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(54) Title: TRANSCRIPTION FACTOR ARNTL2 GENE AND EXPRESSION PRODUCTS THEREOF USED IN THE DIAGNOSIS, PREVENTION, AND TREATMENT OF TYPE 1 DIABETES

(57) Abstract: The present application identifies the involvement of the HIFß- homologous *Arntl2* gene in the control of type 1 (insulin-dependent) diabetes. Accordingly, the present invention provides a method of determining the susceptibility of a subject to developing insulin-dependent diabetes based on the expressing level of the *Arntl2* gene. The present invention also provides a method for identifying compounds effective for treating or preventing insulin-dependent diabetes in a subject in need thereof and a method of treating or preventing insulin-dependent diabetes by administering an effective amount of compound identified by the identification method. The present invention also provides a method of enhancing protection against insulitis progression or autoimmune diabetes development in a subject in need thereof comprising, enhancing expression of the *Arntl2* gene or modulating the expression of target genes thereof.

TITLE OF THE INVENTION

Transcription factor Arntl2 gene and expression products thereof used in the diagnosis, prevention, and treatment of type 1 diabetes

BACKGROUND OF THE INVENTION

Field of the Invention

The present application implicates the involvement of the *HIFß*- homologous *Arntl2* gene in the control of type 1 (insulin-dependent) diabetes. Accordingly, the present invention provides a method of determining the susceptibility of a subject to developing insulin-dependent diabetes based on the expressing level of the *Arntl2* gene. The present invention also provides a method for identifying compounds effective for treating or preventing having insulin-dependent diabetes in a subject in need thereof and a method of treating or preventing insulin-dependent diabetes by administering an effective amount of compound identified by the identification method. The present invention also provides a method of enhancing protection against insulitis progression or autoimmune diabetes development in a subject in need thereof comprising, enhancing expression of the *Arntl2* gene.

Discussion of the Background

Type 1 or insulin dependent diabetes (IDDM) is an autoimmune disease characterized by the progressive destruction of insulin-producing ß-cells of the islets of Langerhans by infiltrating lymphocytes (1, 2). The disease, which affects about 0.3% of the Caucasian population, is both multifactorial and polygenic, with the MHC class II locus and the insulin locus being the two best studied genetic loci (3, 4).

The non-obese diabetes (NOD) mouse (5, 6) is a well-characterized animal model of IDDM. More than twenty murine insulin dependent diabetes susceptibility loci (*Idd*) have been genetically identified (7), but little information has been obtained about the nature of these non-MHC *Idd* genes. Construction of congenic strains, differing from the NOD receiver strain by only a selected genetic region derived from a non-diabetes prone parental donor strain (8, 9), is a widely used approach allowing the definition of disease-related candidate regions. A promising strategy for candidate

gene identification is to combine a variety of phenotypic studies of congenic mice with expression profiling, haplotype and mutational analysis (10-13).

Several *Idd* loci have been identified on mouse chromosome 6. (14-16). Recently, the IDDM associated loci *Idd6*, *Idd19*, and *Idd20* on distal chromosome 6 have been further defined by the analysis of a series of congenic strains, carrying C3H/HeJ genomic material for distal chromosome 6 introgressed onto the NOD/Lt genetic background, with their candidate regions being refined respectively to 4.5, 7 and 4 cM (17).

NOD/Lt alleles at the *Idd6* locus on distal mouse chromosome 6 confer susceptibility to IDDM, whilst C57BL/6, C57BL/10 and C3H/HeJ alleles all confer resistance to diabetes (14, 17, 18). The NOD.C3H congenic strain described in this study carries NOD alleles at both the *Natural Killer* gene complex (18) and the candidate region for the islet-specific BDC-6.9 autoantigen gene (19), which excludes both these loci as responsible for the disease resistance. The *Idd6* candidate region does however overlap with the candidate region for the resistance of immature T-cells to dexamethasone (20-22). *Idd6* has also been suggested to control low rates of proliferation in immature NOD-thymocytes (23).

Recently we have undertaken a detailed phenotypic analysis of the *Idd6* locus containing congenic strain NOD.C3H 6.VIII (17), which shows resistance to the spontaneous development of diabetes. We have shown that this resistance is not ascribable to the resistance of islet ß-cells to immune destruction or to a default in pathogenic T cells. Protection of the congenic strain likely involves changes in the proportions of the various leukocyte subsets infiltrating the pancreatic islet, and in particular that of $CD4^+$ T cells. Critical to our understanding of the reduced diabetes susceptibility of the *Idd6* congenic mice has been our finding that their splenocytes conferred enhanced disease protection in diabetes transfer assays (24).

However, heretofore, there remained a critical need for the identification of specific genes that control and/or regulate type 1 or insulin dependent diabetes. Additionally, heretofore, there remained a critical need for the identification and development of safe therapeutics for treating or preventing type 1 or insulin dependent diabetes.

SUMMARY OF THE INVENTION

To address the aforementioned critical needs, in the present application the inventors describe the transcriptional profiling of all identified transcripts within the *Idd6* interval of the murine model system. A total of six transcripts distributed throughout the interval were found to have strongly altered expression profiles when comparing splenic tissues of the disease protected congenic NOD.C3H 6.VIII and a NOD control strain. Analysis of newly created subcongenic strains showed the presence of at least three diabetes related sub-loci within the *Idd6* locus. The recently identified control of disease protection mediated splenocytes was mapped to a 700 kb interval, which contains the Aryl hydrocarbon receptor nuclear translocator-like 2 (*Arntl2*, *Bmal2*) encoding gene. This candidate gene was strongly upregulated in the NOD.C3H 6.VIII congenic strain and exhibited a large number of sequence polymorphisms and alternative splice variants. *Arntl2* upregulation correlated with the upregulation of the ARNT-binding site containing *Pla2g4a* gene that has recently been shown to be required for protection against insulitis progression and autoimmune diabetes development. Accordingly, the present invention targets *Arntl2* and its downstream targets for controlling type 1 diabetes resistance.

It is an object of the present invention to provide a method of determining the susceptibility of a subject to developing insulin-dependent diabetes by:

a) acquiring a sample from the subject;

b) determining the expression level of the *Arntl2* gene in the sample;

c) comparing the expression level of the *Arntl2* gene determined in (b) with that of the average expression level of the *Arntl2* gene in samples of the corresponding type obtained from the population to which the subject belongs, wherein an expression level of the *Arntl2* gene in the subject that is lower than that of the average expression level of the *Arntl2* gene is correlated with an increased susceptibility in developing insulin-dependent diabetes.

Another object of the present invention is to provide a method for identifying a compound effective for treating or preventing insulin-dependent diabetes in a subject in need thereof by:

a) acquiring a control sample from a diabetes-sensitive NOD mouse;

b) determining the expression level of the *Arntl2* gene in the control sample;

c) administering at least one candidate compound to the diabetes-sensitive NOD mouse;

d) acquiring a test sample from the diabetes-sensitive NOD mouse;

e) determining the expression level of the *Arntl2* gene in the test sample; and

e) comparing the expression level of the *Arntl2* gene determined in (b) with that determined in (e), wherein an increase in the expression level of the *Arntl2* gene in (e) as compared to (b) is correlated with an increase in insulin-dependent diabetes resistance.

It is yet another object of the present invention to provide a method of treating insulin-dependent diabetes in a subject in need thereof by administering an effective amount of a composition containing the compound identified by the method above.

Another object of the present invention to provide a method of preventing insulin-dependent diabetes in a subject in need thereof by administering an effective amount of a composition containing the compound identified by the method above.

It is still another object of the present invention to provide a method of enhancing protection against insulitis progression or autoimmune diabetes development in a subject in need thereof by enhancing expression of the *Arntl2* gene in cells of the subject.

It is yet another object of the present invention to provide a method of enhancing protection against insulitis progression or autoimmune diabetes development in a subject in need thereof comprising modulating expression of a target gene of the *Arntl2* gene in cells of the subject. Within this object, the target genes may be one or more of *Pla2g4a*, *Gpx*, *Chi313*, and *Mpo*.

The above objects highlight certain aspects of the invention. Additional objects, aspects and embodiments of the invention are found in the following detailed description of the invention.

BRIEF DESCRIPTION OF THE FIGURES

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following Figures in conjunction with the detailed description below.

Figure 1 shows genotyping using a large marker panel for distal chromosome 6, which permits estimation of the size of the C3H derived intervals. (A) Cumulative incidence of different mouse strains and (B) diabetes protection in splenocyte co-transfer. P-values are < 0.0001 for 6.VIII, < 0.0001 for 6.VIIIa, 0.01 for 6.VIII c, 0.026 for 6.VIIIc against CO in diabetes incidence; 0.012 for 6.VIII, 0.568 for 6.VIIIa, 0.339 for 6.VIIIb, and 0.048 for 6.VIIIc against CO, and < 0.0001 for CO against diabetogenic splenocytes (Db) in diabetes transfer assay.

Figure 2 shows a map of the C3H derived intervals (grey bars) on distal chromosome 6 contained in the original NOD.C3H 6.VIII and new subcongenic mouse strains 6.VIIIa, 6.VIIIb, 6.VIIIc. The localization of the newly defined candidate region for the splenocyte subphenotype (*Idd6.3*) is indicated by dotted lines. Based on NCBI Human version 35.1 June 2004, Mouse version 34.1 Feb 28, 2005.

Figure 3 shows the relative expression of *Arntl2* (*Bmal2*) gene in strain 6.VIII (white bars) and NOD CO mice (black bars) indicated as arbitrary units. Pooled RNA from spleen and thymus of four pre-diabetic female mice (A) and spleen at different ages and 15 weeks old diabetic mice (B) were analysed for the expression of *Arntl2* by Q-RT-PCR.

Figure 4 shows the transcription profile and architecture of *Bmal2*. (A) Transcript profiles of *Bmal2* in 6.VIII and NOD mice were identical as shown by Northern blotting. 1, thymus; 2, testis; 3, spleen; 4, skeletal muscle; 5, lung; 6, liver; 7, kidney; 8, heart; 9, brain. (B) Three isoforms Bmal2a, Bmal2b, and Bmal2c of 579, 199 and 355 amino acids length, were present in the spleen (bottom). (C) Partial sequences identified in spleen indicate the presence of strain-specific isoforms (top).

Figure 5 shows C3H versus NOD polymorphisms within *Bmal2*. (A) Within the coding sequence of *Bmal2*, six codons at positions 71, 425, 426, 450, 455 and 483, and one synonymous mutation corresponding to codon 94 differed between the 6.VIII and NOD strains. (B) The alignment of partial 3'UTR of the *Bmal2* gene, corresponding to Ensembl Chr.6 sequence positions 147,759,731 to 147,760,164, from strain 6.VIII (upper sequence) and NOD displays significant sequence variation. The length of this region in 6.VIII is 123 bases shorter than in NOD mice.

Figure 6 shows circadian transcription profiles of *Arntl2*, *Arntl1*, *Per1*, and *PAI-1* expression in spleens of single eight weeks old 6.VIII (white circles) and NOD

(black triangles) mice housed under 14 hour light (blank bar) and 10 hour dark cycle (filled bar) which are shown as arbitrary units. ZT= zeitgeber time

Figure 7 shows relative expression in arbitrary units of *Pla2g4a* (A) in the spleens of different aged mice of 6.VIII (white bars) and NOD (black bars) strains. Data were pooled from four pre-diabetic female mice. (B) Circadian profile of *Pla2g4a* in spleen samples of 8 week-old 6.VIII (white circles) and NOD (black triangles) mice kept under 14 hours of light (blank bar) and 10 hours of dark (filled bar). ZT= zeitgeber time.

Figure 8 shows a graphic depiction of information relevant to mBmal2.

Figure 9 shows the transcription profile of mBmal2.

Figure 10 shows the gene structure of mBmal2.

Figure 11 shows the SNPs and Indels of mBmal2. The sequence depicted as "Query" is SEQ ID NO: 35 and the sequence depicted as "Sbjct" is SEQ ID NO: 36.

Figures 12A-B shows the Bmal2 coding region. NOD control is shown in SEQ ID NO: 33, while 6.VIII is shown in SEQ ID NO: 34.

Figures 13A-C shows the designation of the intron and exon portions of the genomic sequence. Figure 13A: 071-E1 (118F-668R) – 3 SNP (Ensembl Chr.6 147727278 to 147727780), Exon: underlined; NOD control is shown in SEQ ID NO: 37, while 6.VIII is shown in SEQ ID NO: 38. Figure 13B: SNP35-38 (SNP35-38-64F+SNP35-38-465R) – 1 SNP (Ensembl Chr.6 147746092-147746427); NOD control is shown in SEQ ID NO: 39, while 6.VIII is shown in SEQ ID NO: 40. Figure 13C: 071-E5 (131F+465R) – 5 SNPs (Ensembl Chr.6 147751781 to 147752273) Exon : underlined ; NOD control is shown in SEQ ID NO: 41, while 6.VIII is shown in SEQ ID NO: 42. Figure 13D: 071-SNP 75-80 (114F+440R) – 1 SNPs (Ensembl Chr.6 starting from the 3rd base 147758806 to 147759169); NOD control is shown in SEQ ID NO: 43, while 6.VIII is shown in SEQ ID NO: 44.

Figure 14 shows the Bmal2 coding region which marked exons. Bmal2 coding region (SEQ ID NO: 45) – exons marked by different colors (CO as example) Bold: the same exon but with splice forms (The part in bold and underlined is spliced out in mBmal2b sequence resulting in the early stop of translation. The present inventors found the same types of sequences in NOD and 6.VIII.) Underline and and

italics with underline: the alternative exons of 6.VIII Italics: the alternative exons of NOD control.

Figures 15A-B shows the Sequencing files (071-43F to 071-2122R) corresponding to Bmal2c. More specifically, these figures show the sequencing data corresponding to Bmal2c by amplification using SEQ ID NO: 31 as the forward primer and the reverse complement of SEQ ID NO: 32 as the reverse primer. Bmal2c was cloned into a pGEM-T vector. The coding region is underlined. NOD control (Fig. 15A) is shown in SEQ ID NO: 46 with the encoded polypeptide appearing as SEQ ID NO: 47, while 6.VIII (Fig. 15B) is shown in SEQ ID NO: 48 with the encoded polypeptide appearing as SEQ ID NO: 49.

Figure 16 shows the 3' UTR (right after the stop codon), the first base corresponds to Ensembl v37 chr.6 147759660. NOD control is shown in SEQ ID NO: 50, while 6.VIII is shown in SEQ ID NO: 51.

Figures 17A-D shows the upstream genomic sequence (primers: 071-16798F+071-17695R). Fig. 17A: Upstream genomic sequence (primers: 071-16798F+071-17695R) - 4 SNPs (6.VIII sequence blast to Ensembl Chr.6 : starting from the 100st base to the end 147715903 to 147716583- did not see the result of 1-99); NOD control is shown in SEQ ID NO: 52, while 6.VIII is shown in SEQ ID NO: 53. Fig. 17B: Upstream genomic sequence (primers: 071-17610F-18412R) - 5 SNPs (Ensembl Chr.6 147716616 to 147717298); NOD control is shown in SEQ ID NO: 54, while 6.VIII is shown in SEQ ID NO: 55. Fig. 17C: Upstream genomic sequence (primers: 071-18235F-19236R) - 2 SNPs (The 1st base=Ensembl Chr.6 147717274 - the sequence has been broken to several pieces in the blast result); NOD control is shown in SEQ ID NO: 56, while 6.VIII is shown in SEQ ID NO: 57. Fig. 17D: Upstream genomic sequence (primers: 071-19129F+071-19896R) - 6SNPs (The 24th nt to the end = Ensembl Chr.6 147718263 to 147718817); NOD control is shown in SEQ ID NO: 58, while 6.VIII is shown in SEQ ID NO: 59.

DETAILED DESCRIPTION OF THE INVENTION

Unless specifically defined, all technical and scientific terms used herein have the same meaning as commonly understood by a skilled artisan in enzymology, biochemistry, cellular biology, molecular biology, and the medical sciences.

All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

The *Idd6* murine type 1 diabetes locus has been shown to control diabetes by regulating the protective activity of the peripheral immune system as demonstrated by diabetes transfer assays using splenocytes. The analysis of three novel subcongenic NOD.C3H strains has confirmed the presence of at least two diabetes related genes within the 5.4 Mb *Idd6* interval with the disease protection conferred by splenocyte co-transfer being located to a 700 kb subregion. This sub-interval contains the circadian rhythm related transcription factor *Arntl2* (*Bmal2*), a homologue of the type 2 diabetes associated *ARNT (HIF1ß)* gene. As shown in the Examples herein, *Arntl2* exhibited a six-fold upregulation in spleens of the NOD.C3H 6.VIII congenic strain compared to the NOD control strain, strain-specific splice variants and a large number of polymorphisms in both coding and non-coding regions. *Arntl2* upregulation was not associated with changes in the expression levels of other circadian genes in the spleen, but did correlate with the upregulation of the ARNT-binding motif containing *Pla2g4a* gene, that has recently been described as being protective for the progression of insulitis and autoimmune diabetes in the NOD mouse. The present application provides that the *HIFß*- homologous *Arntl2* gene is involved in the control of type 1 diabetes.

Both others and we have previously shown that the immune system, notably spleen and thymus, are required for *Idd6* mediated disease susceptibility in the NOD mouse. In the present application a systematic transcriptional profiling approach to genes located within the candidate region for the murine type 1 diabetes locus *Idd6* is described. In a comparison of the disease protected NOD.C3H congenic strain 6.VIII to its NOD control strain, six genes were found to be differentially expressed in the spleen. We mapped the subphenotype of diabetes disease protection in splenocyte co-transfer assays to a restricted interval of 700 kb by analysis of three newly created

NOD.C3H congenic strains. Whilst this region (*Idd6.3*) contains ten transcripts, only the bHLH-PAS transcription factor superfamily member *Arntl2* (*Bmal2*), a component of the circadian clock pathway, was found to be differentially expressed in the disease protected 6.VIII strain.

Arntl2 contains a large number of NOD/C3H polymorphisms within the 5'UTR, exonic, and 3'UTR sequences of its transcript. Several polymophisms in *Arnt2* will lead to changes in its functional domains, and could be expected to influence its dimerization, transcriptional activity and/or specificity. In addition, putative alternative strain-specific splice forms were identified. It has previously been suggested that such alternative splicing of *ARNTL2* (*BMAL2*) might provide tissues with a rheostat capable of regulating CLOCK:BMAL2 heterodimer function across a broad continuum of potential transcriptional activities, and that this might be important in accommodating a variety of metabolic demands and physiological roles (36).

