with ³⁵S according to Wuenschell, et al. (*Proc.Natl.Acad.Sci.,USA*, 83:6193, 1986) procedure. Hydrolyzed fragments of 200 bp were hybridized to coronal sections of a rat cerebellum. Animals were anesthetized under halothane and decapitated. The brain was rapidly frozen in dry ice and coronal frozen sections (12μm) were fixed for 30 min in freshly prepared 4% formaldehyde in phosphate-buffered saline (PBS; 130mM NaCl, 10mM Na phosphate, pH 7.0). The
25 tissue was dehydrated through graded ethanol solutions and stored at -70°C.

5 In order to increase tissue permeability, the sections were submitted to the following pretreatments: rehydration through graded ethanol solutions (5 min each in 95%, 85%, 70%, 50%, and 30% ethanol); PBS (5 min); 0.02N HCl (10 min); PBS (5 min); 0.01% Triton N-101 in PBS (1 min); PBS (2 x 5 min); 1 μ g/ml proteinase K (7.5 min); and glycine (to inhibit proteinase K) in PBS (3 x 5 min). Proteinase K was digested for 30 min at 37°C before use. Sections were then incubated at 37°C in 50% formamide, 750mM NaCl, 25mM EDTA, 0.2% SDS, 0.02% BSA, 0.002% Ficoll, 0.02% polyvinylpyrrolidone, 250 μ g/ml yeast tRNA, 250 μ g/ml poly A, and 25mM PPES (pH 6.8).

10 For the hybridization, 100mM DTT, 10% dextran sulfate, and sense or antisense ³⁵S-RNA were added to the prehybridization solution. An aliquot (50 μ l) of the hybridization solution containing about 3 ng (10⁶ cpm) of probe (sense or antisense) was added onto the slides. Each slide was coverslipped and incubated for 16 hrs at 50°C, following which the siliconized coverslips were removed by brief washing in 4 x SSC (1 x SSC - 150mM NaCl, 60mM Na citrate, pH 7.0).

15 Sections were then treated with ribonuclease A (50 μ g/ml in 0.5M NaCl, 10mM Na thiosulfate, 1mM EDTA, 10mM TrisHCL, pH 8.0) for 20 min at 37°C and rinsed for 2 hrs at room temperature in 2 x SSC, 10mM Na thiosulfate, for 30 min at 55°C.

20 Sections were dehydrated in ethanol, delipidated in xylene, coated with Kodak* NTB2 emulsion and exposed for 10 days at 4°C. The emulsion was developed with Kodak D19, and the tissue counterstained with cresyl violet.

25 Autoradiographic grains were detected using reflected polarized light and grain numbers, densities, and cell areas were determined with an Analytic Imaging Concepts image analyzer system. Due to the low background level, the criteria for defining a cell "labeled" was based on the presence of more than 5 clustered grains. The GAD labeled cells were found scattered throughout the brain, enabling the measurement of the number of grains over individual cells. The boundary of the cell and the area covered by a grain allowed the calculation of

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the number of grains per cell. This analysis was done at a high magnification (800X), using both reflected polarized light and transmitted light to simultaneously visualize the stained cell and the superimposed grains. Numbers are means $\pm$ S.E.M. of "n" cells.

TABLE 2

CELL TYPE	GRAINS/CELL		$\frac{GAD_{67}}{GAD_{65}}$
	GAD_{67} mRNA	GAD_{65} mRNA	
Purkinje	172 $\pm$ 34 (87) ^a	43 $\pm$ 2 (70)	4.0
Golgi II	96 $\pm$ 8 (80)	64 $\pm$ 9 (65)	1.5
Basket	61 $\pm$ 12 (102)	16 $\pm$ 1 (57)	3.8
Stellate	55 $\pm$ 15 (65)	18 $\pm$ 3 (37)	3.1

^a $\pm$ S.E.M. (n)

In all neuronal types GAD_{67} mRNA levels are greater. The observations with *in-situ* hybridization are consistent with previous findings (Nitsch, *J.Neurochem.*, 34:822, 1980; Denner, *et al.*, *J.Neurochem.*, 44:957, 1985; Itoh, *et al.*, *Neurochem. Res.* 6:1283, 1981) that the ratio of PLP dependent to independent GAD activities in the cerebellum is one of the lowest in brain regions tested. In addition, as shown in Table 2, the order of amounts for GAD_{67} mRNA is Purkinje > Golgi II > Basket > Stellate cells; in contrast, for GAD_{65} mRNA, this order is Golgi II > Purkinje > Basket > Stellate cells.

The expression of GAD_{65} and GAD_{67} mRNAs thus differs among classes of neurons. The contribution of each to total GAD activity in turn affects how GABA production is regulated. For example, the substantia nigra contains one of the

highest ratios of PLP-dependent to PLP-independent GAD activities (Nitsch, *J. Neurochem.*, 34:822, 1980). Increasing GABA concentration in the substantia nigra by local injection of inhibitors of GABA catabolism is especially effective in reducing seizure susceptibility (Gale, *Fed. Proc.*, 44:2414, 1985). Experimental animals undergoing seizures induced by PLP-antagonists may therefore be unable to inhibit seizure propagation because of inhibition of GAD₆₅ particularly in nerve terminals within the substantia nigra.

