Aspects of the molecular and functional genetics in T1DM

A study of selected candidate genes

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1. INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an immune mediated disease characterised by selective destruction of the pancreatic beta-cells in the islets of Langerhans leading to lack of insulin production capacity, insulin depletion, hyperglycaemia, diabetic ketoacidosis and death if untreated. Exogenous delivery of insulin is standard care with the aim to obtain near-normalised blood sugar levels thereby preventing the metabolic deroute. Despite insulin-replacement treatment, T1DM patients face the risk of late diabetic complications like severe macro- and microvascular complications resulting in a decreased life-expectancy (Borch-Johnsen, 1989; Ng et al., 2001). However, stringent blood glucose control has shown to reduce the risk of developing late diabetic complications (DCCT, 1993; DCCT, 2003).

There is a between-ethnic group and between-country variation in incidence and prevalence of T1DM (Karvonen et al., 2000). Recently, increasing incidence rates have been demonstrated especially in the eastern parts of Europe and a general tendency of decrease in the age of onset (Green et al., 2000; Green et al., 2001; Gale, 2002). In Denmark the incidence is approx. 16/100,000 per year in the age group 0-15 years (Green et al., 2000; Green et al., 2001), and the prevalence is 0.4% (Christy et al., 1979; Green et al., 1992). A recent publication describing the increasing incidence rates of T1DM in Danish children from 1996 to 2000, suggested the steep increase in the youngest age group to be associated to an increased risk of cohorts born in the beginning of the 1980s (Svensson et al., 2002).

T1DM is an immune-mediated disease. Both cellular (Roep, 2003) and humoral immunity (Notkins et al., 2001) have been detected in T1DM patients. Although autoantibodies to GAD65, IA-2, and insulin are clearly markers for T1DM, today these are believed to be a response of the underlying destructive process and do not contribute to the pathogenesis (Notkins et al., 2001). However, the observation of cellular infiltration of the islet of Langerhans (Gepts, 1965) as well as T-cell immuno-suppression preserving beta-cell function (Feutren et al., 1986) suggest a functional role of the T-cells in T1DM pathogenesis, which has been substantiated (Roep, 2003). Whether the initiation of the selective beta-cell destruction is mediated by T-cells or by cytokines remains controversial (Donath et al., 2003).

The aetiology of T1DM is still incompletely understood, however both genetic and environmental factors are involved. The evidence supporting T1DM being a genetically complex disorder includes:

- increased average risk for siblings of 6% rising with increasing observation time (Lorenzen et al., 1994) compared to 0.4% in the general population (Karvonen et al., 2000)
- increased familiar clustering (Risch, 1987) with a genetic risk ratio (λs) of approximately 15 (6.0/0.4)
- the increased concordance rate for monozygotic twins spanning from 0.27 to 0.53 and from 0.04 to 0.11 for dizygtic twin pairs (Kyvik et al., 1995; Hyttinen et al., 2003)
- HLA identical siblings are 15% concordant (Thomson et al., 1988)

The genetic basis of T1DM is complex and more than 30 chromosomal loci have been linked to T1DM susceptibility, suggesting T1DM being a polygenetic disease and implicated genes are risk modifying. Specific susceptibility/protective genes may not be required or sufficient for disease development; hence the susceptibility genes are commonly occurring alleles of normal genes in an unfavourable combination in individuals at risk (Pociot, 1996). Various environmental factors have been proposed, but so far none – except for vira in a minority of cases – have been shown to initiate or accelerate the development of T1DM (Akerblom et al., 2002; Jun et al., 2003). However, the environmental impact seems to influence the varying disease frequencies from country to country as these differences cannot be explained simply by ethnic differences e.g. migrants from countries with low T1DM frequencies moving to areas with high frequencies are more susceptible than their compatriots (Patrick et al., 1989). Secondly, the incidence increase in most countries over the last decades strongly points to environmental influence.

As of today, most genetic studies within T1DM have been limited to the question of a gene or chromosomal region being associated or linked to T1DM, e.g. candidate genes have been tested for association and various genetic markers for linkage to T1DM. Most genetic studies are conducted to either demonstrate or reject association or linkage of genetic markers to T1DM – only few studies are extended with functional data, e.g. (Pociot, 1996; Vafiadis et al., 1997; Bergholdt et al., 2000; Morahan et al., 2001; Ueda et al., 2003). Moreover, the search for candidate genes has been carried out mainly for genes related to the immune system, as the beta-cell generally has been considered a passive bystander cell to its own destruction.

Thus, the hypothesis underlying this thesis is:

Target organ candidate genes are identified from an experimentally testable pathogenetic model of cytokine mediated beta-cell destruction, Figure 1. Such candidate genes may show inter-individual sequence variation, conferring a genetic risk of or protection against T1DM – alone or in combination. Functional characterisation of such gene variants might show correlation between genetic risk of or protection against T1DM development and beta-cell function.

Hence, this thesis aims at:

- Identifying predisposing T1DM genes with special reference to those selected from an experimentally testable pathogenetic T1DM model of cytokine mediated beta-cell destruction.
- Testing such identified candidate genes for association to diabetes in a Danish T1DM family collection – preceded by a review of investigated candidate genes in T1DM. Finally,
- To investigate inter-individual differences in expression of selected candidate genes by examining mRNA and protein expression pattern in islets from two rat strains and to relate different expression pattern to genetic variation of the encoding genes within the rat strains.

Chapter 2 deals with general aspects regarding genetic studies in T1DM, various ways and approaches to identify genes and chromosomal regions of interest to T1DM. The main findings from these studies are presented in tabulated form.

As a consequence of the relatively limited success from these efforts – especially in identifying minor contributing T1DM genes –

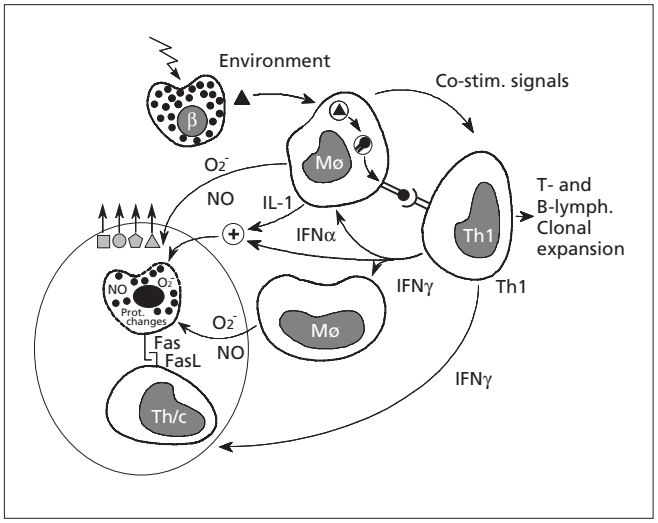

Figure 1. The Copenhagen Model, 1994. An inflammatory model of the pathogenesis of T1DM. The model suggests that environmental factors, e.g. common viruses, (i) induce initial beta-cell damage releasing beta-cell components and/or (ii) induce a MHC Class I restricted presentation of beta-cell antigen – leading to a $CD8^+$ T-cell /MHC Class I restricted beta-cell damage – effected via either cytotoxic cytokines and/or the perforin/granzyme system. Released beta-cell components, possible modified due to e.g. intracellular beta-cell oxidative stress, hence not previously "recognised" by the immune system, are taken up by antigen presenting cells in the islet, where the antigens are processed and presented to $CD4^+$ cells – either in the islet or in regional pancreatic lymph nodes. Activated $CD4^+$ T-cells will recruit and activate specific as well as non-specific inflammatory cells that then build up the inflammatory insulitis infiltrate. The effecter phase of beta-cell destruction is mediated by (i) cytokines via induction of intracellular free radicals and/or proapoptotic signalling selectively in beta-cells and/or (ii) inducing beta-cell expression of Fas, marking the beta-cells for MHC Class II non-restricted $CD4^+$ T-cell killing via interaction between the Fas ligand on $CD4^+$ T-cells and Fas in the beta-cells.

Chapter 3 presents a "combined approach to select candidate genes" as a supplement to identify new candidate genes. This approach ideally comprises (i) theoretical pathogenetical considerations based upon "The Copenhagen Model", (ii) an *in vitro*, functional testable model hereof using expressing profiling of proteins expressed in islets of Langerhans, and (iii) linkage analysis data derived from T1DM genome scans.

As the approach is based upon "The Copenhagen Model", a brief review of cytokine mediated beta-cell destruction introduces this chapter – leading to the selection of the candidate genes to be studied.

In *Chapter 4*, the selected candidate genes of this thesis are evaluated. This comprises genetic studies of identified polymorphisms. Secondly, determination of different mRNA and protein expression patterns of the selected candidate genes in islets from two rat strains as well as associating the expression pattern to inter-individual different genetic variations within the rat strains – will illustrate genetic functionality of the selected candidate genes.

Chapter 5 presents the summary, conclusion and perspectives.

This review will not include a presentation of the genetics of the two most used rodent models for T1DM, the BioBreeding (BB) rat and the Non Obese Diabetic (NOD) mouse. Neither will the different genetic tools for testing heredity of polygenetic disorders or interaction between different loci be discussed in detail and data from the T1DM genome scans will only be discussed when appropriate.

2. PUTATIVE PREDISPOSING GENES TO T1DM

This chapter briefly reviews some general aspects regarding genetic studies in T1DM, various ways and approaches to identify susceptibility genes as well as genetic areas of interest within the genome (e.g. genome scans). Different genetic tests of such genes/genetic markers are briefly touch upon and the main findings from these studies are presented in tabulated form.

2.1. GENERAL ASPECTS

Over the years, many genes have been investigated as predisposing genes to T1DM. Today it is generally considered that the HLA region is the only major genetic contributor along with minor contributions by other genes. However, no gene is neither sufficient nor necessary for T1DM development (Pociot et al., 2002).

In general, at least three aspects need to be considered when conducting genetic studies: (i) identification, characterisation and collection of the population to be studied, (ii) identification of genes or genetic regions to be investigated and, (iii) methods to analyse data.

Ad (i). The *study population* investigated in the papers included in this thesis is derived partly from a national survey obtained in 1990-1991 describing epidemiological parameters of T1DM individuals ageing less than 18 years – a study performed in collaboration with The Danish Society of Diabetes in Childhood and Adolescent (DSBD) (Pociot et al., 1993) – and partly from The Danish Insulin-Dependent Diabetes Mellitus Epidemiology and Genetics Group (DIEGG) in 1994-1999 (Lorenzen et al., 1998) identifying all T1DM probands below the age of 30 years. Population based sampling of probands (the T1DM individual through which the family was identified) and their families in racial/ethnically uniform populations in large sample sizes are important to identify genes with minor contributions (Risch et al., 1998; Altmuller et al., 2001; Cox et al., 2001; Risch et al., 2002). Sample size is particular important when the original data set is stratified for various parameters in order to test for association or linkage in relevant sub-fractions. When the candidate gene approach is undertaken – using either a case-control design or the design using Transmission Disequelibrium Testing of family based data – calculations regarding the power and size of the study population can be performed. The power of the study is determined by e.g.:

– the different allele frequencies of the tested gene(s)
– penetrance of the disease
– the relative disease risk of a given polymorphism

– parameters often unknown beforehand, when testing new polymorphisms. However, papers have been published comparing the power using different analytical approaches e.g. using different subtests of TDT (Deng et al., 2001), and the number needed in TDT testing under various permissions (McGinnis, 2000).

Hence, the power calculation in our negative findings has been performed as follows: Given OR = 1.25 leads to $P_1 = 0.5$ and $P_2 = 0.625$ and hence, p(average): 0.5625.

Standard difference can then be calculated to 0.252.

N = 500/power 80 at 5% level and, N = 350/power 70 at 5% level (Altmann, 1993).

The collected multiplex families are characterised as being either affected sibs, including parents (n = 154) or parent/offspring families (trios) (n = 103) – in total 1143 family members.

Phenotypic characterisation is important to reduce genetic heterogeneity in the population studied. Hence, subsequent stratification of the patient material i.e. by onset of age or HLA-status may furthermore result in more homogeneous classes studied. In T1DM, variation in phenotype may not be a major problem as the clinical presentation of the disease is quite unique. However, the clinical presentation in very young childhood may clinically be slightly different, as the length of the remission period may be shorter or even absent (Bonfanti et al., 1998; Muhammad et al., 1999). This difference could hold a genetic component (Veijola et al., 1995). Age of onset has also been suggested to possess a genetic component (Fava et al., 1998). Another recent study found a lower MZ concordance rate when the index case was diagnosed at 25 years of age or older, suggesting a role for age-related non-genetic dependent factors (Re-

dondo et al., 2001). The probands in the present material are identified in accordance to WHO criteria for T1DM (WHO, 1999). Data have subsequently been stratified according to e.g. HLA status or age of onset.

Ad (ii). Two different forms of *genetic variation* have been used in most studies of genetics in T1DM: (i) single nucleotide polymorphisms (SNP) being one nucleotide substituted by another at the same genomic position, when located in the coding regions may alter the "triplet" and give rise to an amino acid shift. (ii) variable numbers of tandem repeats (VNTR) also called "microsatelites" or "minisatelites" being two (or more) nucleotides repeated for a variable number of times. Microsatelites are typically located in genomic regions between genes and are widely distributed throughout the entire genome. The SNP provides two genetic variants whereas the VNTR may lead to typically 10-15 alleles, thereby being more informative than the SNPs in genetic testing. Today, the localisation and nature of many microsatelites are public available in various databases and have been generated from the world-wide efforts in sequencing the entire human genome. SNPs are also public available e.g NCBI dbSNP database (Sherry et al., 2000) but much of this information is based on comparisons of various submitted base-pair sequences, and many SNPs have not been confirmed (Taillon-Miller et al., 1998; Marth et al., 2001). In the studies included in this thesis we have screened the coding regions for polymorphisms. When doing so, two issues need to be considered:

- Number of chromosomes tested: we have tested in the range of 34 and 40 persons equalling a frequency of minimum 1.25% for the most rare allele if only one copy was identified. Allele frequencies less than 5% were not studied further due to the low chance of detecting such a gene to influence T1DM susceptibility.
- The method used for identification of the polymorphism: as direct sequencing is automated for most procedures today, this would be the method of choice. Previously, we did not have the capacity needed for such an approach, hence we used the technique of Single Stranded Conformational Polymorphism (SSCP), which in our hands had a sensitivity and specificity of 91% and 92%, respectively (Johannesen et al., 2001a).

