

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 March 2003 (06.03.2003)

PCT

(10) International Publication Number
WO 03/019197 A2

- (51) International Patent Classification⁷: **G01N 33/569**
- (21) International Application Number: PCT/IB02/03957
- (22) International Filing Date: 21 August 2002 (21.08.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
0120437.9 22 August 2001 (22.08.2001) GB
- (71) Applicant (*for all designated States except US*): **APODEMUS AB** [SE/SE]; Sibyllegatan 15, S-11442 Stockholm (SE).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **NIKLASSON, Bo** [SE/SE]; Grevgatan 38, S-114 53 Stockholm (SE). **LERNMARK, Åke** [SE/SE]; Birger Jarlsgatan 61, S-216 11 Limhamn (SE).
- (74) Agents: **HALEY, Jame, F., Jr.** et al.; Fish & Neave, 1251 Avenue of the Americas, New York, NY 10020 (US).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— *without international search report and to be republished upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 03/019197 A2

(54) Title: DIABETIC MODEL

(57) Abstract: The invention relates to an animal model for diabetes and a method for obtaining said animal model. The invention also relates to the uses of the animal model for screening for or testing compounds for treating or preventing diabetes symptoms. The invention further relates to an assay for determining an individual's susceptibility to developing diabetes. The invention also relates to nucleic acid molecules isolated from Ljungan virus and to polypeptides encoded by any portion of said nucleic acid molecule.

DIABETIC MODEL

[0001] This application claims priority under 35
U.S.C. § 119 from United Kingdom application number
0120437.9, filed August 22, 2001, incorporated herein
5 in its entirety by reference.

TECHNICAL FIELD OF INVENTION

[0002] The present invention relates to a method for
obtaining an animal model for diabetes. The present
10 invention also relates to the uses of the animal model
for screening for or testing compounds which affect
diabetic symptoms. The present invention also relates
to an assay for determining an individual's
susceptibility to developing diabetes.

15

Background of the Invention

[0003] Diabetes is a disease in which the body does
not produce or use insulin correctly. Insulin is a
hormone that is required to convert sugar, starches and
20 other food into energy needed for daily life. Insulin
is produced by the beta cells in the islets of
Langerhans in the pancreas. Partial or total loss of

- 2 -

these cells will result in partial or total loss of insulin production.

[0004] There are two major types of diabetes.

[0005] Type 1 diabetes is an autoimmune disease in which the body actually fails to produce any insulin. Type 1 disease most often occurs in children and young adults but can develop at any age. Type 1 diabetes is characterized by total loss of beta cells so that the patient requires insulin by injection. Type 1 diabetes accounts for 10-15% of all diabetes. Type 1 diabetes is strongly associated with auto-antibodies and this association has become part of the definition/classification of type 1 diabetes. Type 1 diabetes is discussed in greater detail below.

[0006] Type 2 diabetes is a metabolic disorder resulting from the body's inability to make enough, or properly use, insulin. It is the most common form of the disease. Type 2 diabetes accounts for 85-90% of diabetes.

[0007] The definitions of type 1 and 2 diabetes, however, are changing slowly. Auto-antibodies are found in type 2 diabetes patients and type 2 diabetes is found in increasing numbers in children. As a result, the traditional view of type 1 and 2 diabetes as two different diseases both resulting in increased blood glucose levels is shifting to the view that there is a large grey zone with patients in between the two extremes. This view is important when evaluating the usefulness of different animal models.

[0008] Both genetic and environmental factors are believed to be involved in the development of type 1 (insulin dependent) diabetes (for reviews see Leslie et al., *Diabetologia*, 42, 3-14, 1999; and Schranz et al.,

Diab. Metab. Rev., 14, 3-29, 1998). The HLA Class II region is the strongest genetic component, but other genes and loci have been implicated as contributing to a genetic predisposition to the disease (reviewed in Schranz et al., 1998 (*supra*)). Monozygotic twin studies show only 20-30% concordance of type 1 diabetes indicating a significant contribution of environmental factors (Kyvik et al., *BMJ*, 311, 913-7, 1995). The role of environmental factors is also supported by the fact that more than 85% of new onset patients do not have a first degree relative with the disease (Dahlquist et al., *Diabetologia*, 32, 2-6, 1989).

[0009] Worldwide, there is a large variation in the incidence of type 1 diabetes, ranging from more than 40 patients per 100,000 in Finland to 1-2 cases per 100,000 in Japan (Onkamo et al., *Diabetologia*, 42, 1395-403, 1999). Seasonal variation in incidence rate, together with serological studies, have suggested viral infections as a major environmental risk factor for type 1 diabetes (for reviews see Jun et al., *Diabetologia*, 44, 271-285, 2001; Rayfield et al., *Diab./Metab. Rev.*, 3, 925-57, 1987; and Vaarala et al., *Diabetes Nutr. Metab.*, 12, 75-85, 1999). Congenital rubella virus infection (Menser et al., *Lancet*, *i*, 57-60, 1978) or different members in the enterovirus genus are most often implicated as etiologic agents in diabetes development (Yoon, *Do Viruses Play a Role in the Development of Insulin-dependent Diabetes?*, 1991; Vaarala et al., 1999, (*supra*)). Signs of enterovirus infection during pregnancy (Dahlquist et al., *Diabetologia*, 32, 2-6, 1989; and Hyoty et al., *Diabetes*, 44, 652-657, 1995) and in some infants who developed islet cell autoantibodies and later type 1 diabetes

(Lonrot et al., *Diabetes*, 49, 1314-8, 2000) further supports this hypothesis. Both Coxsackie B and rota virus contain peptide sequences also found in the islet autoantigens glutamate decarboxylase (GAD65) (Kaufman et al., *J. Clin. Invest.*, 89, 283, 292, 1992), the tyrosine-phosphatase like protein IA-2 (Honeyman et al., *Diabetes*, 49, 1319-1324, 2000) or proinsulin (Rudy et al., *Mol. Med.*, 1, 625-33, 1995) suggesting that T lymphocytes recognizing viral antigens may potentially contribute to islet autoimmunity by cross-reactivity or molecular mimicry. Indeed, cross-reactive GAD65 and rubella virus peptides were recognized by T cells in type 1 diabetes patients (Ou et al., *Diabetologia*, 43, 750-62, 2000). Since T cell tests that predict type 1 diabetes are not yet available, standardized tests for GAD65, IA-2 or insulin autoantibodies are useful markers to predict type 1 diabetes (for a review see Gottlieb et al., *Arum. Rev. Med.*, 49, 391-405, 1998). Rota virus seroconversion was reported to be associated with increases in autoantibodies to GAD65, IA-2, and insulin suggesting that this virus infection may trigger or exacerbate islet autoimmunity in genetically susceptible children (Honeyman et al., 2000 (*supra*)). Coxsackie virus-induced diabetes in mice was also associated with the development of GAD antibodies (Gerling et al., *Autoimmunity*, 6 49-56, 1991). It is still controversial, however, whether viruses cause beta cell destruction directly by a cytolytic infection in the islets or indirectly by initiating autoimmunity (Vreugdenhil et al., *Clin. Infect. Dis.*, 31, 1025-31, 2000; and Kukreja et al., *Cell Mol. Life Sci.*, 57, 534-41, 2000).

[0010] Rodents are well-known reservoirs and vectors for viruses causing disease in humans. Puumala virus causing Nephropathia Epidemica (Myhrman, *Nordisk Medicinsk Tidskrift*, 7, 739-794, 1934; and Niklasson et al., *Lancet*, 1, 1012-3, 1984) is one example of an important human pathogen carried by bank voles. It has been demonstrated that the incidence rate of human Nephropathia Epidemica correlates with the vole population density during the previous year (Niklasson et al., *Am. J. Trop. Med. Hyg.*, 53, 134-40, 1995). More recently, statistical evidence suggests that type 1 diabetes in humans also tracks the 3- to 4- year population density cycles of the bank vole with a similar time lag (Niklasson et al., *Emerg. Infect. Dis.*, 4, 187-93, 1998). It also was observed that a high frequency of bank voles trapped in the wild and kept in the laboratory for studies of stereotypic behavior (Schoenecker et al., *Appl. Anim. Behav. Sci.*, 68, 349-357, 2000) develop symptoms of type 1 diabetes, i.e., polydipsia and glucosuria, at a high frequency.

[0011] Currently there are two main animal models of diabetes: the NOD (non obese diabetic) mouse and the BB (bio breeding) rat. Both models involve animals with insulin dependent diabetes. Both of the current models, however, fail to display important symptoms of human diabetes. The NOD mouse, for example, shows gender preferences that are opposite to the human disease (i.e., more females than males develop the disease), develops mild diabetes, requires a long time before developing ketoacidosis, and fails to develop autoantibodies to GAD65, 1A-2 or insulin. The disease is genetically controlled in the NOD mouse and the

cleaner the animal, the higher the frequency of diabetes.

[0012] The BB rat is also no ideal. The animals have lymphopenia controlled by an autosomal mutation on chromosome 4 and the development of autoantibodies in
5 inbred and specific pathogen free BB rats appears negligible. None of these BB rats develop diabetes in association with an infectious agent.

[0013] Thus, there is a need to develop an improved
10 method for obtaining an animal model which displays the features of diabetes for both research and therapeutic purposes.

SUMMARY OF THE INVENTION

[0014] The invention provides an animal model for
15 human diabetes and methods for producing it. The invention also provides methods for screening for or testing compounds which affect diabetic symptoms comprising use of the animal model. The invention further provides an assay for determining an
20 individual's susceptibility to developing diabetes.

BRIEF DESCRIPTION OF DRAWINGS

[0015] Figure 1 shows the histology of the pancreas in bank voles without diabetes (panels a and b as well
25 as e and f) and with diabetes (panels c and d as well as g and h). The histology of non-diabetic bank voles demonstrates well-defined islets of Langerhans surrounded by a conspicuous and delicate capsule. Diabetic bank voles have dramatic islet cytopathology
30 characterized by distinct vacuoles or fatty infiltration of the pancreatic islets. Hematoxylin and Eosin staining are shown in panels a-d while

immunostaining for insulin and glucagon are shown in panels e-h. The immunostained sections demonstrate that the cytopathology affected only insulin positive cells, which were lost resulting in a redistribution of glucagon immunoreactive cells. Size bars are indicated in each panel.

[0016] Figure 2 shows the histology of the pancreas of a diabetic bank vole demonstrating islet infiltration of mononuclear cells following Hematoxylin and Eosin staining.

[0017] Figure 3 shows the histology of the pancreas in bank voles without (panels a and b) and with diabetes (panels c and d) following immunostaining with the mouse 87-012 or 145L antiserum against Ljungan virus. The binding of the mouse antiserum was revealed with red vector staining. The sections were double stained with glucagon antiserum revealed with alkaline phosphatase and tetrazolium blue. The non-diabetic voles did not show binding of mouse anti-Ljungan virus antibodies while the immunostaining against glucagon stained cells in the periphery of the islets (panel a). The islets in diabetic bank voles showed varying degree of vacuolization or fatty infiltration of the pancreatic islets (panels b, c and d). The edges of these lesions are stained indicating the presence of Ljungan virus antigen. The glucagon immunostaining showed redistribution of cells that became more pronounced the greater the lesions.

[0018] Figure 4 shows that the bank voles have autoantibodies against islet cell autoantigens and against Ljungan virus *in vitro* translated antigens. Autoantibodies to GAD65 (panel a), IA-2 (panel b), insulin (panel c) as well as Ljungan virus *in vitro*

translated antigens are shown as in-house relative Units on a log scale (wherein a 1/25 dilution of standard serum is equal to 100 units/ml). Group A animals were caught and bled in the wild and only 4% had diabetes. Group B bank voles were captive and 33% of the animals shown had diabetes. The levels of GAD65 (p<0.0001), IA-2 (p<0.0001) and insulin (p<0.03) autoantibodies were increased in Group B compared to Group A bank voles. The autoantibody levels of both GAD65 and IA-2 were higher in diabetic as compared to non-diabetic Group B bank voles as indicated in the Figure. The data in panel d demonstrate that antibodies to Ljungan virus *in vitro* translated antigens were also increased in diabetic compared to non-diabetic bank voles. Data for individual bank voles are shown.

[0019] Figure 5 shows the sequence similarities and cross-reactivity between GAD65 autoantibodies and mouse or human anti-Ljungan virus antibodies. Sequence comparisons between the predicted amino acid sequence of Ljungan virus (serotype 87-012) and type 1 diabetes associated autoantigens are shown in panel a. The data compares the 87-012 Ljungan virus sequence and regions of potential molecular mimicry to GAD65, IA-2 and insulin. Areas of homology are boxed, with identical amino acids indicated by a dot, similar amino acids are boxed, and non-similar amino acids are plain type. Antibodies against Ljungan virus raised in mice (antiserum 87-012) showed cross-reactivity with human GAD65 (panel b). Radiobinding analysis to the 87-012 antiserum showed concentration-dependent binding of ³⁵S-labeled Ljungan virus *in vitro* translated antigen (x-x) and human (o-o) but not mouse (·-·) GAJD65. The

competition at half maximal binding of the 87-012 antiserum between binding of ³⁵S-labelled human GAD65 and unlabelled Ljungan virus antigen (x-x), human GAD65 (o-o) or human proinsulin (▼-▼) (panel c) demonstrates
5 displacement by unlabelled Ljungan virus *in vitro* translated antigens as well as by recombinant human GAD65. Fig. 5 d. shows binding of different ³⁵S-labeled antigens including Ljungan virus antigen (x-x), human GAD65 (o-o) or mouse GAD65 (·-·) to the type 1 diabetes
10 human serum #591 (panel d). Human and mouse GAD65 bind equally well and there are also significant levels of antibodies detecting the Ljungan virus *in vitro* translated antigens. Competition at half maximal binding to human serum 591 of ³⁵S-labelled Ljungan virus
15 antigen and cold Ljungan virus antigen (x-x), human GAD65 (o-o) or human proinsulin (▼-▼) (panel e) showed displacement of cold Ljungan virus *in vitro* translated antigens but not of cold GAD65 or proinsulin. All radioactivity values in cpm are mean values ± SEM for
20 3-5 experiments. The SEM bars are within the size of the symbols unless indicated.

[0020] Figure 6 shows the results of tests on sera from children with new onset type 1 diabetes indicating the presence of anti-Ljungan virus antibodies. Ljungan
25 virus antibodies were determined in two independent tests by either indirect immunofluorescence of cells used to propagate the virus or by the radioligand binding assay with Ljungan virus *in vitro* translated antigens. The radioligand binding assay correlated to
30 the indirect immunofluorescence test (p<0.001 at 95 confidence interval).

[0021] Figure 7 shows the nucleotide sequence of Ljungan virus 87-012.

[0022] Figure 8 shows the nucleotide sequence of Ljungan virus 145SL.

[0023] Figure 9 shows the nucleotide sequence of Ljungan virus 174F.

5

DETAILED DESCRIPTION OF THE INVENTION

[0024] According to a first embodiment of the present invention, there is provided a method for obtaining an animal model for human diabetes, comprising obtaining a mammal that has been determined to be infected with Ljungan virus.

[0025] The mammal may be any mammal including rodents such as rats, mice, hamsters, guinea pigs, rabbits, bank voles and field voles; cattle such as cows; cats; dogs; and non-human primates. Preferably the mammal is a rodent, a cat or a dog, more preferably the mammal is a rodent, most preferably the mammal is a bank vole.

[0026] It has been found that bank voles having type 1 diabetes are all infected with Ljungan virus and that the presence of Ljungan virus causes or at least contributes to the development of type 1 diabetes.

[0027] The term "Ljungan virus" as used herein refers to any Ljungan picornavirus as defined in International PCT patent application WO 98/11133, the disclosure of which is incorporated herein by reference. Preferably the Ljungan virus is Ljungan virus 87-012, the nucleotide sequence of which is shown in Figure 7; Ljungan virus 145SL, the nucleotide sequence of which is shown in Figure 8; or Ljungan virus 174F, the nucleotide sequence of which is shown in Figure 9.

[0028] The presence of Ljungan virus can be determined using any standard procedure including, but not limited to, virus isolation, detection of Ljungan virus antigen by ELISA or immunohistochemistry using antibody molecules having affinity for Ljungan virus or detection of Ljungan virus specific RNA sequences using PCR or by a labeled nucleic acid probe capable of specifically hybridizing to Ljungan virus nucleic acid. The presence of Ljungan virus also can be determined by detecting for the presence of Ljungan virus antibodies using a suitable test. Suitable techniques for determining the presence of Ljungan virus or anti-Ljungan virus antibodies are described in the examples below.