F. SUBCELLULAR LOCATION OF GAD₆₅ AND GAD₆₇

The distribution of GAD₆₅ and GAD₆₇ was evaluated in the S₂ and synaptosome subcellular fractions. S₂ is a high speed supernatant consisting of the cytosol of all cells in the brain, while the synaptosomal fraction consists primarily of nerve endings (Gray, *et al.*, *J. Anat., Lond.*, 96:79, 1962). For these studies, whole rat brain fractionation was performed as described by Booth and Clark (Booth, *et al.*, *Biochem. J.*, 176:365, 1978). Protein concentrations were determined by Schaffner and Weissman (Schaffner, *et al.*, *Anal. Biochem.* 56:502, 1973). Samples were prepared as described (Kaiser, *et al.*, *DNA Cloning, Vol. 1, A Practical Approach*, D.M. Glover ed. (IRL Press, Oxford, 1985, pp. 38-40), and immunoblotting was done as described above using GAD-6 monoclonal antibody and K-2 antiserum. Equal amounts of protein (16 μ g) were added to each lane. Autoradiography showed a linear response of increasing amount of ¹²⁵I-protein A bound to antibody with protein concentrations of 1, 3, 10, 30, 100 μ gs with both K-2 antiserum and GAD-6 monoclonal antibody (data not shown).

The results showed that GAD₆₇ was present in equal amounts in both fractions. Since the S₂ fraction contains the cytosolic proteins of glial (as well as other non-neuronal) and neuronal cells, the concentration of GAD₆₇ must be greater in neuronal cell bodies than in nerve endings. In contrast, the concentration of GAD₆₅ was greater in synaptosomes than in S₂. These subcellular fractionation experiments suggest that, in contrast to GAD₆₅, a much greater fraction of GAD₆₇

is present in cell bodies of neurons than in nerve terminals. Thus, subcellular fractionation, like immunohistochemistry, shows that GAD₆₅ and GAD₆₇ have different subcellular distributions.

5 *In vivo* experiments utilizing inhibitors of GABA synthesis and degradation have suggested that the GABA pool in neuronal cell bodies is different from that in the nerve terminals (Iadarola, *et al.*, *Mol. Cell. Biochem.*, 39:305, 1981). GABA produced by GAD₆₇ may be involved more in cellular metabolism (for example, in the GABA shunt) and in dendrodendritic synapses. The dendrites of granule cells in the olfactory bulb, which form dendrodendritic synapses with mitral
10 dendrites (Shepard, *Physiol. Rev.*, 52:864, 1972) and probably release GABA (McLennan, *Brain Res.*, 29:177-184, 1971), label intensely with K-2 antiserum. While not shown here, it has also been found greater GAD₆₇ than GAD₆₅ mRNA levels (2-3 fold) in the olfactory bulb. This distribution is consistent with the reported finding that most GAD activity in the olfactory bulb is present in S₂ and
15 P₁ (crude nuclear pellet) and not in synaptosomes (Quinn, *et al.*, *J. Neurochem.*, 35:583, 1980).

The differing subcellular distributions of GAD₆₅ and GAD₆₇ could result from cytoskeletal anchoring or from some unknown protein targeting mechanism. Some cytoskeletal proteins have distributions that resemble GAD₆₅ and GAD₆₇.
20 For example, in cultured sympathetic neurons Peng, *et al.* (*J Cell. Biol.*, 102:252, 1986), demonstrate that 84% of tau is in axons while 100% of MAP-2 is in cell bodies and dendrites. In addition, 43kd protein, a cytoskeletal protein, is thought to anchor the acetylcholine receptor to the underlying membrane cytoskeleton (Flucher, *et al.*, *Neuron*, 3:163, 1989).

EXAMPLE 3DETECTION OF GAD AUTOANTIBODIES IN CLINICAL SPECIMENSA. MATERIALS AND METHODS

5 1. Patient Specimens. Sera from four groups of individuals were selected from a previous study by Atkinson and co-workers (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990). These groups consisted of: Group (1), 1 new onset IDD patients diagnosed according to the established National Diabetes Data Group (NDDG) criteria (Gleichman, *et al.*, *Diabetes*, 36:578-584, 1987) that had been referred to the University of Florida, Diabetes Clinics; Group (2), 5 randomly selected islet
10 cell cytoplasmic antibody (ICA) negative non-diabetic controls without any known family history of autoimmune disease; Group (3), 13 individuals whose sera had been collected 3 to 66 months prior to their documented clinical onsets of IDD; Group (4), non-diabetic controls and relatives, and those who were studied prior to their onsets of IDD; and Group (5), 3 patients at risk for IDDM, but where onset
15 has not yet occurred. This latter group had been ascertained through ongoing prospective ICA screening studies of more than 5000 first degree relative of IDD probands, and 8200 individuals from the general population (of which 4813 were school children).

20 2. Islet Cell Autoantibodies. ICA were assayed by indirect immunofluorescence on blood group O cryocut pancreatic (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990). All results were interpreted on coded samples, with control negative and positive sera in each batch. The degrees of ICA positivity were analyzed with the guidelines established by the Immunology Diabetes Workshop (IDW) for the standardization of ICA (Gleichman, *et al.*, *Diabetes*, 36:578-584, 1987). All positive
25 sera were titered by end point dilution, and the Juvenile Diabetes Foundation (JDF) units were determined by reference to a standard serum previously

calibrated to the international JDF standard of 80 units. In the studies reported here, a positive ICA result was defined by replicate titers of 10 JDF units or greater.