Finally, when a candidate gene has been screened for polymorphisms the identified SNPs should be prioritised according to their putative functional impact of the protein before selecting of which SNP should be tested for association and/or linkage to disease (Tabor et al., 2002). However, even silent mutations may confer considerable impact on protein function due to e.g. involvement in mRNA splicing (Cartegni et al., 2002).

Ad (iii). Two *analytical approaches* have been undertaken in the analysis of T1DM genetics: association of a polymorphism to T1DM tested in case-control designs and association/linkage analysis applied to data generated from T1DM family collections. Linkage means that a marker allele co-segregate with the disease within each family – different families can have different marker alleles segregating with disease – in contrast to association where different families have the same specific marker co-occurring with disease (Field, 2002).

The association test in case-control designs is typically a chi-square test simply testing whether there is a difference in allele or genotype frequencies between cases and controls. The case-control design is straightforward in the sense that only genetic testing of the proband is required and the statistics are simple. When positive association is identified it is considered to be due to linkage disequilibrium between the disease and marker loci. However, especially the control population should be carefully selected in order to mirror the general population best possible and obviously the case population should be phenotypically well characterised and randomly included to avoid selection bias. The case-control design is typically used in the candidate gene approach.

The genetic analyses used in T1DM family collections have either been linkage or association based tests. Linkage analysis is a method to determine whether there is evidence for co-segregation – due to physical linkage on the chromosome – of alleles at a hypothetical disease-susceptibility locus and alleles at a marker locus in families with multiple affected members. Classical linkage requires the collection of families comprising affected and unaffected members in consecutive generations and a defined hypothesis for heredity to be tested. As T1DM is considered a genetic multiplex disease without a known mode of heredity, model-free methods testing linkage has been used in T1DM. The most common model-independent method is the affected sib-pair (ASP) linkage analysis – used in genome scans (see Chapter 2.3). The average proportion of alleles shared in affected sibs is tested against the 50% sharing expected by chance. A higher sharing is indicative of the marker locus also contains a disease locus – hence being linked. The relatively low frequency of affected sib-pair families lead to the development of the Linkage Disequelibrium Test (TDT), as this test uses the information obtained from simplex families ("Trios"). The TDT compares the number of transmitted alleles to non-transmitted alleles from heterozygous parents to affected offspring and is an association test (Spielman et al., 1993). This method was extended to handle multiallelic marker systems (ETDT) (Sham et al., 1995). TDT statistics have become the golden standard for testing candidate genes in family collections for linkage disequilibrium (Spielman et al., 1996), and have been used for testing the candidate genes in the papers included in this thesis.

As transmission distortion in general seems to be evident in humans, all polymorphisms tested by TDT have been performed for affected as well as non-affected individuals, to ensure random transmission to non-affected individuals (Zollner, 2004).

Hence, initially the classical candidate gene approach testing for association in a case-control design was taken – later, ASP linkage analysis of genome scan data and TDT analysis were applied to T1DM family collections.

2.2. THE CANDIDATE GENE APPROACH

The candidate gene approach is a classical strategy. Based upon a pathophysiologically relevant indication allelic variants of such selected genes are tested for either association or linkage to T1DM.

The strength of the candidate gene approach depends upon the model in which it is a candidate. In favour of the candidate gene approach is the testing of the gene encoding the relevant protein in contrast to genomic markers of chromosomal loci as in genome scans. Using the candidate gene approach in a classical association study design, the identification and collection of a large T1DM population are more easily achieved than for a large T1DM family material, whereas the draw back is the risk of selection bias and confounding. Thus, by using the family based design testing candidate genes this potential bias is eliminated. In order to exclude a candidate gene as a susceptibility or protective gene, the search for polymorphisms to be tested can be quite extensive. In addition to the coding region, the functional regulation of the gene can be found 5' in the proximal promoter region and 3' UTR's distant regulatory regions as well as within introns (intron/exon splicing sites) (Cartegni et al., 2002). In a recent review more than 600 positive association studies were reviewed of which only 6 were considered consistently replicated. It was concluded that in order to substantiate association, case-control studies should contain large number of cases and controls tested of uniform ethnical origin and that replication studies seem mandatory (Hirschhorn et al., 2002). However, a rejection of genetic association of a protein does not exclude a pathogenetical relevance of the protein.

This thesis will review the current status of *candidate genes* tested in T1DM at two levels:

- Candidate genes subdivided into categories based upon "The Copenhagen Model":

  - T-cell regulation and inflammation
  - cytokine genes
  - genes relating to deleterious and protective mechanisms in the beta-cell and, finally
  - other tested classical candidate genes in T1DM. The HLA region is described separately, the remaining tabulated and categorised as above described, see Table 2.
- Encoding genes for proteins identified upon the "combined approach to select functionally focused candidate genes" – as previously defined and reviewed in Chapter 4.

2.2.1. HLA genes

The HLA region has been proposed to account for 40-50% of the genetic susceptibility to T1DM (Risch, 1987; Noble et al., 1996). The HLA class II mediated susceptibility/protection seems to be mediated through class II antigen presentation in the islets as well as through the development of central and peripheral tolerance (Lee et al., 2001; Todd et al., 2001).

The human leukocyte antigen (HLA) region is located at the short arm of chromosome 6, 6p21. Its organisation is shown in Figure 2 (HLA overview incl. genes).

The current understanding of HLA-DQ association shows the strongest association for individuals being heterozygous carrying the genotype DQA1*0501-DQB1*0201/DQA1*0301-DQB1*0302 (encoding the DQ2 and DQ8 molecules, respectively) conferring a relative risk of ≥ 10. Likewise, protection from developing T1DM is conferred by the haplotype DRB1*1501-DQA1*0102-DQB1*0602 (DQ6 molecule), which may provide dominant protection over the susceptibility conferred by other HLA genes. Finally, the risk conferred by DQ2 and DQ8 molecules is modified by DR (Thorsby et al., 1993; Boitard et al., 1997; Undlien et al., 1999) which points to a role of the DR locus in susceptibility to T1DM.

The observation that the highest susceptibility are seen for DR3/4 heterozygous, has lead to the hypothesis of transcomplementation allowing for the construction of DQA1*0501-DQB1*0302/DQA1*0301-DQB1*0201 molecules. Linkage studies have also shown the existence of susceptibility genes in the HLA region: of 538 diabetic sib-pairs 54% shared two HLA haplotypes and only 7.3% shared no haplotypes, both frequencies being significantly different from the 25% expected (Payami et al., 1985; Robinson et al., 1993). Recently, the genome scans within T1DM have all demonstrated highly significant LOD-scores for the HLA region, demonstrating linkage to T1DM of the HLA region (see Chapter 2.3 for references). The above listed associations are primarily found in Caucasians, see review by (She, 1996; Zamani et al., 1998) for further explorations into inter-racial differences.

Recently, changes in the frequencies of HLA genotypes over time in Finnish T1DM patients have been reported: the frequency of high risk HLA genotype has decreased from 25.3% to 18.2% while the protective HLA genotypes have doubled comparing data from patients diagnosed before 1965 and after 1990, despite an increase in incidence of 2.5 times during the period from 1966 to 2000 in Finland (Hermann et al., 2003). It is concluded that the environmental pressure has increased resulting in higher penetrance of disease, especially in individuals with protective HLA genotypes.

The functional basis of the HLA class II molecule in T1DM has been related to peptide/antigen binding of the molecule, for review please see (Nepom et al., 1998).

2.2.2. HLA non-DQ/DR genes

Within the HLA region, other genes – apart from the HLA-DQ and DR – have been tested for genetic susceptibility to T1DM.

Based upon a review of the literature, genes tested for genetic susceptibility are listed in Table 1 and Table 2. An evaluation of the genes being demonstrated or rejected as risk modifying genes is based upon the the following criteria:

- The study of a candidate gene must have been consistently replicated at least once, in order to minimize the risk of false positive reports (Lohmueller, 2003).
- A single case/control study should comprise approximately 200 or more cases and a matching number of controls. This number is required to have a power of 80, at the significance level of 0.05, identifying a relative risk of 1.5-2.0, given the frequency of the associated allele in the control group is 0.15-0.60 (Breslow et al., 1987). However, the finding of several minor case/control studies (n: 4-5) uniformly indicating the same result has also been taken into account within the overall evaluation of a gene influencing the risk of T1DM.
- All family based studies are included, as the number of qualified transmissions within the family collection depends on the allelic frequencies of the tested polymorphism and the analysis used.

These simple criteria represent one way to select the more robust candidate genes in T1DM, as a huge number of genes have been tested as candidate genes in T1DM.

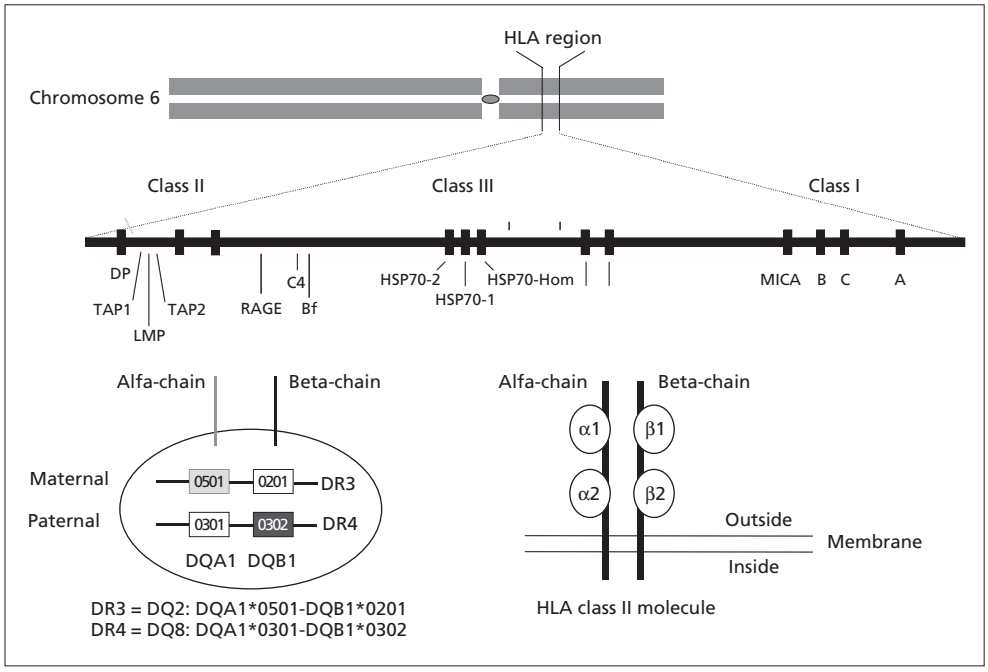

Figure 2. HLA organisation. A simplified illustration of a selection of genes in the HLA region located at the short arm of chromosome 6. None of the tested microsatelites within this region are illustrated. Besides the location of the antigen presenting genes, other genes tested in T1DM are shown. In the lower panels are outlined the structure in a simplified way of the HLA molecule as well as the association between the serological typing of DR3/DR4 and the genomically defined DQ genes. The DQA1*0301 and DQB1*0201 genes are found on the same haplotype (in cis) among Black T1DM patients, while – as illustrated in the figure – they are most often found on different haplotypes (in trans) among Caucasians and Japanese T1DM patients.

Table 1A. The HLA non-DQ/DR genes. Genes demonstrated as having an increasing risk modifying effect in T1DM.

Gene	Position	Association case/control	Association TDT	Confirmed replication	Reference
Bf[1]	6p21.3	Yes (57/342)		Yes	(Kirk et al., 1982)
		Yes (96/115)			(Kirk et al., 1985)
		Yes (217/136)			(Wang et al., 1989)
		Yes (215/192)			(Staneková et al., 1993)
C4[1]	6p21.3	Yes (217/136)		Yes	(Wang et al., 1989)
		Yes (176/92)			(Caplen et al., 1990)
		Yes (48/35)			(Ben-Salem et al., 1991)
		Yes (61/64)			(Segurado et al., 1991)
		Yes (67/73)			(Jenhani et al., 1992)
		Yes (241/140)			(Lhotta et al., 1996)
			No (220 fam)*		(Pani et al., 2002)
MICA	6p21.3	No (241/354)*		Yes	(Nejentsev et al., 2000)
		Yes (162/154)			(Lee et al., 2000)
		Yes (101/110)*			(Kawabata et al., 2000)
		Yes (119/134)	Yes (52 fam)*		(Park et al., 2001)
		Yes (93/108)*			(Shtauvere-Brameus et al., 2002)
		Yes (52/73)			(Sanjeevi et al., 2002)
			Yes (70 fam)*		(Bilbao et al., 2002)
		Yes (95/98)*			(Gambelunghe et al., 2000)
			Yes (78 fam)		(Zake et al., 2002)
		No (98/113)			(Torn et al., 2003)
		Yes (635/503)*			(Gupta et al., 2003)

Table 1B. Genes rejected as having a risk modifying effect in T1DM.

Gene	Position	Association case/control	Association TDT	Confirmed replication	Putative function	Reference
LMP2	6p	No (77/102)*		Yes	Cleaves endogenous antigenic peptides	(Van-Endert et al., 1994)
		No (45/53)				(Kawaguchi et al., 1994)
		Yes (198/192)*				(Deng et al., 1995)
		No (92/117)*				(Chauffert et al., 1997)
		No (285/337)	No (61 fam.)*			(Undlien et al., 1997)
LMP7	6p	Yes (198/192)*		Yes	Cleaves endogenous antigenic peptides	(Deng et al., 1995)
		No (285/337)	No (62 fam)*			(Undlien et al., 1997)
			No (142 fam)*			(McTernan et al., 2000)
		Yes (71/86)*				(Ding et al., 2001)

However, in some cases even replicated results from independent studies of tested candidate genes are contradictory. Hence, in such cases it can be difficult to determine whether the candidate gene is truly associated to T1DM or not – the genetic risk modification of the candidate gene being inconclusive. Within the column "Confirmed replication" (Conf. Rep) these genes are marked "Yes for both outcomes". An explanation of these apparent contradictory results could be due to genetic heterogeneity of disease susceptibility between and within populations e.g. (Metcalfe et al., 1996). The risk modifying effect is considered minimal for these genes. Furthermore, in some cases different genetic variants have been tested within the gene, hence no meta-analysis has been performed.