15 [0029] Use of the mammal obtained by the method according to the first embodiment of present invention as a model for human diabetes has a number of advantages over the prior art animal models of diabetes including:

20 a pathology that includes total destruction of the beta cells without affecting the surrounding pancreas tissue;
no or minor signs of inflammatory cells and only modest insulinitis; and
25 the presence of auto-antibodies used as markers for human type 1 diabetes (antibodies to GAD 65, IA-2 and insulin) in most bank voles obtained by the method according to the first embodiment of the present invention.

30 [0030] The fact that the mammal obtained using the method according to the first embodiment of the present invention has features that mimic the human disease

means that it closely represents the human disease and is therefore a particularly useful model of diabetes.

[0031] Preferably, the method according to the first embodiment of the present invention also comprises
5 determining whether the mammal has high blood glucose levels that can be reduced by insulin and signs of ketoacidosis.

[0032] Preferably, the mammal is a bank vole. The bank vole may be any species of bank vole. Preferably
10 the bank vole is *Clethrionomys glareolus*. The mammal can be male or female. The bank vole may be obtained from the wild or may be the progeny of a bank vole obtained from the wild. It is preferred that the bank vole obtained from the wild is obtained from Denmark,
15 Sweden or Finland. Alternatively, the bank vole may be a laboratory bred bank vole.

[0033] The term "diabetes" as used herein means type 1 or type 2 diabetes or diabetes having a combination of symptoms of both type 1 and type 2 diabetes. The
20 type of diabetes developed by the mammal will depend on the type of mammal. For example bank voles infected with Ljungan virus develop type 1 diabetes, whereas cats and dogs can develop type 1 or type 2 diabetes or diabetes having a combination of symptoms of both type
25 1 and type 2 diabetes. It is currently believed that diabetes in humans is not always type 1 or type 2 diabetes but that diabetes can fall somewhere between the two defined types wherein the individual has some symptoms of both type 1 and type 2 diabetes. The term
30 "diabetes" as used herein refers to diabetes characterized by high blood glucose levels that can be reduced by insulin and signs of ketoacidosis. The presence of auto-antibodies to at least one of GAD65,

IA-2 and insulin is an additionally preferred characteristic of type 1 diabetes according to the present invention. Additional features of diabetes such as hyperlipidemia, slowly progressive increase of hyperglycemia and variable glucosuria as well as symptoms of hyperphagia and obesity also may be present in addition to the characteristics of type 1 diabetes defined above in accordance with the situation in humans.

10 [0034] The term "high blood glucose levels" as used herein means blood glucose levels that are at least 1.5 times as high, more preferably at least 3 times as high and most preferably at least 5 times as high as the mean level of blood glucose found in the corresponding non-diabetic mammals. Non-diabetic mammals are mammals that do not show any symptoms of diabetes such as increased glucosuria. It is particularly preferred that a high blood glucose level is at least 150 mg/dl, more preferably at least 200 mg/dl.

20 [0035] The term "reduced by insulin" as used herein means that the high blood glucose levels can be reduced by the addition of insulin. Preferably the blood glucose levels can be reduced by about 30%, more preferably 60% and most preferably to approximately the level of a non-diabetic mammals by the addition of insulin. As those skilled in the art will appreciate, the reduction in blood glucose levels will vary depending on the amount of insulin given to the mammal.

25 [0036] Signs of ketoacidosis include nausea, vomiting, stomach pain, deep and rapid breathing, flushed face, dry skin and mouth, fruity breath odor, rapid and weak pulse, low blood pressure. Ketoacidosis

can be determined by the measurement of keton bodies in the blood or in plasma or serum.

[0037] The method according to the first embodiment of the present invention preferably additionally
5 comprises modulating the immune system of the mammal to facilitate the development of diabetes. The modulation can be by suppressing or enhancing the immune system. The immune system of the mammal can be modulated by any method including administrating immunosuppressing or
10 immunostimulating agents, altering the diet of the mammal or subjecting the mammal to stress. Preferably the immune system of the mammal is modulated by subjecting the mammal to stress. The mammal can be subjected to any form of stress that affects the immune
15 system of the mammal including keeping the mammal in a cage. In embodiments in which the mammal is a bank vole, it preferably is kept in a cage for at least 2 months, more preferably at least 3 months. Preferably the mammal is kept isolated in its own cage.

20 [0038] The present invention also provides the use a mammal infected with Ljungan virus as a model of diabetes.

[0039] The mammal used as a model of diabetes is preferably obtained by the method according to the
25 first embodiment of the present invention.

[0040] The mammal can be used as a model of diabetes in order to investigate the development and etiology of diabetes. The mammal can also be used to test
30 candidate compounds for their effects on symptoms of diabetes. In particular, a candidate compound can be administered to the mammal and the effects of the compound on symptoms of diabetes, such as blood glucose

levels, signs of ketoacidosis and glucosuria can be measured.

[0041] The mammal can also be used to screen for compounds having an effect on the development of
5 diabetes. Preferably the mammal is used to screen for compounds that prevent the development of, or reduce the symptoms of, diabetes.

[0042] The present invention also relates to the use of compounds identified in the above-described
10 screening in the manufacture of a composition for treating and/or preventing diabetes.

[0043] The present invention also relates to the use of cells obtained from mammals obtained by the method according to the first embodiment of the present
15 invention. The cells can be used in a variety of *in vitro* assays which are well known to those skilled in the art.

[0044] In a second embodiment of the present invention there is provided a method for producing
20 diabetes in a mammal comprising infecting the mammal with a Ljungan virus.

[0045] It has been found that mammals infected with Ljungan virus develop diabetes. The mammal infected with Ljungan virus can be used as a model of diabetes
25 whether or not symptoms of diabetes can be detected.

[0046] The mammal can be infected using any standard technique, including, but not limited to, parenteral routes such as intravenous injection and
intrapertoneal injection. Methods for determining the
30 necessary viral dose leading to the development of diabetes can be easily determined by those skilled in the art. In making such a determination, a number of factors are considered including the species of mammal,

the rate of viral replication, the route of infection, the age and sex of the mammal. Preferably about 1,000 infection units are given to the mammal.

[0047] The mammal infected with Ljungan virus may
5 develop type 1 or type 2 diabetes or diabetes having a combination of symptoms of both type 1 and type 2 diabetes.

[0048] The method according to the second embodiment of the present invention preferably additionally
10 comprises modulating the immune system of the mammal as described above with respect to the first embodiment of the present invention. Preferably the immune system of the mammal is compromised by subjecting the mammal to stress as described above with respect to the first
15 embodiment of the present invention subsequent to infection with the infectious agent.

[0049] By compromising the immune system of the mammal, it has been found that the animals develop diabetes more quickly. Without being bound to any one
20 theory, it is believed that the Ljungan virus can replicate at a faster rate leading to the development of diabetes in a shorter period of time in immune-compromised animals.

[0050] The present invention also provides the use
25 of a mammal infected with a Ljungan virus as a model of diabetes. Preferably the mammal is obtained by the method according to the second embodiment of the present invention.

[0051] The mammal infected with a Ljungan virus can
30 be used as a model to investigate the development and etiology of diabetes. The mammal also can be used to test candidate compounds for their effects on diabetes. In particular, a candidate compound can be administered

to a mammal infected with a Ljungan virus and the effects of the compound on symptoms of diabetes, such as blood glucose levels, signs of ketoacidosis, glucosuria, hyperlipidemia, a slowly progressive
5 increase in hyperglycemia, symptoms of hyperphagia, obesity and insulin resistance, can be measured.

[0052] The mammal infected with a Ljungan virus can also be used to screen for compounds having an effect on the development of diabetes. Preferably the mammal
10 is used to screen for compounds which prevent the development of, or reduce the symptoms of, diabetes.

[0053] The present invention also provides an assay for determining an individual's susceptibility to developing diabetes comprising analyzing a sample from
15 the individual in order to determine if the individual is infected with a Ljungan virus, wherein infection with a Ljungan virus indicates a greater susceptibility to developing diabetes.

[0054] It has been found that children with an
20 increased level of antibodies against Ljungan virus have type 1 diabetes (i.e., serologically positive for Ljungan virus infection). We have determined that a population of children with diabetes has a much higher frequency of being serologically positive for Ljungan
25 virus than in a population of healthy control children.

[0055] The presence of Ljungan virus can be determined using any standard procedure including immunohistochemistry using antibody molecules having
affinity for Ljungan virus or by using a labeled
30 nucleic acid probe capable of specifically hybridizing to Ljungan virus nucleic acid. Alternatively the presence of Ljungan virus can be determined by detecting the presence of anti-Ljungan virus antibodies

using a suitable test. Suitable techniques for determining the presence of Ljungan virus or anti-Ljungan virus antibodies are described in the examples below.

5 [0056] The present invention also provides a method of treating an individual who has developed diabetes or is susceptible to developing diabetes comprising administering an effective amount of a compound which prevents or reduces Ljungan virus-induced diabetes.

10 [0057] Compounds which prevent or reduce the effects of Ljungan virus include antibody molecules having affinity for Ljungan virus or any other anti-viral agents. Methods for producing suitable antibody molecules are well know to those skilled in the art.

15 [0058] The present invention also provides a method of vaccinating an individual against a Ljungan virus infection, thereby preventing, at least in part, the individual developing diabetes.

[0059] Vaccines of the invention may comprise any
20 antigenic portion of the Ljungan virus (e.g. a protein displayed on the surface of the virus) or by using an attenuated form of the Ljungan virus. Methods for producing vaccines based on antigenic components or attenuated forms of the virus are well known to those
25 skilled in the art and are described in a variety of literature know to those skilled in the art (see Textbook Field's Virology by David M. Knipe et al).

[0060] In another aspect, the invention includes
30 nucleic acid molecules isolated from Ljungan viruses or any portion thereof. In some embodiments, the nucleic acid is the one shown in any one of Figures 7, 8 or 9 or any portion thereof.

[0061] The invention further comprises a nucleic acid molecule encoding a Ljungan virus polypeptide, or fragments thereof. In some embodiments, the nucleic acid is operably linked to one or more expression
5 control sequences. In some embodiments, the nucleic acid molecule or fragment is incorporated into a vector. In some embodiments, the vector is an expression vector.

[0062] The invention also provides host cells
10 comprising a nucleic acid or vector of the invention. The host cell can be prokaryotic or eukaryotic. The choice of host cells for expressing a Ljungan virus polypeptide is well-known in the art.

[0063] The invention further provides methods for
15 producing a Ljungan virus polypeptide or fragment thereof comprising culturing host cells of the invention under conditions suitable for the expression of the polypeptide and recovering the polypeptide.

[0064] The invention further comprises a vaccine
20 comprising at least one Ljungan virus polypeptide or an immunogenic fragment thereof. In some embodiments, the vaccine comprises a plurality of Ljungan virus polypeptides or immunogenic fragments thereof. The Ljungan virus polypeptides can be from the same or
25 different strains of Ljungan virus. Vaccines comprising polypeptides from different strains are useful to prevent or inhibit infection by a broader range of Ljungan virus in conditions caused by Ljungan virus infection, including diabetes.

[0065] In some embodiments, Ljungan virus
30 polypeptides are a component of a multivalent vaccine that comprises one or more components from other pathogens, including human pathogens.

[0066] In some embodiments, a vaccine of the invention comprises an adjuvant. Methods for selecting an adjuvant for use in the vaccine are well-known to those of skill in the art.

5 [0067] The invention further provides an antibody that specifically binds Ljungan virus or a Ljungan virus polypeptide. In some embodiments, the antibody specifically binds one or more of the Ljungan virus polypeptides shown in Figures 7-9.

10

EXAMPLES

[0068] In order that this invention may be better understood, the following examples are set forth.

15 These examples are for the purposes of illustration only and are not to be construed as limiting the scope of the invention in any manner.

Materials and Methods

[0069] **Wild caught bank voles (Group A).** Group A
20 bank voles represent 101 animals from a single trapping session. These bank voles were tested at the trap for glucosuria and then euthanized. Heart-blood samples for blood glucose, ketosis, lipids and antibody analyses were taken immediately after the voles were
25 killed. Blood samples were either immediately analyzed for blood glucose and ketones or centrifuged for 25 minutes at 1,000xg and plasma stored at -30°C. Pancreas was dissected and fixed in 4% paraformaldehyde followed by ethanol before being embedded in paraffin.

30 [0070] **Voles caught in the wild and kept in the laboratory (Group B).** In two other trapping sessions, 163 voles were caught and transferred to the laboratory

as previously described (Schoenecker et al., Appl. Anim. Behav. Sci, 68, 339-347, 2000; Schoenecker et al., Appl. Anim. Behav. Sci., 68. 349-357, 2000). The animals were housed individually in small barren cages
5 of transparent plastic (13.5 x 16.0 x 22.5 cm) under conditions of minimum extraneous disturbance and with a twelve-hour light regime (8.00-20.00 h). The cages were supplied with a woodcutting bed, and food (standard rat chow) and water were available *ad*
10 *libitum*. Cage cleaning and body weight measurements were performed once every week. A portion of grain mixture was given when the cages were cleaned. Diabetes development was followed by measurements of water intake, glucosuria, and blood glucose and
15 ketonemia determined after bleeding from the retro orbital plexus. Polydipsic voles were characterized by >21 ml/day water intake compared with non-polydipsic voles for which daily intake did not exceed 12 ml.

[0071] Histological analysis and immunocyto-
20 **chemistry.** Standard hematoxylin and eosin staining was carried out on samples fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5 micron sections, and affixed to slides. Sections were deparaffinized, rehydrated, and stained for three minutes in Gill's
25 hematoxylin and for one minute in Eosin Y. Stained sections were dehydrated and mounted.

[0072] In the immunohistochemistry tests, pancreas fixed in 4% paraformaldehyde and embedded in paraffin were cut into 5 micron thick sections, affixed to
30 slides, deparaffinized and rehydrated. The sections were blocked for 30 min at RT in PBS containing 0.05% Tween 20 (Sigma, St. Louis, MO), 1% BSA (Sigma), 2% normal horse (in the case of staining for Ljungan virus

- 22 -

antisera) or 2% normal goat (in the case of insulin or glucagon staining) serum (Vector Laboratories, Burlingame, CA), and 4 drops/ml Avidin solution (Avidin/Biotin blocking kit, Vector Laboratories). The primary antibody was diluted in PBS with 0.05% Tween 20, 1% BSA, 2% normal serum, and 4 drops/ml Biotin solution (from Avidin/Biotin blocking kit, Vector Labs) to 1:100 (guinea pig anti-insulin and rabbit anti-glucagon, Zymed Laboratories, S. San Francisco, CA) or 1:500 (mouse Ljungan virus antiserum). Slides were exposed to the primary antibody solution for 60 minutes at room temperature or overnight at 4°C. Slides were then washed in PBS, incubated for 30 minutes at room temperature with a biotinylated secondary antibody (goat anti-rabbit IgG, goat anti-guinea pig IgG, or horse anti-mouse IgG (Vector Labs) diluted 1:500 in PBS, and washed again.. The slides were next incubated for 30 minutes at room temperature in alkaline phosphatase streptavidin conjugate (Vector Labs) at a 1:200 dilution, washed in PBS, and reacted with the Vector Red or Vector BCIP/NBT alkaline phosphatase substrate kit. Finally, slides were counter stained with methyl green, dehydrated, and mounted. All slides were coded and scored independently by two readers.

25 **[0073] Immunofluorescence assay for Ljungan virus antibodies.** Sera from children with type 1 diabetes and controls were tested for presence of antibodies to Ljungan virus using an indirect immunofluorescence test (IFT). A previously described IFT protocol (Niklasson et al., J. Infect. Dis., 155, 369-76, 1987) was used to test antibody titers. Briefly, spot slides were prepared by incubating virus in Green Monkey Kidney cells for 8-10 days. At signs of discrete cytopathic

effects (CPE), cells were removed from the flask with a rubber policeman and applied onto microscope slides, air dried, fixed in cold (4°C) acetone and stored at -70°C until used. The titer was determined after
5 incubating the serum, diluted in PBS, on the slides at 37°C for 1 h in a moist chamber and bound antibodies were detected by incubating FITC-conjugated goat anti-human IgG (Sigma, St Louis, MO) for 1 h at 37 °C. Patient and control sera was first tested at a 1:8
10 dilution using three Ljungan virus isolates (87-012, 145SL, 174F). Any sera scoring positive for any of the three isolates were titrated again using all three isolates separately. Patients and controls positive to one or several isolates at a titer of 32 or higher was
15 considered positive.