5 **3. HLA DR Typing.** HLA DR typing was performed as adapted from the method described by Van Rood and Van Leuwen (*Nature*, 262:795-797, 1976), using DR trays (One Lamda Laboratories, Los Angeles, CA).

10 **4. Human Islet Cells.** Human pancreatic islets were isolated from cadaveric pancreases and maintained *in vitro* as previously described (Ricordi, et al., *Diabetes*, 37:413-420, 1988). The islet cells were metabolically labeled with ³⁵S methionine (Amersham, Arlington Heights, IL) *in vitro* (95% air/5%CO₂).

15 **5. Islet Cell Extractions and Immunoprecipitations.** Islet cells were extracted as previously described by Atkinson, et al. (*Lancet*, 335:1357-1360, 1990) with the following modifications. For immunoprecipitation studies, the islet cell lysates were precleared twice by incubation (2h, 4°C) with either control, IDD serum (100μl), or GAD-6 (Chang, et al., *J.Neuro*, 8:2123-2130, 1988) (1μl in 99μl of Tris buffer (Atkinson, et al., *Lancet*, 335:1357-1360, 1990) for every 1000 islets. Immune complexes were then absorbed (1h 4°C) with an excess of protein A Sepharose * CL-4B (Pharmacia, NJ). Aliquot volumes representing 1000 islet cells containing unbound (precleared) lysate were then incubated (12h, 4°C) with either IDD or control sera (25μl), or GAD-6 (Chang, et al., *J.Neuro*, 8:2123-2130, 1988) (1μl in 25μl Tris buffer). Following another incubation with protein A Sepharose CL-4B (1h, 4°C), the complexes were then washed 5 times with 50mM Tris HCL (pH 7.4) with 0.1% SDS, 1.0% Triton X-114, and 2mM EDTA, and then washed again one time in double distilled water. The protein A Sepharose CL-4B was then boiled in Laemmli sample buffer (Laemmli, *Nature*, 227:680-685, 1970), and the samples were subjected to SDS-PAGE and fluororadiography (Kodak, X-omat AR5) using Enhance (New England Nuclear). Alternatively, the autoradiographs were analyzed by a BETAGEN*(Boston, MA) analyzer. Both 64KA positive and negative sera were used in each assay, to serve as interassay controls. All fluororadiographs

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5 were analyzed and rated as positive or negative after comparison with the known interassay controls. Positive serum samples were designated as 1 when a sample resulted in immunoprecipitation of a low intensity 64,000 M_x band, 2 if a moderate intensity band was observed and 3 if the intensity of the immunoprecipitated protein was high. A similar rating procedure was employed for the intensity of bands corresponding to immunoprecipitated ³⁵S-GAD₆₅ and ³⁵S-GAD₆₇.

6. Immunoprecipitations. Immunoprecipitation of bacterial lysates containing ³⁵S-GAD₆₅ or ³⁵S-GAD₆₇, and GAD from human brain homogenate, was completed as described above in immunoprecipitation studies of human islet cell extractions.

10 7. GAD Assays. Human brain homogenates were incubated with patient sera as described above in human islet cells. After absorption and washes, the protein A agarose slurry was aliquoted into three equal volumes and GAD activity was measured as described (Krieger, et al., *Neurochem.* 33:299, 1984). Briefly, Protein A agarose beads were incubated with (1-¹⁴C)-glutamate (Amersham) in a
15 designated incubation mixture (Krieger, et al., *Neurochem.* 33:299, 1984) and production of ¹⁴CO₂ was quantitated by a liquid scintillation counter.

20 8. Production of ³⁵S-GAD₆₅ and ³⁵S-GAD₆₇. Rat GAD₆₅ and GAD₆₇ cDNAs were subcloned into a bacterial expression system as previously described. Labeling of ³⁵S-GADs was completed by pulsing IPTG induced bacterium (growing in Minimal Media) for 15 minutes with TRAN ³⁵S-label (ICN). Cultures were then spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM 2-aminoethylisothiuronium Bromide (AET) and 60mM potassium phosphate, pH 7.1). After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford,
25 *Anal.Biochem.*, 72:248, 1986) in the supernatant (supernatant was stored in aliquots at -70°C).

B. IMMUNOREACTIVITY OF IDDM SPECIMENS

Sera from patients with IDDM were tested for the ability to precipitate GAD from human brain homogenates.

TABLE 3

SERA FROM IDDM PATIENTS IMMUNOPRECIPITATE GAD ACTIVITY

	<u>Patient</u>	<u>IDDM</u>	<u>Pre-IDDM Period¹</u>	<u>64K²</u>	<u>JDF³</u>	<u>GAD Activity⁴ cpm's</u>
5	DA	* ⁵	>24	3	164	13,762
	DC	*	>1	3	20	1,719
	RS	+	5	3	40	588
	NL	+	0	2	80	440
	DM	*	>1	2	10	184
10	C	-	na	0	0	280
	C	-	na	0	0	285
	C	-	na	0	0	325
	C	-	na	0	0	275
	C	-	na	0	0	270

15 ¹ Expressed as months² 64K titers as described in Experimental Methods³ The islet cell antibody test as expressed in
Juvenile Diabetes Foundation (JDF) units⁴ Not adjusted for background20 ⁵ At risk for diabetes (also, failed glucose test)

na - Not applicable

As shown in Table 3, the sera of four (out of five) at risk for IDDM or IDDM patients bound significantly greater amounts of enzymatically active GAD of human brain extracts than sera from control patients. In addition, sera from one of the patients was drawn in a pre-IDDM period, thus autoantibodies to GAD are present prior to the onset of IDDM symptoms (see C below).