Table 1 lists the HLA non-DQ/DR candidate genes. As strong linkage disequilibrium (LD) exists within the HLA region – strong LD exists between studied non-DR/DQ genes in the HLA region and the high risk HLA DR/DQ genes – different strategies have been used to evaluate the independent effect of the studied non-DR/DQ genes. Within the case/control design the use of HLA haplo-identical control subjects and diabetic patients have been used (Deng et al., 1995). Furthermore in the case/control study by Gambelunghe testing the MIC-A gene polymorphism (Gambelunghe et al., 2000), a test for the strongest HLA association was performed as described by (Svejgaard et al., 1994).

Within family studies subset TDT analyses have been performed e.g. (i) comparing the risk conferred by HLA-DQ8 and HLA-DQ2 in the presence/absence of the tested genetic variation as illustrated for HERV-K(C4) (Pani et al., 2002) or (ii) by testing the transmission of parents being homozygous for the high risk DR/DQ *and* heterozygous for the variant in question to affected offspring as illustrated for LMP2 and LMP7 (Undlien et al., 1997).

As previously described, strong linkage disequilibrium exists within the HLA region, making identification of DR/DQ independent contributions of other genes within the HLA region difficult. A pathogenically interesting observation is the association of the diabetogenic TNF haplotype, TNFa2/TNFB*2/HLA-B15 to high TNFα production from macrophages (Pociot et al., 1993). This TNF microsatelite has been shown to be associated to age of onset of T1DM (Obayashi et al., 1999). Furthermore, a retroviral long terminal repeat adjacent to the HLA-DQB1 gene (DQ-LTR13) has been shown to modify T1DM susceptibility on high risk DQ haplotypes (Bieda et al., 2002). Recently, the random marker approach has been applied to the HLA region, identifying susceptibility regions outside HLA class II (Lie et al., 1999; Undlien et al., 2001), and Noble has shown an importance of class I antigens in modulating susceptibility to T1DM (Noble et al., 2002). Support for additional susceptibility genes in the HLA class III region, close to the TNF genes, has been provided by an analysis of the Belgian diabetes registry (Moghaddam et al., 1998).

Apart from determining T1DM risk, the HLA genes have been associated to modulation of clinical features of the disease, e.g. age of onset or outcome of active cellular autoimmunity, see (Bach et al., 2001).

2.2.3. Candidate genes outside the HLA region catagorised according to "The Copenhagen Model"

The major genetic contribution of the HLA region in T1DM has been assessed to approximately 40-50% (Risch, 1987; Noble et al., 1996). Hence, the remaining genetic susceptibility comes from several other minor contributions outside the HLA region. Many different genes have been tested for association and linkage to T1DM. In Table 2 are

Table 1C. Genes having an inconclusive risk modifying effect in T1DM.

Gene	Position	Association case/control	Association TDT	Confirmed replication	Putative function	Reference
HSP70	6p21.3	Yes (176/92) No (47/102)* No (32/31) Yes (114/110)* Yes (112/110) Yes (59/83)		Yes for both outcomes	Beta-cell defence	(Caplen et al., 1990) (Pugliese et al., 1992) (Kawaguchi et al., 1993) (Pociot et al., 1993) (Pociot et al., 1994) (Chuang et al., 1996)
TAP1	6p23.1	No (167/98)* Yes (199/140)* No (129/90)* No (45/53)* No (77/102)* No (92/75)* No (179/200)* Yes (119/92)* No (92/117)* Yes (60/62) No (120/218)* Yes (75/ 80)*		Yes for both outcomes	Facilitates transport of proteins to be MHC presented	(Caillat-Zucman et al., 1993) (Jackson et al., 1993) (Cucca et al., 1994) (Kawaguchi et al., 1994) (Van-Endert et al., 1994) (Nakanishi et al., 1994) (Maugendre et al., 1996) (Ma et al., 1997) (Chauffert et al., 1997) (Yan et al., 1997) (Rau et al., 1997) (Yu et al., 1999)
TAP2	6p btw: DQ-DP	Yes (167/98)* No (254/248)* No (129/90)* No (64/63)* No (45/53)* No (77/102)* No (92/75) Yes (241/208)* No (179/200)* No (92/117)* No (120/218)* Yes (146/90)*	 No (49 fam)* 	Yes for both outcomes	Facilitates transport of proteins to be MHC presented	(Caillat-Zucman et al., 1993) (Rønningen et al., 1993) (Cucca et al., 1994) (Yamazaki et al., 1994) (Kawaguchi et al., 1994) (Van-Endert et al., 1994) (Nakanishi et al., 1994) (Caillat-Zucman et al., 1995) (Jackson et al., 1995) (Maugendre et al., 1996) (Chauffert et al., 1997) (Rau et al., 1997) (Penfornis et al., 2002)

Only results from case/control studies including more than approx. 200 cases and controls as well as all family studies have been included in the evaluation of a gene modifying the risk of developing T1DM – however, the finding of several minor case/control studies (n: 4-5) uniformly indicating the same result has also been taken into account.
The column "Confirmed replication" indicates whether confirmation of the outcome of association/linkage has been obtained for the candidate gene tested. Hence, only genes where the outcome has been confirmed can either be (i) rejected as a candidate gene or (ii) a gene modulating risk of T1DM.
1): The apparent association is not independent of HLA-DQ/DR, as no stratification has been performed.
*): Results stratified for HLA-DQ/DR.
The following genes have been tested, but only as non-replicated studies or in small populations:
AGER (Prevost et al., 1999), BAT2 (Hashimoto et al., 1999), DMB (Esposito et al., 1997), LST-1 (Rau et al., 1995), TNFA (Pociot et al., 1994; Monos et al., 1995; Feugeas et al., 1997; Moghaddam et al., 1997; Obayashi et al., 1999; Gambelunghe et al., 2000; Camacho et al., 2002; Shtauvere-Brameus et al., 2002), TNFB (Monos et al., 1995) – hence the genetic risk modulation being inconclusive.

listed putative candidate genes tabulated according to – but not identified by – "The Copenhagen Model" of pathogenesis to T1DM, as other strategies naturally have been advocated to qualify candidate genes in T1DM than based upon "The Copenhagen Model".

The idea has *not* been to provide the reader with a complete list of published papers in the field, as a meaningful review of a specific gene in T1DM would require a separate up to date search of the literature, but to illustrate the huge effort world wide that has been put into this field – and the relatively sparse outcome.

The criteria for selection of genes in Table 2 are identical to those listed for the HLA non-DQ/DR genes in Table 1.

In conclusion: The success of the candidate gene approach in identifying the HLA region is evident, since the major genetic predisposition to T1DM resides in the HLA region. However, the identification of specific genes inside the HLA region associated and/or linked to T1DM is complicated by strong linkage disequilibrium within this region. Genes outside the HLA region each contributing to a minor degree of the overall genetic predisposition to T1DM have also been identified by means of the candidate gene approach – however, the number of genes and their significance as well as interactions need further exploration. The functional implications of the genetic contributors to T1DM identified so far (HLA, CTLA4 and INS) do not reject "The Copenhagen Model" as a pathogenetic model of T1DM as the immune system as well as the beta-cell are considered to be important in this model. Neither has the identification of genes not influencing the genetic risk of T1DM lead to the rejection of "The Copenhagen Model". The encoding gene to a pathological important protein does not need to be genetically associated to the disease.

In the search for identifying the genetic predisposition of T1DM supplementary model-independent approaches has been initiated, e.g. genome scans.

2.3. GENOME SCANS

As a consequence of T1DM being a polygenetic disorder and the failure of the candidate gene approach to identify all the genetic components conferring increased or decreased risk of T1DM development, new approaches to solve the T1DM genetic puzzle were sought. In the early 1990's, an alternative to the classical candidate gene approach emerged: complete and partial genome scans using polymorphic microsatelite markers spread over the entire genome or specific parts of the genome, in order to identify chromosomal regions linked to the disease.

The obvious strength of using polymorphic markers widely spread over the entire genome is that no *a priori* considerations regarding interesting regions are required; hence, the opportunity of identifying unknown regions of putative importance exists. Furthermore, as for the case/control design testing for association the linkage analysis (examining identity by descent) in affected sibs pairs overcomes the lack of knowledge regarding the mode of inherence of T1DM. Drawbacks, however, are that after identifying a region of

Table 2A. Candidate genes outside the HLA region in T1DM. Genes demonstrated as having an increasing risk modifying effect in T1DM.

Gene	Position	Poly-morph.	Association case/control	Association TDT	Confirmed replication	Func. sign.	Putative function	Reference
Copenhagen Model T-cell regulation and inflammation								
CD4	12p12	5UTR	Yes (199/212)	Yes (220 families) Yes (253 families)	Yes	Allele dose effect	Early phase of T-cell activation and clonal expansion	(Zamani-Ghabanbasani et al., 1994) (Kristiansen et al., 1998) (Kristiansen et al., 2004)
CTLA4	2q33	3UTR and exon1	Yes (616/502) (Lowe et al., 2000)	Yes (one large family) Yes (3671 families)	Yes	Allele dose effect	Down regulation of T-cell function and regulation of immune responses (IDDM12)	For review see: (Kristiansen et al., 2000) (Einarsdottir et al., 2003) (Ueda et al., 2003)
PTPN22	1p13	Exon	Yes (468/609) Yes (1599/1718)	Yes (1388 families) Yes (406 families)	Yes		Negative regulator of T-cell reactivity	(Bottini, 2004) (Smyth et al., 2004) (Onengut-Gumuscu et al., 2004)
Beta-cells INS	11p15.5	Promoter	Yes	Yes	Yes	Different classes: different INS transcription in pancreas and thymus	Autoantigen/ shaping of T-cell repertoire in thymus	For review see: (Pugliese et al., 2002)
Other candidate genes								
IRS-1	2q36	Exon	Yes (307/243)	Yes (140 families) Yes (767 families)	Yes			(Federici et al., 2003) (Morrison et al., 2004)
VDR	12q12-14	Exon/ intron	Yes (157/248) Yes (108/142) Yes (75/57) Yes (108/120) Yes (107/103) Yes (134/132)	Yes (93 families) Yes (152 families) No (147 families) Yes (285 families) No (204 families) Yes (206 families)	Yes		Vit D having immuno-regulatory function	(McDermott et al., 1997) (Pani et al., 2000) (Chang et al., 2000) (Malecki et al., 2000) (Yamada et al., 2001) (Pani et al., 2001) (Guja et al., 2002) (Fassbender et al., 2002) (Eerligh et al., 2002) (Yokota et al., 2002) (Györffy et al., 2002) (Skrabic et al., 2003)

Table 2B. Genes rejected as having a risk modifying effect in T1DM.

Gene	Position	Poly-morph.	Association case/control	Association TDT	Confirmed replication	Func. sign.	Putative function	Reference
Other candidate genes								
AIRE	21q22	Exons	No (224/205) No (235/318)		Yes			(Meyer et al., 2001) (Nithiyananthan et al., 2000)
CCR5	3p21	Deletion	No (115/280) No (93/105)		Yes		Trafficking of leukocytes	(Szalai et al., 1999) (Imberti et al., 1999)
GAD2	10p11-12	Promoter exons and 3UTR		No (186 families) No (58 families) No (1345 families)	Yes	No association to GAD Ab	Autoantibody	(Wapelhorst et al., 1995) (Rambrand et al., 1997) (Johnson et al., 2002)
PTPRN (IA2)	2q35-36.1	Intron	No (139/137)	No (352 families)	Yes			(Esposito et al., 1998) (Nishino et al., 2001)
GC	4q12	Intron, exon	No (181/163) No (181/172) Yes (44/58)	No (152 families)	Yes		Immuno-regulatory function	(Klupa et al., 1999) (Sieradzki et al. 1999) (Pani et al., 1999) (Ongagna et al., 2001)

interest, a major effort has to be put into identifying the pathogenically relevant gene(s) (fine mapping) and subsequent cloning and functional characterisation (positional cloning) as the chosen polymorphic markers used in genome scans typically are located in genetic areas between the coding genes, see Table 3. However, new strategies for positional cloning are continuously emerging, e.g. hierarchical genotyping design using successive rounds of genotyping and analysis by the haplotype pattern mining algorithm (Laitinen, 2004).

Experience from the first complete genome scans in T1DM has

Table 2C. Genes having an inconclusive risk modifying effect in T1DM.