[0074] Radioligand binding assays for GAD65 and IA-2 antibodies. GAD65 and IA-2 antibodies were analyzed as described (Grubin *et al.*, *Diabetologia*, 37, 344-350, 1994; Hampe *et al.*, *J. Clin. Endocrinol. Metab.*, 85,
20 4671-9, 2000; Vandewalle *et al.*, *Diabetes Care*, 20, 1547-1552, 1997). GAD65 and IA-2 antibody levels were expressed in U/ml for GAD65 and IA-2 antibodies using the WHO/JDF standard (Mire-Sluis *et al.*, *Diabetologia*, 43, 1282-1292, 2000).

25 **[0075] Insulin autoantibodies (IAA).** IAA were measured using a method for small plasma/serum samples (Williams *et al.*, *Journal of Autoimmunity*, 10, 473-478, 1997). An in-house serum sample was used as the standard to express the data in arbitrary U/ml.
30 Recombinant human insulin (Novo Nordisk, Copenhagen, Denmark) was used to determine IAA specificity as described (Williams *et al.*, (*supra*), 1997).

[0076] **Radioligand binding assay for Ljungan virus antibodies.** We used the Ljungan virus cDNA (unpublished observation) in the coupled *in vitro* transcription translation assay as described for GAD65 (Grubin *et al.*, (*supra*), 1994; Hampe *et al.*, (*supra*), 2000). The Ljungan virus cDNA was translated into multiple components which were immunoprecipitated with Ljungan virus mouse and guinea-pig antisera (data not shown) as well as from serum for both non-diabetic and diabetic bank voles and new onset type 1 diabetic patients. The human 591 GAD65-positive serum (Mire-Sluis *et al.*, (*supra*), 2000) showed high binding and was used as an in-house standard to express antibody binding levels in arbitrary U/ml.

15 [0077] **Competition experiments.** Competition in binding between radioactive and cold antigens was carried out at half maximal binding of either the Ljungan virus 87-012 mouse antiserum or the 591 human standard serum found to be positive for antibodies against both Ljungan virus *in vitro* translated antigens and GAD65 (Mire-Sluis *et al.*, (*supra*), 2000). Competition for binding of ³⁵S-labeled Ljungan virus *in vitro* translated antigens was carried out with different concentrations of unlabeled Ljungan virus *in vitro* translated antigens, recombinant human GAD65 (DiamydAB, Stockholm, Sweden) or human proinsulin (Elli Lilly Company, Indianapolis, Indiana).

25 [0078] **Type 1 diabetes patients and controls.** A total of 53 children with a median age of 10.1 years (range 2.3-16.4 years of age) were diagnosed with type 1 diabetes at the St Göran Hospital and Astid Lindgren's Children's Hospital between 1992 and 1995. Within two days of diagnosis, blood samples were drawn

for antibody analysis. Healthy children (7 boys, median age 12.6 (7.8 - 16.8 years and 10 girls, median age 13.5 (6.7 - 16.6 years) were recruited from school classes in central Stockholm and children to personnel
5 at the hospital. All children were previously healthy and without present medication. The Ethics Committee at the Karolinska Institute, Stockholm, Sweden, approved the study.

[0079] **Bioinformatics.** To identify regions of high
10 local homology between the virus polyprotein and known diabetes autoantigens, we created a local database of GAD65, IA-2 and insulin sequences and ran stand alone BLAST using software from the NCBI (Altschul et al., Nucleic Acids Res., 25, 3389-402, 1997). Alignments
15 were compiled manually to align regions of similarity onto the Ljungan protein sequence using CLUSTALW to determine similarity between non-homologous residues.

[0080] **Statistics.** The frequency of diabetes in the different groups was analyzed by Fischer exact test or
20 Chi Square tests. Non-parametric tests were used to analyze differences in levels between groups. Spearman's Rank Correlation was used to examine possible correlation between different parameters.

25

Example 1

Obtaining Bank Voles having Type 1 Diabetes

Development of diabetes in trapped bank voles

[0081] Bank voles were trapped from May to November in a forest habitat on the island of Zealand, Denmark.
30 In different continuous trapping sessions of 30 days duration, 100 traditional live traps were set and inspected twice a day. Two groups of bank voles were

analyzed for diabetes and pancreas histology in addition to type 1 diabetes associated autoantibodies against insulin (Williams *et al.*, (*supra*), 1997), GAD65 (Grubin *et al.*, (*supra*), 1994), and IA-2 (Lan *et al.*,
5 DNA and Cell Biology, 13, 505-514, 1994) also known as ICA512 (Rabin *et al.*, J. Immunol, 152, 3183-3187, 1994) as well as antibodies against Ljungan virus (Niklasson *et al.*, Virology, 255, 86-93, 1999). Group A bank voles represents 101 trapped bank voles that were
10 euthanized in the forest for immediate examination of blood glucose, glucosuria, body weight, pancreas histology and antibodies. Group B represents 67 bank voles that were trapped and kept in the laboratory for one month as previously described (Schoenecker *et al.*,
15 Appl. Anim. Behav. Sci., 68, 349-357, 2000). An additional group of 54 animals were examined in Stockholm for insulin sensitivity and pancreas histology before and after diabetes development.

[0082] The data in Table 1 shows the occurrence of
20 diabetes in the two groups of bank voles. In the Group A bank voles (n=101), four female animals were found positive for glucosuria and blood glucose values of 215, 302, 313 and 340 mg/dl, respectively, In the remaining bank voles, the mean blood glucose \pm S.D. was
25 normally distributed at 101 ± 28 mg/dl. The body weight of the trapped voles from group A ranged from 8.5 - 28.4g, the mean value \pm S.D. was 19 ± 5 g. Occasional hyperglycemic and glucosuric bank voles may therefore be trapped in the wild. Whether these four animals had
30 stress-induced hyperglycemia or overt diabetes remains to be established. These results in the Group A bank voles differ markedly from the 67 Group B bank voles that were trapped and kept in standard laboratory mouse

cages for one month before they were tested for diabetes. We observed that 22/67 (33%) of these Group B bank voles had a blood glucose above 200 mg/dl, the range being 211-540 mg/dl. As many as 18/22 (82%) had ketones and were polydipsic. Gender differences are common in both humans (Harris, Diabetes in America, (ed. Harris, M. I.) (National Institutes of Health, Bethesda, 1995) and in animal models of diabetes as well as in captured wild rodents that develop non-insulin dependent diabetes when fed laboratory chow (for a review see (Shafir et al., Diabetes Metab. Rev., 8, 179-208, 1992). The bank voles we captured were also fed laboratory chow but they were not only glucosuric and hyperglycemic but also positive for ketonuria, ketonemia and hyperlipidemia, all suggestive of type 1 diabetes (data not shown). An insulin sensitivity test (Actrapid, Novo Nordisk, Copenhagen, Denmark) was also carried out in 16 randomly selected Stockholm bank voles to exclude diabetes due to insulin resistance. At 60 minutes following insulin, four animals with blood glucose levels above 200 mg/dl experienced 30% decrease in blood glucose, four animals with blood glucose at 120-200 mg/dl showed 60% decrease while eight animals with blood glucose <120 mg/dl showed a 40% decrease in blood glucose. These data indicate that bank voles with varying blood glucose levels are insulin sensitive. We therefore next examined the pancreas histology in non-diabetic and diabetic bank voles to test if the classification of type 1 diabetes was supported by a loss of beta cells.

[0083] **Table 1. The frequency of diabetes in wild caught bank voles and in bank voles kept in the laboratory.**

- 28 -

	Group of bank voles	
	A. Analyzed at trap	B. Trapped and captive
N	101	67
M/F ratio	42/59	29/38
Blood glucose (mg/dl)		
Non-diabetic	101±27	86±24
Diabetic	293±54	346±88
Diabetes n (%)	4 (4%)	22 (33%)
M/F ratio	0/4	14/8

Mean values ± S.D are shown.

Bank voles develop type 1 diabetes because of a specific loss of beta cells.

5 **[0084]** The pancreas of all 101 Group A bank voles showed normal islets as did those of non-diabetic Group B bank voles (Fig. 1). The four hyperglycemic Group A bank voles had no appreciable islet lesions. Immunostaining with insulin and glucagon antibodies

10 showed a normal islet cell distribution with beta cells located in the center surrounded by glucagon-positive cells (Fig. 1a and 1b). In dramatic contrast, Group B bank voles with diabetes had an almost complete loss of centrally located insulin-positive cells that were

15 replaced by prominent vacuolization or fatty infiltration (Fig. 1c and 1d). Islets with infiltrating mononuclear cells were occasionally observed (Fig. 2) but insulinitis was conspicuously absent in the majority of the bank voles. The beta

20 cell destruction was unique to bank voles with diabetes and indicate that the animal should be classified as having type 1 diabetes.

[0085] In order to evaluate whether the Ljungan virus was associated with the islet beta cell lesion we next immunostained the pancreas sections with high titer mouse antisera against Ljungan virus (Niklasson *et al.*, (*supra*), 1999). We used antisera to two distinct Ljungan virus isolates, 87-012 and 145SL and, as controls, eight different antisera prepared with the same procedure against Rift Valley Fever virus, Ockelbo virus, Langat virus and Sindbis virus. Both the 87-012 and the 145SL Ljungan virus antisera at dilution of 1:4000 or higher immunostained islets in diabetic but not in non-diabetic bank voles (Fig. 3) visualizing the presence of Ljungan virus antigen in affected islets. None of the control sera showed immunostaining at a dilution of 1:500 or higher. Furthermore, an analysis of Stockholm bank voles euthanized with variable blood glucose levels after 2-3 months of captivity revealed that the severity of beta cell loss was gradual (Fig. 3 panel b, c and d). Also in these apparently early lesions, a mononuclear cell infiltration was conspicuously absent. Without being bound to any one theory, it is submitted that the beta cell-specific destruction in association with immunoreactive virus antigen suggests that the Ljungan virus might have had a lytic effect on the beta cells, perhaps accelerated by the stress of bringing the bank voles into captivity. Although mononuclear cell infiltration was not a prominent feature of the beta cell destruction it cannot be excluded that Ljungan virus beta cell lysis results in autoantigen presentation that takes place in lymph nodes draining the pancreas or by antigen presenting cells in or around the islets. We therefore next examined the possibility that the development of

diabetes was associated with autoantibodies to the islet autoantigens GAD65, IA-2 or insulin. Autoantibodies to these autoantigens predict type 1 diabetes in humans (reviewed in Schranz *et al.*,
5 (*supra*), 1998 and Leslie *et al.*, (*supra*), 1999) but not in the NOD mouse or BB rat models of type 1 diabetes (Bach *et al.*, *Endocrine Rev.*, 15, 516-542, 1994). The possible presence of autoantibodies to these autoantigens would further support the hypothesis that
10 the bank voles developed type 1 diabetes.

Bank vole diabetes is associated with autoantibodies to GAD65 and IA-2.

[0086] Standardized radioligand-binding assays that
15 detect autoantibodies to GAD65 (Grubin *et al.*, (*supra*), 1994; Hampe *et al.*, (*supra*), 2000), IA-2 (Kawasaki *et al.*, *Diabetes*, 45, 1344-9, 1996; Vandewalle *et al.*, (*supra*), 1997) and insulin (Williams *et al.*, (*supra*), 1997) were used to analyze serum samples from available
20 animals of both Group A and Copenhagen group B bank voles (Fig. 4). Compared to Group A animals, GAD65 ($P<0.001$) but not IA-2 or insulin autoantibodies were increased non-diabetic Group B bank voles. More importantly, however, diabetic group B bank voles had
25 higher GAD65 ($P<0.001$), IA-2 ($P<0.001$) and insulin ($P<0.0346$) autoantibody levels than the non-diabetic voles (Fig. 4). The increased levels of GAD65, IA-2 and insulin autoantibodies further indicates that diabetes in these bank voles should be classified as
30 type 1 diabetes.

[0087] Since Ljungan virus antigen was demonstrated in the islets of diabetic bank voles (Fig. 3) we next determined whether antibodies to Ljungan virus antigens

were associated to GAD65 and IA-2 autoantibodies. A radioligand binding assay, similar to the GAD65 and IA-2 autoantibody assays (Grubin et al., (*supra*), 1994) was developed with ³⁵S-labelled virus antigens generated
5 by coupled *in vitro* transcription and translation using the T7 promoter of the Ljungan virus cDNA (unpublished results). Although the Group A bank vole sera showed a wide range of antibody levels against Ljungan virus antigen (Fig. 4), the mean levels of Ljungan virus
10 antibodies in the non-diabetic Group B bank voles were significantly increased ($P<0.001$). In group B bank voles, the levels of Ljungan virus antibodies were higher in diabetic than non-diabetic animals ($P=0.0015$). Since the diabetic Group B bank voles also
15 showed increased levels of GAD65, IA-2 and insulin autoantibodies, we next tested if they were related to Ljungan virus antibody levels. In the diabetic Group B bank voles, antibody levels against Ljungan virus antigens correlated with levels of GAD65 ($P<0.0001$),
20 IA-2 ($P<0.0001$) and insulin ($P<0.03$) autoantibodies. These associations suggest that Ljungan virus infection may induce an immune response that will also include beta cell autoantigens. Without being bound to any one theory, there seems to be two possibilities. The first
25 possibility is that beta cell destruction is leading to autoantigen presentation in draining lymph nodes; the second possibility is that autoantibodies are formed due to molecular mimicry between virus and the autoantigen. The latter hypothesis was tested by
30 comparing the predicted amino acid sequences of the Ljungan virus cDNA with those of GAD65, IA-2 and insulin.

Ljungan virus molecular mimicry to islet autoantigens

[0088] The comparison between the Ljungan virus amino acid sequence predicted from the cDNA and the GAD65, IA-2 and insulin sequences revealed several potential regions of sequence similarities (Fig. 5a). We searched 1514 picornavirus proteins in Genbank's viral taxonomy at NCBI using stand-alone BLAST and found that these homologies were exclusively found in parechoviruses (echovirus 22/23) isolates (data not shown). The regions indicated for GAD65, 237-241 and 449-452 have been implicated in the middle and C-terminal GAD65 autoantibody binding sites (Schwartz et al., J. Mol. Biol., 287, 983-999, 1999). While the 561-569 is outside, the 964-976 sequence is within reported autoantibody binding sites for type 1 diabetes associated IA-2 autoantibodies (Leslie et al., (supra), 1999). The most interesting significant relationship was between the Ljungan virus antigen and the 45-54 insulin since the 45-54 homology maps to the insulin active site (Steiner et al., Diab. Care, 13, 600-609, 1990). Since antibody levels against Ljungan virus *in vitro* translated antigens correlated to levels of both GAD65 and IA-2 autoantibodies in the diabetic Group B bank voles and because of the significant sequence similarities (Fig. 5a), we next tested whether Ljungan virus antisera would immunoprecipitate any of the islet autoantigens. While labeled mouse GAD65 was not recognized, the mouse Ljungan virus polyclonal antiserum 87-012 (Niklasson et al., (supra), 1999) was capable of immunoprecipitating human GAD65 (Fig. 5b), indicating significant epitope specificity (Hampe et al., (supra), 2000). The Ljungan virus *in vitro* translated antigens immunoprecipitated by the 87-012

Ljungan virus antiserum was reciprocally displaced by both cold Ljungan virus *in vitro* translated antigens and human GAD65 but not by human proinsulin (Fig. 5c). The human type 1 diabetes GAD65 antibody-positive serum #591 showed concentration dependent immunoprecipitation of Ljungan virus *in vitro* translated antigens and both human and mouse GAD65 (Fig 5d). Cold Ljungan virus *in vitro* translated antigens but not GAD65 nor proinsulin, displaced the immunoprecipitation of Ljungan virus *in vitro* translated antigens by the human serum (Fig. 5e). These observations support the possibility of antibody cross-reactivity due to molecular mimicry between Ljungan virus antigen and GAD65 in mice inoculated by Ljungan virus.

15

Example 2

Individuals Infected with Ljungan Virus are Susceptible to Developing Diabetes

[0089] We tested to see if new onset type 1 diabetes children had Ljungan virus antibodies by both standard immunofluorescence and radioligand binding assay with Ljungan virus cDNA *in vitro* translated antigens.