Further experiments (results not presented) showed that the sera of two at risk IDDM patients (DA, DC) immunoprecipitated recombinantly produced ^{35}S -GAD₆₅ whereas recombinantly produced ^{35}S -GAD₆₇ was only recognized by sera of patient DA (and to a lesser degree than ^{35}S -GAD₆₅). Subsequent studies have found larger titers of GAD₆₇ autoantibodies than GAD₆₅ are present in sera of IDDM patients with neuropathic complications (not shown here).

Additional studies using patient DA sera showed the presence of antibodies which recognize specific polypeptides produced in human pancreatic islet cells. Electrophoretic analysis of the bound polypeptides demonstrated the presence of autoantibodies to a 64kD component, as previously shown by others in human IDDM (Baekkeskov, et al., *Nature*, 298:167-169, 1982) and in animal models (Baekkeskov, et al., *Science*, 224:1348-1350, 1984; Atkinson, et al., *Diabetes*, 37:1587-1590, 1988). Prior absorption of these sera with GAD-6 monoclonal, which recognized GAD₆₅ but not GAD₆₇, or with bacterially produced GAD₆₅, abolished the ability of the sera to recognize the 64kD pancreatic polypeptide. The epitopes recognized by autoantibodies to the 64kD autoantigen are thus present in GAD₆₅, indicating that the autoantigen is indeed GAD₆₅. In order to investigate the predictive value of GAD₆₅, sera drawn from patients prior to onset of clinical manifestation of IDDM were tested for autoantibodies to GAD₆₅.

TABLE 4

IDDM PATIENTS ANALYZED FOR AUTOANTIBODIES PRIOR TO THE ONSET OF DISEASE

	<u>Patient</u>	<u>Sex</u>	<u>HLA</u>	<u>Age Onset¹</u>	<u>Pre-IDDM Period²</u>	<u>JDF</u>	<u>64KA³</u>	<u>GAD³₋₆₅</u>	<u>GAD³₋₆₇</u>
5	TA	M	3,2	17	11	20	2	0	1
	CA	F	4,5	38	4	0	1	1	0
	RA	M	2,1	5	34	0	2	1	0
	TB	M	2,4	11	66	40	1	1	0
10	AB	M	N.D.	23	6	160	3	3	2
	VC	F	4,6	15	3	40	1	0	1
	JD	M	6,1	34	25	10	3	1	1
	DR	F	3,4	14	42	320	2	1	0
15	JG	M	3,3	12	8	40	1	0	0
	BR	M	3,3	5	9	0	0	1	1
	KR	F	4,X	34	14	10	3	2	0
	JT	F	4,6	7	10	N.D.	1	1	1

¹ Age of IDDM onset expressed as months² The time interval between sera drawn and IDDM onset expressed as months³ 1 - lowest; 2 - medium; and 3 - highest band intensities

N.D. - not determined

As shown in Table 4, 9 out of 12 specimens (75%) were immunoreactive with ^{35}S -GAD₆₅. In addition, two patients (JA and VC) were immunoreactive to GAD₆₇, but not GAD₆₅ under these conditions. Therefore, in combination, autoantibodies to GAD₆₅ and GAD₆₇ were present in 11 out of 12 (91%) of these patients sera. This finding suggests that although autoantibodies to GAD₆₅ are more common than autoantibodies to GAD₆₇, the use of both recombinant GADs (GAD₆₅ and GAD₆₇) in an assay would allow for greater predictability of IDDM. Previous tests of these sera (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990) demonstrated that 11 out of 12, or 92%, immunoreacted with the ^{35}S -64kD molecule from human pancreatic islet cells. The serum which contained detectable autoantibodies to the 64kD molecule and not GAD₆₅ was a serum which contained the lowest titer (or "1") for the 64kD molecule. Thus, the false negative obtained was due to a lack of sensitivity in this assay. Furthermore, this assay predicted IDDM in one patient (BR) who was negative for 64K.

These results show that the 64kD molecule identified in β -cells of human pancreas is identical in size and antigenicity to rat GAD₆₅. Furthermore, sera drawn from patients prior to IDDM onset contain autoantibodies to GAD₆₅. Consequently, the GAD₆₅ recombinant molecule is of great utility as a diagnostic tool for predicting IDDM. The ability of a physician to diagnose IDDM prior to actual symptoms will no doubt result in a greater extension of time before insulin therapy is needed. The sensitivity of such immunoassays will improve with the use of a recombinant GAD₆₅ of human origin which represents the GAD form present in β -cells of the pancreas.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made without departing from the spirit or scope of the invention.