In the Examples of the present application, it is shown that changes in *Arntl2* transcript levels are not associated with widespread generalized changes in the expression levels of other circadian and hypoxia-induced genes in spleen. The BMAL-CLOCK heterodimers are however known to activate E-box element-dependent transcription (27) and our microarray analysis and quantitative RT-PCR on spleen samples have revealed the *Cytosolic phospholipase A(2)alpha (cPLA(2)alpha)*, which contains an ARNT binding motif, as a potential downstream target of *Arntl2*. The *Cytosolic phospholipase A(2)alpha (cPLA(2)alpha, Pla2g4a)* gene is known to play an important role in arachidonate pathway. Non-obese diabetic (NOD) mice deficient in cPLA(2)alpha show severe insulitis and an increased incidence of diabetes. In the macrophages of these knockout mice, prostaglandin E(2) (PGE(2)) production is decreased and tumour necrosis factor (TNF)-alpha production is increased. Overall the results suggest that cPLA(2)alpha plays a protective role in the progression of insulitis and the development of autoimmune diabetes via suppression of TNF-alpha production from macrophages (37). This observation correlates with our finding that peritoneal macrophages of pre-diabetic 6.VIII mice show a 2.8 fold decrease in Tnf-alpha expression (unpublished data), data that could suggest that *Arntl2* may be involved in the control the Tnf-alpha pathway in macrophages of 6.VIII mice.

A more precise understanding of how the upregulation and polymorphisms of the widely expressed *Arntl2* gene in the 6.VIII strain interact in the regulation of different aspects of the immune system will benefit from additional studies, in particular as it can be expected that the role of *Arntl2* may vary from tissue to tissue and between cell types. For example, it is possible to identify and characterize tissue-specific splice variants that effectuate enhanced effects. In relation with previously described phenotypes for *Idd6* whose alleles appear to be involved in the regulation of proliferation and apoptosis in the thymus (22, 23), it is important to note that *Arntl2* downregulation was found to enhance cell proliferation (26). Another study has identified *Arntl2* as being differentially expressed in various $CD4^+CD25^+$ regulatory T cell subpopulations (38). This finding is of interested because $CD4^+CD25^+$ T cell activity has been found to be modulated by *Idd6* alleles (24).

Of particular interest is recent data showing that a homologue of *Arntl2*, *ARNT* (*HIF1ß*) is associated with type 2 diabetes in both human and mouse and as being essential for normal pancreatic beta cell function and insulin production (39, 40). ARNT, also known as the Hypoxia-Inducible Factor 1, heterodimerizes with both BMAL1 and BMAL2 to regulate gene transcription. These and our data implicating *Arntl2* in type 1 diabetes in the mouse suggest some communality of genetic and molecular pathways of type 1 and type 2 diabetes, and that ARNT like genes may set the clock for mechanisms of disease protection.

In view of the foregoing, the present invention provides a method of diagnosing the susceptibility of a subject to developing insulin-dependent diabetes by:

a) acquiring a sample from said subject;

b) determining the expression level of the *Arntl2* gene in said sample;

c) comparing the expression level of the *Arntl2* gene determined in (b) with that of the average expression level of the *Arntl2* gene in samples of the corresponding type obtained from the population to which said subject belongs, wherein an expression level of the *Arntl2* gene in said subject that is lower than that of the average expression level of the *Arntl2* gene is correlated with an increased susceptibility in developing insulin-dependent diabetes.

Within this embodiment, any mammal may be used as the subject. Examples of mammals suitable for use in the present invention include humans, rats, and mice.

In this embodiment, it is preferred that the *Arntl2* gene is at least 70% homologous, preferably at least 80% homologous, more preferably at least 90% homologous, and most preferably at least 95% homologous to the sequence of SEQ ID NO: 3. Further, it is preferred that the *Arntl2* gene encodes a protein that is at least 70% homologous, preferably at least 80% homologous, more preferably at least 90% homologous, and most preferably at least 95% homologous to the sequence of SEQ ID NO: 4. Still further, it is preferred that the *Arntl2* gene product possess aryl hydrocarbon receptor nuclear translocator activity.

In this embodiment, the term "% homologous" includes "% similarity" and "% identity". Incidentally, it is preferred that the *Arntl2* gene is at least 70% identical, preferably at least 80% identical, more preferably at least 90% identical, and most preferably at least 95% identical to the sequence of SEQ ID NO: 3. Further, it is preferred that the *Arntl2* gene encodes a protein that is at least 70% identical, preferably at least 80% identical, more preferably at least 90% identical, and most preferably at least 95% identical to the sequence of SEQ ID NO: 4. Still further, it is preferred that the *Arntl2* gene product possess aryl hydrocarbon receptor nuclear translocator activity.

Since the *Arntl2* gene is found to be ubiquitously expressed (see, for example, Schoenhard et al, Am. J. Physiol Cell Physiol. 2002 Jul; 283(1):C103-114), within this embodiment, the sample may be obtained from one of several sources. These sources include many other body tissues and/or cells. Particular exemplary cell types include: mucosa, spleen, thymus, blood, and pancreas. The skilled artisan would know how to and would select the most appropriate source for the samples for use in the methods of the present invention.

Preferably, the sample contains at least one type of cells selected from the group consisting of CD4(+) T cells, CD8(+) T cells, B cells, and macrophages. In an aspect of this embodiment, the sample is obtained from the spleen.

Within this embodiment, the sample may be acquired by conventional techniques that are readily known to the skilled artisan. For example, the sample may be obtained by tissue biopsy, a blood sample, or a mucosa sample.

The determination method of the expression level of the *Arntl2* gene may be achieved by any known method. For example, it is possible to quantitate the amount

of *Arntl2* transcripts by quantitative PCR techniques using primers designed based upon the known sequence of the *Arntl2* gene. The artisan is referred to Current Protocols in Molecular Biology, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (2000), among other well known treatises for a discussion of standard PCR protocols. Other quantitation techniques that may be used to effectuate the expression level determination include ELISA and/or Western blot techniques.

Within this embodiment, the expression level of the *Anrtl2* gene of the candidate subject is compared to that of the average expression level of the *Arntl2* gene in splenic samples of the corresponding type (i.e., where the sample from the subject is acquired from the spleen the comparative expression level would be from spleen, etc.) obtained from the population to which said subject belongs. It is also envisioned in the present invention that the sample may be from a variable source (e.g., thymus, spleen, pancreas, blood, mucosa, etc.) while the comparative expression level is that for a known standard source (e.g., blood).

To this end, the basal expression level for each individual member of the population may be obtained via the same procedure as that of the candidate subject. Following collection of a representative number of members in the population to which the candidate subject belongs an average expression level is determined to which the expression level of the candidate subject can be compared. Within this embodiment, it is preferred that the representatives of the population be clinically screened as to their type 1 diabetes status so as to ensure an unbiased population.

As set forth in the Examples of the present application, in subjects that are resistant to diabetes the expression level of the *Arntl2* gene is significantly higher than that found in diabetes-sensitive subjects. As such, where the expression level of the *Arntl2* gene in the candidate subject is lower than that of the average expression level of the *Arntl2* gene this decreased expression may be correlated to an increased susceptibility in developing insulin-dependent diabetes.

In another embodiment of the present invention is a method for identifying a compound effective for treating or preventing insulin-dependent diabetes in a subject in need thereof by:

a) acquiring a control sample from a diabetes-sensitive NOD mouse;

b) determining the expression level of the *Arntl2* gene in said control sample;

c) administering at least one candidate compound to said diabetes-sensitive NOD mouse;

d) acquiring a test sample from said diabetes-sensitive NOD mouse after said administering;

e) determining the expression level of the *Arntl2* gene in said test sample; and

e) comparing the expression level of the *Arntl2* gene determined in (b) with that determined in (e), wherein an increase in the expression level of the *Arntl2* gene in (e) as compared to (b) is correlated with an increase in insulin-dependent diabetes resistance.

In this embodiment, it is preferred that the *Arntl2* gene is at least 70% homologous, preferably at least 80% homologous, more preferably at least 90% homologous, and most preferably at least 95% homologous to the sequence of SEQ ID NO: 1. Further, it is preferred that the *Arntl2* gene encodes a protein that is at least 70% homologous, preferably at least 80% homologous, more preferably at least 90% homologous, and most preferably at least 95% homologous to the sequence of SEQ ID NO: 2. Still further, it is preferred that the *Arntl2* gene product possess aryl hydrocarbon receptor nuclear translocator activity.

In this embodiment, the term "% homologous" includes "% similarity" and "% identity". Incidentally, it is preferred that the *Arntl2* gene is at least 70% identical, preferably at least 80% identical, more preferably at least 90% identical, and most preferably at least 95% identical to the sequence of SEQ ID NO: 1. Further, it is preferred that the *Arntl2* gene encodes a protein that is at least 70% identical, preferably at least 80% identical, more preferably at least 90% identical, and most preferably at least 95% identical to the sequence of SEQ ID NO: 2. Still further, it is preferred that the *Arntl2* gene product possess aryl hydrocarbon receptor nuclear translocator activity.

In a preferred aspect of this embodiment, the control sample is obtained from the spleen of the diabetes-sensitive NOD mouse. Preferably, the control sample contains at least one type of splenic cells selected from the group consisting of CD4(+) T cells, CD8(+) T cells, B cells, and macrophages. Alternatively, it is possible that the foregoing control sample may be obtained from the thymus, pancreas, a blood sample, or a mucosa sample of the diabetes-sensitive NOD mouse.

In a preferred aspect of this embodiment, the test sample is obtained from the spleen of the diabetes-sensitive NOD mouse. Preferably, the test sample contains at least one type of splenic cells selected from the group consisting of CD4(+) T cells, CD8(+) T cells, B cells, and macrophages. Alternatively, it is possible that the foregoing test sample may be obtained from the thymus of the of the diabetes-sensitive NOD mouse. In addition, the test sample may be obtained from other tissue and/or cell sources, such as the pancreas. In addition to a tissue biopsy, the sample may also be acquired from a blood sample or a mucosa sample.

Within this embodiment, the sample may be acquired by conventional techniques that are readily known to the skilled artisan. For example, the sample may be obtained by tissue biopsy, a blood sample, or a mucosa sample.

The determination method of the expression level of the *Arntl2* gene may be achieved by any known method. For example, it is possible to quantitate the amount of *Arntl2* transcripts by quantitative PCR techniques using primers designed based upon the known sequence of the *Arntl2* gene. Examples of suitable PCR primers include the primer pairs represented by SEQ ID NO: 11 (071-248F) and SEQ ID NO: 12 (071-334R) or SEQ ID NO: 13 (AY-56F) and SEQ ID NO: 14 (AY-136R). Additional primers suitable for use are the primer pair: 071-43F - GGGAGGATTGTTAGCACGTCTGTGA (SEQ ID NO: 31) and 071-2122R - the reverse and complementary sequence of 5'-CACTGTACTCTTGAGCACTGTATTG-3' (SEQ ID NO: 32).

The artisan is referred to Current Protocols in Molecular Biology, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (2000), among other well known treatises for a discussion of standard PCR protocols. Other quantitation techniques that may be used to effectuate the expression level determination include ELISA and/or Western blot techniques.

As set forth in the Examples of the present application, in subjects that are resistant to diabetes the expression level of the *Arntl2* gene is significantly higher than that found in diabetes-sensitive subjects. As such, where the expression level of the *Arntl2* gene in the test sample as compared to the control sample has increased due to contact with the candidate compound(s), the increased expression level of the *Arntl2* gene may be correlated with an increase in insulin-dependent diabetes resistance.

In the context of the present invention a difference in expression level is considered to be "significant" when it is a reproducible and noticeable and/or measurable difference. More preferably, the term "significant" refers to a statistically significant difference. As the skilled artisan would appreciate, statistically significance can be determined by any conventional statistical analysis method. The difference is considered statistically significant when the p-value is 5%, more preferably 1%, and most preferably 0.1%.

Within this embodiment, the candidate compound(s) is not particularly limited. It is envisioned that in the present invention the candidate compound(s) may be a drug, a polynucleotide, a polypeptide, immunogenic fragments of polypeptides, a hormone, etc. or a salt thereof. Further, within this embodiment there is no particular limitation on the number of compounds that may be simultaneously administered to the subject. In other words, a compound may be separately administered or multiple compounds may be administered sequentially or simultaneously. Further, the compounds may be administered as pharmaceutical compositions containing one or more pharmaceutically acceptable carriers, diluents, excipients, and adjuvants, or mixtures of the same.

Also, the present method is adaptable to determining the effect of a wide range of dosage forms and amounts. Therefore, it is envisioned that the compound(s) may be administered via any route including orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid or solid form.

The time between the administration of the candidate compound(s) and the recovery of a test sample may range from instantaneous to minutes to hours to weeks. Further, the administration may be either a single administration or may include multiple repeated administration events prior to recovery of a test compound. For example, the present invention embraces repeated individual administration events of the same (or different compounds) once hourly, every four to six hours, twice daily, once daily, once weekly, etc.

The phrase "effective for treating a subject having insulin-dependent diabetes" or the term "treating" as used herein means that the administration of the compound(s) results in a reduction of symptoms associated with insulin-dependent diabetes or of at least one disorder induced, caused or mediated by insulin-induced diabetes by at least

10%, preferably at least 25%, more preferably at least 50%, still more preferably at least 75%, even more preferably at least 80%, yet more preferably at least 90%, and most preferably at least 95%.

The phrase "effective for preventing a subject having insulin-dependent diabetes" or the term "preventing" as used herein means that the administration of the compound(s) results in a reduction in the likelihood that a subject with a propensity of developing or believed to be at risk for developing insulin-dependent diabetes will indeed develop insulin-dependent diabetes. Preferably, in the context of the present invention, this phrase means that the administration of the compound(s) results in the elimination of the likelihood or probability that a subject with a propensity of developing or believed to be at risk for developing insulin-dependent diabetes will indeed develop insulin-dependent diabetes.

In another embodiment of the present invention is a method of treating insulin-dependent diabetes in a subject in need thereof by administering an effective amount of a composition containing a compound(s) that was determined to be effective for treating a subject having insulin-dependent diabetes by the method of the foregoing embodiment.

In another embodiment of the present invention is a method of preventing insulin-dependent diabetes in a subject in need thereof by administering an effective amount of a composition containing a compound(s) that was determined to be effective for preventing insulin-dependent diabetes in a subject in need thereof having by the method of the foregoing embodiment.

As used in the present application, the term "subject in need thereof" is used to designate the subject as being one with a recognized need for prophylactic and/or therapeutic treatment of at least one disorder induced, caused or mediated by insulin-induced diabetes. Within this embodiment and the present invention as a whole, the subject may be any mammal, including by not limited to: a human, a rat, and a mouse.

Within this embodiment, there is no particular limitation on the number of compounds that may be simultaneously administered to the subject. In other words, a compound may be separately administered or multiple compounds may be administered sequentially or simultaneously. Further, the compounds may be administered as pharmaceutical compositions containing one or more

pharmaceutically acceptable carriers, diluents, excipients, and adjuvants, or mixtures of the same.

Also, the present method is adaptable to a wide range of dosage forms and amounts. Therefore, it is envisioned that the compound(s) may be administered via any route including orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid or solid form.

Within this embodiment, the composition may be administered in single or repeated dosages. For example, the composition may be administered once hourly, every four to six hours, twice daily, once daily, once weekly, etc.

Further, the term "effective amount" is any amount that results in the reduction of symptoms associated with insulin-dependent diabetes or of at least one disorder induced, caused or mediated by insulin-induced diabetes by at least 10%, preferably at least 25%, more preferably at least 50%, still more preferably at least 75%, even more preferably at least 80%, yet more preferably at least 90%, and most preferably at least 95%.

However, it is generally preferred that the nature of the compound and the nature of the administration method and dosage be tailored to that determined to be effective by the above-described identification method.

Yet another embodiment of the present invention is a method of enhancing protection against insulitis progression or autoimmune diabetes development in a subject in need thereof comprising modulating expression of a target gene of the *Arntl2* gene in cells of said subject.

Suitable targets within the scope of the present invention include *Pla2g4a*, *Gpx*, *Chi313*, and *Mpo* (see Example 8).

Therefore, in still another embodiment of the present invention is a method of enhancing protection against insulitis progression and/or autoimmune diabetes development in a subject in need thereof by enhancing expression of the *Arntl2* gene in the cells of said subject.

This embodiment is based on the observation in the Examples below that *Arntl2* upregulation was not associated with changes in the expression levels of other circadian genes in the spleen, but did correlate with the upregulation of the ARNT-binding motif containing *Pla2g4a* gene, that has recently been described as being

protective for the progression of insulitis and autoimmune diabetes in the NOD mouse. The present application provides that the *HIFß-* homologous *Arntl2* gene is involved in the control of type 1 diabetes. As such, enhancing the expression of the Arntl2 gene in the cells of a subject would be expected to upregulate the expression level of the *Pla2g4a* gene, which in turn would enhance protection against insulitis progression and/or autoimmune diabetes development.

In accordance with the definition of "subject in need thereof" defined above, within this embodiment, the subject may be any mammal, including but not limited to: a human, a rat, and a mouse.

In this embodiment, it is preferred that the *Arntl2* gene is at least 70% homologous, preferably at least 80% homologous, more preferably at least 90% homologous, and most preferably at least 95% homologous to the sequence of SEQ ID NO: 1. Further, it is preferred that the *Arntl2* gene encodes a protein that is at least 70% homologous, preferably at least 80% homologous, more preferably at least 90% homologous, and most preferably at least 95% homologous to the sequence of SEQ ID NO: 2. Still further, it is preferred that the *Arntl2* gene product possess aryl hydrocarbon receptor nuclear translocator activity.

In this embodiment, the term "% homologous" includes "% similarity" and "% identity". Incidentally, it is preferred that the *Arntl2* gene is at least 70% identical, preferably at least 80% identical, more preferably at least 90% identical, and most preferably at least 95% identical to the sequence of SEQ ID NO: 1. Further, it is preferred that the *Arntl2* gene encodes a protein that is at least 70% identical, preferably at least 80% identical, more preferably at least 90% identical, and most preferably at least 95% identical to the sequence of SEQ ID NO: 2. Still further, it is preferred that the *Arntl2* gene product possess aryl hydrocarbon receptor nuclear translocator activity.

In this embodiment, it is preferred that the *Arntl2* gene is at least 70% homologous, preferably at least 80% homologous, more preferably at least 90% homologous, and most preferably at least 95% homologous to the sequence of SEQ ID NO: 3. Further, it is preferred that the *Arntl2* gene encodes a protein that is at least 70% homologous, preferably at least 80% homologous, more preferably at least 90% homologous, and most preferably at least 95% homologous to the sequence of SEQ ID

NO: 4. Still further, it is preferred that the *Arntl2* gene product possess aryl hydrocarbon receptor nuclear translocator activity.