Children with type 1 diabetes have Ljungan virus antibodies

[0090] The commonly used indirect immunofluorescence virus antibody test was first compared to the radioligand binding assay for Ljungan virus antigen antibodies (Fig. 6). There was a significant correlation (Spearman Rank Sum correlation) between the two assays ($P < 0.001$). Compared to the 17 healthy control children, the children with new onset Type 1 diabetes had increased levels of Ljungan virus

30

antibodies scored in the immunofluorescence assay ($P < 0.001$) (Fig. 6). These data indicate that children with new onset diabetes may have been exposed to Ljungan virus.

5

Discussion

[0091] The examples provide evidence that wild caught bank voles may develop type 1 diabetes associated with specific beta-cell destruction, insulinitis and autoantibodies to GAD65 and IA-2. Our
10 observation that it was possible to detect Ljungan virus antigen in affected pancreatic islets showing gradual destruction and end-stage fatty degeneration also indicates that this virus causes or at least contributes to the loss of beta cells. In addition,
15 the diabetic bank voles had increased levels of antibodies to Ljungan virus cDNA *in vitro* translated virus antigens. The levels of these antibodies were also found to correlate to the levels of autoantibodies to both GAD65 and IA-2. These data indicate that
20 diabetes observed in both captured bank voles and in bank voles born to captive animals represents type 1 diabetes. Although the histology of the islets in diabetic voles may be consistent with an acute lytic effect and presence of viral antigen, it cannot be
25 excluded that beta cells may also have been lost by an ensuing T cell or antibody-mediated cellular toxicity. Also consistent with type 1 diabetes were our observations that islet beta cells were specifically lost. The prominent vacuolization or fatty
30 infiltration seems unique to the bank voles diabetes and differ from other virus causing diabetes in rodents where insulinitis is seen more often. In particular, the rare occurrence of islets infiltrated with mononuclear

cells suggest that the bank vole islet lesion is less associated with insulinitis compared to other diabetogenic virus (Jun *et al.*, (*supra*), 2001; Rayfield *et al.*, *Diabetes*, 27, 1126-1140, 1978; Vaaralae *et al.*, (*supra*), 1999).

[0092] Diabetes in bank voles was first described during a study of stereotypic behavior in bank voles (Schoenecker *et al.*, *Appl. Anim. Behav. Sci.*, 68, 349-357, 2000) When captured in the wild, brought to the laboratory to be kept in standard laboratory mouse cages, and fed laboratory chow, bank voles developed polydipsia and glucosuria. Diabetes was detected in 4/101 Group A animals that were euthanized in the forest at the site of the trap which was different from the 22/67 bank voles kept in the laboratory. Our data suggest that the diabetes symptoms in these animals fulfill current classification criteria for autoimmune type 1 diabetes in humans (Mellitus *et al.*, *Diabetes Care*, 20, 1183-1197, 1997). The diabetic bank voles sustain hyperglycemia, ketonemia, ketonuria, hyperlipidemia and weight loss, all criteria that are consistent with type 1 diabetes classification. In addition, the diabetic bank voles had increased levels of both GAD65, IA-2 and insulin autoantibodies. These autoantibodies predict type 1 diabetes in humans (Verge *et al.*, *Diabetes*, 45 , 926-933, 1996); Bingley *et al.*, *Diabetes Care*, 22, 1796-801, 1999) and confirm the type 1 diabetes classification.

[0093] The short period of time it took for bank voles to develop diabetes after capture, and the complete islet beta cell destruction in diabetic voles associated with positive immunostaining for Ljungan virus antigen suggest that acceleration of an existing,

low-level viral infection may induce disease. While the exact mechanism of such a process remains unclear, our initial studies of diabetic bank voles indicate that stress is involved in diabetes development. Early
5 experimental stress may lead to increases in adult adrenocortical stress responses (King *et al.*, *Horm. Behav.*, 36, 79-85, 1999) and such stress responses may act as a stimulus of virus replication in beta cells. This speculation is supported by the observation that
10 stress induced by swimming increased the frequency of diabetes in our wild caught bank voles (data not shown). Stress has also been implicated in human type 1 diabetes since negative life events increased the risk for childhood type 1 diabetes (Thernlund *et al.*,
15 *Psychological Stress and the Onset of IDDM in children*, 18, 1995; Hagglof *et al.*, *Diabetologia*, 34, 579-83, 1991; Dahlquist *et al.*, *Diabetologia*, 34, 757-762, 1991). Similar relationships could be relevant to our bank voles and therefore aid in understanding the
20 etiology of diabetes.

[0094] It is now possible to identify other mechanisms that induce Ljungan virus-associated diabetes in bank voles and to conduct studies on intervention and protection, for example by anti-viral
25 agents or reducing responses to stress.

[0095] It has also been found that very high Ljungan virus antigen antibody levels are observed in some of the non-diabetic Group A bank voles. This may reflect a neutralizing and protective Ljungan virus immune
30 response with implications for future vaccination approaches.

[0096] Taken together we have demonstrated, first, that bank voles develop diabetes that fulfills the

criteria for type 1 diabetes: diabetic animals showed persistent hyperglycemia associated with weight loss, ketosis and hyperlipidemia (data not shown) as well as insulin dependence associated with specific beta-cell
5 destruction and insulinitis. Second, diabetic voles had increased levels of autoantibodies to GAD65 and IA-2, and that these autoantibodies correlated to Ljungan virus antigen antibodies. Third, the association between Ljungan virus and bank vole diabetes was
10 supported by the presence of Ljungan virus antigen detected by immunocytochemistry in affected diabetic bank vole islets. Fourth, there was significant molecular mimicry between the Ljungan virus polyprotein and GAD65, IA-2, and insulin islet autoantigens,
15 illustrated by GAD65 cross reactivity of high titer mouse and guinea-pig Ljungan virus antisera. Finally, a relationship between Ljungan virus infection and human type 1 diabetes was indicated by increased levels of Ljungan virus antibodies in children with newly
20 diagnosed type 1 diabetes.

Example 3

Data on diabetes mellitus in other mammals than bank voles

25 1. Field Voles (*Microtus agrestis*)

[0097] The field vole develops clinical diabetes with symptoms of polydipsia and polyuria identical to bank voles. Ljungan virus has been isolated from field voles with type 1 diabetes trapped in Sweden. It is
30 likely that field voles are just as good an animal model for type 1 diabetes as the bank vole.

2. Cats

[0098] Between 1 in 50 - 500 will develop the disease in a lifetime. The clinical disease mimics in some animals type 1 diabetes and in some animals type 2
5 diabetes. Amyloid deposits localized to the islets of Langerhans are typical of type 2 diabetes mellitus. However, diabetic cats most commonly have pancreatic islet destruction associated with pancreatic amyloidosis and are insulin deficient like type 1
10 diabetes. The disease occurs in all ages of the cats but the majority of diabetes affect older cats (Westermarck et al., PNAS USA, 84, 3881-5, 1987; Johnson et al., Veterinary Pathology, 22(5):463-8, 1985; and Yano et al., Veterinary Pathology, 18(3) : 310-5,
15 1981). We have investigated the pancreas tissue of diabetic type 1 and type 2 as well as normal cats using immunohistochemistry (IHC). As in the bank voles Ljungan virus could be detected in the destroyed islets of the pancreas in the diabetic type 1 and 2 cats but
20 not in the normal cats (Bo Niklasson unpublished observations). This is very strong evidence suggesting that Ljungan virus cause diabetes in cats.

3. Dogs

25 [0099] Between 1 in 50 - 500 will develop the disease in a lifetime. A review of the literature has shown that over half of the documented diabetic dogs are type I diabetes. However, type 2 is also common in dogs with obesity (Stogdale L. Cornell Veterinarian.
30 76(2):156-74, 1986). We have investigated the pancreas tissue of diabetic type 1 and type 2 as well as normal cats using immunohistochemistry (IHC). As in the bank

- 39 -

voles Ljungan virus could be detected in the destroyed islets of the pancreas in the diabetic type 1 and 2 dogs but not in the normal dogs (Bo Niklasson unpublished observation). This is very strong evidence
5 suggesting that Ljungan virus caused the disease.

4. Guinea Pigs

[0100] Spontaneous diabetes mellitus in guinea pigs, parallels in many ways the syndrome known as juvenile
10 diabetes mellitus in man: elevated blood glucose levels; reproductive dysfunction in the female; degranulation and severe cytoplasmic vacuolation of beta cells, severe fatty degeneration of acinar cells, and hyperplasia of the islets of the pancreas; and a
15 high frequency of abnormal pancreatic secretions (Lang *et al.*, *Diabetes*, 25(5):434-43, 1976). The severity of pathologic changes in the pancreatic islets parallel, in general, the severity of the clinical symptoms. The other clinical parameters of note are elevated serum
20 triglycerides, normal serum but elevated aortic cholesterol, and absence of ketonemia or ketonuria. Microangiopathy, another characteristic of juvenile diabetes mellitus in man was demonstrated as a significant increase in the thickness of the basal
25 membranes in peripheral capillaries. A glomerular lesion encountered in some of the diabetic guinea pigs was shown to be similar to the glomerular sclerosis seen in human diabetics. Although a definitive etiologic agent was not identified, the disease was
30 clearly contagious in origin.

5. Rabbits

[0101] Spontaneous diabetes mellitus has been observed in a female New Zealand white rabbit. Three groups of animals could be identified. Some animals had overt diabetes characterized by fasting hyperglycemia and depressed intravenous glucose stimulated serum insulin levels (Conaway *et al.*, Clinical & Experimental, 30(1):50-6, 1981). This abnormality is seen between 1 and 3 years of life. Another group of animals developed abnormal glucose disposal with normal or slight elevations in fasting serum glucose levels. Glucose stimulated insulin levels are also significantly lower in the rabbits with abnormal glucose disposal. The remaining animals exhibit no apparent abnormalities of glucose metabolism. Despite marked increases in serum and urinary glucose, only mild ketonemia was observed. The relatively late onset of diabetic symptoms, lack of obesity, severe hyperglycemia, and depressed insulin secretion without ketoacidosis make this a model with many of the characteristics of insulin responsive diabetes as seen in non-obese human adults.

6. Hamster

[0102] Chinese hamsters spontaneously develop diabetes mellitus and cardiomyopathy. The diabetic hamsters, shows body weight loss, hyperglycemia (mean fasting plasma glucose 402 mg/dl), hypoinsulinemia, hyperlipidemia and ketonemia. The diabetic hamsters showed reduced activities of cytoplasmic glycolytic key enzymes: hexokinase, pyruvate kinase and phosphofructokinase; increases in cardiac glycogen and glucose-6-phosphate contents; and a 40% decrease in cardiac ATP content, indicating decreased energy

production. An accumulation of myocardial triglyceride and cholesterol was found in the diabetic hamsters (Eto *et al.*, *Diabetes Research & Clinical Practice*, 3(6):297-305, 1987).

- 5 [0103] Syrian hamsters infected with rubella virus passaged in beta-cells also develops diabetes that closely parallels the diabetes observed with congenital rubella (Rayfield *et al.*, *Diabetes*, 35(11): 1278-81, 1986). The hamsters develop hyperglycemia and
- 10 hypoinsulinemia, which are sustained throughout the 15 week study period. A mononuclear infiltration of the islets, isolation of rubella virus from whole pancreas, the presence of viral antigen in beta-cells by immunofluorescence localization, and cytoplasmic islet
- 15 cell antibodies (40%) are demonstrated. These data suggest that an autoimmune process and diabetes develop after rubella virus infection in neonatal hamsters. This model may uncover the precise mechanism by which rubella virus induces similar disease in humans.
- 20 [0104] All documents referred to in the above description are incorporated herein by reference.

What is Claimed is:

1. A method for obtaining an animal model for human diabetes, comprising obtaining a mammal and determining whether the mammal is infected with a Ljungan virus.

2. The method according to claim 1, wherein the method additionally comprises determining whether the mammal has high blood glucose levels that can be reduced by insulin and signs of ketoacidosis.

3. The method according to claim 1 or claim 2, wherein the method additionally comprises testing for the presence of autoantibodies to at least one of GAD65, IA-2 and insulin.

4. The method according to any one of claims 1 to 3, wherein the method additionally comprises modulating the immune system of the mammal to facilitate the development of diabetes.

5. The method according to claim 4, wherein the immune system of the mammal is affected by subjecting the mammal to stress for at least about 2 months.

6. The method according to claim 5, wherein the mammal is subjected to stress by keeping it in a cage.

7. The method according to any one of the previous claims, wherein the mammal is a rodent.

8. The method according to any one of the previous claims, wherein the mammal is a bank vole.

9. The method according to claim 8, wherein the bank vole is *Clethrionomys glareolus*.

10. The method according to claim 8, wherein the bank vole is obtained from the wild or is the progeny of a bank vole obtained from the wild.

11. The method according to claim 10, wherein the bank vole is obtained from Denmark, Sweden or Finland.

12. A method for using a mammal having high blood glucose levels that can be reduced by insulin and signs of ketoacidosis as a model of diabetes.

13. The method according to claim 12, wherein the mammal was obtained by the method according to any one of claims 1 to 10.

14. The method according to claim 12 or claim 13, wherein the mammal is used to screen for compounds having an effect on symptoms of diabetes.

15. The method according to claim 14, wherein the mammal is used to screen for compounds which prevent or reduce the symptoms of diabetes.

16. A method for using compounds identified by the method according to claim 15 in the preparation of a composition for treating and/or preventing diabetes.

17. A method for treating a mammal comprising the step of infecting the mammal with a Ljungan virus and the mammal developing diabetes.

18. The method according to claim 17, additionally comprising suppressing the immune system of the mammal.

19. The method according to claim 18, wherein the immune system is suppressed by subjecting the mammal to stress subsequent to infection with the infectious agent.

20. The method according to any one of claims 17 to 19, wherein the mammal is a cat, dog or bank vole.

21. The method according to any one of claims 17 to 19, wherein the mammal is a bank vole.

22. A method of using a mammal infected with a Ljungan virus as a model of diabetes.

23. A method of using a mammal obtained by the method according to any one of claims 17 to 21 as a model of diabetes.

24. The method according to claim 22 or claim 23, wherein the mammal is used to screen for compounds having an effect on symptoms of diabetes.

25. The method according to claim 24, wherein the mammal is used to screen for compounds which prevent or reduce the symptoms of diabetes.

26. A method for using compounds identified by the method according to claim 25 in the preparation of a composition for treating and/or preventing diabetes.

27. An assay for determining an individual's susceptibility to developing diabetes comprising the steps of analyzing a sample from the individual in order to determine if the individual is infected with a Ljungan virus, wherein infection with a Ljungan virus indicates a greater susceptibility to developing diabetes.

28. The assay according to claim 27, wherein the sample is analyzed in order to determine the presence of a Ljungan virus.

29. A method of treating an individual who has developed diabetes or is susceptible to developing diabetes comprising administering an effective amount of a compound which prevents or reduces the effects of a Ljungan virus, which causes, at least in part, diabetes.

30. A method of vaccinating an individual against a Ljungan virus infection, thereby preventing, at least in part, the individual developing diabetes.

31. An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence as shown in Figure 7;

(b) the nucleotide sequence as shown in Figure 8;

(c) the nucleotide sequence as shown in Figure 9; and

(d) a fragment of a nucleotide sequence of (a)-(c).

32. A polypeptide encoded by the nucleic acid molecule according to claim 31.

33. An antibody that specifically binds the polypeptide according to claim 32.

34. The antibody according to claim 33 which is monoclonal.

35. A vaccine comprising a component selected from:

(a) a polypeptide according to claim 32
and

(b) an antibody according to claim 33.

36. A method for treating or preventing Ljungan virus infection and diabetes comprising administering a composition selected from:

(a) a polypeptide according to claim 32;

(b) an antibody according to claim 33;
and

(c) a vaccine according to claim 35.