CLAIMS:

1. An isolated cDNA sequence consisting essentially of the cDNA sequence of Figure 2 or Figure 3.
5
2. A host cell transformed or transfected with the isolated cDNA sequence of claim 1.
3. A biologically functional plasmid or viral DNA vector including the
10 isolated cDNA sequence of claim 1.
4. A host cell stably transformed or transfected with a DNA vector according to claim 3.
- 15 5. An isolated cDNA sequence consisting essentially of a portion of the cDNA sequence of Figure 2 or Figure 3 which encodes at least one epitope for autoantibodies to GAD₆₅.
6. The isolated cDNA sequence of claim 5 comprising a cDNA sequence
20 encoding about the first 100 amino acids of the N-terminus of GAD₆₅.
7. A host cell transformed or transfected with the isolated cDNA sequence of claim 5.
- 25 8. A method of providing GAD₆₅ polypeptide having at least one epitope for autoantibodies to GAD₆₅ comprising:
 - (a) providing a host cell which replicates and expresses an intron-free DNA sequence consisting essentially of a portion of the cDNA sequence of Figure 2 or Figure 3 which encodes said at least one
30 epitope;

37

- (b) growing the host cell; and
- (c) recovering the GAD₆₅ polypeptide.

9. The method of claim 8, wherein the GAD₆₅ is human GAD₆₅.

5

10. A method of detecting autoantibodies to GAD₆₅ which comprises:

- (a) contacting a sample with a recombinantly produced GAD₆₅ polypeptide wherein the polypeptide is substantially free of non-GAD eukaryotic polypeptides and is encoded by a portion of the cDNA sequence of Figure 2 or Figure 3;

10

- (b) incubating the components of step (a) for a period of time and under conditions sufficient for said autoantibodies to bind to the polypeptide;
- (c) separating the autoantibodies bound to the polypeptide from the sample; and

15

- (d) detecting the presence of the autoantibodies bound to the polypeptide.

11. The method of claim 10, wherein the GAD₆₅ is human GAD₆₅.

12. The method of claim 10, wherein the sample is from a human.

20

13. The method of claim 10, wherein the step of detecting utilizes a detectably labeled protein capable of binding to the autoantibody.

14. The method of claim 13, wherein the binding protein is a detectably labeled second antibody.

25

15. The method of claim 14, wherein the detectable label is selected from the group consisting of a radioisotope, a fluorescent compound, a colloidal metal, a chemiluminescent compound, a bioluminescent compound and an enzyme.

16. The method of claim 15, wherein the GAD₆₅ is human GAD₆₅.

17. The use of a composition for ameliorating an autoimmune response in a patient; said composition comprising:

- 5 (1) a therapeutically effective amount of a recombinant GAD₆₅ polypeptide, wherein the GAD₆₅ polypeptide binds with a receptor, is substantially free of non-GAD eukaryotic polypeptides, and is encoded by a portion of the cDNA sequence of Figure 2 or Figure 3; and
- (2) a pharmaceutically acceptable carrier.

10

18. The use of claim 17, wherein the receptor is selected from the group consisting of an antibody and T-helper cells.

19. The use of claim 17, wherein the autoimmune response is associated
15 with diseases selected from the group consisting of IDDM and stiff-man disease.

20. The use of claim 17, wherein the composition includes a pharmaceutically acceptable carrier which is adapted for parenteral administration.

20 21. The use of claim 20, wherein the composition is adapted for parenteral administration by subcutaneous, intramuscular, intraperitoneal, intracavity, transdermal, or intravenous injection.

22. The use of claim 17, wherein the composition is adapted to provide
25 about 0.01 mg/kg/dose to about 2000 mg/kg/dose.

23. The use of claim 17, wherein said recombinant GAD₆₅ polypeptide is therapeutically labeled.

24. The use of claim 23, wherein the therapeutic label is selected from the group consisting of a radioisotope, a drug, a lectin, and a toxin.

25. A kit useful for the detection of autoantibodies to GAD₆₅, comprising a compartmentalized carrier to receive in close confinement therein one or more containers wherein:

(a) a first container contains recombinantly produced GAD₆₅ encoded by at least a portion of the cDNA sequence of Figure 2 or Figure 3; and

(b) a second container contains a detectably-labeled second antibody, wherein the second antibody binds an epitopic determinant present on the autoantibody.

26. The kit of claim 25, wherein the GAD₆₅ is bound to a carrier.

27. The kit of claim 26 wherein the carrier to which the GAD₆₅ is bound is insoluble.

28. The kit of claim 25, wherein the first container further contains GAD₆₇.

29. An isolated cDNA sequence consisting essentially of the complement of any DNA sequence which hybridizes to the cDNA sequence of Figure 2 or Figure 3 under stringent conditions.

30. A cDNA sequence comprising a cDNA sequence of Figure 2 or the complement to any DNA sequence which hybridizes to the sequence of Figure 2 under stringent conditions.

31. A cDNA sequence comprising a cDNA sequence of Figure 3 or the complement to any DNA sequence which hybridizes to sequence of Figure 3 under stringent conditions.

32. A host cell transformed or transfected with the cDNA sequence of claim 30 or 31.

5 33. A biologically functional plasmid or viral DNA vector including the cDNA sequence of claim 30 or 31.

34. A host cell stably transformed or transfected with a DNA vector according to claim 33.

10

35. A cDNA sequence consisting essentially of a cDNA sequence encoding a polypeptide having an amino acid sequence possessing at least one epitope for autoantibodies of GAD₆₅; said cDNA sequence being selected from a sequence of Figure 2 or Figure 3 or the complement to any DNA sequence which hybridizes to the
15 sequence of Figure 2 or Figure 3 under stringent conditions.

36. The cDNA sequence of claim 35 comprising a cDNA sequence encoding about the first 100 amino acids of the N-terminus of GAD₆₅.

20 37. A host cell transformed or transfected with the cDNA sequence of claim 35.