In this embodiment, the term "% homologous" includes "% similarity" and "% identity". Incidentally, it is preferred that the *Arntl2* gene is at least 70% identical, preferably at least 80% identical, more preferably at least 90% identical, and most preferably at least 95% identical to the sequence of SEQ ID NO: 3. Further, it is preferred that the *Arntl2* gene encodes a protein that is at least 70% identical, preferably at least 80% identical, more preferably at least 90% identical, and most preferably at least 95% identical to the sequence of SEQ ID NO: 4. Still further, it is preferred that the *Arntl2* gene product possess aryl hydrocarbon receptor nuclear translocator activity.

As stated above, since the *Arntl2* gene is found to be ubiquitously expressed (see, for example, Schoenhard et al, Am. J. Physiol Cell Physiol. 2002 Jul; 283(1):C103-114), within this embodiment and the present invention as a whole, the sample may be obtained from one of several sources. These sources include many other body tissues and/or cells. Particular exemplary cell types include: spleen, thymus, blood, mucosa, and pancreas.

In an aspect of this embodiment, the sample is obtained from the spleen. Preferably, the sample contains at least one type of splenic cells selected from the group consisting of CD4(+) T cells, CD8(+) T cells, B cells, and macrophages.

Within this embodiment, the sample may be acquired by conventional techniques that are readily known to the skilled artisan. For example, the sample may be obtained by tissue biopsy, a blood sample, or a mucosa sample.

As a means of enhancing expression of the *Arntl2* gene, the following methods may be mentioned, but are not intended to be an exhaustive list of suitable methods:

- overexpression by gene therapy;
- administration of specific drugs that inhibit Arntl2 protein degradation;
- therapy to produce a stable form of *Arntl2*.

As used herein, the term "*Arntl2*" is used to designate the polynucleotide sequence of SEQ ID NO: 1 obtained from mice and SEQ ID NO: 3 obtained from humans and homologous sequences coding for polypeptides with the same function as the polypeptides shown by SEQ ID NO: 2 or 4. Specifically, the term "*Arntl2*" is used

to designate the open-reading frame, inclusive of exons and introns. However, it is to be recognized that the protein encoded by the *Arntl2* gene would constitute only the exonic regions. Where necessary to distinguish, the present application refers to the mouse *Arntl2* gene as "*mArntl2*" and the human *Arntl2* gene as "*hArntl2*."

The present invention also includes polynucleotides that hybridize to the complement of the polynucleotide sequence of *Arntl2*, or homologs and/or fragments thereof, under stringent conditions.

The terms "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a polynucleotide will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing).

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 5X SSC, preferably 1X to 2X SSC, (20X SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 68°C, preferably 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA--DNA hybrids, the Tm can be approximated from the equation of Meinkoth and Wahl,

Anal. Biochem., 138:267-284 (1984): Tm =81.5°C+16.6 (log M)+0.41 (%GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Tm is reduced by about 1°C for each 1% of mismatching; thus, Tm, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with approximately 90% identity are sought, the Tm can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize hybridization and/or wash at 1, 2, 3, or 4°C. lower than the thermal melting point (Tm); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (Tm); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (Tm). Using the equation, hybridization and wash compositions, and desired Tm, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a Tm of less than 45°C (aqueous solution) or 32°C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (2000).

In the context of the present invention, a polynucleotide sequence is "homologous" with the sequence according to the invention if at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95% of its base composition and base sequence is identical to the sequence according to the invention (i.e., *Arntl2*).

Another object of the present invention are the polypeptide sequences encoded by *Arntl2*, or a homolog thereof. The polypeptides of the present invention exhibit aryl hydrocarbon receptor nuclear translocator activity.

According to the invention, a "homologous protein" or "homologous polypeptide" is to be understood to comprise proteins (polypeptides) which contain an amino acid sequence at least 70 % of which, preferably at least 80 % of which, more preferably at least 90%, most preferably at least 95% of which corresponds (i.e., is identical and/or similar) to the amino acid sequence encoded by *Arntl2*. It is particularly preferred that the homologous protein retain at least 50%, preferably at least 70%, more preferably at least 80%, most preferably at least 90% of the residual activity of the wild-type hydrocarbon receptor nuclear translocator activity. The homologous proteins embrace homologous and non-homologous amino acid substitutions, as well as polymorphs and alternative spliced variants.

The expression "homologous amino acids" denotes those that have corresponding properties, particularly with regard to their charge, hydrophobic character, steric properties, etc.

Homology, sequence similarity or sequence identity of nucleotide or amino acid sequences may be determined conventionally by using known software or computer programs such as the *BestFit* or *Gap* pairwise comparison programs (GCG Wisconsin Package, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711). *BestFit* uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of identity or similarity between two sequences. *Gap* performs global alignments: all of one sequence with all of another similar sequence using the method of Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970). When using a sequence alignment program such as *BestFit*, to determine the degree of sequence homology, similarity or identity, the default setting may be used, or an appropriate scoring matrix may be selected to optimize identity, similarity or homology scores. Similarly, when using a program such as *BestFit* to determine sequence identity, similarity or homology between two different amino acid sequences, the default settings may be used, or an appropriate scoring matrix, such as *blosum45* or *blosum80*, may be selected to optimize identity, similarity or homology scores. Sequence alignments can

also be performed using the Align.ppc program (Mac Molly TetraLite, Mologen) or ClustalW.

One skilled in the art is also aware of conservative amino acid replacements such as the replacement of glycine by alanine or of aspartic acid by glutamic acid in proteins as "sense mutations" which do not result in any fundamental change in the activity of the protein, i.e. which are functionally neutral. It is also known that changes at the N- and/or C-terminus of a protein do not substantially impair the function thereof, and may even stabilize said function.

The term "isolated" means separated from its natural environment.

The term "polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, and can denote an unmodified RNA or DNA or a modified RNA or DNA. Further, this term embraces recombinant polynucleotides. Of course, this term also embraces salt forms thereof.

The term "polypeptides" is to be understood to mean peptides or proteins that contain two or more amino acids that are bound via peptide bonds. Further, this term embraces recombinant polypeptides. Of course, this term also embraces salt forms thereof.

The present inventors have sequenced the murine gene *Arntl2* in the NOD/Lt and C3HHeJ strains and identified polymorphisms between the diabetes sensitive strain NOD/Lt and the diabetes resistant strain C3HHeJ (see Example 6 and Figures 8-17). These polymorphs form a part of the present invention. The reference sequence NM_172309 refers to the diabetes-resistant C57BL/6J strain. It should be noticed that the present inventors have also identified alteration of transcription levels of *Arntl2*, which is upregulated in the diabetes resistant congenic NOD.C3H strain 6.VIII.

The above written description of the invention provides a manner and process of making and using it such that any person skilled in this art is enabled to make and use the same, this enablement being provided in particular for the subject matter of the appended claims, which make up a part of the original description.

As used above, the phrases "selected from the group consisting of," "chosen from," and the like include mixtures of the specified materials.

Where a numerical limit or range is stated herein, the endpoints are included. Also, all values and subranges within a numerical limit or range are specifically included as if explicitly written out.

The above description is presented to enable a person skilled in the art to make and use the invention, and is provided in the context of a particular application and its requirements. Various modifications to the preferred embodiments will be readily apparent to those skilled in the art, and the generic principles defined herein may be applied to other embodiments and applications without departing from the spirit and scope of the invention. Thus, this invention is not intended to be limited to the embodiments shown, but is to be accorded the widest scope consistent with the principles and features disclosed herein.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples, which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLES

Material and Methods

RNA preparation, cDNA synthesis and microarray analysis -

Total RNA was prepared using RNABle (Eurobio). Random cDNA synthesis was carried out on 6 µg DNAseI treated total RNA using SuperScript™ II reverse transcriptase (Invitrogen) according to the manufacturer's conditions. For microarray experiments, RNA quality was examined using an Agilent 2100 Bioanalyser (Agilent). DNA-microarrays (8k mouse cDNA, Agilent) were hybridised using 10 µg of total RNA transcribed in the presence of Cy3-dCTP or Cy5-dCTP, respectively. Data were from four individual experiments, each including a dye swap, were analysed using Feature Extraction and Rosetta resolver software ($p<0.05$) and annotated using SOURCE software (provided by the Genetics Department, Stanford University).

Northern blot and RACE experiment -

Total RNA of various tissues from the 6.VIII and NOD control strains was separated in TBE on 1% agarose gels containing 1% formaldehyde and transferred on Hybond N+ membranes (Amersham). Northern blots were hybridized using a 3' NOD cDNA fragment amplified with the *Arntl2* specific primers AY-555F 5'-AGGCAACACCAGAGCACTGA-3' (SEQ ID NO: 5) and AY334R 071-334R 5'-GCCAGGATTACAAAGTGTGCAC-3' (SEQ ID NO: 6). 5' and 3' RACE experiments were performed using both total spleen RNA extracted from NOD CO and 6.VIII strain and the GeneRacer Kit (Invitrogen).

Quantitative PCR -

Quantitative PCR was performed on an ABIPRISM 7700 Sequence detector using the SYBR Green PCR Master Mix (PE Biosystems) according to the manufacturer's conditions. Primers were designed using PrimerExpress software and used at optimal concentration. Quantification of the amplification product was carried out using the Standard curve method. For the circadian rhythm analysis we used the ΔCT method and the *Gapdh* gene expression as reporter. Sequences of the oligonucleotides used were as follows:

Gene	Primer name	primer sequence
Bcat1	030-263F	GGAGTGACCAGGCAGAGCA (SEQ ID NO: 7)
	030-344R	CATCCATGGTGAGGTGTCTCTC (SEQ ID NO: 8)
Las1	032-138F	CTGTGACCTTGATTCAGGATGC (SEQ ID NO: 9)
	032-218R	GGACTTTGTTCATGCCACAGG (SEQ ID NO: 10)
Arntl2	071-248F	GGTGACAGAGTCCTTGCCTAGC (SEQ ID NO: 11)
	071-334R	GCCAGGATTACAAAGTGTGCAC (SEQ ID NO: 12)
	AY-56F	GTGGCTATGGGACGGTTGC (SEQ ID NO: 13)
	AY-136R	AGTTATGAACTCAGCCGGTCTCA (SEQ ID NO: 14)
BE647206	082-258F	TGAAGAAAAGCAGCCTTCCTTAG (SEQ ID NO: 15)
	082-338R	GGTAGGCACGTCCATTAAGGAG (SEQ ID NO: 16)
Mlstd1	098-660F	AAAGCCTTGGGAGAGATAGTAGTGC (SEQ ID NO: 17)
	098-740R	TGCTCCCACTATGGATGGC (SEQ ID NO: 18)
mCG1027210	106-497F	GGTCGTTCATCCTCAGTCCAC (SEQ ID NO: 19)
	106-578R	TGCAGGTGTGAAGTTTTATATCCAG (SEQ ID NO: 20)
Arntl1	mBmal1-224F	GCCCAAAGAGGACTCATCCC (SEQ ID NO: 21)
	mBmal1-304R	CGATCATTCGACCTATTTTTCCTG (SEQ ID NO: 22)
Per1	mPer1-377F	TATTCCCTACCCCACCTAGTTATCC (SEQ ID NO: 23)
	mPer1-460	AGGGCGAGTGGGAAGCAG (SEQ ID NO: 24)
PAI-1	PAI-147F	GTCTGCATCCCTGTATGTCAGG (SEQ ID NO: 25)
	PAI-236R	CCACTAGGCGGCAGTGTGA (SEQ ID NO: 26)
Pla2g4a	2329F	TCGTTGCTCTGTTTCCCTCA (SEQ ID NO: 27)
	2425R	ATCATCCCAGCACAGAAATTACAC (SEQ ID NO: 28)
Gapdh	GAPDH-RT-F	TGCACCACCAACTGCTTAG (SEQ ID NO: 29)
	GAPDH-RT-R	GATGCAGGGATGATGTTC (SEQ ID NO: 30)

Sequence analysis -

DNA fragments were amplified and sequenced from genomic DNA or cDNA of the NOD.C3H strain 6.VIII and the NOD control mice. Polymporphisms were identified by sequence alignment using Megalign (DNASTAR Inc.). Potential transcription factor binding sites were identified by using the MatInspector program, which is available from Genomatix Software GmbH (Munich, Germany) (41).

Construction of mouse strains -

The subcongenic strains were constructed by intercrossing the *Idd6* congenic NOD.C3H 6.VIII strain (6.VIII) and the NOD control congenic strain (CO), both originally derived from crosses between C3H/HeJ and NOD/Lt mice (17). Male mice heterozygous for the *Idd6* interval were then backcrossed to the CO strain. Recombinant offspring were selected using the polymorphic markers D6*Mit*14, D6*Mit*15, D6*Mit*294 and D6*Mit*304. The corresponding subcongenic intervals were fixed by intercrossing of the heterozygous offspring resulting from a backcross to the CO strain.

Diabetes assessment and transfer assays -

Spontaneous diabetes incidence was monitored weekly from 10 to 30 weeks of age by assessment of glucosuria (Diabur test, Roche). Splenocyte co-transfer was performed by transferring 10^7 splenocytes from diabetic NOD mice together with 2x 10^7 splenocytes from seven week old mice of various mouse strains onto five week old NOD/Scid mice. Cumulative diabetes incidence was monitored weekly throughout 10 weeks post transfer.

Statistical analysis -

Statistics were performed by Kaplan-Meier estimation and log-rank test for group comparison. Pooled data from quantitative RT-PCR were compared as mean +/- standard deviation.

Example 1: Refinement of the Idd6 interval by haplotype mapping -

The original microsatellite based genotyping of the diabetes-resistant *Idd6* congenic strain NOD.C3H 6.VIII (strain 6.VIII) indicated that the C3H introgressed donor sequence was located at the end of chromosome 6, distal to the microsatellite marker D6*Mit*113 (17, 25). Random sampling of potential SNPs listed in the genomic databases identified four polymorphisms located between bps 144,874,468 and 144,874,516 on mouse chromosome 6. These SNPs included a SNP at bp position 144,874,516 associated with a silent amino acid exchange in the *Sox5* gene, located distal to D6*Mit*113 (Ensembl mouse database for *Mus musculus*) (Table 1). The mapping of these newly identified SNPs allowed the *Idd6* locus to be restrained from a 6.1 Mb to a 5.4 Mb interval lying in between the *Sox5* locus and the telomere of mouse chromosome 6.

Table 1 - Genes that are significantly differentially expressed in the diabetes-resistance strain 6.VIII compared to the diabetes-sensitive NOD mice at 6-7 weeks old of age. Data were pooled from the analysis of three or four pre-diabetic animals showing no signs of insulitis progression. The sequence positions shown are according to the NCBI build m34.

Gene Name	**Position in Chr. 6 (*Idd6* subinterval)**	**Fold change in spleen 6.VIII/CO**
Bcat1	145,845,604-145,922,395 (*Idd6.2*)	- 2.1 ± 0.4
Casc1	146,021,369-146,057,345 (*Idd6.2*)	+ 2.9 ± 0.43
Arntl2	147,726,464-147,759,659 (*Idd6.3*)	+ 6.7 ± 1.62
BE647206, AW120472	148,167,817-148,169,401 (*Idd6.1*)	+ 7.2 ± 4.31
Mlstd1	148,976,675-149,116,580 (*Idd6.1*)	+ 1.5 ± 0.3[(a)]
mCG1027210	149,535,041-149,574,578 (*Idd6.1*)	absent in the NOD strain

(a) The expression of Mlstd1 in strain 6.VIII at 4 weeks of age is 2.5 fold higher that in NOD mice.

Example 2: Construction and diabetes incidence of subcongenic strains -

In order to further refine the type 1 diabetes associated *Idd6* candidate region localising within 4 cM (5.4 Mb) of distal mouse chromosome 6, we constructed a series of subcongenic strains by intercrossing the *Idd6* congenic NOD.C3H 6.VIII strain (6.VIII) and the NOD control congenic strain (CO), that were originally derived from crosses between the C3H/HeJ and NOD/Lt mouse strains. Heterozygous male mice resulting from the intercross were then again backcrossed to the CO strain and recombinants were selected amongst the offspring using the polymorphic markers D6*Mit*14, D6*Mit*15, D6*Mit*294 and D6*Mit*304 (Figure 1). Out of the 200 BX1 animals that were tested three were found to be recombinants. The corresponding subcongenic intervals were fixed by further backcrossing to the CO strain and by intercrossing of the heterozygous recombinant animals. Genotyping using a large marker panel for distal chromosome 6, as shown in Figure 1, allowed the estimation of the size of the C3H derived intervals. We noticed that all three breakpoints for recombination were located between the markers D6*Mit*294 and D6*Mit*373, suggesting that this region may recombine more frequently than others within the *Idd6* interval.

We tested the diabetes incidence weekly for all three subcongenic strains in parallel to the parental strains over a period of 30 weeks (Figure 2). In female mice, all the newly created strains were protected compared to the CO strain, although each strain was slightly less protected than the 6.VIII strain. Data were similar for male mice, although male mice of each strain developed less diabetes than female mice. This result led us to conclude that at least two intervals (*Idd6.1* and *Idd6.2*) and several genes in the *Idd6* region contribute to the overall diabetes protection of the 6.VIII strain (Table 1).

Example 3: Analysis of inhibition of diabetes transfer -

We have previously shown that *Idd6* modifies suppression of diabetes in co-transfer assays when using splenocytes. We tested whether this splenocyte sub-phenotype segregates with one or other of the newly derived C3H derived sub-intervals. A total of 2 x 10^7 splenocytes from 7 week-old mice were injected into NOD/Scid recipient mice together with 10^7 total splenocytes from diabetic mice. As

expected, injection of the diabetogenic cells alone resulted in the rapid induction of diabetes in the NOD/Scid recipient. Co-transfer of splenocytes inhibited significantly the diabetes transfer in all the groups tested (Figure 2). As previously described, stronger protection was found with the 6.VIII splenocytes than with CO splenocytes. Similar significant protection was found for strain 6.VIIIc, but not for either strains 6.VIIIa or 6.VIIIb. Strain 6.VIIIc differs from strain 6.VIIIa by only a 700 kb C3H derived interval (*Idd6.3*) lying between the markers D6*Mit*294 (147.2 Mb, C3H allele in strain 6.VIIIc, NOD allele in 6.VIIIa) and D6*Mit*373 (147.9 Mb, C3H allele in both 6.VIIIc and 6.VIIIa).