Fig. 1A

Fig. 1C

Fig. 1E

Fig. 1G

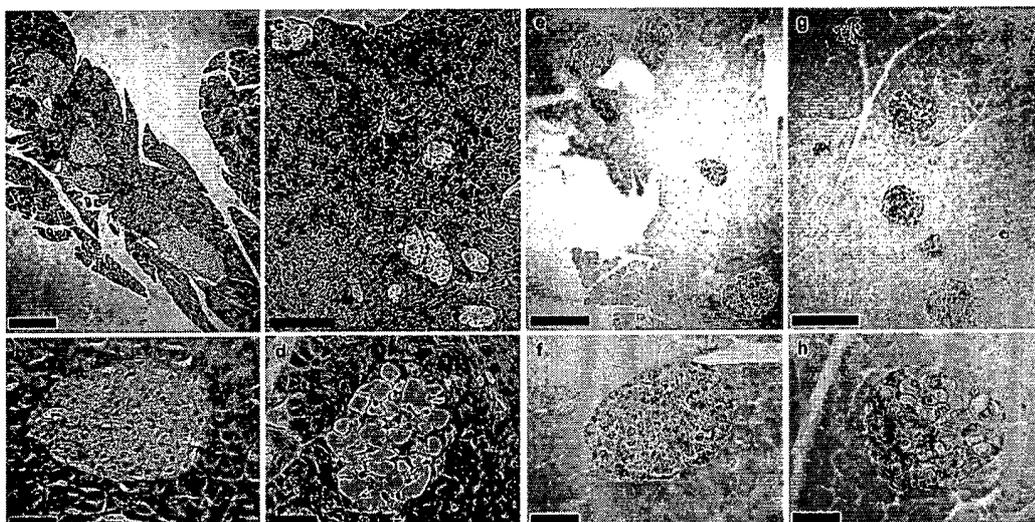


Fig. 1B

Fig. 1D

Fig. 1F

Fig. 1H

Fig. 1

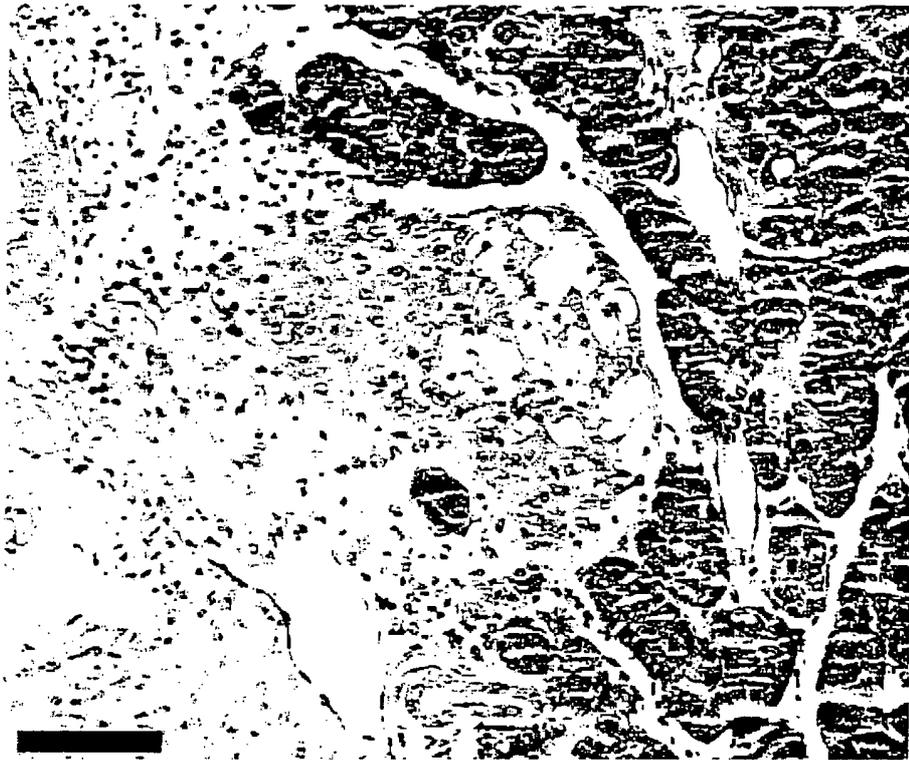


Fig2

Fig. 3A

Fig. 3B

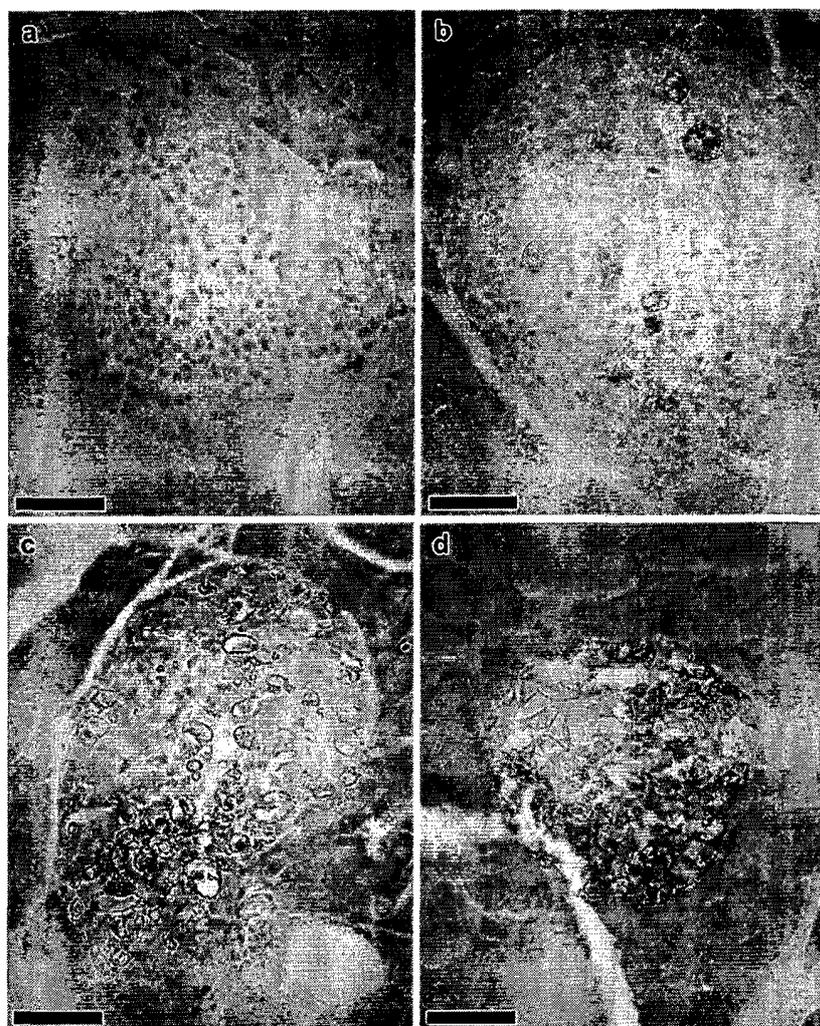


Fig. 3C

Fig. 3D

Fig. 3

FIG. 4.

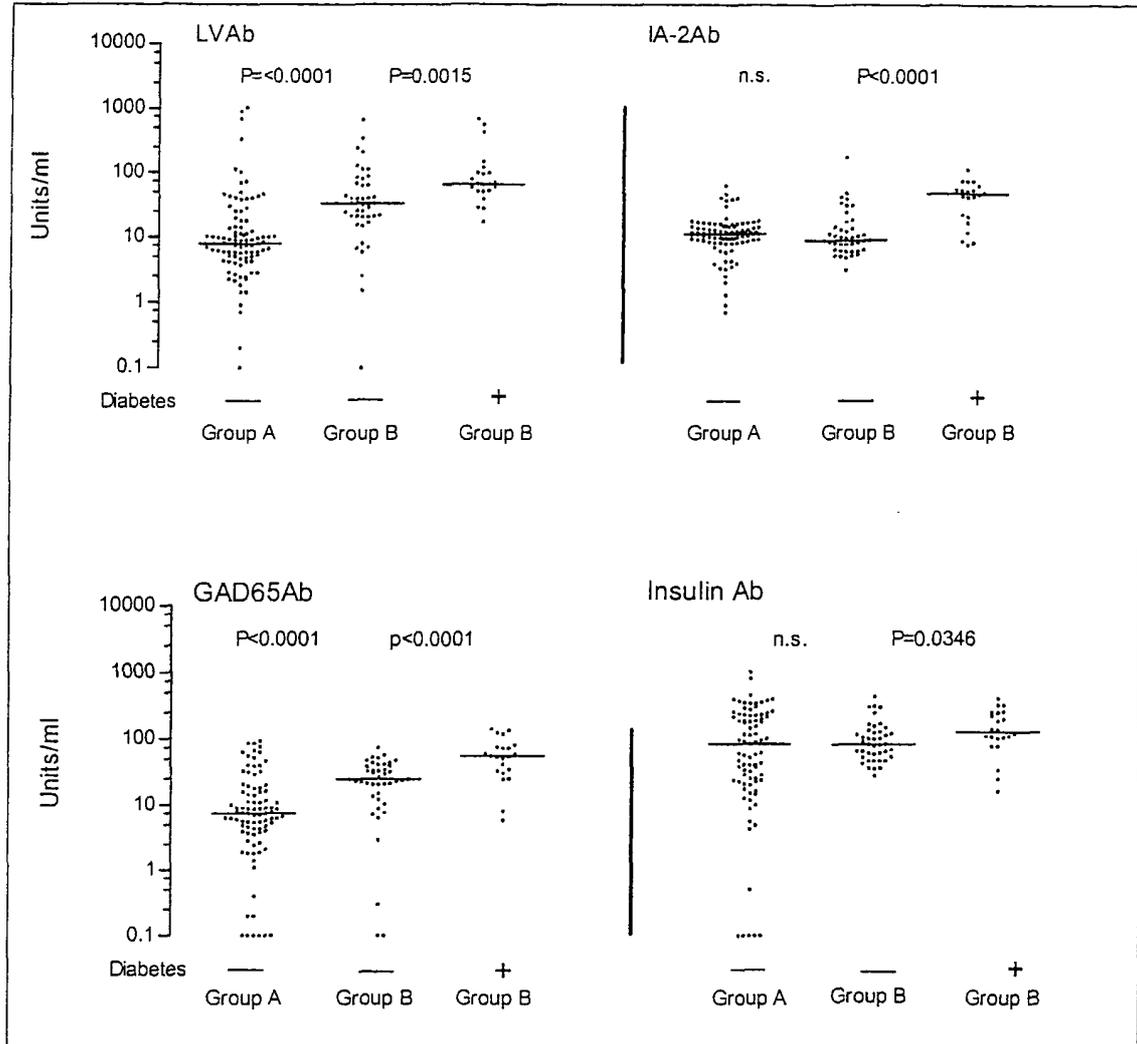


Fig. 5 a Ljungan virus molecular mimicry to islet autoantigens.

87012	121	QSRYFA	A V R C S F I Q V	QLNVN I G S A G C L I	A A Y M P K T A H D	H M N T Y T F G S Y T N L P H V L M N A A	180
GAD65	443		*L Q ** R * V J *				452
IA2	561				**V L * Q ** *		569
87012	721	SFRC P N F F F P L P A P R E A T S R S I L E R V D E A	V A E E L E A V L E A	R T P D A P L R L K F N P E D P L K Q L			780
IA2	964		*v ** * [V N] * I * K *				976
87012	1081	P M E Q M R Q E G W R E F N D V S M S F R H V E W W L T M F K K V Y N V L K S	I F A P S	I E Q K A V D W I D R N Q E Y I			1140
GAD65	237		** S *				241
87012	1259	T G S E F M D G Y I G Q Q I H I I D D A G Q N R E E K D L A L L C Q C I S S V P F T V P M A D L T	E X G T F Y T S K	I V			1318
INS	45		* R * F * * * P *				53