38. The isolated cDNA sequence of claim 30 which encodes at least one epitope for autoantibodies.

25

39. The isolated cDNA sequence of claim 38 comprising a cDNA sequence encoding about the first 100 amino acids of the N-terminus of GAD₆₅.

40. A host cell transformed or transfected with the isolated cDNA
30 sequence of claim 38.

41. A method of providing GAD₆₅ polypeptide having at least one epitope for autoantibodies to GAD₆₅ comprising:

- 5 (a) providing a host cell which replicates and expresses an intron-free DNA sequence consisting essentially of a portion of the complement to any DNA sequence which hybridizes to the sequence of Figure 2 which encodes at least one epitope.
- (b) growing the host cell; and
- (c) recovering the GAD₆₅ polypeptide.

10

42. The isolated cDNA sequence of claim 31 which encodes at least one epitope for autoantibodies.

43. The isolated cDNA sequence of claim 42 comprising a cDNA
15 sequence encoding about the first 100 amino acids of the N-terminus of GAD₆₅.

44. A host cell transformed or transfected with the isolated cDNA sequence of claim 42.

20

45. A method of providing GAD₆₅ polypeptide having at least one epitope for autoantibodies to GAD₆₅ comprising:

- 25 (a) providing a host cell which replicates and expresses an intron-free DNA sequence consisting essentially of a portion of the complement to any DNA sequence which hybridizes to the sequence of Figure 3 which encodes at least one epitope.
- (b) growing the host cell; and
- (c) recovering the GAD₆₅ polypeptide.

GGGCGTGGGGGTGAGCCGAAGCAGCTTCCCCGAGCCACTCGGAGGCGACAGCGCCA
10 30 50
GACTAGCAGAACCCATGGCATCTCCGGCTCTGGCTTTTGGTCTTCCGATCTGAAGATG
70 90 110
S G D P E N P G T A R A W C Q V A Q K F
GCTCTGGGGATCCTGAGAACCCGGGAACAGCGAGAGCCTGGTGGCAGGTGGCCCAAAGT
130 150 170
T G G I G N K L C A L L Y G D S E K P A
TCACGGGCGGCATCGGAAACAAGCTATGCGCTCTGCTCTACGGAGACTCTGAGAAGCCAG
190 210 230
E S G G S V T S R A A T R K V A C T C D
CAGAGAGCGGGGAGCGTGACCTCGGGGCGCCACTCGGAAGGTGGCCTGCACCTGTG
250 270 290
Q K P C S C P K G D V N Y A L L H A T D
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310 330 350
L L P A C E G E R P T L A F L Q D V M N
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370 390 410
I L L Q V V V K S F D R S T K V I D F H
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430 450 470
Y P N E L L Q E Y N W E L A D Q P Q N L
ATTACCCCAATGAGCTTCTTCAAGAGTATAATTGGGAATTGGCAGACCAACCGCAAATC
490 510 530
E E I L T H C Q T T L K Y A I K T G H P
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550 570 590
R Y F N Q L S T G L D M V G L A A D W L
CCCGATATTTTAAATCAGCTGTCTACCGGATTGGATATGGTTGGATTAGCAGCAGATTGGT
610 630 650
T S T A N T N M F T Y E I A P V F V L L
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670 690 710
E Y V T L K K M R E I I G W P G G S G D
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730 750 770
G I F S P G G A I S N M Y A M L I A R Y
ATGGAATCTTTTCTCTGGTGGTGGCATCTCCAACATGTACGCCATGCTCATTGCCCGCT
790 810 830
K M F P E V K E K G M A A V P R L I A F
ATAAGATGTTTCCAGAAGTCAAGGAAAGGGGATGGCGCGGTGCCAGGCTCATCGCAT
850 870 890
T S E H S H F S L K K G A A A L G I G T
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910 930 950
D S V I L I K C D E R G K M I P S D L E
CAGACAGCGTATTCTGATTAAATGTGATGAGAGAGGGAATGATCCCATCTGACCTTG
970 990 1010
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1150 1170 1190
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1210 1230 1250
M M G V P L Q C S A L L V R E E G L M Q
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1270 1290 1310
S C N Q M H A S Y L F Q Q D K H Y D L S
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1330 1350 1370
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1390 1410 1430
L M W R A K G T T G F E A H I D K C L E
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1450 1470 1490
L A E Y L Y N I I K N R E G Y E M V F D
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1510 1530 1550
G K P Q H T N V C F W F V P P S L R V L
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1570 1590 1610
E D N E E R M S R L S K V A P V I K A R
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1630 1650 1670
M M E Y G T T M V S Y Q P L G D K V N F
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1690 1710 1730
F R M V I S N P A A T H Q D I D F L I E
TCTTCCGCTATCTCAAAACCTGCAAGCACTCAGCAAGACATTGACTTCTCATTTG
1750 1770 1790
E I E R L G Q D L *
AAGAAATCGAACGCTGGGACAAGATTGTAATCACTTTGCTCACCACAACTTTTCAGTTCT
1810 1830 1850
CTAGGTAGACAGCTAAGTTGTCAAACTGTGTAATGTATTTGTAGTTTGTTCAGAGT
1870 1890 1910
AATTCTATTTCTATATCGTGGTGTACAGTAGAGTCCAGTTTAAAA
1930 1950