Gene	Position	Poly-morph.	Association case/control	Association TDT	Confirmed replication	Func. sign.	Putative function	Reference
Copenhagen Model T-cell regulation and inflammation								
CD3	11q23	intron	Yes (168/89) No (24/49) Yes (199/212) No (403/446)	 No (120 families)	Yes for both outcomes		T-cell	(Wong et al., 1991) (Timon et al., 1991) (Zamani-Ghabanbasani et al., 1994) (Pritchard et al., 1995)
TCR	14q11.2 7q34 7p15-p14	RFLP's	Yes (118/126) No (50/48) Yes (50/94) No (72/97) No (73/45) No (164/193) No (56/48) Yes (102/163) Yes (198/84) No (125/78) Yes (75/84)	 No (29 families) No (36 families) No (10 families) No (5 families) No (21 families)	Yes for both outcomes		T-cell function	(Millward et al., 1987) (Bhatia et al., 1988) (Ito et al., 1988) (Sheehy et al., 1989) (Niven et al., 1990) (Concannon et al., 1990) (Reijonen et al., 1990) (Aparicio et al., 1990) (McMillan et al., 1990) (Field et al., 1991) (Avoustin et al., 1992) (Hibberd et al., 1992) (Kelly et al., 1993) (Martínez-Naves et al., 1993) (McDermott et al., 1996)
IFNG	12q14	Intron 1, CA-repeat	Yes (175/267) No (266/195) Yes (168/110) Yes (236/104) No (206/160)	No (153 families)	Yes for both outcomes	2-allele: increased in vitro expression	Cytotoxic to beta-cells	(Awata et al., 1994) (Pociot et al., 1997) (Jahromi et al., 2000) (Tegoshi et al., 2002)
IL1B	2q12-q22	Exon	Yes (90/48) No (112/110) Yes (312/171)	 No (245 families)	Yes for both outcomes	Allele dosage effect on LPS stimulation on IL-1 secretion	Effector molecule, acting on β-cells, co-stimulatory cytokine for T-cells, macrophages	(Pociot et al., 1992) (Pociot et al., 1994) (Kristiansen et al., 2000) (Krikovsky et al., 2002)
IL1RI	2q12-q22	Promoter	Yes (112/110) Yes (262/189) Yes (351/254)	 No (97 families) No (245 families) Yes (253 families)	Yes for both outcomes	Allele dosage effect		(Pociot et al., 1994) (Bergholdt et al., 1995) (Metcalfe et al., 1996) (Kristiansen et al., 2000) (Bergholdt et al., 2000)
Cytokines								
IL10	1q31-32	Promoter	No (437/307) Yes (128/107) Yes (207/160)	 No (204 families)	Yes for both outcomes		Immuno-suppressive	(McCormack et al., 2001) (Guja et al., 2002) (Ide et al., 2002) (Tegoshi et al., 2002)
IL12B	5q31.1-q33.1	3'UTR, promoter, intron	 No (470/544) No (120/330)	Yes (249 + 120 families) No (387 families) Yes (364 families) No (307 families) No (337 families + 795 families)	Yes for both outcomes	1-allele increased expression No functional significance	Influence on T-cell function	(Morahan et al., 2001) (Johansson et al., 2001) (Nisticò et al., 2002) (Davoodi-Semiromi et al., 2002) (McCormack et al., 2002) (Bergholdt et al., 2004)
Other candidate genes								
ICAM1	19p13	Exon 6	Yes (164/171) No (218/212)	 Yes/No (559 families)	Yes for both outcomes		Regulation of leukocyte circulation and homing	(Nishimura et al., 2000) (Nejentsev et al., 2000) (Kristiansen et al., 2000)
IGH	14q32	RFLP Microsat	Yes (101/114)	No (101 families) Yes/No (351 and 241 families)	Yes for both outcomes			(Veijola et al., 1996) (Field et al., 2002)

generated quite different results with only few consistently identified chromosomal regions contributing to the risk of T1DM. The HLA region (IDDM1) has been identified within all complete human genome scans within T1DM (Davies et al., 1994) (Hashimoto et al., 1994; Concannon et al., 1998; Mein et al., 1998; Nerup et al., 2001). The VNTR at the 5' end of the insulin gene (IDDM2) has also demonstrated to confer risk of T1DM in two genome scans and several association studies. As these two regions together only can account

Table 2C. Continued.

Gene	Position	Poly-morph.	Association case/control	Association TDT	Confirmed replication	Func. sign.	Putative function	Reference
Other candidate genes								
NeuroD/ beta2	2q32	Exon	No (160/124) No (146/268) Yes (60/174) No (87/114) No (234/383) Yes (105/122) Yes (80/121) Yes (285/289)	Yes (138 families) No (2434 families)	Yes for both outcomes	No association of poly-morphisms to insulin promoter activity	Regenera-tion/ differentiation of beta-cells Positional cloning of IDDM7	(Owerbach et al., 1997) (Marron et al., 1999) (Iwata et al., 1999) (Dupont et al., 1999) (Awata et al., 2000) (Hansen et al., 2000) (Yamada et al., 2001) (Mochizuki et al., 2002) (Cinek et al., 2003) (Vella et al., 2004)

Notes to Table 2:

Only results from case/control studies including more than approx. 200 cases and controls as well as all family studies have been included in the evaluation of a gene modifying the risk of developing T1DM – however, the finding of several minor case/control studies (n: 4-5) uniformly indicating the same result has also been taken into account.

The column "Confirmed replication" indicates whether confirmation of the assertion of association/linkage has been obtained for the candidate gene tested. Hence, only genes where the assertion has been confirmed can be either (i) a gene modulating risk of T1DM or (ii) rejected as a candidate gene.

The following genes have been tested, but only as non-replicated studies or in small populations:

– "The Copenhagen Model" (T-cell regulation and inflammation): CD28 (Ihara et al., 2001; Wood et al., 2002), FAS (Nolsoe et al., 2000), FASL (Nolsoe et al., 2002),
– Cytokines: IL1RN (Pociot et al., 1994; Kristiansen et al., 2000), IL4R (Reimsnider et al., 2000; Bugawan et al., 2001; Mirel et al., 2002), IL4 (Jahromi et al., 2000; Reimsnider et al., 2000; Ohkubo et al., 2001), IL6 (Jahromi et al., 2000), IL12R (Tabone et al., 2003), IL18 (Kretowski et al., 2002), TNFR2 (Rau et al., 1997),
– Beta-cells: BCL2 (Komaki et al., 1998; Heding et al., 2001), GCK (Bain et al., 1992; Rowe et al., 1995; Lotfi et al., 1997), IRF1 (Johannesen et al., 1997), IRF2 (Field et al.), NF B (Hegazy et al., 2001; Gylvin et al., 2002), NOS2 (Johannesen et al., 2000b; Johannesen et al., 2001a), SOD2 (Pociot et al., 1993; Pociot et al., 1994; Furuta et al., 2001; Savostianov et al., 2002).
– Other candidate genes: AIR1 (Sartoris et al., 2000), CCR2 (Szalai et al., 1999), FADD (Eckenrode et al., 2000), GAD1 (Rambrand et al., 1997), GALN (Eckenrode et al., 2000), GALNT3 (Kristiansen et al., 2000), GCGR (Gough et al., 1995), HOXD8 (Owerbach et al., 1997), ICOS (Ihara et al., 2001), IDDMK1,222 (Kinjo et al., 2001), IGFBP5 (Owerbach et al., 1997), Kidd (Barbosa et al., 1982; Hodge et al., 1983; Olivès et al., 1997), LCK (Nervi et al., 2002), NAT2 (Mrozikiewicz et al., 1994; Korpinen et al., 1999), NHE1 (Dubouix et al., 2000), NQO1 (Kristiansen et al., 1999), NRAMP1 (Esposito et al., 1998; Takahashi et al., 2001; Bassuny et al., 2002), OAS (Hitman et al., 1989; Field et al., 1999), PAI1 (Mansfield et al., 1994), PARP (Delrieu et al., 2001), PPAR (Ringel et al., 1999), SEL1L (Larsen et al., 2001; Pociot et al., 2001), SOX13 (Argentaro et al., 2001), TCF7 (Noble et al., 2001), – hence, the genetic risk modulation being inconclusive.

Some of the conflicting results are due to different tested polymorphisms within the same genes. Positive association are indicated if the association are significant after stratification/identified in a subpopulation.

for a λ_s of 4-5 (Todd et al., 1997) of the total λ_s of 15, the remaining genetic susceptibility is located elsewhere (Lernmark et al., 1998).

A major problem in the genome scans using linkage analysis is the limited power to map genes with a weak genetic component, e.g. testing relatively small sample sizes only detect disease genes with a genotypic risk ratio of more than 4 (the increased chance that an individual with a particular genotype has the disease); – hence, increasing the chance of identifying minor genetic components would require very large family materials (1000s of ASPs) (Risch et al., 1996). This can partly explain the deviating results obtained in the different genome scans indicating the importance of sample size. Other possible explanations of the variation observed in the results between the different genome scans are (i) genetic heterogeneity (an apparently uniform phenotype being caused by two or more different genotypes), (ii) differences in disease phenotype: age of onset, presence/absence of IDDM-associated auto antibodies at onset, other autoimmune diseases, gender-specific effect (iii) ethnic origin, (iv) gene to gene and gene to environmental interactions being different in various populations and (v) variation due to random chance (She, 1998; Altmuller et al., 2001; Cox et al., 2001). In an attempt to overcome these drawbacks of complete genome scans, linkage disequelibrium analysis has been taken into use, as a tool to confirm and fine map susceptibility intervals. This approach has successfully been used for e.g. IDDM12/CTLA4 (Nistico et al., 1996; Marron et al., 1997; Kristiansen et al., 2000; Ueda et al., 2003).

On the other hand, besides suggesting chromosomal regions of importance in modifying genetic risk, the genome-scan data potentially exclude chromosomal regions as disease modifying.

In the future, there is an urgent need for collaboration world wide within this area in order to increase the number of tested families. This can be done by two separate approaches (i) pooling of existing data set and (ii) identification and sampling of new families. Finally, stratification of genome-scan data has been proposed to identify various interactions between different loci, as initially proposed by Cox (Cox et al., 2001; Nerup et al., 2001). This approach has lead to the identification of an increased LOD score on chromosome 6q27 from 0.94 to 3.69 when conditioned for age at onset less that 11 years in the combined UK and US family material (Cox et al., 2001), and in the Scandinavian genome scan evidence of heterogeneity was

Table 3. Results from the two recent genome scans in T1DM. The chromosomal regions are selected as those having a MLS of more than 1.5. as suggested by (Cox et al., 2001).

	Nerup et al., 2001 (n = 408)	Cox et al., 2001 (n = 767)	Putative genes*
IDDM1	6p21.3	6p21.3	HLA-DQ
IDDM2		11p15.5	INS 5' VNTR
IDDM5	6q25	6q25	ESR1/MnSOD
IDDM7		2q31	HOXD8
IDDM8	6q27	6q27	
IDDM10		10p11	
IDDM12		2q33	CTLA4
IDDM13	2q34	2q34	IGFBP2, IGFBP5,
IDDM15		6q21	distinct from HLA, neonatal diabetes
IDDM17		10q25	
		1q42	
	2q11		
	4p16		
	5q11.2		
	12p13		
	16p13.1-p11	16p13.1-p11	
		16q22-q24	
		17q25	
		19q11-q13	

*) The LOD-score peaks span in average 20-40 cM (Concannon et al., submitted) covering the listed putative genes (Pugliese et al., 2003).

demonstrated with markers at 16p and in the HLA DR3/4 group (Nerup et al., 2001).

A spin-off from the genome scans has been the opportunity to compare genome scan data obtained from different autoimmune mediated diseases in order to identify shared loci within e.g. lupus erythematosus, multiple sclerosis and Crohn's Disease (Becker et al., 1998; Becker, 1999). Overlapping regions at chromosome 2q (CTLA4), 6p (HLA), 11p, and Xp have been reported and may lead to identification of common pathogenically pathways encoded by genes within these regions.

In conclusion: The initial high expectations of whole genome scans to enlighten the genetic puzzle of T1DM have not been fully met as only few genetic regions have been consistently identified. The limitations of genome scans possibly responsible for these findings e.g. population and ethnic differences and imperfect statistical and analytical methods have led to initiatives of large scale sampling of affected sib pair families and pooling of existing data. However, pooling of data worldwide does not exclude population based differences. Secondly, it may turn out, that the major benefit from T1DM genome scans is to exclude certain genomic areas as potential candidate gene containing areas. However, genome scans might exclude a chromosomal region being important in gene-to-gene interactions, hence, the analytical methods need to include such interaction analyses. New analytical methods should be introduced e.g. haplotype interactions (Zhang, 2003) and non-model based analytical methods e.g. data mining (Pociot et al., 2004) in which non-genetic factors may also be included in the analyses. Finally, as the genome scans generate data from affected individuals and do not include data from non-affected – no protective chromosomal regions are identified.

2.4. OTHER APPROACHES

In order to limit the genetic variation in the study population, diabetes related genes have been searched for within: (i) populations with few founders and no mixing to other populations (e.g. Arab families, IDDM17), and (ii) T1DM encountered in rare genetic syndromes (e.g. mitochondrial disorders, Downs Syndrome, Friedreisch's ataxia, Wolcott Rallison Syndrome and Wolfram Syndrome (Watkins et al., 1998)) in order to examine common diabetes associated genes. Finally, animal models spontaneously developing disease as homologous genes/chromosomal regions may be of interest regarding human diabetes.

In order to study the effect of limited population mixing in a population with a common ancestor, a genome scan of an Bedouin Arab family with a high prevalence of T1DM has been performed (Verge et al., 1998), identifying a locus mapping to the long arm of chromosome 10 (10q25) (IDDM17) being in linkage to T1DM. At this locus, increased LOD scores were observed near the reported location of this putative IDDM17 locus when conditioning the analysis for DR3 positive individuals in the combined UK/US data set (Cox et al., 2001). So far no candidate gene has been identified within this region.

Wolfram's syndrome is an autosomal recessive disorder defined by the occurrence of young-onset diabetes mellitus and progressive bilateral optic atrophy; neurological symptoms and predisposition to psychiatric disease may also associate to the diagnosis (Swift et al., 1998). Linkage of the wolfram syndrome to the short arm of chromosome 4 (D4S431) was established in 1994 (Polymeropoulos et al., 1994). Within the Scandinavian genome scan of T1DM, evidence of linkage to chromosome 4p16.1 was found, particular in the subset of Danish families (Nerup et al., 2001). In a Danish study, additional markers to those used in the Scandinavian genome scan further confirmed linkage to this region, however the 15 new polymorphisms identified did not show linkage to T1DM in the Danish population (Larsen et al., 2004). These results are indicative of a role of yet unidentified polymorphisms of the WFS1 gene in the development of common T1DM.