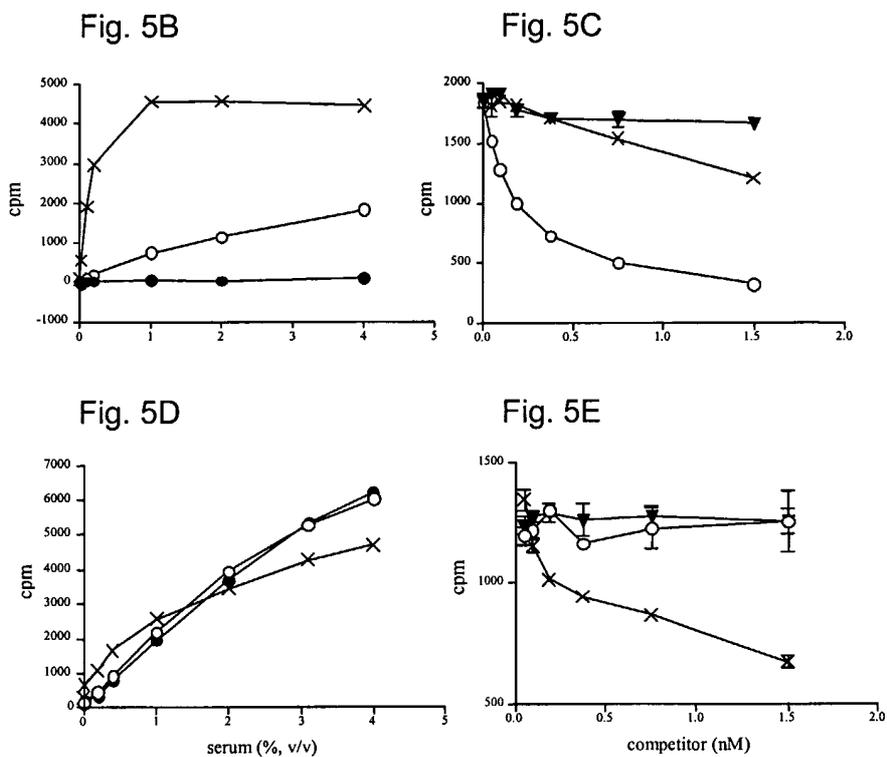


Fig. 6

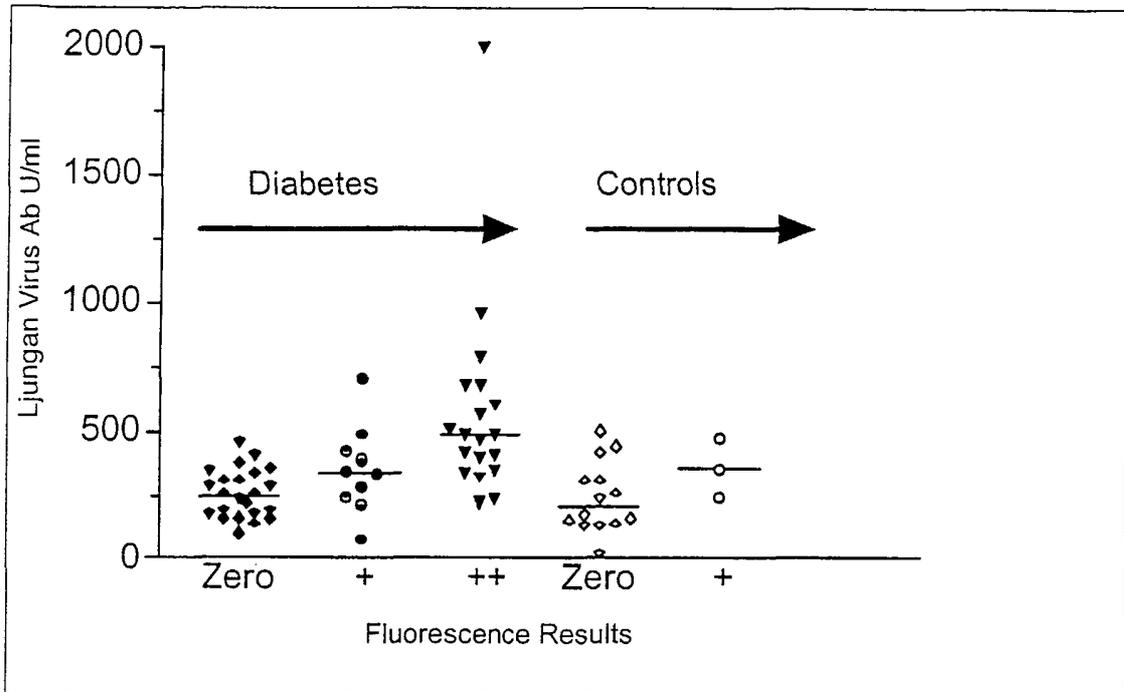


Fig. 7

TTTGAAAGGGGTCTCCTGGTGGGGTGGGTACACTTCTCGCTCGATGAGTGGGGGTGTGGCTC
ATTGCCACACCTGGTTGGTCCCAGGTTTCATACAATAACCATCAATAAACTTTTAACATCT
AAGATAGTATTATCCCATACTAGACTGGACGAAGCCGCTTGGAATAAGTCTAGTCTTATCTT
GTATGTGTCTGCACTGAACTTGTTTCTGTCTCTGGAGTGTCTTACACTTCAGTAGGGGCTG
TACCCGGGCGGTCCCCTCTTACAGGAATCTGCACAGGTGGCTTTCACCTCTGGACAGTGC
ATTCCACACCCGCTCCACGGTAGAAGATGATGTGTGTCTTTGCTTGTGAAAAGCTTGTGAAA
ATCGTGTGTAGGCGTAGCGGCTACTTGAGTGCCAGCGGATTACCCCTAGTGGTAACACTAGC
CTCTGGGCCCCAAAAGGCATGTCATTTGACCACTCAGGTACACAACCCCACTGATGCACACGC
TTAGTAATGGCTTAGTAACAAACATTGATTGATCATTGAAAGCTGTTAGGAGGTTTAGGTA
TGACGGGCTGAAGGATGCCCTGAAGGTACCCATAGGTAACCTTAAGCGACTATGGATCTGAT
CAGGGGCCACCATGTAACACATGGGTAGAAGTCTTCGGACCTTGGGTAAAAAACGTCTAG
GCCGCCCCACAGGGATGTGGGTTTTCCCTTATAACCCCAATATTGTATAATGGCTGCATC
CAAATGAATCCCGTTGGCAACCTGCTCTCCACAGTCTCCTCAACCGTTGGATCTCTTCTAC
AAAACCCCTCTGTTGAAGAAAAGGAAATGGATTCTGACCGTGTGCTGCCTCTACTACAACC
AATGCTGGTAATTTGGTACAAGCTTCTGTGGCCCCAACTATGCCTGTCAAGCCAGATTTTAA
AAACACAGATGATTTCTTGTCCATGAGCTACCGTTCAACAACAGCCCCAACCAACCCCAAA
AAATGGTTCACCTTGGCACATGGCACTTGGACAATAATCAGCACAGACAGGCATTGGTCCGA
TCAATTACTCTACCGCAGGCATTTGGCCCAATCAAGATTTTCCGCATGGGGGCAGTCTCGT
TATTTTGCAGCAGTGCCTGTGGCTTTTCAATTCAAGTACAGTTGAATGTTAACATCGGTTT
TGCTGGTTGCTTGATTGCCGCTACATGCCAAAGACGGCCCATGATCATATGAACACCTATA
CTTTTGGTTCTTACACCAACCTGCCACATGTTTTGATGAATGCGGCAACGCATCCAGGCT
GATCTCTATATACCCTATGTTTTTAATCATAACTATGCAAGAACTGATTTCAGATGATTTAGG
AGGCATTTACATTTGGGTTTGGTCAGCTCTCACAGTTCATCAGGTTACCTACAACAGTGG
ATGTGACCATTTTTGGTTCATTACTCGACTTGGATTTTTCAGTGTCTCGTCTCTGTTGCA
GACACAGTAATTTACACACAAGGGAAAAGAAGTGTTCGAAAGACCAAGACATCAAAGTTCAA
ATGGGTGAGGAATAAAATTGACATAGCTGAAGGTCCAGGAGCAATGAACATCGCTAATGTTT
TCTCCACAACCTGGTGGTCAAACCTATTGCCTTGGTTGGTGAAGAGCATTCTATGACCCAAGA
ACAGCTGGTGTGCTGCACTGAGGTGCAAGGACCTCATGGAGATCGCCAGAATGCCAAGTGTGT
CTTAGGAGAGAGTACTGAACCAGATGGTTCGAAGGGGCTATTTACCTGGTCCACATAAATCT
CACCTGTTAATTGGGTTTTTGTGACCACATTTATTTGAAAACATGCCCAATCTGAGATTA
TTCTCCTCTGTTACAACCTATTGGAGGGGCTCCTTTGTTATTAACTGACAGTTTATGCGTC
AACTTTCAACAAAGGACGCTTGAGGATGGCATTTTTTCCCAAACAGAGAAGGCGCCTACACAC
AGGATGAAGCTCAGAACGCAATCTTTGTTGTGTGTGATATAGGCTTGAATAATACCTTTGAG
ATGACCATCCCCTACACTTGGGGCAACTGGATGAGACCAACAAGAGGAACTCCTTGGGACA
TCTGAGGATTGATGTGTTGAACCGTCTTACATAACAAGTTCCTCCCAAATGCAGTCAACT
GCATTTCTCAGATTAAGATGGGGATGATGCAATGTTTCATGGTGCCTACCACATCTAATCTA
GTTTGGCAAGGTCTGCACTCATGGGTTTTCAGAAATGGACTTGGTGGACTCTCTGACAAATCC
AGACGAGATACAAGACAATGAGGAAATACAAACCCAAAATGTGGAGGCTGCACAAGGGGAAG
AAGCTGCGACTGAAGTTGGTCTTAGGGCAACAGAAAATGATGGCAGTCTTTTCAGAACAATG
AATATGAGTCAACCCATGTTCCCTGAATTTTAAAGAAGCATAAAGTCAACATCTATGCAGCATC
CCATACCAAAGTTGATCATATTTTTGGAAGAGCTTGGGCAGTGGGGTTTTTAAACACAGAAA
CAGCTGCCATACAAAATTTGATTTGCACTTTCCAACCTCTACTCATGGTGCATTGTCTAGA
TTTTTCTGCTTCTGGACTGGAGAGTTAAATATTACATTTTGAATGTGTCAACCACAAATGC
CTTTTTGAAAGTTGCTCACACATGTTTTGGCACTGATTCTGGAATTGCCCGACAGCTACTT
TGGAATCAAATGGAACAATGATTATACCACCAAACGAGCAAATGACACTTGTGTACCCTAT
TATCTGAGGTTCCATTAAGATGTGTTAAAGTTTCAGACAGGAATTCAGCCGACTTGGTTC
TCTCTTACACAGGCTGTGGGCAGAACAACTCTAATCGGGTACAAATCTTTGTGAGCTTCC
GCTGTCTAATTTTTTTCTTCCCACTACCTGCGCCAGGGGAAGCCACGTCTCGAAGCATATTG
GAACGAGTGGATGAAGCAAATGCGGAAGAAGTGAAGCTGTCTTGAAGCTAGAACACCAGA
TGCACCGCTCCGCTCAAGTTAATCCAGAAGATCCTTTGAAACAATTCGGGGAGGCGGCCA

9/16

Fig. 7 Cont.

AGGCTTACTTTAATAATAATGCACAGTGATGAAATGGATTTTGCCGGGGGGAAATTTTTGAAC
CAATGTGGTGACGTGGAAACCAACCCAGGCCCTGACATTGAGTTGGTCTATAAAAAACAGAGG
CTTCTATAAGCATTATGGAGTTAGATTTGGTGGTCATATCTACCACTTGAATTCACAAGACA
TTCTGTCAACCGCAATTACAGGCAAGTCTGACTTCATTAAGGAAGAAGATGATGGCAAATGG
GTTTCATGCTATGACAGCACCATTGGACTACTTTACTGAAAAGTACATCAATTCAATGGTTGG
CTCCAAACACATCTTTTCCGCCACCTCCAATTGTGAGACCATTGCCAGAGATCTTTTCCCAG
GGAGAAAGGAAATAACTCAGTCCAAAGCCTTGGGGATTATTGGGGTCATCTTGTGTGTCAGCC
TCTCTTCTTTTCAATTGCTTGTGTACCCTGGGATTATTCCCTCACTTCAAACCTGTTTATAACCA
ATCCATTGAAGGTGATGCTTCTGGCCTCACACTCCTAAGTCAAAGATGCATGACTTTTTTTTT
CCAATACAATGTGTGAAACTTTTAATAATGATCTTGTTAAGTTTATTATTAAGATTTTGGTG
CGGCTTTTGTGCTACATCGTTCCTTTATTGCCATGCACCAAATATGCTGACAACCATGTGTCT
GGGAACTCTTCTTGTTTTGGACATTACAACCTTGTGAAATCTTGTCTGCTAACACCAAAGCAC
TCTTTCAGGCATTGGTTGATGGTGTGATGTGAAGAGTCTTGTCTGGAAAATTGCTGAGAATATG
CAATTTGCCCAATCCAAGGATGAACAAGCTGAGGACATGGCAGCAACCTTCAACTTTGCCTC
TGACATGGTTAATTTTGTGCCAATGGAACAGATGAGACAAGAAGGCTGGAGAGAATTTAATG
ATGTTTCTATGTCCTTTCGGCATGTTGAATGGTGGCTGACTATGTTCAAAAAGGTGTACAAT
GTTCTGAAAAGTATTTTTGCTCCTAGTATTGAACAGAAGGCTGTTGATTGGATAGATCGCAA
TCAAGAGTACATTGCCGATGTTTTGGACCATGCTTCCAACATCATCATAAAAAATGAAGGATC
CAAAGAACAGGGAGAGCATCAACCATTAGTGAATACTTTGAGGTTTTGAAACAACATAAAGC
CAATTGTGTCCCTTTGCATGAAGGTTGCCCCCTCCACTAAGTTTTCTCTCAAGTGTTTAGA
ATCTATTCTGAAATGATGAGGGTCAATGTTAGAGTGCCTGCGAATACTGACTTGACTAGACT
GGAACCCATTGGCATTGGGTTTCTAGTGAGCCAGGACAGGGTAAATCATTCTTTACACATA
TGTTGAGTACCTGTCTTCTGAAGTCCCTGCAATTTAGAAGGAATTTATAACCAACCCACAGGT
TCAGAATTTATGGATGGCTATATTGGGCAGGACATTCATATCATAGATGATGCAGGGCAAAA
TAGAGAGGAAAAAGATTTGGCCTTGTGTGTGAGTGTATTTCTTCTGTGCCTTTTACTGTTT
CAATGGCAGATTTGACAGAGAAGGGCACTTTTTTATAACAAGCAAAATTGTGATTGCCACCACC
AATAAATTTGATTTTACCTCAATGGTTTTGACAGATCCAGCAGCCCTTGAAAGGAGATTTCC
GTTCCACTTGCGCATTAGAGCTGTAGCCAGCTATTCGCGCAACAACAACAACTAGATGTGGCCC
GTTCAATGGCGGCCATGGCAGATGGTTCTTGTGTTGGAATATTCCACAGATGGTGGTAGGGCT
TGGAAGACCCTCTCCATGGATGAACTTGTGAAACAAATCACGGCAGTTTACACACAGAGGTC
AGATGCCCTTATGGTTTTGAAAAGGAAGTTGAATACCATCAGGAACGAAATGAGCCCTGGAT
CATCCACCGGCAGGATTTTGAACCCTTAGAGGAAACACTCTGTGCTTTGGAACGTCGCTTT
GGTCAACTTGCTGATAGTCTTAAAGACAACCTATCATAAAACAGCTGATGAGTTGATTGAAGC
TATAGAAGATATGATGGCACCGTCACAGAGCCCTTTTGCATGCTTTGCTGAATCCTATCGAC
CCACCATTAAATATACTGCCAGTGATAAAGTTAAATCATGGGTTAAAAATCATATGAATAGA
TGGAAGAATTTGTAATGAGAAATAAAGGCTGGTTTACACTTTTTTCTGTGCTCTCATCATT
TCTCTCGATTCTTACTCTTGTCTATTTACACTATAAAAAGGAGAAGAAAGAGGAAGAGAGAC
AGGAGCGGGCTTACAACCCTCAAACCTGCAATTTCTAAGAAGGGGGTAAGCCTAAGCTCTCA
TTAGTGAAAACCACAACTTTGTTAATGAAGCACCTTATATGCAAGATCTTGAACATTGCTT
TGCACAAACGGCCTACATTTCTTCTCAGAAACCCCAAGATATAATACATTGTGCTGCCTTGA
GTGAAGACACCATTTTGGTTTATGGACATTCTCAGTTTTATTTTAAACCGCTATGAGGACCTG
CGGTTACATTTTAAAGGGGCCATTTTTCCCATAGAAGGGGGAAAAATTTCTCAAGTTACCGT
GAATGGACAGCCTATGGATTTGATACTTGTGAAGATAGATAAACTTCCAATAACATTTAAAA
ATTATACAAAATATTATACAACCTGAGGTTGGCAAGGAAACTCTTTTAAATTTGGAATTCAGAA
AAGGGCAGGTTGGCCATGCCTGTTCAATGTGTGGCTCCGGCTGGTCCGGTGGAGACAATGGA
AGGAACAATTACTCATAAGACCTATTATATAAAGTGGCATCAAAAAAAGGAATGTGTGGGG
GCCTTTTGGTCACTAGAGTGCATGGCACATTCAAGGTTCTGGGAATGCACATTGCAGGCAAT
GGGCAAGTTGCACGAGCTGCAGCAGTTCACCTTTATATCCAATGGTGCAGCTGGCTTTATGGA
TCAAGGTGTTGTTGTGGCCAAAGAAAAGTTACAAAAGCCCATTTATTTGCCATCTAAGACAG
CTTTGAATCCCAGTCCCTTGAATGGTGTAGTCCCCGTGAAAATGGAACCAGCTGTGCTTAGT
CCTCATGACACCAGGCTTGAAGTCATCATGCCAGCGTGTGTGAAAACAGCGGCAGCTAAGTA
TAGAGTTAATATTTTTAATCCTGATTTTGGAGATTTGGGAGAGAGTGGTGGATGAGTTAAAAAT

10/16

Fig. 7 Cont.

CAAAGTTT TAGAACCAAACCTTGGAATTCATAAACATGTCTCTTTTCAGAAAGCAGTTTCAGGGT
TTCTCCTCCCTTTTCATCTCTTGATCTTTCCACATCCCCAGGACAAAAGTATGTTGAAAAGGG
TATGAAGAAGAGAGATCTTTTGTCCACTGAGCCATTTTGGATGCATCCTCAATTGGAAGGTG
ATGTTAAAGATATACTTGGGGCCGTTTACTCTGGTAAAAAGCCCCATACATTTTTTTGCTGCA
CATTTGAAAGATGAGTTGCGCAAAAAGAAAAGATTGCGCAAGGAAAAGACCCGCTGCATTGA
AGCCTGTTCAATTGACTATGTGATTGCCTACAGAGTTGTAATGTCCTCACTCTATGAGGCAA
TCTATCAAACCTCCGGCTCAGGAGTTGGGCCTGGCAGTGGGAATGAATCCCTGGACAGATTGG
GATCCAATGATCAATGTTTTGCAGCCATATAACTATGGCCTGGATTACTCATCTTATGATGG
CAGCCTTTCTGAACAACTGATGAGATATGGAGTGGAAATACTTGCCTACTGTCATGAACAAC
CAGAGGCTGTAATGATTCTTCATGAACCTGTTATAAACTCTCAACACCTTGTGATGGATGAA
ATCTGGCATGTGAATGGTGGAAATGCCCTCAGGGGCCCCATGTACAACCTGTGCTAAATCCAT
ATGCAATCTGCTAGTTTGTACATATTTGGCCTATGAGCAGAGCTTGGATATTGAGGTGTTAC
CCATTGTTTATGGAGATGATGTAATTTTTCTGTTTCATCCCCCTTTGGATGCTGAATACTTG
GTTGAGAGTGCAGCCAAAATTTTGGAAATGGAAGTGACCTCATCAGATAAATCTGGTCCCCC
TAAACTTTTGAAAATGGATGAGATTGAATTTTTAAAGAGGACAACAATAATTTTTCTGGCT
CCACCTACAAGGTGGGGCCCTTGAGCCTGGATACCATGGAACAACATATTATGTGGATGAAG
AATTTGGAAACCTTTCCAGAACAACCTTGTTAGTTTTGAGAATGAATTGGTGTTCATGGGAA
AGAAATTTATGATGATTATAAAAAATAGGTTAATCCTATTTTGAATCAATGGCGAGTGTGCA
TGCAGGACTATGAAGTGGCCCTGCATCGCATGCTACGCTATGTTTTTGAATTGAATTGATTTA
GTTTGATTTTGATTTTATTAGCTTTAGTTTATGTAAGTTAGAATTAGATTATTTTAGTTTAG
TTTTAAAGATTTTGATTTGATTGAATTTGGCCCAACAATC