Example 4: Transcriptional profiling of genes in the Idd6 interval -

Diabetes associated genes are expected to be either functional coding sequence variants or to show differential regulation between diabetes sensitive and a diabetes resistant strains. Detection of functional coding variants would require extensive sequencing efforts throughout the entire 5.4 Mb *Idd6* candidate interval, which contains some hundred potential genes, which would likely through up a very large number of sequence variants for evaluation. We turned therefore first to expression analysis for the identification of potential candidate genes responsible for the susceptibility to IDDM. Potential mouse transcripts within *Idd6* were identified from the Celera and public databases. Additional information was obtained by examination of the syntenic region to *Idd6* in the human genome, which maps to the 12p11-p12.2 chromosomal region (NCBI version 35.1, Figure 1).

Since our previous results had indicated that splenocytes contribute to the disease regulation mediated by *Idd6*, the expression profiles of potential transcripts in the spleen were examined. Those genes that were expressed in the spleen were then analysed by real time RT-PCR for differential expression in the diabetes-sensitive NOD mice and the diabetic-resistant congenic strain 6.VIII. Spleen samples from both four weeks old and six to seven weeks old mice were chosen in order to capture genes showing differences during the primary stages of disease progression before the onset of overt diabetes. Six transcripts were found to have such differential expression in spleen. These genes were *Bcat1*, *Csac1* (*Las1*), *Arntl2* (*Bmal2*), a gene of unknown

function represented by two EST clones BE647206 and AW120472, *Mlstd1* (*Msl2*), and the predicted transcript mCG1027210 (Celera database) (Table 1).

The 700 kb *Idd6.3* interval contains a total of ten genes (Figure 1, Table 2) with seven transcripts, *4933424B01Rik*, *Tm7sf3*, *Stk381*, *LOC232534*, *1700023A16Rik*, *Ppfib1*, and *2210417D09Rik*, being unlikely candidates for IDDM because of their known role or inappropriate expression pattern. None of the genes showed however differential expression between the 6.VIII and CO strains except for the *Arntl2* (*Bmal2)* (brain-muscle- ARNT-like protein 2) gene that was six-fold overexpressed in spleens of the 6.VIII strain. We turned therefore to a detailed analysis of *Arntl2.*

Table 2 - Candidate genes in the *Idd6.3* candidate region. If not otherwise stated, information concerning the expression profile was obtained from the NCBI GEO and the MGI databases. Names of known genes are indicated in bold.

Transcripts in the *Idd6.3* interval M. musculus Genome (Build 34.1), Chromosome: 6, Map: rna			
start- stop	**Accession**	**Locus**	**Description**
147470323-147498450	NM_138757.1	4933424B01Rik	**RIKEN cDNA 4933424B01 gene** strongly expressed in brain and mammary gland
147498626-147519888	NM_026218	**Fgfr1op2**	**FGFR1 oncogene partner 2** ubiquitous, fusion protein associated with myeloproliferative syndrome (42)
147523051 - 147555191	NM_026281.1	**Tm7sf3**	**Tm7sf3: Transmembrane 7 superfamily member 3** highly in diencephanlon; mainly kidney expressed (43)
147563270-147571291	NM_025315.1	**Surb7**	**SRB7 (supressor of RNA polymerase B) homolog (S. cerevisiae)** highest in brain stem; downregulated in spleen nockout embryonic lethal (44)
147645809-147699737	NM_172734.2	Stk38l	**serine/threonine kinase 38 like** highly in bladder and olfactory system
147703404-147704208	XM_620385.1	LOC232534	similar to 40S ribosomal protein S2
147726464-147759659	NM_172309.1	***Arntl2/ Bmal2***	**aryl hydrocarbon receptor nuclear translocator-like 2** (see detailed analysis herein)
147777200-147798519	XM_132958.2	1700023A16Rik	**RIKEN cDNA 1700023A16 gene** male genital specific
147815629-147958590	NM_026221.1	**Ppfibp1**	**PTPRF interacting protein, binding protein 1 (liprin beta 1)** **RIKEN cDNA 1700034J05 gene** absent in lymph nodes and thymus
147959104-147960052	NM_025620.1	2210417D09Rik	**RIKEN cDNA 2210417D09 gene** intestine and stomac restricted expression

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Example 5: Expression and transcript analysis of the *Arntl2* candidate gene -

The *Arntl2* gene encodes a basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) transcription factor and has been functionally linked to circadian clock mediated activities and to the regulation of cell proliferation (26). The *Arntl2* gene was expressed in significantly higher amounts in spleen samples obtained from either 4 weeks old or 6-7 weeks old diabetes-resistant strain 6.VIII animals than from diabetes-sensitive NOD mice (Figure 3A). Thymi obtained from the same groups of animals showed a similar tendency, although with only two-fold difference, between the strain 6.VIII and NOD mice (Figure 3B). Lymphocyte subsets, including B cells, CD4+ T cells, CD8+ T cells, CD4+CD25+ regulatory T cells showed similar expression differences to the whole tissue preparations. Further expression profiling of spleens showed that differential expression of *Arntl2* was independent of the age of the animals and maintained from two to twelve weeks of age as well as in diabetic animals (Figure 3C). These results suggest that the differential expression of *Arntl2* in the two mouse strains is independent of disease progression.

The detailed transcript pattern of *Arntl2* in multiple tissues was examined in strain 6.VIII and NOD mice. For most of the organs examined such as brain or lung, the major transcripts identified were 9 kb or 0.6 kb in size. The transcript profiles of the spleen and thymus were however surprisingly varied compared to those of other organs, and additional 3.9 kb and 1.6 kb transcripts were found in both spleen and thymus. A 1.4 kb transcript was exclusively found in thymus. The common 0.6 kb transcript, present in most of organs, was not detected in the thymus (Figure 4). Such complex transcription profiles with transcripts specific to the spleen and thymus may indicate a specific role for the *Arntl2* gene in the immune system.

Prior studies have identified two protein products of *Bmal2*, Bmal2a and Bmal2b (27) containing respectively 579 and 199 amino acids. While examining the transcripts expressed in spleen of strain 6.VIII and NOD CO mice, a third putative alternative spliced variant, *Bmal2c*, was identified in a 5' RACE experiment. This transcript initiated within an intron, 100 nucleotides upstream of the start of exon 7 in the consensus mRNA (AY005163, *Arntl2a* mRNA). This transcript encoded a 355 amino acid protein containing only the C-terminal half of the full-length protein and was missing both the bHLH and PAS-A domains (Figure 4B). All these transcripts

were identical between 6.VIII and NOD spleen, but the analysis of partial cDNA sequences amplified from nested primers indicated the existence of transcripts specific for strains 6.VIII and NOD, generated by differential exon use (Figure 4C).

Example 6: Sequence polymorphisms in the *Arntl2* gene -

To validate *Arntl2* as a candidate gene, we analysed the sequence of its coding, 3' UTR and 5' UTR regions for polymorphisms between strain 6.VIII and NOD CO mice. Within exonic regions, one synonymous polymorphism at the wobble position of amino acid 94 (6.VIII: A and NOD mice: G), five non-synonymous polymorphisms and one insertion/deletion were identified (Figure 5A). The leucine residue located within helix I of the HLH region at amino acid position 71 in strain 6.VIII has been replaced by a methionine in NOD mice. This leucine residue, which is highly conserved in the bHLH family, serves as an important contact site by interacting with residues in helix II in the formation of the helix structure, and is also involved in protein dimerisation (28, 29). The other polymorphisms identified were all located between the PAS-B domain and C terminus. Three serine residues in the strain 6.VIII at amino acid position 425, 426 and 455 were all replaced by glycine residues in the NOD mice. In NOD mice, the valine residue at position 450 in strain 6.VIII was replaced by isoleucine, and the glutamic acid at position 483 was deleted.

The 3' UTR sequences of the *Arntl2* gene also showed striking variation. Multiple long insertion/deletions lying between the sequence position 147,759,748, located some 90 nucleotides distal to the stop codon of *Arntl2*, and the position 147,760,154 on mouse chromosome 6, resulted in major variation of the lengths of the DNA fragments in 6.VIII (409 bp) and in NOD (529 bp). Numerous base substitutions were also identified (Figure 5B).

Analysis of the EST clone BY242187 allowed the identification of the upstream 5'UTR sequence of *Arntl2*. Two additional exons were identified between positions 147,716,951 and 147,717,036 (E1') and between positions 147,723,827 and 147,723,947 with the E1' exon locating about 9.4 kb upstream of the ATG. The position of the splice donor of the initial exon of AY005163 was found at position 147,726,370, some 95 nucleotides upstream of the ATG. No polymorphisms were

identified within the 5'UTR region. However, numerous SNPs were identified adjacent to the E1' exon.

The foregoing sequences are and explanatory information is provided in Figures 8-17, which correspond to the following:

Figure 8 shows a graphic depiction of information relevant to mBmal2.

Figure 9 shows the transcription profile of mBmal2.

Figure 10 shows the gene structure of mBmal2.

Figure 11 shows the SNPs and Indels of mBmal2. The sequence depicted as "Query" is SEQ ID NO: 35 and the sequence depicted as "Sbjct" is SEQ ID NO: 36.

Figures 12A-B shows the Bmal2 coding region. NOD control is shown in SEQ ID NO: 33, while 6.VIII is shown in SEQ ID NO: 34.

Figures 13A-C shows the designation of the intron and exon portions of the genomic sequence. Figure 13A: 071-E1 (118F-668R) – 3 SNP (Ensembl Chr.6 147727278 to 147727780), Exon: underlined; NOD control is shown in SEQ ID NO: 37, while 6.VIII is shown in SEQ ID NO: 38. Figure 13B: SNP35-38 (SNP35-38-64F+SNP35-38-465R) – 1 SNP (Ensembl Chr.6 147746092-147746427); NOD control is shown in SEQ ID NO: 39, while 6.VIII is shown in SEQ ID NO: 40. Figure 13C: 071-E5 (131F+465R) – 5 SNPs (Ensembl Chr.6 147751781 to 147752273) Exon : underlined ; NOD control is shown in SEQ ID NO: 41, while 6.VIII is shown in SEQ ID NO: 42. Figure 13D: 071-SNP 75-80 (114F+440R) – 1 SNPs (Ensembl Chr.6 starting from the 3rd base 147758806 to 147759169); NOD control is shown in SEQ ID NO: 43, while 6.VIII is shown in SEQ ID NO: 44.

Figure 14 shows the Bmal2 coding region which marked exons. Bmal2 coding region (SEQ ID NO: 45) – exons marked by different colors (CO as example) Bold: the same exon but with splice forms (The part in bold and underlined is spliced out in mBmal2b sequence resulting in the early stop of translation. The present inventors found the same types of sequences in NOD and 6.VIII.) Underline and and italics with underline: the alternative exons of 6.VIII Italics: the alternative exons of NOD control.

Figures 15A-B shows the Sequencing files (071-43F to 071-2122R) corresponding to Bmal2c. More specifically, these figures show the sequencing data corresponding to Bmal2c by amplification using SEQ ID NO: 31 as the forward primer and the reverse complement of SEQ ID NO: 32 as the reverse primer. Bmal2c

was cloned into a pGEM-T vector. The coding region is underlined. NOD control (Fig. 15A) is shown in SEQ ID NO: 46 with the encoded polypeptide appearing as SEQ ID NO: 47, while 6.VIII (Fig. 15B) is shown in SEQ ID NO: 48 with the encoded polypeptide appearing as SEQ ID NO: 49.

Figure 16 shows the 3' UTR (right after the stop codon), the first base corresponds to Ensembl v37 chr.6 147759660. NOD control is shown in SEQ ID NO: 50, while 6.VIII is shown in SEQ ID NO: 51.

Figures 17A-D shows the upstream genomic sequence (primers: 071-16798F+071-17695R). Fig. 17A: Upstream genomic sequence (primers: 071-16798F+071-17695R) - 4 SNPs (6.VIII sequence blast to Ensembl Chr.6 : starting from the 100st base to the end 147715903 to 147716583- did not see the result of 1-99); NOD control is shown in SEQ ID NO: 52, while 6.VIII is shown in SEQ ID NO: 53. Fig. 17B: Upstream genomic sequence (primers: 071-17610F-18412R) - 5 SNPs (Ensembl Chr.6 147716616 to 147717298); NOD control is shown in SEQ ID NO: 54, while 6.VIII is shown in SEQ ID NO: 55. Fig. 17C: Upstream genomic sequence (primers: 071-18235F-19236R) - 2 SNPs (The 1st base=Ensembl Chr.6 147717274 - the sequence has been broken to several pieces in the blast result); NOD control is shown in SEQ ID NO: 56, while 6.VIII is shown in SEQ ID NO: 57. Fig. 17D: Upstream genomic sequence (primers: 071-19129F+071-19896R) - 6SNPs (The 24th nt to the end = Ensembl Chr.6 147718263 to 147718817); NOD control is shown in SEQ ID NO: 58, while 6.VIII is shown in SEQ ID NO: 59.

Example 7: Circadian regulation of the Arnt2 gene and the circadian genes -

The circadian expression of *Arntl2* (*Bmal2*) in spleen was examined in mice housed under a cycle of fourteen hours artificial light and ten hours obscurity. These settings were identical to that used when diabetes incidence was monitored. Whilst splenic expression of *Bmal2* oscillated moderately during the day, the differences in transcript level between strain 6.VIII and the NOD control were maintained over the whole 24-hour period (Figure 6A). The strain difference in *Bmal2* transcript levels suggested a possible alteration in the expression of other circadian genes regulated by Bmal2. *Arntl1* (*Bmal1*), a master circadian gene and a close homolog of *Bmal2*, oscillated with a cycle which showed lowest expression at the beginning of the dark

phase and highest at the beginning of light (Figure 6B). No significant differences were found for either *Bmal1* or for *Per1*, which is negatively regulated by *Bmal1* (Figure 6C). Similar results were obtained for other circadian genes involved in the autoregulatory feedback loop such as *Per2*, *Per3,* and *Dec1*.

In addition, the *plasminogen activator inhibitor 1* (*PAI-1*) gene, a downstream circadian output gene regulated by Bmal2 *in vitro* (30), did not display strain specific differences in its transcription level (Figure 6D). Hypoxia-inducible factor-1α (Hif-1α), a protein capable of heterodimerizing with *Arntl2 in vitro* (31), mediates expression of *Adrenomedullin*, which is in turn involved in T cell survival (32, 33). Analysis of *Adrenomedullin* expression failed to reveal differences between the 6.VIII strain and the NOD control spleens. We conclude that the up-regulation of *Bmal2* in strain 6.VIII does not lead to a general alteration in transcription levels in the spleen of other circadian and hypoxia-induced genes.

Example 8: Cytosolic phospholipase A2 is a potential downstream target of *Arntl2* -

From microarray experiments using pooled spleen samples from four eight-week old pre-diabetic females we concluded that the replacement of the 5.4 Mb *Idd6* interval by C3H alleles resulted in a deregulation of about 5% of the transcriptome in 6.VIII mice compared to CO mice. We selected 7 downregulated and 14 upregulated transcripts with known or potential immune function to test whether their expression difference in spleen of 6.VIII mice would correlate with that of *Arntl2*.

Real-time RT-PCR using pooled RNA from 6-7 week-old females confirmed the microarray results for two of the downregulated and nine of the upregulated genes that showed fold changes in excess of 1.5. Highest upregulation in the 6.VIII mice was found for the *Chitinase 3-like 1* (*Chi3l1*) (5.6 fold), the *Myeloperoxidase* (*Mpo*) (2.1 fold), and *Cytosolic phospholipase A2* (*Pla2g4a*) (1.7 fold) genes. When the genes were subject to detailed transcriptional analysis using spleen samples from mice of different ages, the hypoxia-involved *Pla2g4a* gene (34, 35) showed a particular interesting expression as it was, like *Arntl2*, upregulated in strain 6.VIII at all ages (Figure 7). A two-fold upregulation was also measured when spleen samples from strain 6.VIIIc were compared to 6.VIIIa samples, confirming that the upregulation was at least to some extent directly to factors lying within *Idd6.3*.

A potential ARNT binding site (TGCGTG) was identified +101 to +106 of its transcription start site, which indicated that *Pla2g4a* might be a direct target of *Arntl2*. Similar to *Arntl2*, *Pla2ga4a* expression was upregulated in different splenic cell population, including CD4(+) T cells, CD8(+) T cell, B cells, and macrophages. We analysed its circadian profile and showed that whilst the expression of *Pla2g4a* oscillated mildly, the variation between strain 6.VIII and CO mice was maintained throughout the day (Figure 7). Interestingly, the circadian profile of *Pla2g4a* whilst very similar to that of *Arntl1* (*Bmal1*) and *Arntl2* (*Bmal2*), was clearly different from those of *Per1* and *PAI-1*. This suggests that *Pla2g4a* circadian expression correlates with that of the *Bmal1* and *Bmal2* and that it may be regulated by these transcription factors.

Example 9: Establishment of cellular *ex vivo* systems for testing of the *Arntl2* gene –

The present inventors were interested in exploring the adequacy of *ex vivo* systems in the characterization of the candidate gene. To this end the present inventors have undertaken studies on the RAW264.7 cell macrophage line and several other currently used mouse cell lines. The present inventors have been able to show that these cell lines can be used for the systematic testing of RNAi constructs cloned into the pSUPER vector system (Oligogene) prior to their sub-cloning into lentiviral vectors. The RAW264.7 is of particular interest for some of these studies because it can also be used for functional studies of the mediated Arntl2 pathways.

In these experiments, transient transfection using the lipofection method (jetPEI™ transfection reagent, Polyplus) of the RAW264.7 cell line results in about a 60% reduction of gene expression, when tested on *Arntl2*. Stable integrants can be expected to show about 90% reduction of expression. *Arntl2* downregulation resulted in deregulation of other genes, known to be involved in diabetes development.

Numerous modifications and variations on the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the accompanying claims, the invention may be practiced otherwise than as specifically described herein.

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CLAIMS

1. A method of determining the susceptibility of a subject to developing insulin-dependent diabetes comprising:

a) acquiring a sample from said subject;

b) determining the expression level of the *Arntl2* gene in said sample;

c) comparing the expression level of the *Arntl2* gene determined in (b) with that of the average expression level of the *Arntl2* gene in samples of the corresponding type obtained from the population to which said subject belongs, wherein an expression level of the *Arntl2* gene in said subject that is lower than that of the average expression level of the *Arntl2* gene is correlated with an increased susceptibility in developing insulin-dependent diabetes.

2. The method of Claim 1, wherein said subject is a human.

3. The method of Claim 1, wherein said *Arntl2* gene is at least 90% homologous to the sequence of SEQ ID NO: 3.

4. The method of Claim 1, wherein said *Arntl2* gene is at least 95% homologous to the sequence of SEQ ID NO: 3.

5. The method of Claim 1, wherein said sample comprises splenic cells.

6. The method of Claim 5, wherein said splenic cells are at least one type selected from the group consisting of CD4(+) T cells, CD8(+) T cells, B cells, and macrophages.