Sim: M. Bunnell

M A S
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 70 90 110
 G T A R A W C Q V A Q K F T G G I G N K
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 130 150 170
 L C A L L Y G D A E K P A E S G G S Q P
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 370 390 410
 K S F D R S T K V I D F H Y P N E L L Q
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 430 450 470
 E Y N W E L A D Q P Q N L E E I L M H C
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 550 570 590
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 610 630 650
 M F T Y E I A P V F V L L E Y V T L K K
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 1630 1650 1670
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 1690 1710 1730
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 D L *
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 1930 1950 1970
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 1990 2010 2030
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 2050 2070 2090

Sim: M. Lunn

Gap Weight: 3.000 Average Match: 0.540
 Length Weight: 0.100 Average Mismatch: -0.396

Quality: 856.2 Length: 585
 Ratio: 1.464 Gaps: 0
 Percent Similarity: 97.436 Percent Identity: 96.068

4817.Pep x Hgt2.Pep

August 22, 1990 08:20 ..

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1 MASP GSGFWSFG SEDGSGDPENPGTARAWCQVAQKFTGGIGNKLCALLYG 50
1 MASP GSGFWSFG SEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLYG 50
51 DSEKPAESGGSVTSRAATRKVACTCDQKPCSCPKGDVNYALLHATDLLPA 100
51 DAEKPAESGGSQPPRAAARKAACACDQKPCSCSKVDVNYAFLHATDLLPA 100
101 CEGERP TLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150
101 CDGERP TLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150
151 DQPQNLEEILTHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200
151 DQPQNLEEILMHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200
201 NTNMF TYEIA PVFV LLEYVTLKQMR EII GWPGSGDGIFSPGGAISNMYA 250
201 NTNMF TYEIA PVFV LLEYVTLKQMR EII GWPGSGDGIFSPGGAISNMYA 250
251 MLIARYKMFPEVKEKGMAAVPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300
251 MMIARFKMFPEVKEKGMAALPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300
301 LIKCDERGKMIPSDLERRILEVRKQKGFVPFLVSATAGTTVYGAFDPLLAV 350
301 LIKCDERGKMIPSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLAV 350
351 ADICKKYKIWMHVDAAWGGGLMSRKHKWKLNGVERANSVTWNP HKMMGV 400
351 ADICKKYKIWMHVDAAWGGGLMSRKHKWKLNGVERANSVTWNP HKMMGV 400
401 PLQCSALLVREEGLMQSCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHV 450
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501 HTNVCFWYIPPSLRTLEDNEERMSRLSKVAPVVIKARMEYGTTHVSYQPL 550
551 GDKVNFFRMVISNPAATHQDIDFLIEETIERLGQDL 585
551 GDKVNFFRMVISNPAATHQDIDFLIEETIERLGQDL 585