11/16

Fig. 8

TTTGAAAGGGGTCTCCTGGTGGGGTGGGTACGTCTCTCGCTCATTGAGTGGGGGCGTGGCTC
ACCAACCACACCTGGTTGGTCCCCAGGTTTCATGCAATAACCACTTTTTGTAATCTTTACATC
TAAGCTTAATTCACCCACTAGAAGTGGACGAAACCGCTTGAATAAGTTTGGTTCTCTCTTG
AGTGTGTTTTGTGTTAGCATAATTTCTGTCTCTAGAGTGCTTTACACTCTAGTAGGGGCTGT
ACCCGGGCGGTCCCCTCTTACAGGAATCTGCACAGGTGGCTTTACCTCTGGACAGTGCA
TTCCATACCCGCTCCACAATAGAAGATGATGTATATCTTTGTTTGTGAAATGCTCATGAAAC
GTGTGTGTAGGCGTAGCGGCTACTTGAATGCCAGCGGAACCCCCCTAGTGGTAACACTAGCC
TCTGGGCCAAAAGGCATGTCTCTGACCATTCAAGTACACAACCCCACTGATACACACATTT
AGTAATGGCTCAGTAATGGACATTGATTGATCATCAGACAATTGTTAGGAGGCCTAGGTATG
ACGGGCTGAAGGATGCCCTGGAGGTACCCCGCAGGTAACCTTAAGAGACTGTGGATCTGACCA
GGGGCCACCATGGAAACATGGGTAGAAGTCTTCGGACCTTGGGTTAAAAACGTCTAGGCC
CGCCCCCACAGGGATGTGGGGTTTCCCTTATAACCCCAATATCACATTATGGCTGCAACCA
AGATGAATCCCGTTGAGAATCTTCTTTCTACTGTCTCCTCCACCGTTGGCTCACTGCTACAA
AATCCACCATGGAAGAAAAGGAAATGGACTCAGATCGTGTTGCCGCATCCACCACTACTAA
CGCTGGAATGTAGTTCAGGCTTCAGTTGCCCCACCATGCCAATTAACCCAGATTTCAAGA
ACACGGATAACTTTTTGTCAATGAGTTATAGCCCAAATACTGCACCTACAAATCCAACAAAA
ATGGTACATTTGGCTAATGGAACATGGACTACATCGCAGCATCGACAGTCTTTGGTTGCATC
GATTCAGCTACCACAGGCATTTTGGCCCAATGAACGCTATCCGGCTTGGGGTCAATCACGCT
ATTTTGCTGCAGTCCGATGTGGCTTTCATATTCAGGTTCAATTGAATGTTAACATTGGCTCA
GCAGGTTGTTGATAGCTGCCTATATGCCCAAAGTGCACATGATCATATGGATACATATAC
ATTTAGTTCCTACACCAATTTGCCTCATGTTCTGATGAA TGCTGCCACCACGTCTCAGGCTG
ATTTGTATATAACCCTATGTGCATAATCATAATTATGCAAAGACAGATTCAGATGACTTGGGT
GGTATATACATTTGGTGTGGTCTGCCCTCACAGTTCATCAGGTTCTCCGACAACCTGTTGA
TGTCACAATTTTTGGCTCCTTGCTTGACTTGGACTTCCAGTGCCCTAGACCACCAGGTGCTA
ATACTGTCATATTTACACAAGGCAAAGAAGTGCAGGAAAACCAAAGCAACAAAATTTAAA
TGGACAAGGAATAAAATAGACATTGCTGAAGGTCTTGGCGCTCTTAATATTGCCAATGTCTT
GTCTACTACAGGGGGCCAAACTGTTGCCCTCGTTGGGGAAAGAGCTTTCTACGATCCCAGAA
CTGCAGGAGCCGCTGTGCGGTGTAAGGATTTGATGGAATGCCAGAATGCCATCAGTCTAT
AAGGGGAGAGAACTGAACCTGGAGGAACTAATGGCTATTTTCAATGGTCTCATAACGACTC
CCCTATAAATTTGGGTTTTTGACGGGGAAATTCATTTGGAAGACATGCCCAATCTAAATTTGT
TTTCTCATGCTATAACTATTGGAGAGGCTCAATTGTTTTGAAACTCACTGTGTATGCATCA
ACCTTTAACAAGGGTAGATTGAGAATGGCCTTCTCCCAAATCATGATGCAAGGTACACAGA
GGAAGAAGCACAAAATGCCATCTTCATGGTGTGTGATATTGGGCTCAACAACACTTTTGAAA
TGACCATCCCATACACCTGGGGAAACTGGATGAGACCAACTAGGGGATCTGTCAATTGGATGG
CTTAGGATTGATGTTTTGAATCGCCTCACTTATAACAGTTCCTCACCCAATGCTGTTAATTG
CATTCTTCAGGTTAAAATGGGGAATGATGCCAAATTTATGGTACCACCACATCTAACATTG
TGTGGGAAGGTCTCCACTCATGGGGGTCTGAGATGGACTTACTGGACAGTTTGGATAATCCA
GAAGACATTCAAGATATGGAGGAACCAGAATCTGAAAATGTGGAGGCCGCACAAGGAGAGGA
AGCCGCCACTGCCGTTGGCCTTCGAGCCACCGAAAAATGATGGATCCCTATCTGAACAACAAA
ACATGGCACAACCAATGTTTTTGAATTTTAAGCAACATAGAGTGGACATTTACTCTGCTTCC
CACACCAAAGTTGACCATATTTTTGGTAGGGCGTGGGCAGTGGGAATTTTTAATGTGACTAA
TGCTAATATATCCAAATTTGACCTTAACTTTCCACAACCCACACATGGTGCATTGTGTGCT
TCTTCTGTTTCTGGACGGGAGAGCTTAACTTGCATATTTTTGAACATTTCTTCTTCCAATGCT
CCAGTCAAAGTTGCTCACACATGGTTTGGCACAGATTCAGGCATTGCCAGGACTGCAACTTT
GGAATCAAACGGGGTTATCATCATAACCACCAAATGAGCAAATGACACTCTGCATACCCTATT
ATTCTGAGGCACCATTGCGCTGTGTTAAGGGGCCACATTCAGCTGGTGGTGGATTGGGCTCA
ATTTTCACACAGTGTATTGGCAACAGCGTTAATAACAGGATTCAAATTTTTGTTAGTTTTCG
CTGCCCAAACCTCTTTTTTCCCCTTCCCTGCACCCCATGAGGCTTCTTCAAGGTCAATTTTGC
AGAGAATTTCCACTGCTAGCGCAGATGAGTTAGAAGCTGTCTTGGACGCAAAAACACCTGAT

12/16

Fig. 8 Cont.

GCTCCTGTGCGCTTGTGCTACCAACCAGAGGATCCTTTGAGACAACCTTAGGGAGGCAGCTAA
GGCATATTTCAATATTATGCACAATGATGAGATGACTATTCTGAGGTAAATTCTTGAATC
AGTGTGGTGTGATGTGGAGTCCAATCCAGGTCCTGATATTGAATTAGTCTATAAGAACAGAGGC
TTTTATAAACATTATGGGGTTAGGTTTGGTGGCTTTATTTACCATCTTAATTCACAAGACAT
TTTGTGACAGCCATCACTGGAAAATCAGACTTCATAAAAGAGGAAGATGATGGTAAATGGA
CACATGCTATGACTGCACCCCTGGATTATTTTACTGAGAAGTATGTGAAATCAATGGTTGGT
TCAAAACACATTTTTTCCGCCACATCAAATGTGAAACCATTGCCAGGGATTGTTTTCCAGG
AAAGAAGGAGATTAGTCAATCTAAAGCTTTGGGTATTATTGGTGTGATCCTTCTTTCTGCAT
CTCTTTTATCCCTACTTGCCGTTCCATGGGATTATTCCTCACTTCAGACAGTTTATAATCAA
TCAATTGAAGGGGATGCTTCAGGCTTAACACTTTTGAGCCAGAGATGCATGACTTTTTTTTC
CAATACCATGTGTGAAACTTTTAATAATGATCCTTGTGAAGTTTATAATTAAGATTTTAGTTA
GGCTTCTTTGCTATATTGTTCTTTATTGTGATGCCCTAATATGCTTACAACAATGTGTTTA
GGCACCCCTTTGGTTTTGGATATTACCACATGTGAGATTTTATCAGCCAATACAAAGGCCCT
GTTTCAAGCTCTTCTTGATGGAGACGTCAAGAATTTGGTTTTGGAAGATTGCAGAGAACATGC
AGTTTGCCAGTCTACAGATGAGCAGGCAGAGGAAATGGCTGCCACCTTTTCATTTGCCAAA
GACATGGTTGACATTCATCCAATTGGGGCTGAGCCATTTCAAACCAAGGCTTTAGGGAGTT
TAATGATGTGTCAATGTCTTTCCGCCACATTGAATGGTGGCTTACAATGTTTTAAGAAAGTTT
ACAATGTTCTTAAGGGCATTTTCTCTCCATCCATTGAGCAGAAAGCGGTGGCGTGGTTGGAT
CGCAACCAAGAATATGTTGCATCAATCTTAGATCATTGCTCTGACATGATTATCCGCATGAA
AGACCCAAAACAACAGCGGAACCCCAAGACCATTGAAGAATATTTTGATGTGTTAAAGAAAA
TGAAGCCCTTGGTGTCACTCTGCATTAAGTTGCCCCGTCAACAAAGTTTTTCATCCCAAGTG
TTTAGGTTGTATTAGAGCTAATGAAGGTTAATGTTAGAGTGCCGTTAACACAGATCTCAC
ACGCATTGAGCCAATTGGTGTGTGGATCTCCAGTGAGCCAGGTCAGGGAAAATCTTTCTTTA
CTCACATGCTTAGCACTTCACTTTTGAAAAGTTGTAATTTGGATGGGGTGTATACCAATGCC
ACAGGCTCTGAGTTTATGGATGGATATGTTGGTCAAGATATACACATTATTGATGATGCAGG
ACAAAATCGGGAAGAGAAGGATTTGGCTCTGCTGTGCCAGTGCATCTCATCTGTGCCATTTA
CTGTACCTATGGCTGATCTAACAGAGAAAGGGACATTTTATACCAGCAAGATTGTTATTGCC
ACAACCAACAAGAGTGATTTCAATTGCATGGTTTTGACAGATCCAGCTGCTCTAGAGAGGCG
TTTCCCATTTAATTTGAGAATTAGGGCAGTTAAAAGTTTTATGAATAAGGACAGAAAGTTGG
ATGTGCCAAGATCAATGGGAGCCATGGCAGATGGATCCTGCTGGGAGTGCTCTATGGACTAT
GGCAGAACCTGGAACACCGTGGTGTGAGAGATCTTGTGAAACAAATAACAGAAATGTATAA
ACAAAGAGATGATGCCCTGACTGTTTTGGAAGTATAAGTTAAATCAGATTAGGAATGAGATGT
CCCCTGGTGACTCAATTGGCCGCATTCTCGATCCAATGGAGGAGACACTCTGTTCAATTGGAG
CGCAGGTTTGGCCAGTTGGCAGATAGTCTTAGAGAAAATTACCATAGGACAGCTGATGAACT
AATTGAAGTTATAGAAGACATGATGGCACCAGGGAATAGTCCCTTTGCATGCTTCGAAAGTG
TAGCACCATCACTTAAACCAAGAACAGCTTGTCAAAAAGTTAAAGATTGGGTAAAACAACAC
ATGATTAGATGGGGCAACTTTGTGATGAGGAATAAAGGCTGGTTTACACTTTTTTTCTGTACT
TTCATCTTTTCTTCAATTCTTACTCTTGTTTATTTACATTATAAAAAAGAGAAAAAAGAGG
AAGAAAGACAAGAGCGGGCTTACAACCCTCAAACCTGCAACTCCCAAGAAGGGGGGTAAGCCA
AAGCTCTCTTTGGTAAAAACTACAAATTTTATAAATGAGGCACCATATATGCAGGATTTGGA
ACACTGCTTTGCCAAACAGCCTACATTTTCATCCCAGAGACTCAGGATATAATTCATTGTG
CTGCCTTGTGTGAGGATACCATTTTGGTTTATGGACATTCACAATTTTATTTTAAACCGCTAT
GAAGATTTGCGGTTACATTTTAAAGGAGCCATTTTTCCTATTGAGGTTGAAAAAATTTTACA
AGTTACTGTGAATGGGCAGCCGATGGATTTGATTCTTGTAAAATAGACAAAACCTTCCATAA
CCTTTAAAAATTATACCAAATATTACACAACCTGAAATTTGGGAAGGAACTCTTTTAAATTTGG
AATTCTGAGAAAGGGAGACTGGCTATGCCAGTCCAATGTGTTGCCCCGGCTGGACCGGTGGA
AACAATGGAAGGCACCATCACTCATAAAACCTATTCTTACAAAGTGGCATCAAAGAAAGGCA
TGTGCGGTGGACTCCTAGTTACTAGAGTGAATGGAACATTTAAGGTTTTGGGGATGCACATT
GCTGGGAACGGACAGGTTGCGCGGGCCGACGAGTTCACTTCATTTCAAATGGGGCTAGTGG
TTTTATGGATCAGGGGGTTGTGGTTGCAAAAGAGAAGATGCAGAAACCAATTTATTTGCCAT
CTAAAACAGCACTAAATCCTAGCCCTTTGAATGGTGTGTGCCCCTGAAGATGGAGCCTGCA
GTTCTTAGCCCTCATGATGTTAGACTTGAAGTGATTATGCCAAGCGTGGTTAAAAATGCAGC

13/16

Fig. 8 Cont.

AGCCAAGTACAGAGTTAACATCTTCAACCCAGATTTTGAAATCTGGGAGAGGGTGGTTGATG
AATTGAAAGCAAGGTTTCGATCTAAGCTTGGCATAACAAACATGTTTCTCTTCAAAAGGCT
GTGCAAGGTTTTTCCCTCCCTTTTCGTCTCTTGATCTTTCTACCTCTCCAGGGCAAAGTATGT
TGAAAAGGAATGAAGAAAAGGGATCTTTTGTCCACTGAACCATTTTGGATGCATCCTCAAT
TGGAAAGTGATGTTAAAGATATACTTGGGGCAGTTTATTCTGGTAAGAAACCCACACATTT
TTTGCTGCCCACTTGAAAGATGAGTTGCGCAAGAAGGAAAAGATTGCGCAAGGAAAGACCCG
CTGCATTGAAGCATGTTCAATTGATTATGTTATTGCCATAGAGTTGTGATGTCCTCTCTCT
ATGAGGCAATTTATCAAACCCAGCTCAAGAATTGGGCTTGGCAGTGGGAATGAATCCCTGG
ACAGATTGGGATCCAATGATTAATGTTTTGCAGCCTTATAATTATGGTTTAGATTATTCATC
CTATGATGGCAGTCTTTCTGAACAGTTAATGAGATATGGTGTGAAATACTTGCTTATTGTC
ATGAGCAACCAGAAGCTGTGATGATTCTCCATGAGCCAGTTATAAATTCGCAACACCTTGTG
ATGGATGAAATCTGGCATGTAAATGGAGGAATGCCCTCAGGAGCCCCATGTACAACCTGTGCT
AAACTCTATATGTAATTTGCTGGTTTGTACATATTTGGCTTATGAGCAGAGTTTGGACATTG
AGGTGTTGCCTATTGTTTATGGGGATGATGTGATTTTTTCTGTTTCTTACCATTGGATGCT
GAATATTTGGTTCAGAGCGCTGCCAAAATTTTGGAAATGGAAGTGACATCATCAGATAAATC
TGGCCCCCAAACCTTTTGAAAATGGATGAGATTGAATTTTTAAAGAGGACAACAAAATTTT
TTCCCGGCTCCACCTATAAGGTGGGGCCTTGAGCCTGGATAACCATGGAACAACACATTATG
TGGATGAAGAATCTGGAAACCTTTCCAGAACAACCTTGTTAGCTTTGAAAATGAGTTGGTGT
GCATGGGAAAGAAATTTATGATGATTATAAAAATAGGTTTAATCCTATTTTGAATCAATGGC
GAGTGTGCATGCAGGACTATGAAGTGGCTCTGCATCGCATGCTACGCTATGTTTTTGAATTAG
ATTGATTTAGTTTGATTTTGATTTTATTAGTTTTATTTTAGGTTAGAATTAGATTATTTAG
TTTAGTTTTAAGGATTTTGATTTGATTGAATTTGGCCCAACCAATC