Regarding the main candidate gene loci in the NOD mouse, please see the following reviews: (Wicker et al., 1995; Todd et al., 2001; Serreze et al., 2001). Special focus has been set upon loci of disease protection (Todd et al., 2001; Adorini et al., 2002). In the paper of Kloting et al., the disease associated chromosomal regions within the BB rat have been reviewed (*Iddm1, Iddm2* and *Iddm3*) as well as alleles within diabetes-resistant BB rats contributing to insulin-dependent type 1 diabetes mellitus (Kloting et al., 2003) are described. These studies have mainly confirmed association to the MHC complex.

In conclusion: The use of clinical syndromes comprising immune-mediated diabetes mellitus, the study of isolated populations and animal models of diabetes, have been used as a supplement to the candidate gene approach and genome scans within the general population in order to identify common genetic disposition to T1DM.

Conclusion from Chapter 2

The genetic predisposition to T1DM is complex and despite major efforts to identify the genetic disposition to T1DM many questions still remain. Both the candidate gene approach and whole genome scans have been applicated in the search for T1DM genetic predisposition, however the results so far have been incomplete. Inconsistency between the results obtained from the different genome scans and the partial overlap of the genome scan findings to the results generated by the candidate gene approach are future challenges. Putative explanations could be different markers used in the genome scans as well as the markers used in the genome scans being too far apart – hence, the small chromosomal regions harbouring the candidate genes are missed. In the future, there is a need for sampling large ethnically homogeneous population based T1DM family collections to expand the genome scans by using SNP's or haplotype Tag SNP's and to refine the statistical methods for evaluation of the candidate genes, e.g. to include interaction with other genes or environmental factors.

Finally, new approaches for candidate gene identification may supplement the search for T1DM modifying genes. *In vitro* data derived from a functional testing of the target organ based upon 'The Copenhagen Model' will be proposed for selection of new candidate genes. In contrast to e.g. the genome scans, this approach allows the identification of protective candidate genes, as the functional testing will illuminate a putative race between deleterious and protective mechanisms in the target organ.

3. "THE COPENHAGEN MODEL" – A WAY TO SELECT CANDIDATE GENES

3.1. A COMBINED APPROACH TO SELECT CANDIDATE GENES

As previous strategies to identify susceptibility genes in T1DM have not succeeded in clarifying the genetic predisposition to T1DM, new strategies may provide additional information. Due to the possibility of gaining more detailed information regarding intracellular processes by the protein and mRNA expressing profiling technologies, a broader understanding of the cytokine mediated beta-cell destruction has become possible.

Hence, a combination of various strategies, all pin-pointing towards the same candidate gene, increases the a priori chances of identifying genes affecting T1DM susceptibility. The different strategies used in this combined approach to select candidate genes are based upon:

- Theoretical pathogenetical considerations derived from "The Copenhagen Model",
- An *in-vitro*, testable model hereof – focusing at the beta-cell – using expressional profiling: As cytokine induced beta-cell destruction may play a role in the pathogenesis of T1DM (Bergholdt et al., 2003) IL-1β induced altered protein expression in

beta-cells reflects putative pathogenetic mechanisms involved in cytokine induced beta-cell destruction. It has been speculated, that in T1DM the beta-cell destruction is not only dependent upon an auto-aggressive immune response – the beta-cells themselves may also influence the outcome (Andersen, 1999). Hence, islet proteins identified as having a changed expression level due to cytokine exposure qualify as putative candidate genes: Firstly, in contrast to the classic candidate gene approach, where subsequent functional evaluation of novel genetic variations is standard, candidate genes identified by an altered expression profile after cytokine exposure have been selected upon a functional basis. However, to what extent such altered expression can influence the outcome of the cytokine exposed beta-cell needs to be evaluated in subsequent functional analyses, e.g. in over-expression studies. Secondly, such genes are focused, as only target organ proteins are considered.

– Linkage analyses data derived from T1DM genome scans.

This approach has been advocated as a general way to identify susceptibility genes in genetically complex diseases (Hirschhorn et al., 2002) and specifically for T1DM (Pociot et al., 2002) (see Figure 3).

As this approach is based upon "The Copenhagen Model" – cytokine induced beta-cell destruction – and a functional evaluation hereof by use of expressing profiling, these two topics are summarised below. The data from T1DM genome scans are reviewed in the previous chapter. In the end of this chapter, the selection of three candidate genes based upon the combined approach and the strategy for their evaluation are outlined.

3.1.1. Expression profiling

The development of two different technologies provides the possibility to gain insight into the expression profiling of cellular systems at different levels: (i) proteome analysis, e.g 2D-protein gel analysis combined with mass spectrometry as protein identification and (ii) transcriptome analysis e.g. microarray or genechip array technology, for review plase see (Jungblut et al., 1999; Celis et al., 2000; Lockhart et al., 2000; Karlsen et al., 2001). In short, these two complementary technologies aim at identifying and quantifying gene transcripts at the mRNA expression (transcriptome analysis) or the protein level including posttranslational protein modifications (proteome analysis) in order to obtain further insight into pathological and pathogenetic mechanisms of different diseases and/or altered physiological conditions (e.g. toxicology). Examples of application areas within human diseases have been leukaemia, breast-, colorectal- and bladder cancers, and heart diseases, e.g. dilated cardiomyopathy and atherosclerosis. Within these diseases various prognostic markers and different transcription factors of putative pathogenetic relevance have been identified.

The technologies comprise obvious advantages as they mirror the intracellular changes in expression within the target organ or cellular system in much more detail than other methods are capable of. The microarray or gene chip arrays can display several thousands of Expressed Sequence Tags (EST) or known mRNA's at the same time making comparisons to different conditions possible by analysing the change in the expression level. A draw back of microarray compaired to 2-dimentional protein gel analysis is that not all mRNAs present in a cell are translated into protein (Gygi et al., 1999) and mRNAs encode for unmodified pre-forms of proteins. On the other hand, 2-dimentional protein gel analysis is able to detect the proteins as well as identifying post-transcriptional modified proteins which is very important, as (i) it is the proteins that initiate and run the cellular processes, not the mRNA – and (ii) posttranscriptional changes e.g. phosphorylation often activate inactivated cytosolic proteins. However, it is only a part of the total number of proteins present in a cell preparation that is actually displayed at a protein gel e.g. proteins with high and low molecular weight as well as membrane bound proteins are missed. General drawbacks of both methods are (i) they represent snap shots of processes that are dynamic in nature as they only reflect the cellular status at a defined time point or period, (ii) they do not allow for discrimination between primary and secondary events or elucidation of putative interactions.

Results from expressing profiling in insulin producing cells: So far, 7 papers have been published applying the *proteome analysis* at cytokine or NO-donor treated insulin producing cell lines or islets of Langerhans (Andersen et al., 1995; Andersen et al., 1997; Christensen et al., 2000; John et al., 2000; Mose-Larsen et al., 2001; Sparre et al., 2002; Nielsen, 2004). Based upon "The Copenhagen Model", it has been attempted to categorise the identifications from these studies into the following main areas: (i) cytokine-signalling, (ii) energy generation, (iii) NO-production, (iv) insulin production/beta-cell function, (v) apoptosis and, (vi) defence/repair. *Transcriptome data* have been obtained using either RINm5F cells, primary rat beta-cells, INS-1 cells or NHI-glu/NHI-ins cell lines exposed to various combinations of cytokines (Rieneck et al., 2000; Cardozo et al., 2001a; Cardozo et al., 2001b; Kutlu et al., 2003; Nielsen et al., 2004). Comparing the data generated using these two methods has revealed only partial overlap. Possible explanations for the different findings can be different cellular sources, variation in cell phenotype and experimental settings, and the biphasic effect of

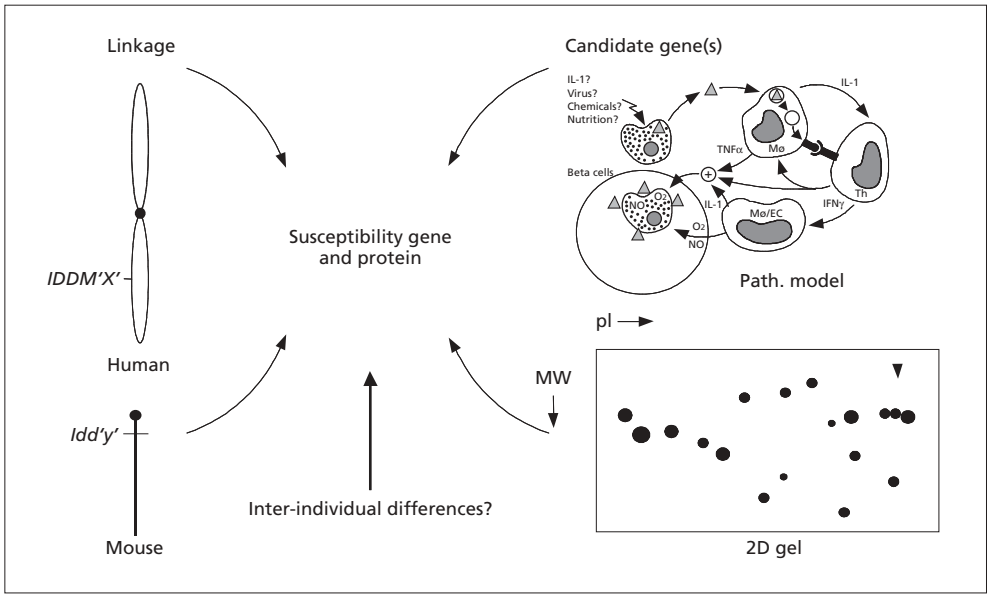

Figure 3. "The Combined Approach to Select Candidate Genes". The candidate genes are focused, as they are related to the target organ and they are selected upon a functional basis – only genes encoding proteins with an altered expression within islets following cytokine exposure are considered.

IL-1 – some cells may be stimulated other suppressed. Finally, not all mRNA changes lead to altered protein expressions. The results will be discussed in more detail in relevant chapters.

In conclusion: These novel and powerful technologies are promising and may add new valuable information to cytokine mediated beta-cell destruction and increase our understanding of biology in general. Naturally, there are obstacles: the generation of huge amounts of data requires development of new software, further insight in bioinformatics, standardisation of normal expression levels in various tissues e.g. target cells, and finally the limitation of the techniques of showing a static picture of a dynamic process. However, the approach of combining transcriptome analysis with serial experiments and cluster analysis has been attempted in order to include a dynamic dimension to this technology (Kutlu et al., 2003). Finally, it seems more relevant to select putative candidate genes based on the data generated from the changed protein expression in the target organ than using altered mRNA in the target as the altered protein expression pattern reflects the functional significant processes best.

3.2. ASPECTS OF CYTOKINE MEDIATED BETA-CELL DESTRUCTION

This chapter reviews some aspects of cytokine mediated beta-cell destruction in order to give background (i) for the functional selection of parameters and expressed mRNA and/or protein transcripts when comparing cytokine exposed islets from two genetically different rat strains (a signalling factor, deleterious as well as protective molecules), and (ii) for the selection of the tested candidate genes. The apoptotic and necrotic process of beta-cells initiated by cytokines is depicted within the iNOS chapter.

3.2.1. Cytokines in beta-cell destruction

The observations by Gepts in 1965 (Gepts, 1965) of lymphocytic infiltration within the islets of Langerhans (insulitis) seen in newly diagnosed T1DM patients were demonstrated *in vivo* and *in vitro* to correlate to immune mediated beta-cell destruction (Nerup et al., 1971). In 1974 association of the HLA system to type 1 diabetes became evident (Nerup et al., 1974), and in 1985 it was suggested that soluble mediators of the immune system liberated during the inflammatory process were beta-cell cytotoxic (Mandrup-Poulsen et al., 1985). IL-1β was identified as being the single cytokine which alone could impair beta-cell function (Bendtzen et al., 1986; Mandrup-Poulsen et al., 1986a; Mandrup-Poulsen et al., 1986b). IL-1 was shown to be selectively beta-cell cytotoxic (Mandrup-Poulsen et al., 1987a; Sandler et al., 1989; Helqvist et al., 1991b), an effect intensified by INFγ and TNFα (Mandrup-Poulsen et al., 1987b; Eizirik, 1988).

In animal models spontaneous developing diabetes, cytokines were identified in the insulitis lesion in the NOD mouse and BB rat (IL-1, TNFα, IFNα and IFNγ, IL-6 and IL-12). Transgenic NOD mice or BB rats expressing IL-4 under the rat insulin promoter (RIP) were protected against diabetes development, and expression of IL-4 and IL-10 was observed in NOD mice protected from diabetes development by various treatments such as oral administration of insulin, injection of CFA or intraperitoneal injections of long-lasting IL-10 preparation. Further, blocking cytokines by either anti-cytokine antibodies (against IFNγ, IL-6, TNFα) or blocking cytokine receptors (by either soluble IL-1 receptor or IL-1Ra) or disrupting cytokine genes (IL-12 and INFγ) have been reported to delay and/or decrease diabetes incidence in NOD mice. Recently, it has been shown that NOD mice being deficient of the IL-1R demonstrated slowed progression to diabetes (Thomas et al., 2004). Finally, over-expression of various cytokines in beta-cells (IFNγ, IFNα) under the RIP in non-diabetic-prone mice resulted in severe lymphocytic islet infiltration and diabetes; whereas beta-cell expression of IL-2, IL-6, IL-10 and TNFα induced insulitis without causing overt diabetes. For more details, please see review (Rabinovitch et al., 1998b).

Histopathology of the islet has identified antigen presenting macrophages (MHC class II positive) and $CD4^+$ T helper cells (Th) as the first cells to infiltrate the islet in the BB rat, NOD mouse and the low-dose streptozotocin animal models of diabetes (Kolb-Bachofen et al., 1988; Lee et al., 1988; Hanenberg et al., 1989). The end-stage infiltrate comprises large number of macrophages, $CD8^+$ cytotoxic T-cells (Tc) and CD4+ T-helper cells (Th), as well as B-lymphocytes (Kay et al., 1991; O'Reilly et al., 1991; Thivolet et al., 1991; Bach, 1994). This lead to the hypothesis of an initiating phase, characterised by antigen presentation and recognition, followed by a perpetuation and amplification phase in which the infiltrate builds up during the insulitis process (Nerup et al., 1994). The role of the $CD8^+$ T-cell in the initiating phase is controversial, as the $CD8^+$ T-cell has been suggested to be necessary but not sufficiently early in the initiating phase (Serreze et al., 1997; DiLorenzo et al., 1998) whereas NOD mice lacking beta-cell class I expression show both initiation and progression of the benign insulitis process (Hamilton-Williams et al., 2003). However, recently a paper demonstrating over-expression of SOCS-1 leading to protection of $CD8^+$ T-cell mediated beta-cell destruction indicates a role of cytokines in $CD8^+$ function in beta-cells destruction (Chong et al., 2004).