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FIGURE 4

Sim 2' M. Burney

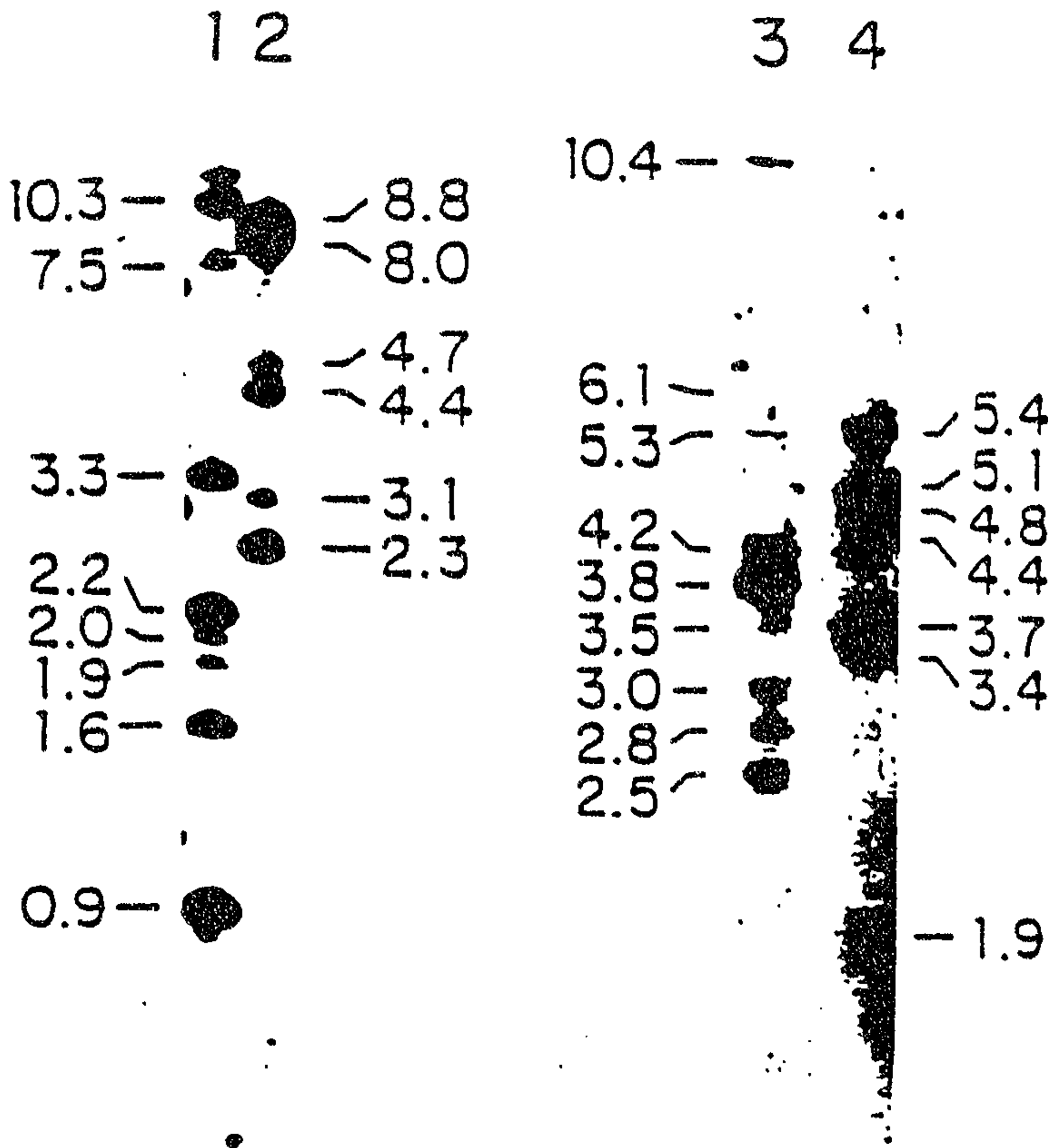


FIGURE 6



FIGURE 5

Sim. of Burns

1 2 3 4 5 6 7 8 9 10 11 12

2040555

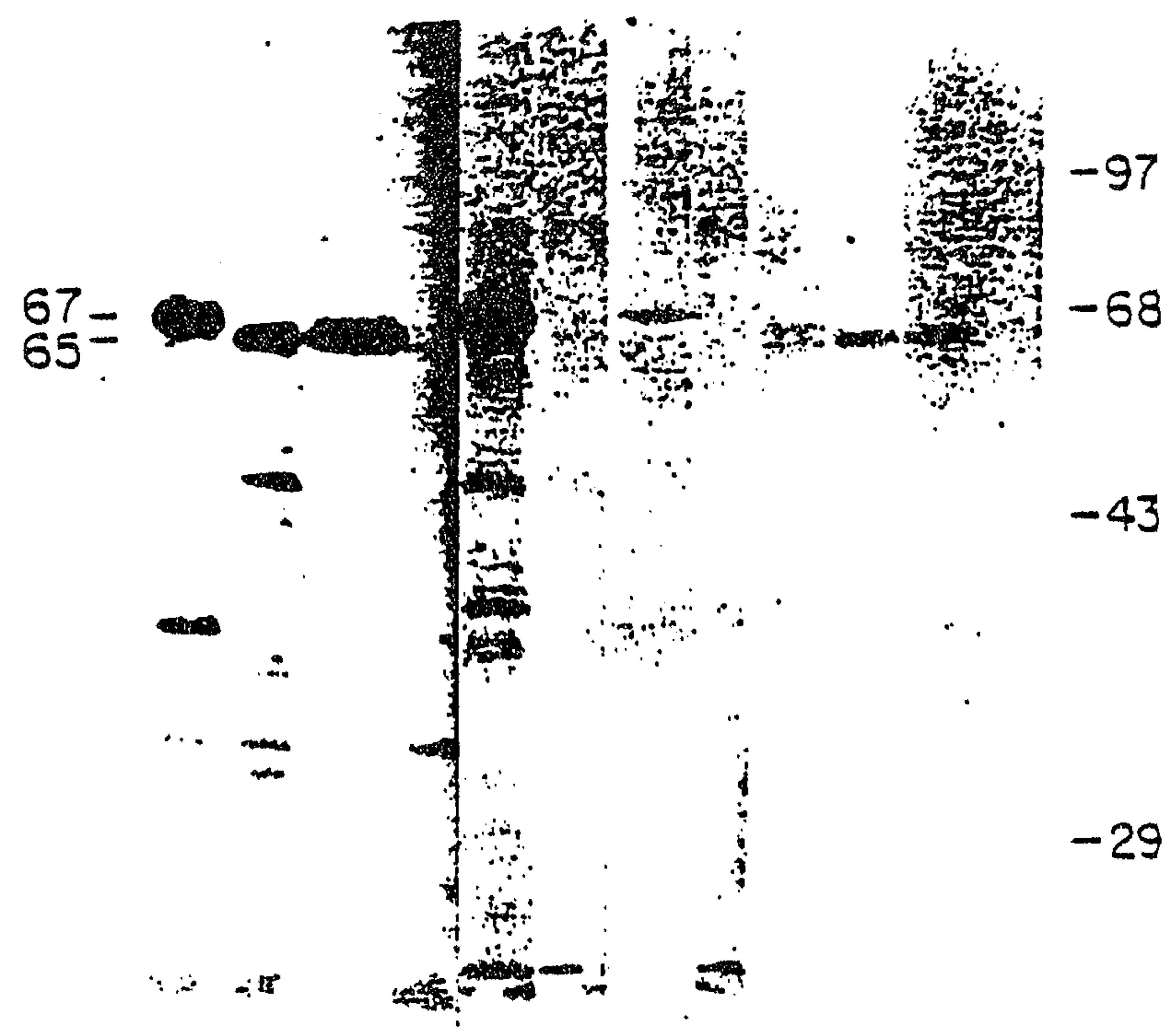


FIGURE 7

Sim. 3' of Curran