7. A method for identifying a compound effective for treating or preventing insulin-dependent diabetes in a subject in need thereof comprising:

a) acquiring a control sample from a diabetes-sensitive NOD mouse;

b) determining the expression level of the *Arntl2* gene in said control sample;

c) administering at least one candidate compound to said diabetes-sensitive NOD mouse;

d) acquiring a test sample from said diabetes-sensitive NOD mouse after said administering;

e) determining the expression level of the *Arntl2* gene in said test sample; and

e) comparing the expression level of the *Arntl2* gene determined in (b) with that determined in (e), wherein an increase in the expression level of the *Arntl2* gene in (e) as compared to (b) is correlated with an increase in insulin-dependent diabetes resistance.

8. The method of Claim 7, wherein said control sample is acquired from the spleen of said diabetes-sensitive NOD mouse.

9. The method of Claim 8, wherein said test sample is acquired from the spleen of said diabetes-sensitive NOD mouse.

10. The method of Claim 7, wherein said control sample is acquired from the thymus of said diabetes-sensitive NOD mouse.

11. The method of Claim 10, wherein said test sample is acquired from the thymus of said diabetes-sensitive NOD mouse.

12. The method of Claim 7, wherein said *Arntl2* gene is at least 90% homologous to the sequence of SEQ ID NO: 1.

13. The method of Claim 7, wherein said *Arntl2* gene is at least 95% homologous to the sequence of SEQ ID NO: 1.

14. The method of Claim 7, wherein said sample comprises at least one type of splenic cells selected from the group consisting of CD4(+) T cells, CD8(+) T cells, B cells, and macrophages.

15. The method of Claim 7, wherein said determining comprises quantitative PCR.

16. The method of Claim 15, wherein said quantitative PCR utilizes the primer pair represented by SEQ ID NO: 11 and SEQ ID NO: 12.

17. The method of Claim 15, wherein said quantitative PCR utilizes the primer pair represented by SEQ ID NO: 13 and SEQ ID NO: 14.

18. The method of Claim 15, wherein said quantitative PCR utilizes the primer pair represented by SEQ ID NO: 31 and the reverse complementary sequence of SEQ ID NO: 32.

19. A method of treating insulin-dependent diabetes in a subject in need thereof comprising administering an effective amount of a composition comprising a compound identified by the method of Claim 7.

20. The method of Claim 19, wherein said subject in need thereof is a human.

21. A method of preventing insulin-dependent diabetes in a subject in need thereof comprising administering an effective amount of a composition comprising a compound identified by the method of Claim 7.

22. The method of Claim 21, wherein said subject in need thereof is a human.

23. A method of enhancing protection against insulitis progression or autoimmune diabetes development in a subject in need thereof comprising, enhancing expression of the *Arntl2* gene in cells of said subject.

24. The method of Claim 23, wherein said cells are splenic cells.

25. The method of Claim 24, wherein said splenic cells are at least one type selected from the group consisting of CD4(+) T cells, CD8(+) T cells, B cells, and macrophages.

26. The method of Claim 23, wherein said *Arntl2* gene is at least 90% homologous to the sequence of SEQ ID NO: 3.

27. The method of Claim 23, wherein said *Arntl2* gene is at least 95% homologous to the sequence of SEQ ID NO: 3.

28. The method of Claim 23, wherein said method is a method of enhancing protection against insulitis progression.

29. The method of Claim 23, wherein said method is a method of enhancing protection against autoimmune diabetes development.

30. A method of enhancing protection against insulitis progression or autoimmune diabetes development in a subject in need thereof comprising modulating expression of a target gene of the *Arntl2* gene in cells of said subject.

31. The method of Claim 30, wherein said target gene is selected from the group consisting of *Pla2g4a*, *Gpx*, *Chi313*, and *Mpo*.

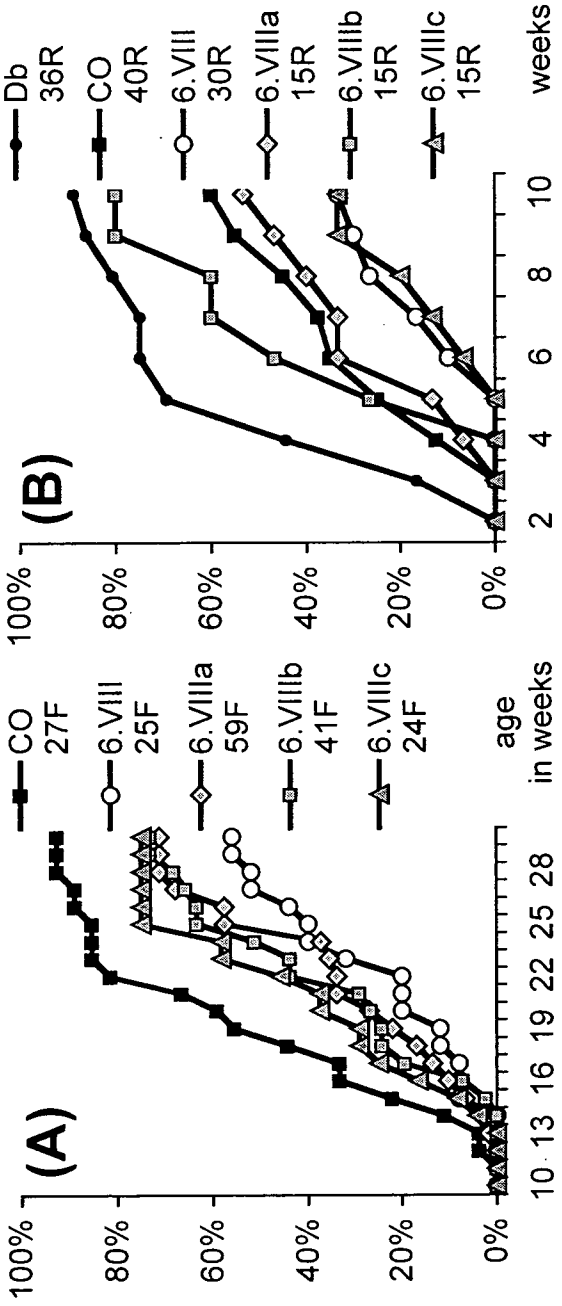
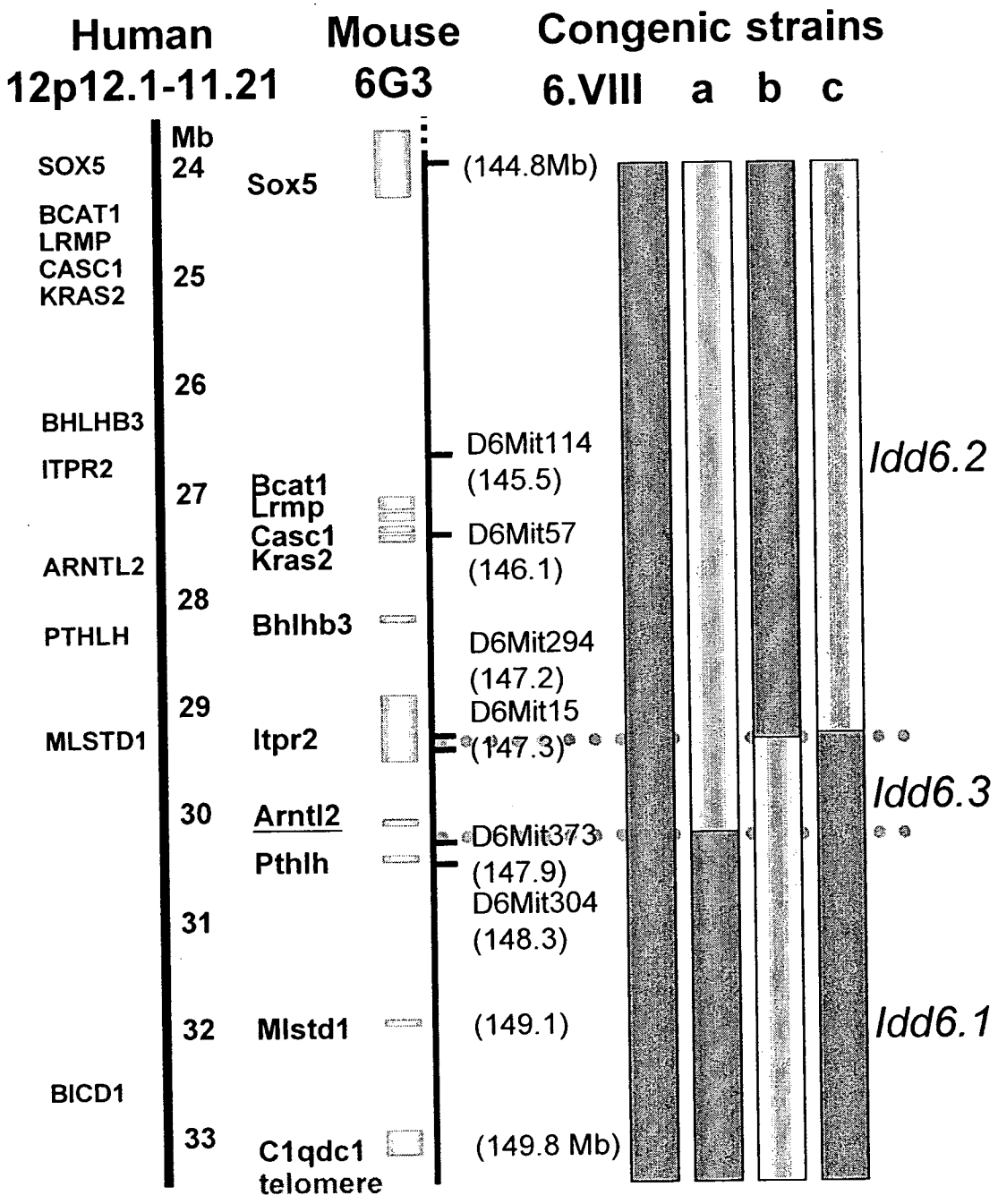

(A)
(B)
100%
80%
60%
40%
20%
0%
CO 27F
6.VIII 25F
6.VIIIa 59F
6.VIIIb 41F
6.VIIIc 24F
Db 36R
CO 40R
6.VIII 30R
6.VIIIa 15R
6.VIIIb 15R
6.VIIIc 15R
10 13 16 19 22 25 28
2 4 6 8 10
age in weeks
weeks

Figure 1


Human
12p12.1-11.21
Mouse
6G3
Congenic strains
6.VIII a b c
Mb
SOX5
24
Sox5
(144.8Mb)
BCAT1
LRMP
CASC1
KRAS2
25
26
BHLHB3
ITPR2
D6Mit114
(145.5)
Idd6.2
27
Bcat1
Lrmp
Casc1
Kras2
D6Mit57
(146.1)
ARNTL2
28
Bhlhb3
PTHLH
D6Mit294
(147.2)
29
D6Mit15
MLSTD1
Itpr2
(147.3)
Idd6.3
30
Arntl2
D6Mit373
Pthlh
(147.9)
D6Mit304
31
(148.3)
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(149.1)
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BICD1
33
C1qdc1
telomere
(149.8 Mb)

Figure 2

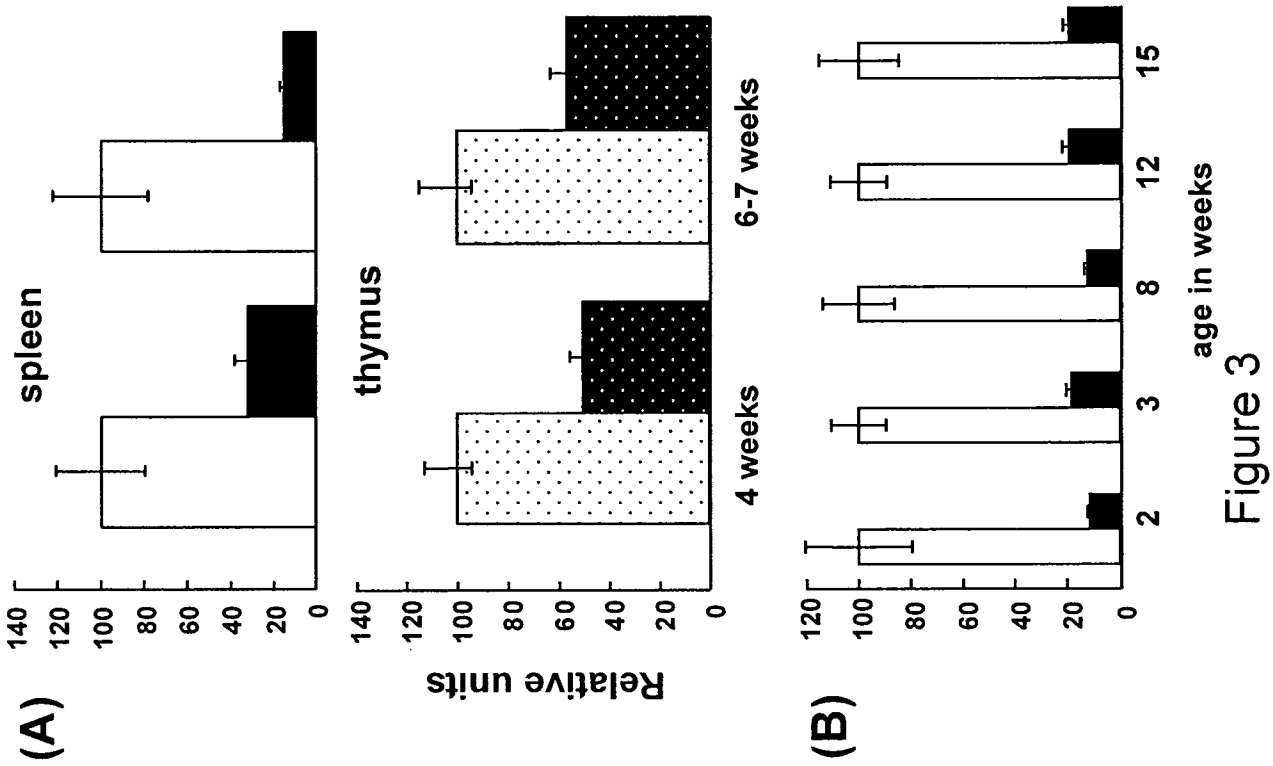

(A)
spleen
thymus
Relative units
4 weeks
6-7 weeks
0
20
40
60
80
100
120
140
(B)
age in weeks
2
3
8
12
15

Figure 3

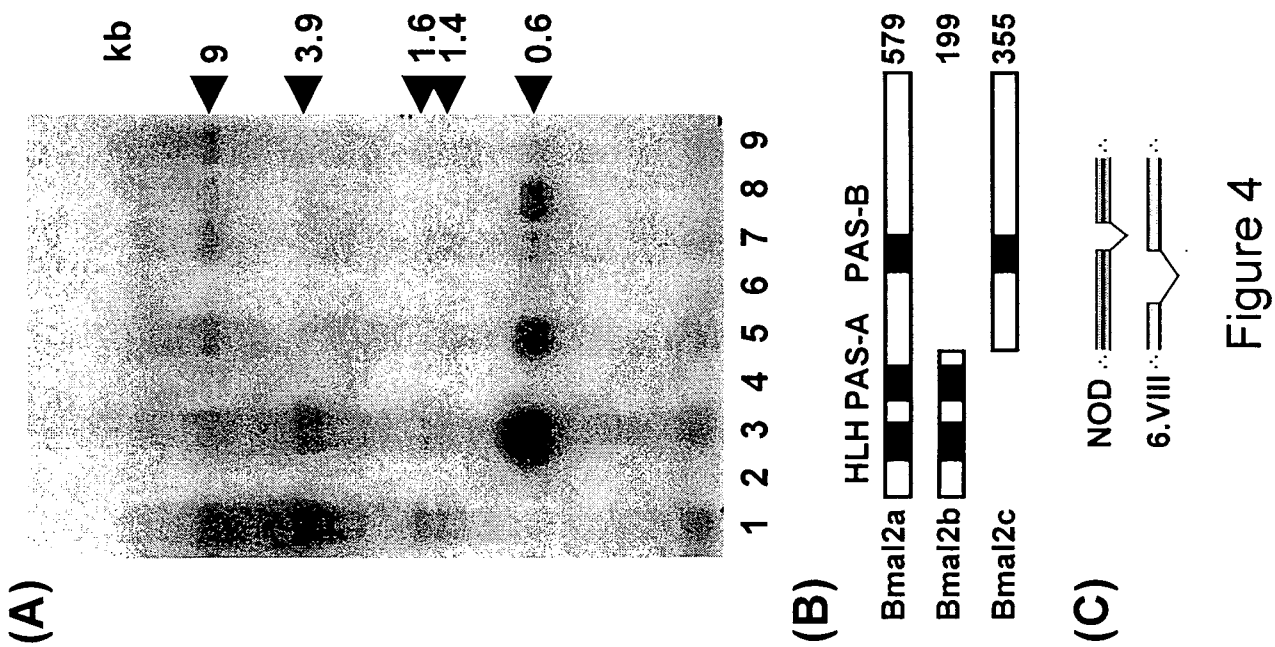
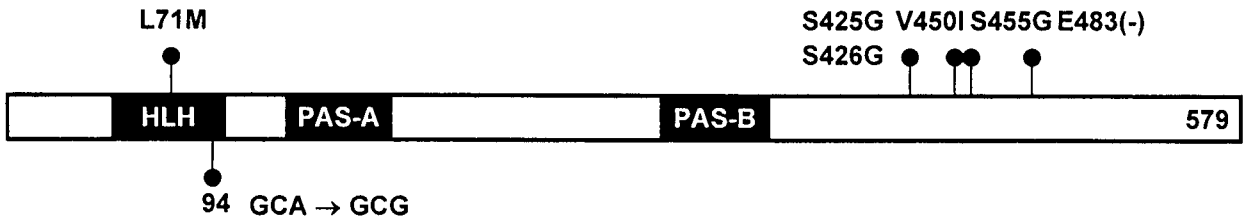

(A)
kb
9
3.9
1.6
1.4
0.6
1 2 3 4 5 6 7 8 9
(B)
HLH PAS-A PAS-B
Bmal2a
579
Bmal2b
199
Bmal2c
355
(C)
NOD
6.VIII

Figure 4

(A)

Helix I:

RDKMNHLIQKLSSMI



(B)

147,759,731

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TGTGCGGAGTCACACATGCTCCTCCAAAGAGAAACAGCCAAGTTTATCAATCCCTCTTTACACAGTGAGAAGGCAGCTTG
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GGGGTCAGGCTGCCATATTTTTGCTAAAATATTCTGACCAAAAACTGCTACCAACCATATTGTTAGGGCTTTTTTTT- - - -
GGGGTCAGGCTGCCATATTTTTGCTAAAATATTCTGACCAAAAACTGCTACCAACCATATTGTTAGGGCTTTTTTTTTTA