Transplantation experiments using mixed syngenic and xenograft islets in C57BL/6 mice have been used for evaluating the effect of locally sustained exposure of islets to cytokines *in vivo*. The xenograft response elected a cellular infiltrate dominated by the presence of macrophages, $CD4^+$ T-lymphocytes and eosinophils with only a small number of $CD8^+$ cells. Within the mixed xenogenic/syngenic islet graft, irreversible impairment of first and second phase insulin response was seen, contrasting the observations of no structural or functional impairment in allogenic/syngenic islet grafts (Korsgren et al., 1994). The latter could be due to low intra-islet IL-1 production, as within the allograft rejection only very few macrophages are seen (Simeonovic et al., 1990).

At the time of the first reports of the proposed role of cytokines in beta-cell destruction, Mosmann et al suggested a dividing of the $CD4^+$ T helper cells into two populations with contrasting and cross-regulating cytokine profiles Th1: secreting IL-2, TNFβ and INFγ leading to a cell mediated (type IV) delayed hypersensitivity reaction, and Th2: secreting IL-4, IL-5, IL-6, and IL-10 mainly initiating antibody formation and inhibition of cell mediated immunity (Mosmann et al., 1986; Liblau et al., 1995). On the basis of the cytokine profiles identified above being able to either promote or inhibit diabetes development, these cytokine profiles have subsequently been characterised as being either (i) Th1 associated with a destructive insulitis process or (ii) Th2 associated with a benign insulitis process (Rabinovitch, 1994c; Charlton et al., 1995; Liblau et al., 1995; Kolb, 1997). Today, it is generally accepted that cytokine mediated beta-cell destruction is related to a Th1 associated cytokine profile. However, in MLD-STZ induced diabetes reduction and upregulation of Th2-type cytokines were more strongly associated to susceptibility and resistance, respectively, than upregulation of Th1-type cytokine levels (Müller et al., 2002).

3.2.2. Functional changes induced by cytokine exposure in beta-cells

Rat islets exposed to cytokines were used in the initial *in vitro* studies. Exposing *rat islets* to IL-1β as a single agent was initially shown to:

- inhibit glucose stimulated insulin release (Mandrup-Poulsen et al., 1986a), (pro)insulin as well as total protein biosynthesis (Spinas et al., 1987)
- decrease oxidative metabolism (Sandler et al., 1987) and glucose oxidation at the mitochondrial level and consequently decrease ATP production and Ca^{++} uptake (Sandler et al., 1991)
- increase DNA damage and reduce DNA content (Sandler et al., 1987; Johannesen et al., 1990; Delaney et al., 1993)

leading to the destruction and death of the beta-cells. The IL-1 mediated beta-cell destruction has been proposed to be due to production of toxic substances in the beta-cell (Thomas et al., 2002) and released within islets from bystander cells (activated MØ and endothelial cells) (Kroncke et al., 1991; Steiner et al., 1997). These effects of IL-1 were intensified by IFNγ and TNFα (Mandrup-Poulsen et al., 1987b; Eizirik, 1988). However, in *purified single rat beta-cells:* IL-1 failed to destruct the beta-cells (Ling et al., 1993; Hoorens et al., 2001) whereas a mixed cytokine exposure lead to destruction (Hoorens et al., 1999; Pavlovic et al., 1999b; Liu et al., 2000; Hoorens et al., 2001; Liu et al., 2001).

Initially, most studies using *human islets* describe neither any cytotoxic effect (Kawahara et al., 1991; Eizirik et al., 1993c; Rabinovitch et al., 1994a) nor decreased accumulated or glucose stimulated insulin release after exposure of IL-1 (Mandrup-Poulsen et al., 1987a; Vara et al., 1994). However, beta-cell destruction due to direct IL-1 exposure alone has been indicated (Giannoukakis et al., 2000) and glucose induced IL-1β production in human islets reduced stimulated insulin secretion and increased apoptosis in beta-cells (Maedler et al., 2002). Finally, Zumsteg and co-workers have shown IL-1 mediated inhibition of glucose stimulated insulin release from human islets (Zumsteg et al., 1993), whereas other studies have shown the need of cytokine mixture to induce beta-cell destruction (Rabinovitch et al., 1994a; Delaney et al., 1997; Hoorens et al., 2001). Using *monolayer human beta-cells/single cell preparations enriched in beta-cells* (FACS): IL-1 alone did not cause destruction (Hoorens et al., 2001) contradictory to the increased ^{51}Cr release following IL-1 exposure demonstrated by (Rabinovitch et al., 1990), and contrasting the destructive effect of cytokine mixture exposure (Delaney et al., 1997; Hoorens et al., 1999; Hoorens et al., 2001).

Hence, evaluating beta-cell functional data after cytokine exposure, the experimental setting needs to be taken into account, e.g. (i) when comparing single beta-cell preparations from rats and humans the purity in rats is reported as more than 92% (Hoorens et al., 1999) contrasting 69-82% for the human enriched beta-cells single cell preparations (Delaney et al., 1997; Hoorens et al., 1999), and (ii) when comparing whole islets to single cell preparation as the yield of single cells only represents a small fraction of the total number of beta-cells (Pipeleers et al., 1985), and it should be considered that these pure beta-cells after FACS purification, might represent a selected, resistant "survivor population" of beta-cells. Furthermore, the Ca^{++} concentration of the culture media influences the effect of IL-1 in mouse islets (Helqvist et al., 1989). Moreover, islet isolation procedures vary slightly from laboratory to laboratory and from isolation of rat and human islets (accepting cold-preservation hours) (Keymeulen et al., 1998). Obviously, the use of single cell preparations has the advantage of studying beta-cell specific effects, however the experimental set-up might be too simple to illustrate the pathology of the cytokine mediated beta-cell destruction *in vivo.*

In conclusion: In both rat and human islets, exposure to cytokine mixture has been shown to impair beta-cell function demonstrated as e.g. inhibited insulin release, destruction of DNA and induced cytotoxicity. Within islets, beta-cells added bystander cells (MØ and endothelial cells) or in single beta-cell preparations grown in high density, the IL-1 mediated beta-cell destruction is suggested to be due to high local production of toxic substances e.g. NO, contrasting the failure of IL-1 induced beta-cell destruction in single cell preparations grown in low density. Furthermore, different experimental settings and conditions as well as islet/beta-cell handling should be kept in mind when comparing data. An altered proteome profile within these settings may be demonstrated and associated to the outcome.

3.2.3. Intracellular cytokine induced pathways

Cytokine receptors on beta-cells provide the basis of cytokine induced signalling in beta-cells (Dinarello, 1997). A review of the signal transduction pathways for IL-1, IFNγ and TNF has been given by Eizirik and Mandrup-Poulsen in (Eizirik et al., 2001b) and recently in (Donath et al., 2003) (see Figure 4).

In short:

(i) IL-1: Binding of IL-1 to the IL-1R1 allowing docking of the IL-1 receptor accessory protein mediates signal transduction through three major pathways: activation of (a) nuclear factor kB (NFκB), (b) mitogen activated protein kinases (MAPK) and (c) protein kinase C (PKC). However, involvement of G-proteins in IL-1 induced NO release and subsequent demise of the pancreatic beta-cell has been suggested (Tannous et al., 2002).

(ii) IFNγ: Interaction between IFNγ and IFNγ receptor 1 leads to the activation of the Janus tyrosine kinases 1 and 2 (JAK1 and JAK2), followed by activation of the signal transducer and activator of transcription 1 molecules (STAT1). STAT1 being a transcription factor translocating to the nucleus and through binding to gamma-activated sites (GAS) initiates transcription of many (hundreds) genes. Interferon regulatory factor 1 (IRF-1) a transcription factor as well,

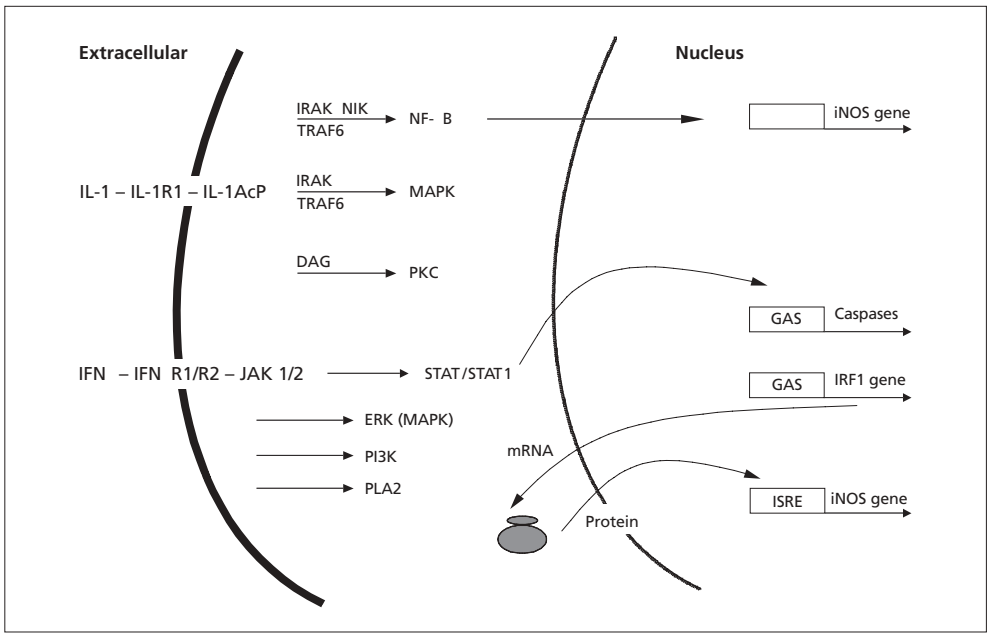

Figure 4. A simplified illustration of relations for IL-1, IRF-1 and iNOS. The pathway for TNF has not been included in the figure, see text for details.

being one of the STAT1 activated genes is subsequently expressed and binds to interferon-stimulated response elements (ISRE) in other genes e.g. iNOS. Further, STAT1 regulates caspase expression and thereby influence the cellular response to pro-apoptotic stimuli.

(iii) TNF: signals through the TNF receptor. TNF belongs to a large family, also containing e.g. FasL. Two TNF receptors exists, p60 and p80, the first containing the death domain (DD), that when activated subsequently leads to activation of e.g. NFκB, MAPKs and/or caspase mediated apoptosis. The effect of TNF will not be discussed further as the selected signalling transduction candidate gene (IRF-1) mainly operates in the IFN signalling cascade.

3.2.3.1. Nuclear Factor kappa Beta (NFκB) and Interferon Regulating Factor-1 (IRF-1)

In rat islets, activation of NFκB is required for IL-1 induced iNOS expression (Saldeen et al., 1994; Bedoya et al., 1995; Flodstrom et al., 1996a; Darville et al., 1998). In unstimulated cells, NFκB is located inactively in the cytoplasma due to binding to the inhibitor IkB. IL-1 mediated signalling leads to phosphorylation and degrading of IkB allowing NFκB to translocate to the nucleus (Gilmore, 1999), bind and initiate or adjust the promoter activity of promoters containing a NFκB binding sites (Pahl, 1999). Expression profiling studies also detect an increased expression of NFκB after cytokine exposure (Rieneck et al., 2000; Mose-Larsen et al., 2001; Cardozo et al., 2001a; Cardozo et al., 2001b). Besides being implicated in the transcription of iNOS, NFκB has been associated to other inflammatory response genes (Tak et al., 2001) like: MnSOD (Darville et al., 2000), Fas (Darville et al.), A20 (Grey et al., 1999) and IkB (Cardozo et al., 2001a). Hence, NFκB regulates the expression of several response genes which have been suggested to be stimulus and cell-type specific (Karin et al., 2000). A substantiation of the role of NFκB in cytokine mediated beta-cell destruction came from a study, blocking the NFκB translocation into the nucleus by infecting rat beta-cells with a non-degradable mutated form of IkB. Cytokine induced iNOS and Fas expression was inhibited and beta-cell survival was significantly improved (Heimberg et al., 2001). Furthermore, inhibition of NFκB in insulin producing MIN6 cells provided partial protection of IL-1β/IFNγ/TNFα induced apoptosis, indicating a role of NFκB in apoptosis signalling (Baker et al., 2001). Finally, NFκB1 (p50) deficient mice are not susceptible to multiple low-dose streptozotocin-induced diabetes (Mabley et al., 2002).