Fig. 9

TTTGAAAGGGGTCTCCTGGTGGGGTGGGTACACTCCTCGCTCAATGAGTGGGGGTGTGGCTC
ATTGCCACACCTGGTTGGTCCCAGGTTTCATACAATAACCATCAATAAACTTCTCAACATC
TAAGCTACTACTATCCCACACTAAACTGGACGAAGCCGCTTGAATAAGTCTAGTTTCATTC
TGTGTGTGTTTTGCACTGAAATTATTTCTGTCTCTGGGGTGTCTTACACTTCAGTAGGGGCT
GTACCCGGGCGGTCCACTCTTTCACAGGAATCTGCACAGGTGGCTTTCACCTCTGGACAGTG
CATTCCACACCCGCTCCACAGTAGAAGATGATGTGTGTCTTTGCTTGTGAAAAGCTTGTGAA
AATCGTGTGTAGGCGTAGCGGCTACTTGAGTGCCAGCGGACTACCCCTAGTGGTAACACTAG
CCTCTGGGCCCAAAGGCATGTCAATTGACCACTCAGGTACACAACCCAGTGATGCACACG
TCTAGTAACGGCTTAGTAACGAGCATTGATTGATCATTGAAAAGCTAGGAGGTTTAGGT
ATGACGGGCTGAAGGATGCCCTGAAGGTACCCATAGGTAACCTTAAGCGACTATGGATCTGA
TCAGGGGCCACCATGTACTACATGGGTAGAAGTCTTCGGACCTTGGGTTAAAAACGTCTA
GGCCCGCCCCCACAGGGATGTGGGGTTCCCTTATAACCCCAATATTGTATAATGGCTGCA
TCCAAAATGAATCCCGTTGGCAACCTGCTTTCACAGTCTCCTCAACCGTTGGATCTCTTCT
ACAAAACCCCTCTGTTGAAGAAAAGGAAATGGATTCTGACCGTGTGCTGCCTCCACCACGA
CCAATGCTGGTAATTTGGTGCAAGCTTCTGTGGCTCCAACCATGCCTGTAAAACCAGACTTT
AAGAACACAGATGACTTCTTGTCCATGAGCTACCGCTCAACAACGGCCCCAACCAACCCGAC
AAAAATGGTTCACTTAGCGCATGGAAGTTGGACAATAATCAGCACAGACAGGCATTGGTTG
CATCAATTACCCTACCACAGGCATTCTGGCCCAATCAAGATTTTCCAGCATGGGGGCAATCT
CGCTATTTTGCAGCAGTGCCTGTGGCTTTCATATACAAGTGCAGTTGAATGTTAACATTGG
TTCTGCCGGCTGCTTGATTGCCGCATACATGCCAAAGACGGCCCATGATCATATGGGTACCT
ATACTTTTGGCTCCTACACCAACCTGCCACATGTTTTGATGAATGCAGCAACGCATCTCAG
GCTGATCTCTATATACCCTATGTTTTAATCACAATTATGCACGAACTGATTCAGATGACTT
AGGAGGTATTTACATTTGGGTATGGTTCAGCTCTCACAGTTCATCAGGTTCCACTACTACAG
TGGATGTCACCAATTTTGGTTCACTACTCGACTTAGATTTTCAATGTCCTCGTCCCCCTGGA
GCAGCCACAGTAATCTACACACAAGGGAAGAACTGTTGAAAGACCAAACATCAAAGTT
TAAATGGGTGAGGAATAAAATTGACATAGCTGAAGGCCAGGAGCAATGAACATTGCTAATG
TTCTCTCCACAACCTGGCGGTCAAATATTGCCCTTGGTTGGTGAAGAGCATTTCTATGACCCA
AGAACAGCTGGTGTGCTGAGTAAGGTGCAAAGATCTCATGGAGATCGCCAGAATGCCGAGTGT
GTTCTTGGGAGAGACTGAACCAGATGGTTCGAAGAGGCTATTTTACCTGGTCCACATACAA
TCTCACCTGTTAATTTGGGTCTTTGATGATCATATTTATTTAGAAAATATGCCCAATTTGAGA
TTGTTTTCTCTTGTATAATTATTGGAGAGGGTCTTTTGTATTAAATTAACAGTCTATGC
ATCAACTTTCAACAAAGGACGCTTGAGGATGGCATTCTTCCCAAACAGAGAGGGCGCCTACA
CACAGGATGAAGCCCAGAATGCAATCTTTGTTGTCTGTGATATAGGCCTGAATAACACTTTT
GAGATGACCATCCCCTACACTTGGGGCAATTGGATGAGGCCAACAAGAGGGAATTCCTTGGG
ACATTTGAGGATTGATGTGCTGAATCGTCTCACATACAACAGTTCCTCCCCGAATGCAGTCA
ACTGCATTCTCAGATCAAGATGGGAGATGATGCAATGTTTATGGTGGCCACCACATCTAAT
CTAGTTTGGCAAGGCCTACATTCCTGGGGTTCAGAAATGGACCTGGTGGACTCCCTTGACAA
TCCAGAAGAGATACAGGATAATGAGGAAATACAACTCAGAATGTGGAGGCAGCACAAAGGG
AAGAAGCTGCAACAGAAGTTGGACTTAGGGCTACAGAAAATGATGGTAGTCTTTCAGAACAA
CTGAATATGAGTCAACCCATGTTCTTGAATTTCAAGAAGCATAAAGTTAACATCTATGCAGC
ATCTCACACTAAAGTTGATCATATTTTGGCAGAGCTTGGGCAGTAGGAGTTTTTAATACAG
AAACAGCTGCCATACAAAAATTTGATTTGCATTTTCCAACCTCTACCCATGGTGCATTATCT
AGATTTTTCTGTTTTTGGACTGGAGAAGTGAACATTCACATCTTGAATGTGTCAACCACAAA
TGCATTCTTGAAGTTGCTCACACATGGTTTTGGCACTGATTCTGGAATTGCTCGGACAGCCA
CTTTGGAATCAAATGGAACAATGATTATACCACCAAATGAGCAAATGACACTCTGTGTGCC
TATTATTCTGAGGTCCCATTAAGATGTGTTAAGGGCTCAGACAGGAATTCAGCCGGTCTTGG
TTCTCTTTTACACAAGCTGTAGGCAGAACAATTTCCAATCGGGTTCAAATTTTTGTGAGCT
TCCGCTGTCTAATTTTTCTTCCCACTACCCGCGCCCAGAGAAGCCACGTCCCGAAGCATA

15/16

Fig. 9 Cont.

TTGGAACGAGTGGATGAAGCGAATGCAGAAGAAGCTTGAAGCTGTTTTGGAAGCTAGAACACC
AGATGCGCCGCTCCGCCTCAAATTTAATCCAGAAGACCCCTTGAAACAATTGCGGGAAGCGG
CTAAGGCCACTTTAATATAATGCACAGTGATGAAATGGATTTTGCCGGGGGAAATTTTTG
AATCAATGTGGTGATGTGGAAGCTAACCCAGGCCCTGACATTGAGTTGGTCTATAAAAACAG
AGGCTTTTATAAACATTATGGGGTTAGATTTGGTGGCTATATCTACCATTGGAATTCACAGG
ATATTCTGTCAACTGCAATTACAGGCAAGTCTGATTTTCAATTAAGGAGGAAGATGATGGCAAA
TGGGTTTCATGCTATGACGGCACCCTGGATTATTTTACTGAAAAGTACATCAATTCATGGT
TGGTTCCAAACATATTTTTTCCGCCACCTCCAATTGTGAGACCATTGCCAGAGACCTTTTCC
CAGGGAGAAAGGAAATAACTCAGTCCAAAGCCTTGGGAATTATTGGGGTCAATTTTGTGTCA
GCCTCTCTTCTTTCTTGCTTGCTGTACCCTGGGATTATTCCTCACTTCAAAGTGTATATA
CCAATCCATTGAAGGTGACGCTTCTGGCCTCACACTTTTAAAGTCAAAGATGCATGACTTTTT
TTTCTAACACAATGTGTGAAACCTTTAATAATGATCTTGTTAAGTTTATTATTAAGATTTTG
GTGCGGCTTTTGTGCTACATCGTTCTCTATTGCCATGCACCAAATATGCTGACAACTATGTG
TCTGGGAACTCTTCTGTTTTGGACATTACAACCTTGTGAAATCTTGTCTGCCAACACCAAAG
CACTCTTTCAGGCATTGGTTCGATGGTGATGTGAAGAGTCTTGTCTGGAAAATTGCTGAAAAC
ATGCAGTTTGCCCAATCCAAAGATGAACAAGCAGAGGAAATGGCGGCAACCTTCAACTTTGC
TTCTGATATGGTTAATTTTTGTGCCAATGGAACAGATGAGACAAGAAGGCTGGAGAGAATTTA
ATGATGTTTTCTATGTCCTTCCGGCATGTAGAATGGTGGCTGACCATGTTTTAAAAAAGTGTAT
AATGGTCTGAAAAGTATTTTTGCACCTAGTATTGAACAGAAGGCTGTTGATTGGATAGATCG
CAATCAAGAATATATTGCCGATGTTTTGGACCATGCTTCCAACATCATTATAAAAATGAAGG
ACCCAAAAGAAGCAGCGGAAAGCATTAAACCATTAGTGAATACTTTGAAGTTTTGAAGCAATTA
AAGCCAATTGTGTCTCTTTGCATGAAGGTTGCTCCCTCCACTAAGTTTTCTCTCAAGTGT
TAGAATTTATTCTGAAATGATGAAGGTTAATGTTAGAGTGCCCTGCAAATACTGACTTGACCA
GATTGGAACCCATTGGCATTGGGTTTTCTAGTGAGCCAGGACAGGGTAAATCATTTTTTACA
CACATGTTGAGCACCTGCCTTTTAAAACTCTGCAATTTAGAGGGAATTTATACCAACCCAC
TGGGTGAGAATTTATGGATGGTTATATTGGACAGGACATCCATATTATAGATGATGCAGGGC
AAAACAGGGAGGAAAAGATTTAGCCTTGTGTGCCAGTGTATTTCTCTGTGCCTTTTACC
GTCCCAATGGCAGATTTGACAGAGAAGGGCACTTTTTACACAAGTAAAATTGTGATTGCTAC
CACCAATAAATTTGATTTTACATCAATGGTTTTGACAGATCCAGCAGCTCTTGAAAGGAGGT
TCCCGTTTCATTTGCGCATTAGAGCTGTAGCCAGCTACTCGCGCAATAATAAATTAGATGTG
GCCCCCTCAATGGCAGCCATGGCTGATGGCTCTTGCTGGGAATACTCTACAGATGGTGGTAG
GGCTTGGAAGACTCTGTCCATGGATGAACTTGTGAAACAGATTACGGCAGTCTATACACAGA
GATCAGATGCCCTTATGGTTTTGAAAAGGAAGTTAAACACCATTAGGAATGAAATGAGTCCT
GGATCCTCCACCGGTAGGATCTTTGAACCCTTGGAGGAAACACTTTGTGCTCTGGAACGCCG
CTTTGGTCAACTTGCTGATAGCCTTAAAGACAATTACCACAAAACAGCTGATGAGCTGATTG
AGGCTATAGAAGATATGATGGCACCATCACAGAGCCCTTTTGCATGCTTTGCAGAATCCTAT
CGGCCACCATTAAATACACTGCCAGTGATAAAGTTAAATCCTGGGTCAAAAATCATATGAA
TAGATGGAAAGAGTTTGTAATGAGAAATAAAGGCTGGTTTACTTTTTCTGTGCTTTTCA
CTTTCTTTCAATTCTTACTCTTGTTTACTTGCATTATAAAAAGGAAAAGAAAGAGGAAGAG
AGACAAGAGCGAGCTTACAACCCTCAAACCGCAACTTTTAAAGAGGGGGGTAAGCCCAAGCT
CTCATTGGTGAAAAATACAAATTTTGTAAATGAAGCACCCCTATATGCAAGATCTTGAACACT
GTTTTGCACAAACAGCCTACATCTCATCTTCAGAGACCAGGATATAATACATTGTGCTGCT
TTGAGTGAAGACACCATCTTGGTTTATGGACACTCCAGTTTTATTTTAAACCGCTATGAAGA
TCTGCGGTTGCATTTTAAAGGGGCCATTTTTCTATAGAAGGGGGGAAAATCTCTCAAGTTA
CTGTGAATGGGCAGCCCATGGATTTAATTCCTTGTGAAAATAGATAAACTTCCAATAACATTT
AAAAATTATACAAAGTACTATACAACTGAGGTTGGTAAGGAAACACTCTTAATTTGGAATTC
AGAGAAAGGCAGATTGGCTATGCCTGTTCAATGTGTAGCCCCGGCTGGTCCGGTGGAAACAA
TGAAGGAACAGTCACCCACAAGACCTATTATACAAAGGTGGCATCAAAAAAGGAATGTGT
GGGGTCTCTTGGTTACTAGAGTGCACGGCACATTTAAGGTTTTAGGAATGCACATTGCTGG
CAATGGACAAGTTGCACGAGCCGCAGCAGTCCACTTTATATCCAATGGGGCTGCTGGCTTTA
TGGATCAGGGTGTGTGTGGCCAAGGAAAATTGCAGAAGCCATTTATTTGCCATCCAAG
ACAGCCTTGAATCCTAGTCCCTTGAATGGAGTAGTTCCTGTGAAAATGGAGCCAGCTGTGCT

16/16

Fig. 9 Cont.

TAGTCCTCATGATACCAGGCTTGAAGTTGCCATGCCAGTGTTGTGAAAACAGCAGCAGCCA
AGTATAGAGTTAACATTTTCAACCCTGACTTTGAGATTTGGGAGAGAGTTGTGGATGAGCTA
AAGTCAAGGTTTAGATTTAAACTTGGGATTCTATAACATGTTTCTTTCCAAAAGCAGTTCA
GGGTTTTTCTTCTCTTTCATCTCTTGGATCTTTCCACTTCTCCAGGACAAAAGTATGTTGAAA
AAGGCATGAAGAAGAGAGATCTTTTATCCACTGAACCATTTTGGATAACATCCTCAATTGGAA
AATGATGTTAAAGATATACTTGGGGCTGTTTATTCTGGCAAAAACCCCATACATTTTTTGC
TGCCCATTTGAAAGATGAATTGCGCAAAAAGAAAAGATTGCACAAGGCAAGACCCGCTGCA
TTGAAGCCTGCTCAATTGACTATGTGATTGCCTATAGAGTTGTAATGTCTCTCTCTATGAG
GCAATCTATCAAACCCAGCTCAGGAATTAGGCTTGGCAGTGGGGATGAATCCCTGGACAGA
CTGGGATCCAATGATTAATGTTTTGCAACCATATAATTATGGTTTGGATTATTCATCTTATG
ATGGCAGTCTTTCTGAGCAGCTGATGAGGTATGGTGTGGAAATACTTGCCTATTGTCATGAA
CAACCAGAGGCTGTGATGATTCTTCATGAACCAGTTATAAACTCACAAACCTTGTGATGGA
TGAAATTTGGCATGTAAATGGAGGAATGCCCTCAGGAGCCCATGTACAACCTGTGCTAAATT
CYATATGCAATCTGCTGGTTTGTACATATTTGGCTTATGAGCAAAGTTTGGATATTGAGGTG
TTGCCCATTTGTTTATGGAGATGATGTGATTTTTTCCGTTTCTCCCTTTGGATGCTGAATA
TCTGGTTTCCAGAGTGCAGCCAGAAATTTTGGGATGGAAGTGACCTCATCAGATAAATCTGGTC
CCCCAAGACTTTTGAAAATGGATGAGATTGAATTTTTAAAGAGGACAACAAAATTTTTTCT
GGCTCCACCTATAAGGTGGGGCCTTGGCCTGGATAACCATGGAACAACATATTATGTGGAT
GAAGAATTTGGAAACCTTTCCAGAACAACCTGTTAGCTTTGAAAATGAGTTGGTGTTCATG
GGAAAGAAATTTATGATGATTATAAAAGTAGTTTTAATCCTATTTTGAATCAATGGCGAGTG
TGCATGCAGGACTATGAAGTGGCCCTGCATCGCATGCTACGCTATGTTTTTATTAAATGA
TTTAATTTGATTTTATTGTTAGTTTTAGTTTTAAGTAAGTTAGAATTAGATTATTTAAT
TTAGCTTTAAAGATTTTATTGATTGAATTTGGCCACCAATC