- - - - - - - - - - - - - - - - AAATATATATATACT- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - TTTGTT- - - - -
CATTTATTTATTTACTATATGTAAGTACACTGTAGCTGTCCTCAGATACTCCAGAAAAGGGAATCAGATTTCGTTGGGGA

- - - - - - - - - - - - - - - - - - - - - - - - - - - - - TTTGAGTTTGGGATTTTTG- - - - - - - - - - - - - - - - TTTTGTTC- - - - - - -
TGGTTGTGTGCCACCATGTGGTTGCTGGGATTTGAACTCGGGACTTTCGGAAGAGCAGTCGGCGCTCTTAACCACTGAGC

TGTCT- - - - GTTTTCTGTTTTTTGTCTTCAAGACAGGGTTTCTCTGTGTAGCCCTGGCTGTCTTGTAACTCACTCTGTAGA
CATCTCACCAGCCCCTGTTCTTGTTTTCAAGACAAGGTTTCTCTGTGTATCCCTGGCTGTCTTGTAACTCACTCTGTAGA

CCAGGCTGGCCTTGAACTCAAAGATCCGCCTGCCTC- - - TTCCTCCCAAGTGTTGAGATTAAAGCCATATATCACAATTC
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147,760,164

Figure 5

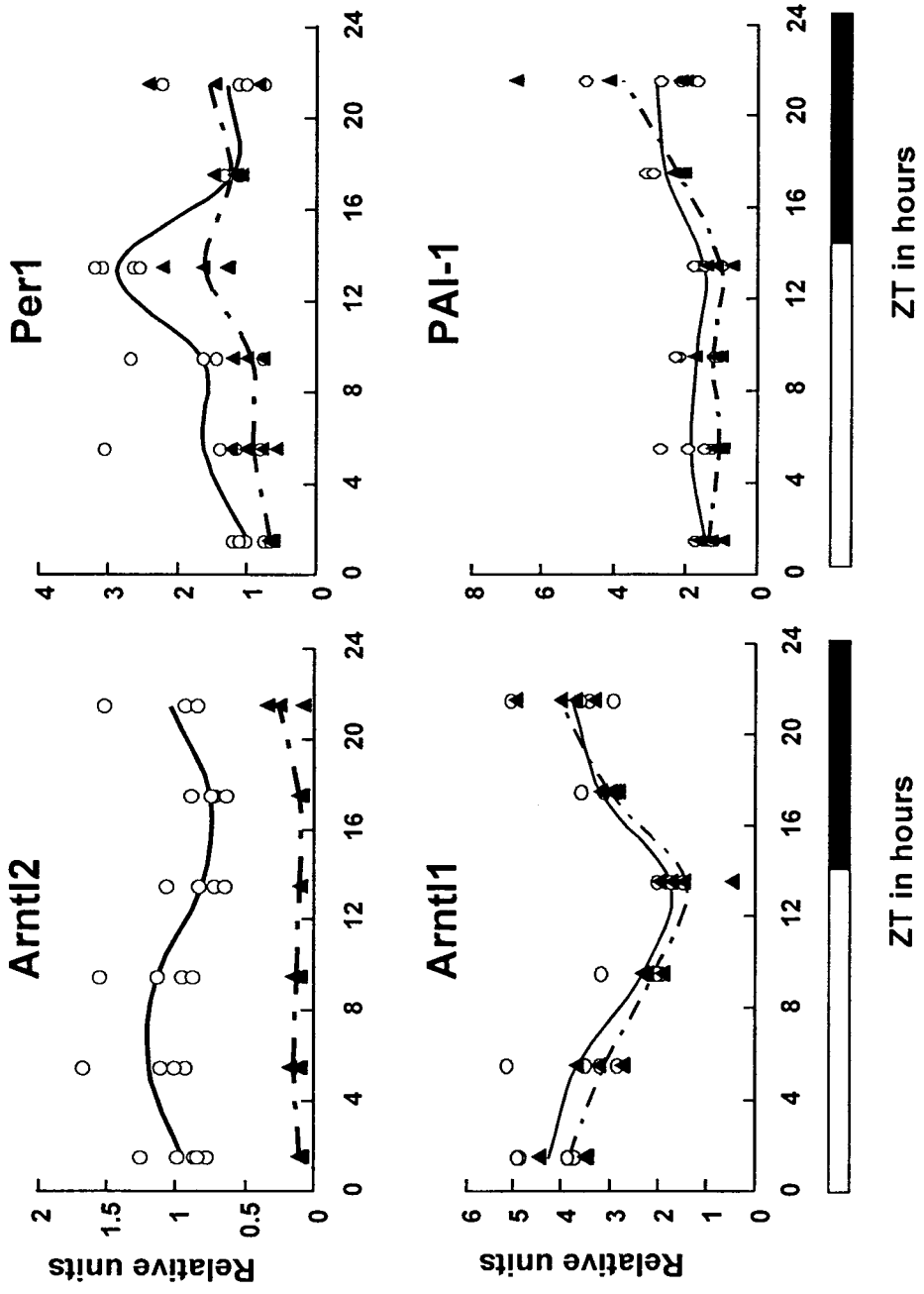

Per1
PAI-1
Arntl2
Arntl1
Relative units
ZT in hours
ZT in hours

Figure 6

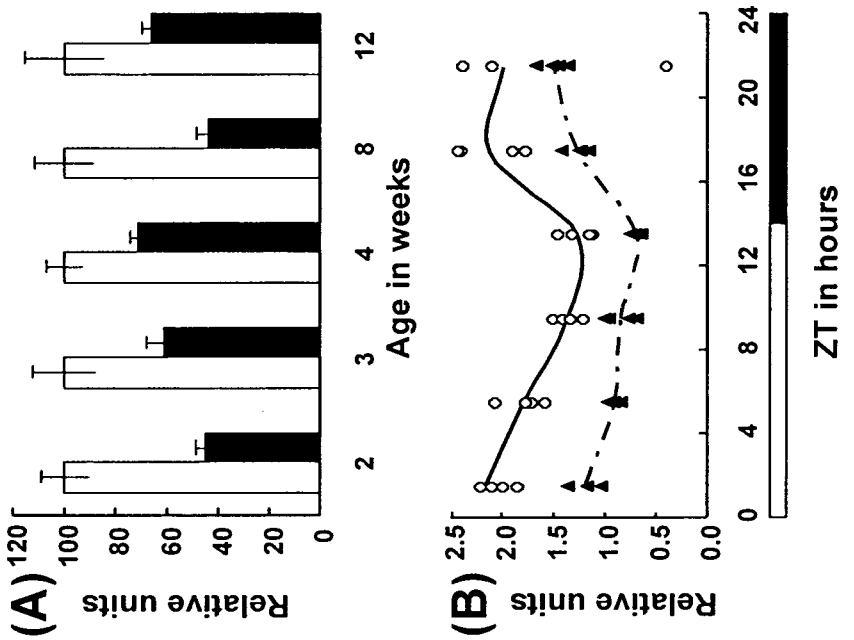
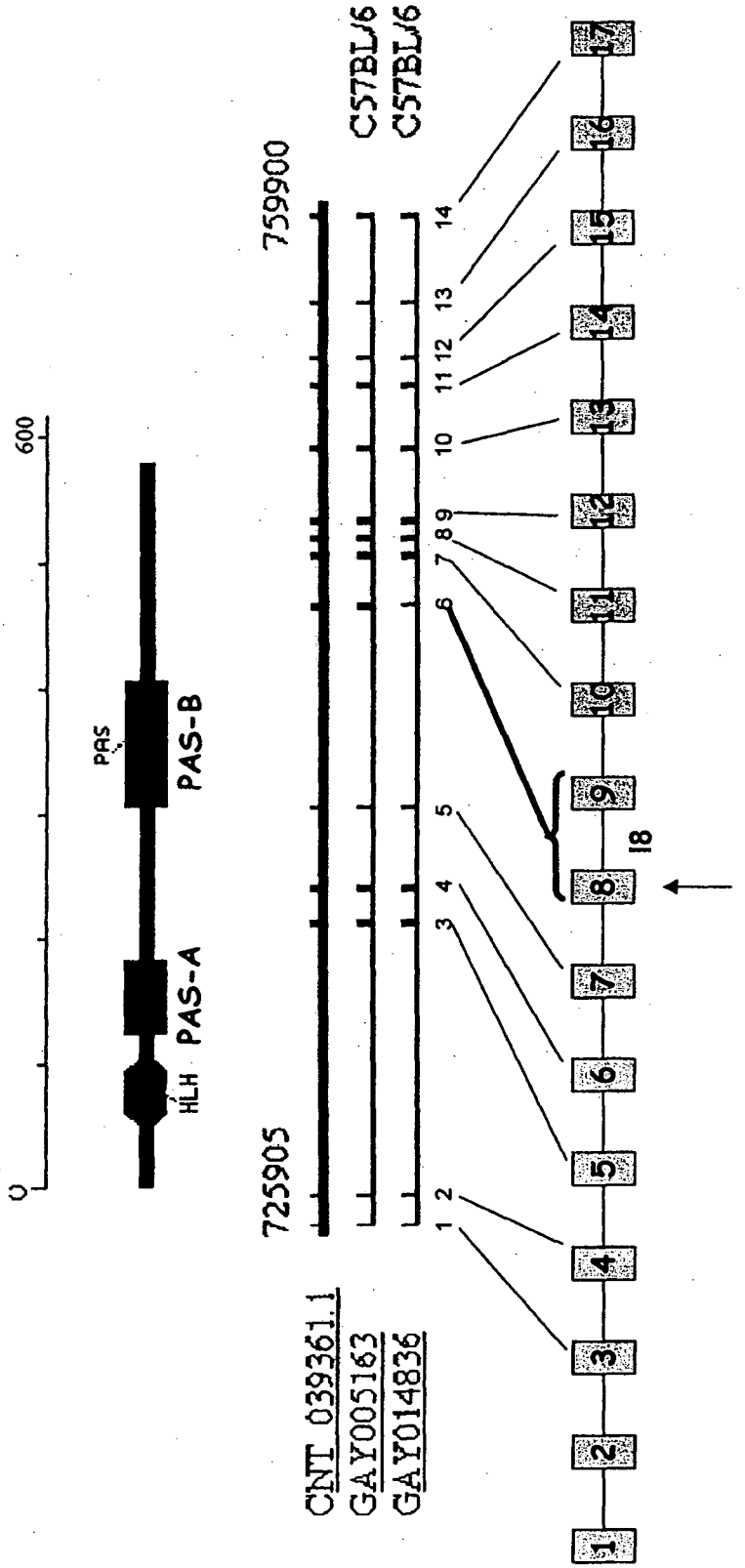

(A)
Relative units
120
100
80
60
40
20
0
2
3
4
8
12
Age in weeks
(B)
Relative units
2.5
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
ZT in hours

Figure 7


0
600
HLH
PAS
PAS-A
PAS-B
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759900
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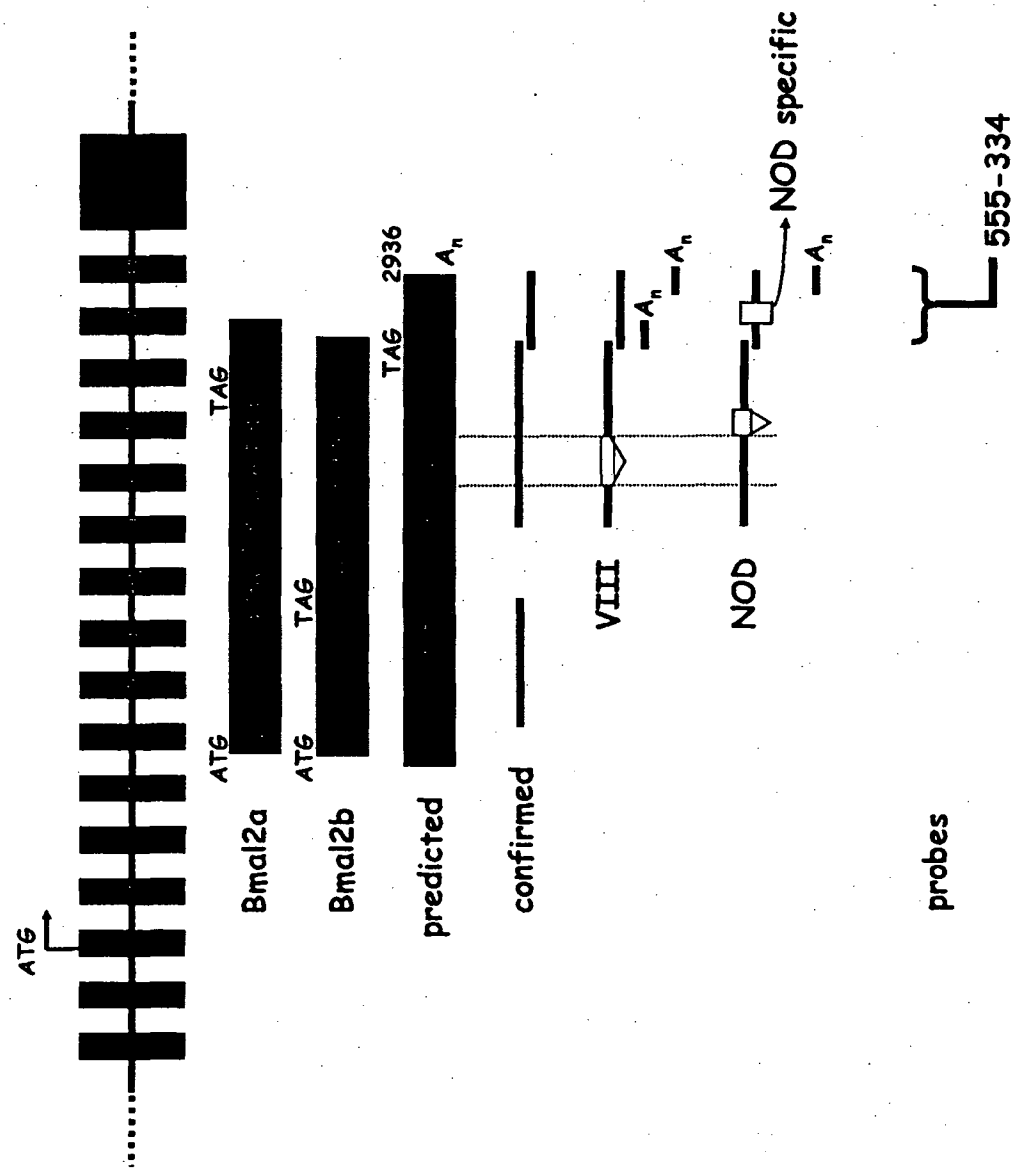
GAY005163
GAY014836
C57BL/6
C57BL/6
1 2
3 4
5
6
7 8 9
10
11 12
13
14
I8

Figure 8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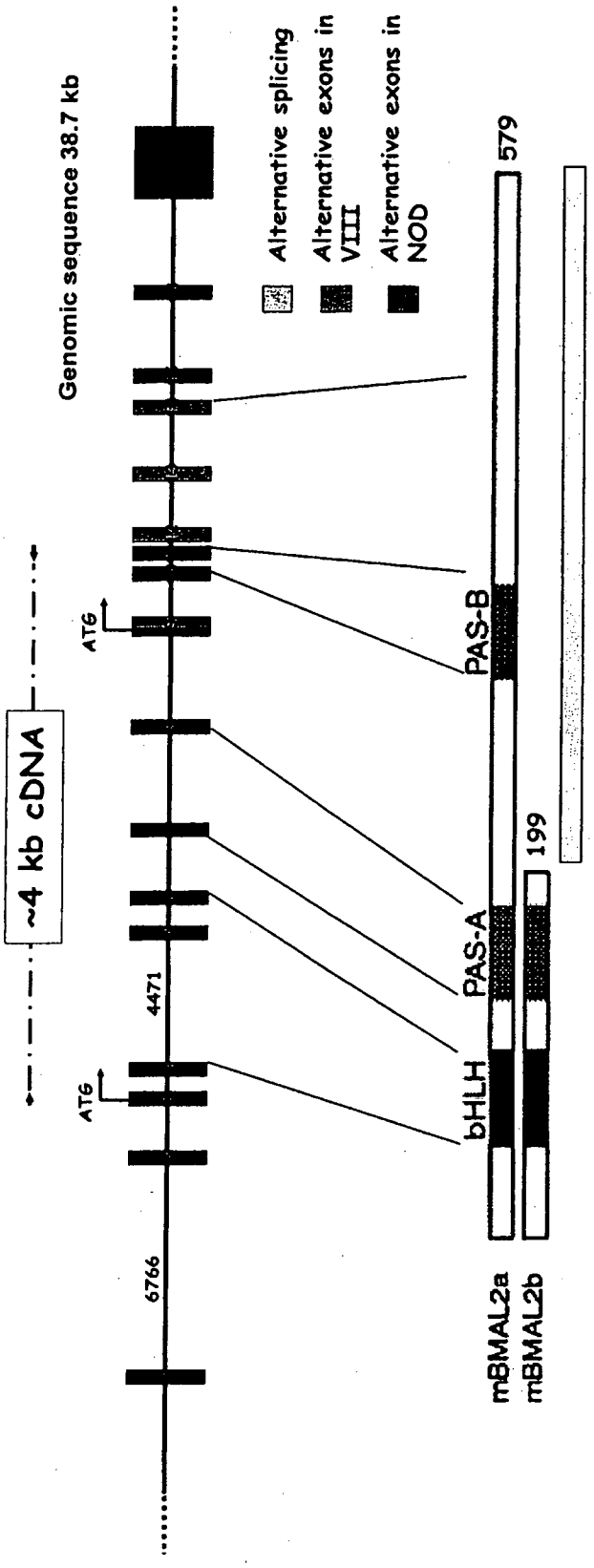
Bmal2a
ATG
TAG
Bmal2b
ATG
TAG
predicted
2936
A_n
confirmed
VIII
A_n
A_n
NOD
NOD specific
A_n
probes
555-334