Inhibition of NFκB by pyrrolidine dithiocarbamate (PDTC) in human islets treated with IL-1β/IFNγ/TNFα lead to inhibition of nitrite production (Flodstrom et al., 1996a), suggesting a role of NFκB in iNOS expression in human islets as well. However, IL-1 alone also enhanced NFκB activation, but failed to induce iNOS expression in human islets (Flodstrom et al., 1996a) indicating that NFκB is a necessary but not sufficient factor in inducing iNOS expression. As blockage of general protein synthesis by cyclohexamide has shown to inhibit iNOS mRNA transcription in insulin-producing RINm5F and HIT-cells (Eizirik et al., 1993b) activation of protein synthesis of another transcription factor seems necessary, since activation of NFκB is not dependent upon active protein synthesis (Grimm et al., 1993). As (i) IL-1 alone induces NFκB (Flodstrom et al., 1996a) and IRF-1 (Johannesen et al., 2001b) in rat islets, (ii) IRF-1 requires de novo protein synthesis and has been suggested to play a role in IL-1 mediated NO production in rat islets (Akabane et al., 1995), and (iii) macrophages from IRF-1 deficient mice did not produce NO at immuno-stimulation (Kamijo et al., 1994), IRF-1 expression was suggested as the "missing link" in IL-1β exposed human islets for iNOS expression and NO production (Eizirik et al., 1996c). On the other hand, recent studies using islets from IRF-1-/- mice *in vitro* and *in vivo* have provided conflicting results. *In vitro* studies using both FACS purified beta-cells and whole islets from IRF-1-/- mice suggested that IRF-1 expression, probably within the non-beta-cells present in whole islets, was involved in *cytokine* induced islets cell damage (Pavlovic et al., 1999b). Contradictory, when islets from IRF-1-/- mice were allografted into *alloxan* induced diabetic recipient mice, reduced graft survival time was observed compared to IRF1+/+ control islets, suggesting a possible protective role of IRF-1 in this *in vivo* model (Gysemans et al., 2001). However, recently, Baker et al have demonstrated that cytokine stimulated IRF-1 deficient islets express a T-cell chemotaxin (inducible protein (IP)-10) in higher concentrations – possibly leading to homing of T-cells and higher local cytokine concentrations – than wild type animals (Baker et al., 2003). This finding suggests an explanation of the paradox between the ability of IRF-1-/- islets to resist cytokine induced destruction *in vitro* and the observed accelerated graft failure *in vivo.*

Such experiments using single gene knock-out animals in very exact experimental designs asking specific questions give equally specific answers, illustrating that only very specific conclusions can be drawn and that caution regarding extrapolation to more general conclusions should be taken. However, IRF-1 seems to play a role in the overall outcome of cytokine exposed beta-cells, and this possible dual effect of IRF-1 is in line with data for NFκB, as this transcription factor also has been shown to possess a role in both deleterious and protective mechanisms mediated by cytokines (Heimberg et al., 2001; Cardozo et al., 2001b). Such dual effect could limit the use of knock-ins or knock-outs regarding any putative dynamic function or critical time-window of action for the gene or protein in question.

3.2.3.2. Mitogen Activated Protein Kinase (MAPK)

As suggested above, cytokine mediated NO-independent beta-cell destruction exists in which MAPK signalling has been involved and suggested to lead to apoptosis mediated beta-cell destruction (Mandrup-Poulsen, 2001). The IL-1β induced MAPK activity in rat islets has shown to be synergistically increased by TNFα and IFNγ (Andersen et al., 2000). The following members of the MAPK family have been identified in beta-cells (Welsh, 1996; Larsen et al., 1998): (i) extracellular regulated signal-kinase (ERK) mainly activated by mitogens, growth factors and cellular stress, (ii) p38, and (iii) c-jun N-terminal kinase (JNK) both activated by cellular stress: e.g. cytokines and irradiation (Widmann et al., 1999).

JNK has been identified as a MAPK mediator to induce apoptosis, as inhibition of JNK reduces IL-1 mediated apoptosis in beta-cell lines (Ammendrup et al., 2000; Bonny et al., 2001). Activation of JNK may be regulated by Ca^{++} influx through high voltage-activated (HVA) Ca^{++} channels, as blockage of HVA channels has been found to significantly reduce IL-1 stimulated JNK activity in beta-cells (Størling et al., 2001). Activation of the caspase cascade has been suggested to be MAPK mediated in beta-cells (Eizirik et al., 2001b) and by IFNγ induced ICE expression (caspase 1) in rat and human islets (Karlsen et al., 2000). Caspases are present as inactive precursors, which when activated leads to cleavage of many proteins resulting in dismantling of the cell (Thornberry et al., 1998).

Cross-talk between MAPK signalling and NO mediated beta-cell destruction has been described as the induction of iNOS – mainly being dependent upon NFκB – also was shown to be regulated by p38 and ERK (Larsen et al., 1998; Bellmann et al., 2000). Furthermore, cytokine produced NO may positively feed-back the MAPK signal (Binzer et al., 2001), possibly explaining the protracted activation of MAPK in cytokine exposed beta-cells (Larsen et al., 1998).

In conclusion: In beta-cells cytokines elicit a variety of different signalling pathways, some leading to NO production resulting in necrotic as well as apoptotic cell death. Elucidating the functional relevance of a single factor e.g. NFκB or IRF-1, caution needs to be taken regarding (i) the efficacy and specificity of chemical blocking of the factor, and (ii) the read-out (usually being a parameter further downstream in the pathway) due to redundancy in such complex pathways.

3.2.4. Protective mechanisms

That the beta-cell is not a passive bystander cell to its own destruction is illustrated by the various defence mechanisms activated by the beta-cell when exposed to toxic stimuli – a race between the deleterious and protective mechanisms is induced. However, a reduced stress-induced defence capacity in the beta-cell has been demonstrated (Welsh et al., 1995b; Lenzen et al., 1996; Burkart et al., 2000) and this may lead to higher susceptibility for destruction of the beta-cell compared to other cell-types (Andersen, 1999) – hence, reduced protective capacity may add to the fact, that in beta-cells the deleterious mechanisms prevail. Concordant results have been provided from a clinical setting, as the total antioxidant status was lower in ICA-positive compared to ICA-negative first degree relatives to T1DM patients (Rocic et al., 1997), and the total antioxidant activity was lower in T1DM patients compared to healthy controls (Maxwell et al., 1997).

From the expression profiling studies various cytokine or NO protective gene-transcript and/or proteins have been demonstrated, e.g. catalase, ceruloplasmine, GADD-153, Gas 5, –6, gluthathione S-transferase, gluthathione peroxidase, glutamine –γ glytanyl transferase, heme oxygenase, HSP 27, –40, –70, metallothionein, MnSOD, MX 1, SOD-B (for references see Chapter 3.1.1. "Expressing profiling"). Only a selection – those that specifically have been evaluated in insulin producing cells or in islets – will be further described here.

Heat shock proteins have been demonstrated as being one of the protective molecules in islets exposed to cytokines. The function of *HSP70* in general has been associated to chaperoning (Bukau et al., 1998; Nollen et al., 1999) and in cellular defence HSP70 has been proposed to participate in repair of damaged nuclei as most HSP70 is found in the nuclei after heat shock (Welch et al., 1991). HSP70 has also been suggested to protect mitochondrial function against oxidative injury, as heat shock induced HSP70 prevented H_2O_2 induced mitochondrial damage (Polla et al., 1996). Finally, HSP70 has been shown to provide cellular protection by interfering with apoptosis induction (Buzzard et al., 1998; Jaattela et al., 1998) possibly by inhibition of JNK and p38 (Gabai et al., 1997; Mosser et al., 1997).

In rat islets expression of a cytokine induced protein with a molecular weight of approximately 72 kDa was initially demonstrated by Helqvist et al. (Helqvist et al., 1989), and subsequently the identity of HSP72 was confirmed in IL-1 exposed mouse and rat islets (Eizirik et al., 1990; Helqvist et al., 1991a; Welsh et al., 1991b). The HSP72 expression was exclusively found in FACS sorted beta-cells and not in alfa-cells (Strandell et al., 1995). A protective role of HSP72 against the deleterious effect of IL-1 in islets was shown by liposomal delivery of HSP72 into rat islets (Margulis et al., 1991). Heat shock treatment induced increased resistance in rat islets against NO, oxygen radicals and STZ toxicity *in vitro* (Bellmann et al., 1995), and over-expression of HSP70 conferred resistance against NO induced (NO-donor) cell lysis (approximately 50% reduction) in RINm5f cells (Bellmann et al., 1996). In contrast, when HSP70 over-expressing RIN cells were stimulated with cytokines an enhanced p38 MAP kinase dependent increase in nitrite production was seen (Bellmann et al., 2000). This apparent paradox of HSP70 possessing chaperone as well as cytokine properties has been suggested to be due to intracellular vs. extracellular actions of HSP70, as extracellular acting HSP70 has been shown to stimulate cytokine production (Asea et al., 2000).

Another stress protein, *heme oxygenase* (HSP32), has also been found to be upregulated selectively in IL-1 stimulated rat islets beta-cells (Helqvist et al., 1991a; Strandell et al., 1995). Heme oxygenase has been shown to be induced and cause cytoprotection of beta-cells and other cells exposed to NO (Motterlini et al., 1996).

Besides induction of stress proteins, low expression of the *antioxidant enzymes* MnSOD, catalase and gluthathione peroxidase (GSH) in rodent islets (Lenzen et al., 1996; Tiedge et al., 1997) have been associated with increased susceptibility to free radicals, as antioxidant administration or over-expression of antioxidant enzymes reduced cytokine induced beta-cell function and/or destruction in rodent and human islets (Sumoski et al., 1989; Welsh et al., 1994; Tiedge et al., 1997; Tiedge et al., 1998; Tiedge et al., 1999; Lortz et al., 2000; Moriscot et al., 2000). Selective increase in MnSOD was found in FACS purified beta-cells and not in alfa-cells contrasting identical levels in both cell types when unstimulated (Strandell et al., 1995). In RIN cells, IL-1 elicited a parallel time-dependent iNOS and MnSOD mRNA expression (Bigdeli et al., 1994). Blockage of IL-1 induced iNOS expression by aminoguanidin (AG) did not inhibit MnSOD mRNA expression, and the NO-donor SNP did not induce MnSOD mRNA expression (Bigdeli et al., 1994) indicating MnSOD being upregulated by IL-1 independently of NO. Inhibited gene transcription by actinomycin D blocked the expression of both iNOS and MnSOD in contrast to inhibition of protein synthesis by cyclohexamide blocking only iNOS expression (Bigdeli et al., 1994) – indicating different mechanisms or pathways controlling the expression of iNOS and MnSOD. This was confirmed by the observation that MnSOD expression was independent of NFκB activation (Bedoya et al., 1995). However, over-expression of MnSOD reduced cytokine-induced activation of NFκB by more than 80% associated with iNOS activity at basal/unstimulated level and a significantly reduced iNOS protein expression compared to control (Azevedo-Martins, 2003). Generally, this low antioxidant defence capacity of beta-cells has been considered to be an important aspect of oxygen free-radical induced damage leading to beta-cell death (Ho et al., 1999).

Differences in the protective capacity between human and rodent islets have been demonstrated. Human islets have been shown to be more resistant than rodent islets to damage from NO (Eizirik et al., 1994c) alloxan, hydrogen peroxide and stretozotocin (Eizirik, 1996b). Moreover, the basal content of HSP70 and the activity of catalase and SOD have been demonstrated to be higher in human islets compared to rat and mouse islets (Welsh et al., 1995b; Burkart et al., 2000). Other protective factors may influence the observed species differences as (i) the degree of resistance to NO, alloxan and streptozotocin has been described as highest in human, less in mouse and lowest in rat, (ii) the expression of HSP70 demonstrated to be highest in human, less in rat and mouse, and finally (iii) the activity of catalase and SOD observed to be highest in human, less in rat and lowest in mouse (Eizirik, 1996b).

In cytokine stimulated FACS purified beta-cells and RINm5F cells, HSP70 has been demonstrated to up- and down-regulate by use of 2D-gel protein and mRNA array analyses, respectively (Rieneck et al., 2000; Mose-Larsen et al., 2001; Cardozo et al., 2001a). Increased expression of MnSOD was identified in cytokine treated RINm5F cells, whereas blockage of NFκB reduced both the expression of HSP70 and MnSOD as well as iNOS in FACS purified rat beta-cells replicating the finding of NFκB being involved in destructive as well as protective signalling pathways (Cardozo et al., 2001b). Moreover, beside the up-regulation of MnSOD and HSP70 in cytokine exposed primary rat beta-cells, downregulation of gas6 and glutathione peroxidase both representing defence/repair genes was demonstrated in beta-cells (Cardozo et al., 2001a).

Taken together, the beta-cell possess a variety of different protective capacities. These can be activated (i) directly by cytokine signalling pathways and (ii) indirectly by cytokine mediated formation of free radicals. Species and possibly strain dependent protective capacities seem to exist, although beta-cells in general seem to have low basal antioxidant levels.

Conclusions from cytokine beta-cell destruction: Since the first version of "The Copenhagen Model" of cytokine mediated beta-cell destruction was proposed, the effects of cytokine exposure to beta-cell have been extensively studied. New technologies have been used and have generated much new information. However, although the understanding of the complexity of the involved processes and their interactions has expanded significantly but has not yet been fully depicted, the initial proposed idea of a cytokine mediated race between deleterious and protective mechanisms within the beta-cell still stands.

3.3. SELECTION OF PROTEINS/GENES USING "THE COMBINED CANDIDATE GENE APPROACH"

On the basis of the above described "Selected candidate gene approach": (i) "The Copenhagen Model", (ii) the functional derived expressional data thereof and (iii) various genome scans, three proteins have been selected for evaluation in this thesis: inducible nitric oxide synthase (iNOS), interferon regulating factor 1 (IRF1), and mortalin.

- The *iNOS* was chosen since:
  - NO was the first major cytokine mediated effector molecule identified in rat islets leading to selective beta-cell destruction
  - the possibility to further characterise the role of iNOS in two rat strains previously identified as being differently sensitive to IL-1β, and
  - the un-revealed impact of iNOS/NO in human beta-cell destruction/T1DM.
- As *IRF1* has been shown to be involved in the cytokine mediated activation of iNOS lead to genetic characterisation of IRF1 and a descriptive role of IRF1 in the rat strains, and finally,
- *Mortalin* being newly identified at the 2D protein gels and hypothesized to play a role in "The Copenhagen Model" of T1DM due its role in senescence and mitochondrial function. Furthermore, the gene encoding for mortalin was located to a genetic region shown to be linked to other autoimmune diseases.

Much information regarding the role of iNOS and NO in cytokine mediated beta-cell destruction was available at the time of selection – less information was available for IRF-1 and none for mortalin. This differentiated knowledge prior to initiation of the studies for each of the selected proteins was also considered in order to demonstrate all parts of the genetic and functional characterisation – although not necessarily for the same protein (Table 4).