Figure 9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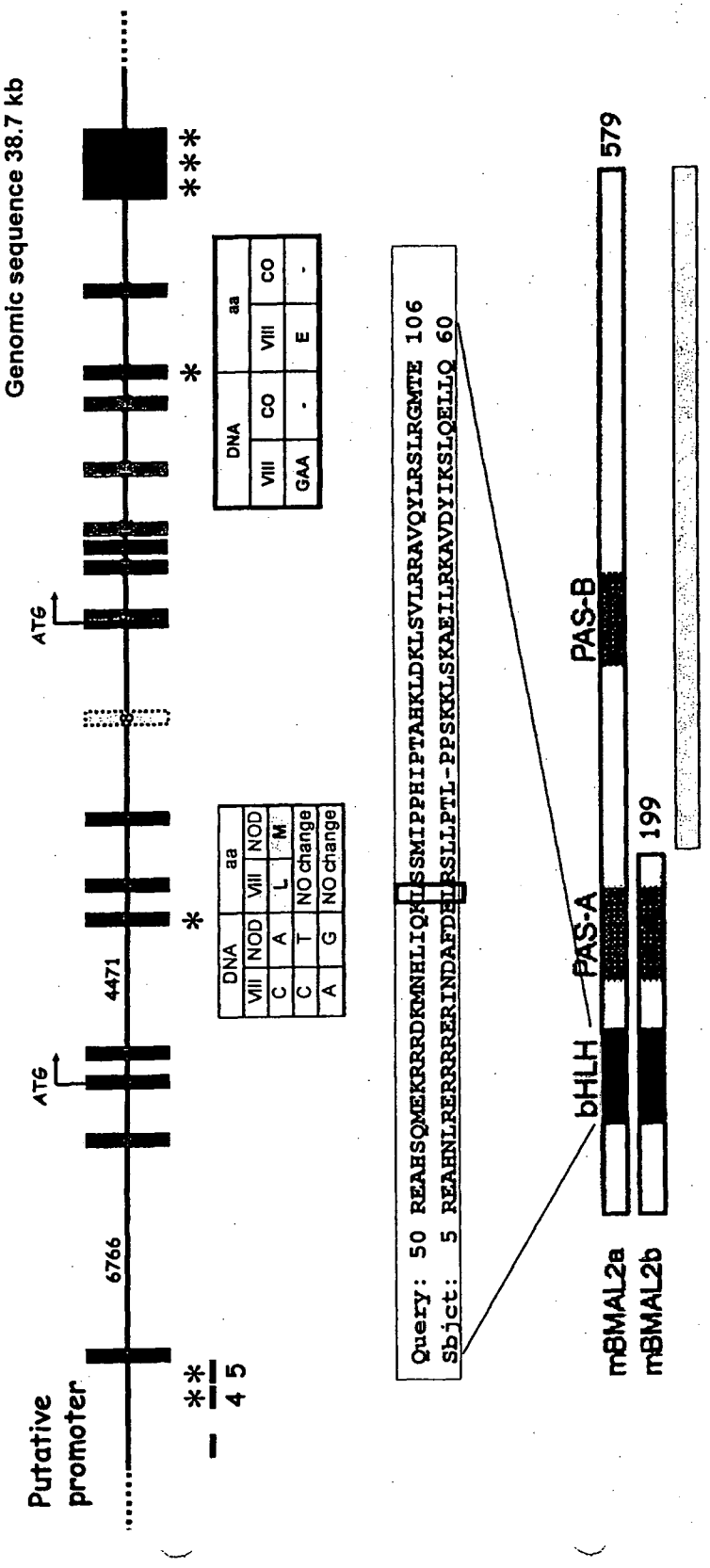
Alternative splicing
Alternative exons in VIII
Alternative exons in NOD
~4 kb cDNA
ATG
ATG
4471
6766
PAS-B
PAS-A
bHLH
579
199
mBMAL2a
mBMAL2b

Figure 10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Putative promoter
ATG
ATG
6766
4471
-45
***
*
*
DNA
aa
VIII
NOD
VIII
NOD
C
A
L
M
C
T
NO change
NO change
A
G
DNA
aa
VIII
CO
VIII
CO
GAA
-
E
-
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bHLH
PAS-A
PAS-B
579
199
mBMAL2a
mBMAL2b

Figure 11

NOD control (1737 nts)

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CTGCATTCATGAATTACCTCGAAGCAGAGGGGGGCCTGGGTGACCCTGGGGACTT
CAGTGACATCCAGTGGGCACTCTAG

Figure 12A

6.VIII (1740 nts)

ATGGAGTTTCCAAGGAAACGCAGAGGCAGAGATTCCCAGCCACTCCAGTCAGAA
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GAGTGAGTGGTTCAGCTTCACAAACCCTTGGACCAAAGAGCTGGAGTACATTGTG
TCTGTCAACACGTTGGTTTTGGGGCGCAGTGAGACCAGGCTGTCTTTGCTTCAGT
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CAAATGAGGTTCTGAGTTTACAGAGATTACACTCTTCATCCCCAGAAGATGCAAAC
CCTTCAGAAGAAGTGAGAGATGACTGCAGTGTAAACGGTGGGAGCGCCTATGGG
CCTGCATCCACTAGGGAGCTTTTTGCAGTGAGTCCTTCTAAAACAGAGGTCCTGG
AGGCTGCCAGGCAACACCAGAGCACTGAACCCGCCCACCCTCACGGACCACTTC
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CATGGCTGCATTCATGAATTACCTCGAAGCAGAGGGGGGCCTGGGTGACCCTGGG
GACTTCAGTGACATCCAGTGGGCACTCTAG

Figure 12B

NOD control (504 bases)

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GTGGAAAGTCTTCCCCAGAATCCCTTTGCCTCTCTTCTTTCAACAAGAACAGGAGT
ATCGGCGCCCAGTGGCATCAGGTAAGTTTCCCTGCTGATCTCCCTGCCATGGAAGA
GGCTGCTCCTCAGAGTTTCGCCCTGGCCCAGTTCTCCTCTGCTCCCACCATTTTAT
GTGATTTCTCAGACTGTAGTTCTTGCCAAATGCTGGATGTTTGTGATATCTCACCCA
GGGTGAGAAAGGCAGTCCAGGGACGTATGAGCATTTTTGTGCCCATAGCCCCAGA
CTTTTTTTAATACTTGTAAAGAAGATCCCATGAGAATGGCCACTTTCTTGTGGTCCT
ATTGGCTTTGGGTAGATAGCTGATCCTCTCAAAGAGATGGTGACCAGATTCCTGTG

6.VIII (504 bases)

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TGATTTCTCAGACTGTAGTTCTTGCCAAATGCTGGATGTTTGTGATATCTCACCCAG
GGTGAGAAAGGCAGTCCAGGGACGTATGAGCATTTTTGTGCCCATAGCCCCAGAC
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TTGGCTTTGGGTAGATAGCTGATCCTCTCAAAAAGATGGTGACCAGATTCCTGTG

Figure 13A

NOD control (332 bases)

GCACACACAGGCATGCACTTGGTACATAGACATTCACTTGGATGAACACAACACA
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AGACCAGGCTGGCTTTGAACTCACTCCCTGAAGGAAGTCTTAAGATAAATTGCAA
GTGTTTTAAATTGTTGAGTGTGGTGATCCATGCCTGCAACTCCAACACTCTAAAGT
TTGAGCCCCCCACCCCCCAGACCTACACTCTCTCCTCGAAAACAAAACACTG

6.VIII (332 bases)

GCACACACAGGCATGCACTTGGTACATAGACATTCACTTGGATGAACACAACACA
CATAAAATAAACGTAGTTTTTAAAAGTTAAAGAAATAATGATCTAACCAGTTTTTAA
TTTTTGTTTGAGACAGGTTTTACTATGTAGCCTTTGCTGGTCTGTAGCTCCTTATGT
AGACCAGGCTGGCTTTGAACTCACTCCCTGAAGGAAGTCTTAAGATAAATTGCAA
GTGTTTTAAATTGTTGAGTGTGGTGATCCATGCCTGCAACTCCAACACTCTAAAGT
TTGAGCCCCCCACCCCCCAGACCTACACTCTCTCCTCGAAAACAAAACACTG

Figure 13B

NOD control (493 bases)

TGAGACCACTGGGGCATTTGCCATAGATCCTGATCTTAGCTGAAGTGAACAATAAA
ATACAAATGAGTGGAAATTTGGCAAATCAAATACTTGGAGCCAACATGGATGCATT
AATAGATTCCCTGCCCCTCAGGGAGTGTCATGTGCTTTTCTGACCTCCAGGGGGCG
CAGTGAGACCAGGCTGTCTTTGCTTCAGTGCGGCGGCAGCAGCCAGTCCTCGGA
AGGTAAGACGGAGCTTCAGGTCAGGCGTGGTAACTCAGATGCATCATCCGGTAAG
GATTCCATGAAGACGTGCTTTTCTGTCTAAGAATGGAGAAACTTTCCAGGGAAAG
TTAGAAAACATTTTGACTGGACAGGTCCGTGCACTGGGCTATAGGAGGAGATAGG
GGCAGGAGAGGTGGTTCAGAAATTCCGGGCCCTTCTTGTATACAGATACTGATCTG
AGTTTAATCTCCAAAACATACATGAAGGTAGAAGGAAAGGACTGACATCA

6.VIII (493 bases)

TGAGACCACTGGGGCATTTGCCACAGATCCTGATCTTAGCTGAAGTGAACAATAA
AATACAAATGAGTGGAAATTTGGCAAATCAAATACTTGGAGCCAACGTGGATGCA
TTAATAGATTCCCTGCCCCTCAGGGAGTGTCATGTGCTTTTCTGACCTCCAGGGGG
CGCAGTGAGACCAGGCTGTCTTTGCTTCAGTGCAGCAGCAGCAGCCAGTCCTCGG
AAGGTAAGACGGAGCTTCAGGTCAGGCGTGGTAACTCAGATGCATCATCCGGTAA
GGATTCCATGAAGACGTGCTTTTCTGTCTAAGAATGGAGAAACTTTCCAGGGAAA
GTTAGAAAACATTTTGACTGGACAGGTCCGTGCACTGGGCTATAGGAGGAGATAG
GGGCAGGAGAGGTGGTTCAGAAAGTCCGGGCCCTTCTTGTATACAGATACTGATC
TGAGTTTAATCTCCAAAACATACATGAAGGTAGAAGGAAAGGACTGACATCA

Figure 13C

NOD control (367 bases)

TCGCTCACTGAGACTCTCCTCCCAGGTTCTAGATTGCAGCAAATTGACATTTAAAG
CTAAGACTTGGGGGCTGGAGAAATGTCTCAATGGCTAAGAGCACTGACTGTTCTT
CCGAGAGGATCTGGGTTCAATCTCCAGCACCCACATGGCAGCTCACCACTGTCTG
TACCTCCAATATTTGACACCCTCACATCAGACATATGTGGAGGCAAAACACCAATG
CAGATAAAATAAAATAAAACGCTAAGCCTCACCCAGGTTTACCTCTTGTATCCCGG
GTTGGGCTCTCACATGTCTGTAGCTAAAGATCACCTGGAACTTCTGGTTCTCCCAC
CTCATGCTGGGGTTGCAGGTGTATACCACCATG

6.VIII (367 bases)

TCGCTCACTGAGACTCTCCTCCCAGGTTCTAGATTGCAGCAAATTGACATTTAAAG
CTAAGACTTGGGGGCTGGAGAAATGTCTCAATGGCTAAGAGCACTGACTGTTCTT
CCGAGAGGATCTGGGTTCAATCTCCAGCACCCACATGGCAGCTCACCACTGTCTG
TACCTCCAATATTTGACACCCTCACACCAGACATATGTGGAGGCAAAACACCAATG
CAGATAAAATAAAATAAAACGCTAAGCCTCACCCAGGTTTACCTCTTGTATCCCGG
GTTGGGCTCTCACATGTCTGTAGCTAAAGATCACCTGGAACTTCTGGTTCTCCCAC
CTCATGCTGGGGTTGCAGGTGTATACCACCATG

Figure 13D

Bmal2 coding region – exons marked by different colors (CO as example)

NOD control (CO, 1737 nts)

ATGGAGTTTCCAAGGAAACGCAGAGGCAGAGATTCCCAGCCACTCCAGTCAGAATTCATGACA
GACACAACAGTGGAAAGTCTTCCCCAGAATCCCTTTGCCTCTCTTCTTTCAACAAGAACAGGAGTA
TCGGCGCCCAGTGGCATCAGGGAAGCTCACAGCCAGATGGAAAAGCGTCGGAGAGACAAGAT
GAACCATCTGATTCAGAAAATGTCATCTATGATCCCTCCACACATCCCCACGGCCCACAAACT
GGACAAGCTCAGCGTCTTGAGGAGGGCGGTGCAGTACTTGAGGTCTCTGAGAGGCATGACAG
AGCTTTACTTAGGAGAAAACTCTAAACCTTCATTTATTCAGGATAAGGAACTCAGTCACTTAATCCTC
AAGGCAGCAGAAGGCTTCCTGTTTGTGGTTGGATGCGAAAGAGGGAGAATTTTTTACGTTTC
TAAGTCTGTCTCCAAAACACTGCGTTATGATCAGGCTAGCTTGATGGGACAGAATTTGTTTGACT
TCTTACACCCAAAAGACGTCGCCAAAGTAAAGGAACAACTTTCTTGTGATGGTTCACCAAGAGAGA
AACCTATAGACACCAAAA<u>CCTCTCAGGTTTACAGTCACCCCCACACTGGGCGACCACGCGTGC</u>
<u>ATTCTGGCTCCAGACGATCTTTCTTCTTTAG</u>AATGAAGAGCTGTACCGTCCCTGTCAAAGAAG
AGCAGCCATGCTCGTCCTGCTCAAAGAAGAAAGACCATAGAAAATTCCACACCGTCCATTGCAC
TGGATACTTGAGAAGCTGGCCTCTGAATGTTGTTGGCATGGAGAAAGAGTCGGGTGGTGGGAAGGA
CAGCGGTCCTCTTACCTGCCTTGTGGCTATGGGACGGTTGCATCCATACATTGTCCCTCAAAAGAGT
GGCAAGATCAACGTGAGACCGGCTGAGTTCATAACTCGCTTCGCAATGAACGGGAAATTCGTCTAT
GTTGACCAAAGGGCAACGGCAATTTTAGGATACCTGCCTCAGGAACTTTTGGGAACTTCATGT
TATGAATATTTTCATCAGGATGACCACAGTAGTTTGACTGACAAGCACAAAGCAG*<u>TTCTGCAGA</u>*
<u>GTAAGGAGAAAATACTTACAGACTCATACAAATTCAGAGTGAAGGATGGTGCCTTCGTGACTCTGAAGAGT</u>
<u>GAGTGGTTCAGCTTCACAAACCCTTGGACCAAAGAGCTGGAGTACATTGTGTCTGTCAACACGTTGGTTTT</u>
<u>GGGGCGCAGTGAGACCAGGCTGTCTTTGCTTCAGTGCGGCGGCAGCAGCCAGTCCTCGGAAG</u>*<u>ACTC</u>*
ATTTAGACAATCCTGCATCAATGTGCCCGGCGTATCCACGGGGACCATCCTTGGTGCTGGGGGTATTGGA
*ACAGATATTGCAAATGAGGTTCTGAGTTTACAGAG*ATTACACTCTTCATCCCCAGAAGATGCAAACCCTT
CAGAAGTGAGAGATGACTGCAGTGTAAACGGTGGGAGCGCCTATGGGCCTG**CATCCACTAGGGAG**
CTTTTTGCAGTGAGTCCTTCTAAAACAGAGGTCCTGGAGGCTGCCAGGCAACACCAGAGCAC
TGAACCCGCCCACCCTCACGGACCACTTCCCAGTGACAGTGCCCAGCTGGGTTTTGATGTCCTG
TGTGACAGTGACAGCATAGACATGGCTGCATTCATGAATTACCTCGAAGCAGAGGGGGGCCTGGGT
GACCCTGGGGACTTCAGTGACATCCAGTGGGCACTCTAG

Figure 14

NOD control (1280 bases)

GGGAGGATTGTTAGCACGTCTGTGATTATTCTGTTCTCATTCCTGCTGCTGTGA
AAGCCTCTCAGGTTTACAGTCACCCCCACACTGGGCGACCACGCGTGCATTCT
GGCTCCAGACGATCTTTCTTCTTTAGAATGAAGAGCTGTACCGTCCCTGTCAAA
GAAGAGCAGCCATGCTCGTCCTGCTCAAAGAAGAAAGACCATAGAAAATTCC
ACACCGTCCATTGCACTGGATACTTGAGAAGCTGGCCTCTGAATGTTGTTGGCA
TGGAGAAAGAGTCGGGTGGTGGGAAGGACAGCGGTCCTCTTACCTGCCTTGT
GGCTATGGGACGGTTGCATCCATACATTGTCCCTCAAAAGAGTGGCAAGATCA
ACGTGAGACCGGCTGAGTTCATAACTCGCTTCGCAATGAACGGGAAATTCGTC
TATGTTGACCAAAGGGCAACGGCAATTTTAGGATACCTGCCTCAGGAACTTTT
GGGAACTTCATGTTATGAATATTTTCATCAGGATGACCACAGTAGTTTGACTGA
CAAGCACAAAGCAGTTCTGCAGAGTAAGGAGAAAATACTTACAGACTCATAC
AAATTCAGAGTGAAGGATGGTGcctTCGTGACTCTGAAGAGTGAGTGGTTCAGC
TTCACAAACCCTTGGACCAAAGAGCTGGAGTACATTGTGTCTGTCAACACGTT
GGTTTTGGGGCGCAGTGAGACCAGGCTGTCTTTGCTTCAGTGCGGCGGCAGC
AGCCAGTCCTCGGAAGACTCATTTAGACAATCCTGCATCAATGTGCCCGGCGTA
TCCACGGGGACCATCCTTGGTGCTGGGGGTATTGGAACAGATATTGCAAATGA
GGTTCTGAGTTTACAGAGATTACACTCTTCATCCCCAGAAGATGCAAACCCTTC
AGAAGTGAGAGATGACTGCAGTGTAAACGGTGGGAGCGCCTATGGGCCTGCA
TCCACTAGGGAGCTTTTTGCAGTGAGTCCTTCTAAAACAGAGGTCCTGGAGGC
TGCCAGGCAACACCAGAGCACTGAACCCGCCCACCCTCACGGACCACTTCCC
AGTGACAGTGCCCAGCTGGGTTTTGATGTCCTGTGTGACAGTGACAGCATAGA
CATGGCTGCATTCATGAATTACCTCGAAGCAGAGGGGGGCCTGGGTGACCCTG
GGGACTTCAGTGACATCCAGTGGGCACTCTAGCATTTTGGCTTTGTACTTTAAC
ATGAGAATCATTTCAGAGTGTTTCATTGACAAAACACTGTACTCTTGAGCACTG
TATTG

Figure 15A

6.VIII (1283 bases)

GGGAGGATTGtTAGCACGTCTGTGATTATTCTGTTCTCATTCCTGCTGCTGTGAA
AGCCTCTCAGGTTTACAGTCACCCCCACACTGGGCGACCACGCGTGCATTCTG
GCTCCAGACGATCTTTCTTCTTTAGAATGAAGAGCTGTACCGTCCCTGTCAAAG
AAGAGCAGCCATGCTCGTCCTGCTCAAAGAAGAAAGACCATAGAAAATTCCA
CACCGTCCATTGCACTGGATACTTGAGAAGCTGGCCTCTGAATGTTGTTGGCAT
GGAGAAAGAGTCGGGTGGTGGGAAGGACAGCGGTCCTCTTACCTGCCTTGTG
GCTATGGGACGGTTGCATCCATACATTGTCCCTCAAAAGAGTGGCAAGATCAA
CGTGAGACCGGCTGAGTTCATAACTCGCTTCGCAATGAACGGGAAATTCGTCT
ATGTTGACCAAAGGGCAACGGCAATTTTAGGATACCTGCCTCAGGAACTTTTG
GGAACTTCATGTTATGAATATTTTCATCAGGATGACCACAGTAGTTTGACTGAC
AAGCACAAAGCAGTTCTGCAGAGTAAGGAGAAAATACTTACAGACTCATACA
AATTCAGAGTGAAGGATGGTGCCTTCGTGACTCTGAAGAGTGAGTGGTTCAGC
TTCACAAACCCTTGGACCAAAGAGCTGGAGTACATTGTGTCTGTCAACACGTT
GGTTTTGGGGCGCAGTGAGACCAGGCTGTCTTTGCTTCAGTGCAGCAGCAGC
AGCCAGTCCTCGGAAGACTCATTTAGACAATCCTGCATCAATGTGCCCGGCGTA
TCCACGGGGACCGTCCTTGGTGCTGGGAGTATTGGAACAGATATTGCAAATGA
GGTTCTGAGTTTACAGAGATTACACTCTTCATCCCCAGAAGATGCAAACCCTTC
AGAAGAAGTGAGAGATGACTGCAGTGTAAACGGTGGGAGCGCCTATGGGCCT
GCATCCACTAGGGAGCTTTTTGCAGTGAGTCCTTCTAAAACAGAGGTCCTGGA
GGCTGCCAGGCAACACCAGAGCACTGAACCCGCCCACCCTCACGGACCACTT
CCCAGTGACAGTGCCCAGCTGGGTTTTGATGTCCTGTGTGACAGTGACAGCAT
AGACATGGCTGCATTCATGAATTACCTCGAAGCAGAGGGGGGCCTGGGTGACC
CTGGGGACTTCAGTGACATCCAGTGGGCACTCTAGCATTTTGGCTTTGTACTTT
AACATGAGAATCATTTCAGAGTGTTTCATTGACAAAACACTGTACTCTTGAGC
ACTGTATTG

Figure 15B

NOD control (CO)

CATTTTGGCTTTGTACTTTAACATGAGAATCATTTCAGAGTGTTTCATTGACAAAAC
ACTGTACTCTTGAGCACTGTATTGGTACATTTATCTCTTATTACTAGTCTACTGACTT
TTATAATATATCTGCCCTTTATTCTCACTGGGATGTGCGGAGTCACACATGCTCCTCC
AAAGAGAAACAGCCAAGTTTATCAGTCCCTCTTTACACAGTGAGAAGGCAGCTTG
GGGGTCAGGCTGCCATATTTTTGCTAAAATATTCTGACCAAAAACTGCTACCAACC
ATATTGTTAGGGCTTTTTTTTTTACATTTATTTATTTACTATATGTAAGTACACTGTAG
CTGTCCTCAGATACTCCAGAAAAGGGAATCAGATTTCGTTGGGGATGGTTGTGTGC
CACCATGTGGTTGCTGGGATTTGAACTCGGGACTTTCGGAAGAGCAGTCGGCGCT
CTTAACCACTGAGCCATCTCACCAGCCCCTGTTCTTGTTTTCAAGACAAGGTTTCT
CTGTGTATCCCTGGCTGTCTTGTAACTCACTCTGTAGACCAGGCTGGCCTTGAACT
CAAAGATCTGCCTGCCTCCTCTTCCTCCCAAGTGTTGAGATTAAAGCCATACATCA
CAATTCCCAGCTTA

6.VIII

CATTTTGGCTTTGTACTTTAACATGAGAATCATTTCAGAGTGTTTCATTGACAAAAC
ACTGTACTCTTGAGCACTGTATTGGTACATTTTTTATCTCTTATTACTAGTCTACTGA
CTTTTATATATCTGCCCTTTATTCTCACTGGGATGTGCGGAGTCACACATGCTCCTC
CAAAGAGAAACAGCCAAGTTTATCAATCCCTCTTTACACAGTGAGAAGGCAGCTT
GGGGGTCAGGCTGCCATATTTTTGCTAAAATATTCTGACCAAAAACTGCTACCAAC
CATATTGTTAGGGCTTTTTTTTAAATATATATATACTTTTGTTTTTGAGTTTGGGATTTT
TGTTTTGTTCTGTCTGTTTTCTGTTTTTGTCTTCAAGACAGGGTTTCTCTGTGTAGC
CCTGGCTGTCTTGTAACTCACTCTGTAGACCAGGCTGGCCTTGAACTCAAAGATCC
GCCTGCCTCTTCCTCCCAAGTGTTGAGATTAAAGCCATATATCACAATTCCCAGCTT
A

Figure 16

NOD control (774 bases)

TTACTGGAGGGATTACAGATAGGTGGGTTTGGAGTCACCCGGTTGGAGGAGGAAA
GACAGGGGAAGGTGGAAGAACGGGAAGAGAAAGGAAGCTGCCATGAGAGGAGA
TGGACCACAAGCACGTGGCCAGGAGAAACAGCAAGTATCCCGGGTACATCCCTG
GGGAGGTAGCCAGGCCAGCAGTTAGAAGAGTAGATTAGGGGTGACCTCCCAGTAA
TTTTCAAAGCCAAATAAAATAACCATAGTTTGAGTCTCATTTATTTGTAAGCTAGTC
TGGGATAAGCTTACATTGCTTGCACTACAAGATTTCCTCCGGTTCTGCAGTGCTCAT
TTCTTAGACTTTCCCCGACTGAGAAAACAGAAGCGAATGGGTGGGTGTTCATTCA
TTTCGGCGGTGGCTGAGGGAATGGCTGGGTGACAGAGGTAAGGGCAGCATTGTG
CAGTCAGGACAGGATAACTCTGCCTCGCTGCTGGAGTGGAACTTCGATCAAGCTC
CTGTCCACGAGGCTTGGTCTCTCTCTGAACAACGGTCTGTCTAGAAAGCCAGAGT
TTCGGGCGACCTGGGTGGATCTCCGACCTATGCGGCAGAGTCGGTGAGTTCTTTCT
GAGCTGTAGTTTATTAGCTTTCGGGGTTTTGAGTGGTTTTTGCGTGTGATAGGAAC
AGCAGCTTGGGAACCCATTTGCCACACTGTATTAACTAAGAAGGTCCACACCCTCT
TGCCCTCTGGACCTAACAAAAAGAGTGTGCGACCCAGTCCTTGGAGATCCAGTGA
C

6.VIII (775 bases)

TTACTGGAGGGATTACAGATAGGTGGGTTTGGAGTCACCCGGTTGGAGGAGGAAA
GACAGGGGAAGGTGGAAGAACGGGAAGAGAAAGGAAGCTGCCATGAGAGGAGA
TGGACCACAAGCACGTGGCCAGGAGAAACAGCAAGTATCCCGGGTACATCCCTG
GGGAGGTAGCCAGGCCAGCAGTTAGAAGAGTAGATTAGGGGTGACCTCCCAGTAA
TTTTCAAAGCCAAATAAAATAACCATAGTTTGAGTCTCATTTATTTGTAAGCTAGTC
AGGGATAAGCTTACATTGCTTGCACTACAAGATTTCCTCCAGTTCTGCAGTGTTCA
TTTCTTAGACTTTCCCCGACTGAGAAAACAGAAGCGAATGGGTGGGTGTTCATTC
ATTTCTGGCGGTGGCTGAGGGAATGGCTGGGTGACAGAGGTAAGGGCAGCATTGT
GCAGTCAGGACAGGATAACTCTGCCTCGCTGCTGGAGTGGAACTTCGATCAAGCT
CCTGTCCACGAGGCTTGGTCTCTCTCTGAACAACGGTCTGTCTAGAAAGCCAGAG
TTTCGGGCGACCTGGGTGGATCTCCGACCTATGCGGCAGAGTCGGTGAGTTCTTTC
TGAGCTGTAGTTTATTAGCTTTCGGGGTTTTGAGTGGTTTTTGCGTGTGATAGGAA
CAGCAGCTTGGGAACCCATTTGCCACACTGTATTAACTAAGAAGGTCCACACCCT
CTTGCCCTCTGGACCTAACAAAAAGAGTGTGCGACCCAGTCCTTGGAGATCCAGT
GAC

Figure 17A

NOD control (683 bases)

ATTTATGTGGGCAGAGTGAGTGTAACTGAGGAATCCCCCTCGCGACCGTAGGATG
AAGGGAGCTCCGGAGCTCCAAGGAGTCTTGGTTACATATGTGCCTAACTGTTACA
GGAGTCCCTGGGGCGGTTGTAAAAAGAAAAGGGTCAGGACCACAGACCAGTCTT
TCTTGCACCATGAAGGGTCTCCCTGTCCTCCACCTCTTGCTTTTCCTCTTGCCTTTT
CAGCGCCACTGAGGGCGTTCCTCTTGGTTTAGGGCGGATCTCAACGCTGCTCCGC
CAGCCTCTGCGCTTGGGGCTCTGCTGAGGCTCCGCCGGTGCGGGTGCCTTGTCCC
GCCCGGGGCTGGCTAGGGCTGCCGGGCCTGCGATGCCCAAGCCCTGTGCGCCAG
GGCCGAATGCAGGGAGCCGCGGGGCTGCAGCTCCAGGTGAGTGGCTTGTCGAGA
CCCCGAAACTGTGTCGGGCGTCCTTGACAGCAAGCCAGTACCCCATTCCAAGCTG
GGGGAGGGGTCCCCGCATGTCTACACCAGGGTCTCATCTGATGGCTGTGGATCAA
GGCGGTAGGTTCGCACAGCCCCAGCGCAGTTGTATCACCTCCGGGACCCAGTCCG
GCGTGTGGGGTCCAGTCCCAGCTGCGTCCTACAGTGGGCGAGCTGCGGGTACAAT
CCAGAGTCTGCATACTCTACAGTT

6.VIII (683 bases)

ATTTATGTGGGCAGAGTGAGTGTAACTGAGGAATCCCCCTCGCGACCGTAGGATG
AAGGGAGCTCCGGAGCTCCAAGGAGTCTTGATTACATATGTGCCTAACTGTTACAG
GAGTCCCTGGGGCGGTTGTAAAAAGAAAAGGGTCAGGACCACAGACCAGTCTTT
CTTGCACCATGAAGGGTCTCCCTGTCCTCCACCTCTTGCTTTTCCTCTTGCCTTTTC
AGCGCCACTGAGGGCGTTCCTCTTGGTTTAGGGCGGATCTCAACGCTGCTCCGCC
AGCCTCTGCGCTTGGGGCTCTGCTGAGGCTCCGCCCGTGCGGGTGCCTTGTCCCG
CCCGGGGCTGGCTAGGGCTGCCGGGCCTGCGATGCCCAAGCCCTGTGCGCCAGG
GCCGAATGCAGGGAGCCGCGGGGCTGCAGCTCCAGGTGAGTGGCTTGTCGAGAC
CCCGAAACTGTGTCGGGCGTCCCTGACAGCAAGCCAGTACCCCATTACAAGCTGG
AGGAGGGGTCCCCGCATGTCTACACCAGGGTCTCATCTGATGGCTGTGGATCAAG
GCGGTAGGTTCGCACAGCCCCAGCGCAGTTGTATCACCTCCGGGACCCAGTCCGG
CGTGTGGGGTCCAGTCCCAGCTGCGTCCTACAGTGGGCGAGCTGCGGGTACAATC
CAGAGTCTGCATACTCTACAGTT

Figure 17B

NOD control (867 bases)

TCCAGAGTCTGCATACTCTACAGTTTGCTCCTCTCTGGAAGAAGCACCAGGATCCA
GGGTGCTGCTGGAGGCCTCGTTGTGCTGCGTTCCAATGGCCAAGTCCAGGCCTGG
GGAGTGCAGAGGCGCGCGCAAGCACCCTCTTATCACGGTCTCTCTGGGCTTGTGG
TGGGAATGGGGAAGCAAACCTCTTATCTTCCGGGGCTGGGTATTGAACCCAGGAC
TCCAGGCTAGTACTGTAATCTCTGCTCCTTTCATTTTGTGAGTCAAGTTGGGAGTTT
TGCCTCACCTGTCCTTTCTGTTGCTCTCAGTGATAAACTGGGAGGTGTCTCCCTGG
GAACATGGTGACCATTATTTCACAGAGGATAATCATAAAAAGCTGTTATTTATGGCA
GGCGGAAGTCAGGAAGAAAAGAACACACCAATAAATAAAAGCAGGCATGGAAGT
CAAGGTCACAGTTATTTTGATTAACAGACTAGCTGGCTGACTGAGATTGATGCTTG
ATTGATTGGTTGATTGATTGGTTGATTGGTTGATTGATTGATTAATTGATTGATTGAT
GTGTAGCCTTTGGCTAGCCTGTAACTTGCTGTACCACCAACTTAACCTGGACTTGC
ATCTGAAGAGCTGGCTACAGGTTTGTCTCATCTTTCTGGTGTAACTTTAATCAATGA
TGGGGAAATTTCTGTCTGTGACCTTTCAAGGGTTCCTGTGGTCTCCCTGCCTGCAT
GGTGCTCAAATCCATTTTGCTAAAAAGAAGGTCCCTTCTTGCTCTAAGCTCTGCTT
CACTCTCTTCTGGGTGCCTCAGTTTCCTCCCTGTAATATGGGGGTAATGACTACATA
GGTCTCTGCATTCTGCAACTGTAAGG

6.VIII (867 bases)

TCCAGAGTCTGCATACTCTACAGTTTGCTCCTCTCTGGAAGAAGCACCAGGATCCA
GGGTGCTGCTGGAGGCCTCGTTGTGCTGCGTTCCAATGGCCAAGTCCAGGCCTGG
GGAGTGCAGAGGCGCGCGCAAGCACCCTCTTATCACGGTCTCTCTGGGCTTGTGG
TGGGAGTGGGGAAGCAAACCTCTTATCTTCCGGGGCTGGGTATTGAACCCAGGAC
TCCAGGCTAGTACTGTAATCTCTGCTCCTTTCATTTTGTGAGTCAAGTTGGGAGTTT
TGCCTCACCTGTCCTTTCTGTTGCTCTCAGTGATAAACTGGGAGGTGTCTCCCTGG
GAACATGGTGACCATTATTTCACAGAGGATAATCATAAAAAGCTGTTATTTATGGCA
GGCGGAAGTCAGGAAGAAAAGAACACACCAATAAATAAAAGCAGGCATGGAAGT
CAAGGTCACAGTTATTTTGATTAACAGACTAGCTGGCTGACTGAGATTGATGCTTG
ATTGATTGGTTGATTGATTGGTTGATTGGTTGATTGATTGATTAATTGATTGATTGAT
GTGTAGCCTTTGGCTAGCCTGTAACTTGCTGTACCACCAACTTAACCTGGACTTGC
ATCTGAAGAGCTGGCTACAGGTTTGTCTCATCTTTCTGGTGTAACTTTAATCAATGA
TGGGGAAATTTCTGTCTGTGACCTTTCAAGGGTTCCTGTGGTCTCCCTGCCTGCAT
GGTGCTCAAATCCATTTTGCTAAAAAGAAGGTCCCTTCTTGCTCTAAGCTCTGCTT
CACTCTCTTCTGGGTGCCTCAGTTTCCTCCCTGTAAGATGGGGGTAATGACTACAT
AGGTCTCTGCATTCTGCAACTGTAAGG

Figure 17C

NOD control (577 bases)

ACACACACACACACACACACACTAGGTGGGGCCAAACATAACTGCCTGCTCAGTT
AACATTTTATGAGAGCAGCTTATGGATTAGACACTGATGCTGAGCCTGCTGCCCTG
CCCAGCAGTTGAGCATCCTCTTGGAAGACGGTCCCAGAACCCTTGGAGCTAGAGG
GAGGACCCTTCCAGAAAGCCACACACTGGCCAGAATCGCCTGCAGGGGCAGCTT
CTTTAATAGCATCTCACCTCCTCCCCTTCTCAGCTGCTCATCTCTTCTGTGATGTTAC
TGGACCTAGCTCTTTTGAGTTGTCGTTTTTGCAACGGTGGTGGCTTAAATTCATGC
TCCTGACTCAGAAGGCATCCCAATCTGCTTTGCATAAACTGTTGTTCCTGGCCTGT
GTGTCCTAGGCAGTTCCTTCCAGGAGTGCATCACACAAGCTAATCCAGGATGGGC
TTGGGCCCTGACTTGCTCTGGTGTGGGGATCGAGTTCATTAGTCAGCCCAGAACC
CTCTCGACATTATCAGGGACTCAGTGTTGGCCTATAAGGCCCAAAGGAAGCTGAG
CCATCTGCCTGCCCTGAAGGTT

6.VIII (577 bases)

ACACACACACACACACACACACAAGGTGGGGCCAAACATAACTGCCTGCTCAGT
TAACATTTTATGAGAGCAGCTTATGGATTAGACACTGATGCTGAGCCTGCTGCCCT
GCCCAGCAGTTGAGCATCCTCTTGGAAGACGGTCCCAGAACCCTTGGAGCTAGAG
GGAGGACCCTTCCAGAAAGCTACACAGTGGCCAGAAGCGCCTGCAGGGGCAGCT
TCTTTAACAGCATCTCACCTCCTCCCCTTCTCAGCTGCTCATCTCTTCTGTGATGTT
ATTGGACCTAGCTCTTTTGAGTTGTCGTTTTTGCAACGGTGGTGGCTTAAATTCAT
GCTCCTGACTCAGAAGGCATCCCAATCTGCTTTGCATAAACTGTTGTTCCTGGCCT
GTGTGTCCTAGGCAGTTCCTTCCAGGAGTGCATCACACAAGCTAATCCAGGATGG
GCTTGGGCCCTGACTTGCTCTGGTGTGGGGATCGAGTTCATTAGTCAGCCCAGAA
CCCTCTCGACATTATCAGGGACTCAGTGTTGGCCTATAAGGCCCAAAGGAAGCTG
AGCCATCTGCCTGCCCTGAAGGTT

Figure 17D