In order to evaluate candidate genes for association to T1DM, it is necessary to obtain information of the DNA sequence of the selected gene in order to identify testable genetic variations within the collected population. Secondly, to examine and understand a functional relevance of these genetic variants in-depth knowledge of regulatory mechanisms becomes mandatory.

Hence, the selected proteins were characterised in the following way:

- The gene encoding the protein was evaluated for genetic polymorphisms and these were tested for linkage using (E)TDT analyses in a nationwide Danish T1DM family collection. Regarding IRF-1, a previously described polymorphism was examined within the family collection.
- In an attempt to compare the protein and mRNA response in two genetically different rats – expression of selected transcripts in cytokine exposed isolated islets from two rat strains (not spontaneously developing diabetes) was characterised. After genetic characterisation of the rat iNOS promoter from both strains, the iNOS promoters were functionally tested and associated to the expressed iNOS level.
- Ideally, over-expressing of the selected protein in a beta-cell line followed by cytokine exposure is warranted to further characterise the effect of the protein in cytokine mediated beta-cell destruction. However, the rat mortalin over-expression studies performed in a mouse-fibroblast cell line (NIH-3T3) illustrated the effect of high mortalin expressional levels *per se* and allowed for comparison to over-expression studies of human and mouse mortalin.

3.4. METHODS

Within the genetic orientated papers: cloning, screening for and verification of polymorphisms as well as establishing typing assays – generally accepted techniques and methodology were used and are not discussed further. The genetic analyses have concentrated upon association and linkage to T1DM in a Danish nationwide collection by methods previously described. Papers focusing on islet responsiveness to cytokines and functional evaluation of expression of selected proteins were also based upon classical and accepted methodology. However, promoter activity assays and real-time PCR have been introduced in the laboratory and used according to the manufacture's description and guidelines. Naturally, the use of promoter assay in cell lines comprises confounders: in cell lines – not fully resembling the relevant naïve cell type – possibly employment of different signalling transduction pathways might affect the regulation of the promoter in question. The systematic confounding factors in reporter assays being inter- and intra-assay transfection efficacy, inter-assay differences in reporter signal due to e.g. variation in plasmid DNA constructs and quantification, have been controlled to the extent possible in the studies.

Conclusion from Chapter 3

"The combined approach to select candidate genes" has the strength of employing multi-string identification of susceptibility genes – one being the response of the target organ to cytokine exposure. Three candidate genes have been selected. The following chapter presents the evaluation in terms of (i) association to T1DM in a nationwide Danish T1DM family collection preceeded by a seach for genetic variants within the genes, (ii) expression of these genes in cytokine exposed islets from two rat strains in order to study inter-strain target organ responses of cytokine mediated beta-cell destruction. Finally, (iii) an over-expression study of rat mortalin demonstrates the effect of mortalin *per se* and allowed for comparison to over-expression studies for human and mouse mortalin, although over-expressing of the selected protein in a beta-cell line followed by cytokine exposure is warrented to further characterise the effect of the protein in cytokine mediated beta-cell destruction.

Table 4. The selected candidate genes.

	Identified at 2D gels	Putative relevance in "The Copenhagen Model"	Characterization in rats	Chromosome assignment in humans	Linked region in T1DM GS	Genetic association to T1DM
iNOS	No*	Beta-cell cytotoxic	Strain dependency: **Yes** Over expression studies: Yes Promoter assays: **Yes**	17q11.2	Yes (isolated population)	**Yes**
Mortalin	Yes	Involved in cellular fate/ apoptosis?	Strain denpendency: **Yes** Over expression studies: **Yes**	5q31.1		**No**
IRF-1	No*	TF involved in cytokine signalling (iNOS)	Strain denpendency: **Yes** Over expression studies: No	5q31		**No**

The statements in bold represent the main findings of the studies included in this thesis.

*) iNOS and IRF-1 have subsequently been identified using mRNA array technology (Rieneck et al., 2000; Cardozo et al., 2001a; Cardozo et al., 2001b; Kutlu et al., 2003).

4. GENETIC AND FUNCTIONAL ANALYSIS OF THE SELECTED CANDIDATE GENES

In this chapter the selected candidate genes – iNOS, IRF-1 and mortalin – will be reviewed. As most of the papers contributing to this thesis describe aspects of rat and human iNOS in cytokine mediated beta-cell destruction as a model for T1DM, the review of the iNOS gene and protein comprises the majority of this chapter. Hence, the section "Modes of cytokine mediated beta-cell destruction" has been included here as the chapter has special focus on iNOS/NO in beta-cell destruction. Genetic characterisation of iNOS, IRF-1 and mortalin within the Danish nationwide T1DM collection will be demonstrated, descriptive evaluation of the expression in cytokine exposed islets from two rat strains, as well as functional evaluations of the candidate genes expressed in the rat.

4.1. THE INDUCIBLE NITRITE OXIDE SYNTHASE (iNOS)

Nitric oxide (NO) formation is generated by the enzymes of nitric oxide synthase (NOS) family converting L-arginine to citruline and NO. It is a potent biologic mediator of diverse physiologic and pathophysiologic effects. It has been implicated in blood pressure regulation, neurotransmission, antimicrobial defence mechanisms, modulation of inflammatory response (Moncada et al., 1991) and autoimmunity (Bogdan, 1998; Singh et al., 2000) in part by modulating the Th1/Th2 response (Taylor-Robinson et al., 1994; Nukaya et al., 1995; Wei et al., 1995; Kolb et al., 1998; Niedbala et al., 1999). The NOS family comprises: neuronal NOS (nNOS or NOS1), the inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). The nNOS and eNOS are constitutively expressed (cNOS), named after the cells in which they were originally discovered, synthesise NO dependent on calmodulin (CaM) and Ca^{++} (Nathan et al., 1994), function in signal transduction cascades by linking temporal changes in Ca^{++} level to NO production, and serves as activators of soluble guanylate cyclase (Ignarro et al., 1995). Both cNOS isoforms participate in homeostatic cell to cell signalling and are regulated independently of the inflammatory responses (Bredt et al., 1991; Sessa et al., 1992). Induced NO production was initially identified in LPS stimulated MØ (Stuehr et al., 1985), and iNOS expression requires de novo protein synthesis following cellular stimulation by LPS or cytokines (Hughes et al., 1990; Eizirik et al., 1991). The enzyme is predominantly soluble (Hevel et al., 1991), and binds CaM tightly even in absence of Ca^{++}, hence being Ca^{++} independent (Cho et al., 1992). Furthermore, the enzyme produces much larger amounts of NO when stimulated than the cNOS's (Cho et al., 1992). Once produced, NO quickly ($T_{1/2}$ are seconds) undergoes spontaneous oxidation to the inactive metabolites nitrite and nitrate (NO_2^- and NO_3^-). iNOS has been implicated in numerous human diseases, including neurodegenerative, autoimmune, cardiovascular, inflammatory and a number of human cancers, for review see (Kröncke et al., 1998). Even though iNOS expression appears to have many beneficial roles in the acute septic response (e.g. hepatoprotection (Taylor et al., 1998)), over-expression can be detrimental (Szabo et al., 1994; Cobb et al., 1996).

4.1.1. Modes of cytokine mediated beta-cell destruction

In general, cytokine mediated beta-cell destruction involves the toxicity of generated free radicals (FR) e.g. reactive oxygen species (ROS), which have the capacity to oxidize and thereby damage cellular components (Chapple, 1997). Besides the toxic effect of ROS, oxidation of proteins may turn them into autoantigens thereby initiating/continuating the immune reaction toward the beta-cell (Karlsen et al., 1998). In rat islets, the majority of the inhibitory effects and toxicity of cytokines are believed to be mediated by expression of free radicals generated in the beta-cells – e.g. the nitrous oxide radical formation – by expression of the inducible form of nitric oxide synthesis (iNOS) and consequently the synthesis of nitric oxide (NO) (Southern et al., 1990), reviewed by (Nerup et al., 1994; Mandrup-Poulsen, 1996; Eizirik et al., 1996c; Eizirik et al., 1997).

The toxic effects of cytokines exposed to beta-cells have been shown to lead to necrosis as well as apoptosis. This chapter will demonstrate that NO can lead to both forms of cell deaths. Besides the induction of ROS – mainly leading to necrosis, the transcription factors JNK and NFκB have been demonstrated to be essential regulators of cytokine signalling leading mainly to apoptotic cell death (See 3.2.3 "Intracellular cytokine induced pathways"). Furthermore, cytokines have been shown to activate Ca^{++} channels and caspases inducing apoptotic cell death (for review see also (Bergholdt et al., 2003)).

4.1.2. Nitric Oxide – necrosis and apoptosis – in cytokine mediated beta-cell destruction

Necrosis is usually the result of acute cellular dysfunction in response to massive cell injury caused by sudden severe ischemia, chemical, physical or thermal injury, leading to loss of the selective permeability of the cell membrane (Majno et al., 1995). Due to disruption of membranes, cellular content and material are released to the exterior triggering an acute inflammatory response by attracting pro-inflammatory cells (Gores et al., 1990; Haslett, 1992). Necrosis is generally considered a passive, non-energy dependent process associated to rapid cellular ATP depletion. In islets exposed to IL-1 a down-regulation of an ATP- syntheses subunit has been observed (Mose-Larsen et al., 2001; Sparre et al., 2002), however, whether this is a primary or secondary effect is unknown.

As previously described, NO was the first effector molecule identified to mediate the deleterious effects of cytokine mediated beta-cell destruction as a pathogenetic model for T1DM (Southern et al., 1990). This has further been substantiated by:

- exposure of NO donors to rat islets (Kroncke et al., 1993; Cunningham, 1994; Sjöholm, 1996; Eizirik et al., 1996a) and beta-cells (Dimatteo et al., 1997) leading to beta-cell destruction
- over-expression of the iNOS gene under the RIP (Takamura et al., 1998) mimicking IL-1 mediated beta-cell destruction, and
- blockage of NO production (Andersen et al., 1996) protecting IL-1 exposed beta-cells. Moreover,
- using the mRNA display technique in cytokine exposed insulin producing cells up-regulation of iNOS and AS (see below) was observed (Rieneck et al., 2000; Cardozo et al., 2001a; Cardozo et al., 2001b; Nielsen et al., 2004).

As iNOS catalyses the reaction arginine to citrulline and NO, the amount of generated NO depends upon availability of arginine. Transportation of arginine has been demonstrated to cross the cell membrane into the cell by use of the transport system y+CAT and being synthesised intracellularly from citrulline by argininosuccinate synthase (AS) (citrulline-NO cycle) or by protein degradation (Morris et al., 1994). IL-1 has been shown to increase the expression of AS (Flodström et al., 1995), to inhibit the enzyme arginase converting arginine to arnithine and urea (Cunningham et al., 1997), and to increase the transport of arginine into rat beta-cells (Flodstrom et al., 1999a). All these IL-1 mediated effects increase iNOS substrate availability securing the NO production.

NO has been identified to:

- nitrosylate the Fe-S center of the aconitase enzyme in the Krebs cycle thereby inactivating its function (Welsh et al., 1991a) and
- induce DNA strand breaks in mitochondrial DNA (Wilson et al., 1997), probably contributing to the initially described IL-1 mediated reduction of glucose oxidation and mitochondrial organelle dysfunction. Furthermore, NO has been shown to
- induce nuclear DNA strand breaks in rat islets (Delaney et al., 1993), a process activating the enzyme poly(ADP-ribose) polymerase (PARP), participating in DNA repair process but consuming nicotinamide adenine nucleotide (NAD) thereby further depleting the cell of energy. In rat and man, nicotinamide, an in-

hibitor of PARP activation, has been shown to reduce cell loss in islets exposed to NO donors (Radons et al., 1994; Eizirik et al., 1996a). Finally, PARP deficient mice have been shown to be less sensitive to NO and FOR mediated cell death (Heller et al., 1995).

Besides the functionally inhibitory and deleterious effects of NO itself, reaction between NO and superoxide leads to the toxic radical peroxynitrite ($ONOO^-$) reported to be an even more potent oxidant and cytotoxic mediator than superoxide or nitric oxide (Szabó, 1996). Peroxynitrite has been identified in NOD mice infiltrated by mononuclear cells (Suarez-Pinzon et al., 1997) and inhibition of iNOS and scavenging peroxynitrite prevented diabetes development in NOD mice (Suarez-Pinzon et al., 2001) as well as in the multiple low-dose streptozotocin induced diabetes model (Mabley et al., 2004). Peroxynitrite has also been identified in human islets exposed to cytokine mixture (Lakey et al., 2001). Human islets exposed to peroxynitrite display acute DNA strand break and decreased glucose metabolism leading to cell death (Delaney et al., 1996).

In addition to the direct tissue damage mediated by NO, a report has suggested that NO may enhance and/or preserve the Th1 cytokine profile in the NOD mouse. The Th1 response is activated by IL-12, a MØ cytokine, and suppression of the IL-12 production in NOD may inhibit the progress of the initial benign Th2 insulitis to the destructive Th1 insulitis process, reduce iNOS mRNA expression and decrease diabetes incidence, for review see (Rothe et al., 1999). Thus it seems that NO production facilitates and maintains the destructive Th1 insulitis process. Finally, it has been shown that activated MØ facilitates islet destruction by $CD8^+$ T-cells through a NO synthesis-dependent pathway (Gurlo et al., 1999).

Hence, NO or derivatives thereof lead to acute cellular dysfunction depleting the cell of energy. Results from iNOS -/- transgenic mice have suggested that NO is predominantly involved in necrosis and not apoptotic cell-death (Liu et al., 2000). However, the same study also recognised the existence of NO-independent effector mechanisms as beta-cell destruction was only partly protected in the iNOS -/- beta-cells following cytokine exposure.

Apoptosis or programmed cell death is an energy requiring process (Cummings et al., 1997), naturally occurring during embryogenesis and in normal tissue turnover and constitutes a common mechanism of cell replacement, tissue remodelling and removal of damaged cells (DeLong, 1998). Morphologically, apoptosis is characterised by condensation and margination of the chromatin towards the nuclear membrane, cellular shrinkage, detachment from neighbouring cells, inter-nucleosomal DNA fragmentation and formation of "apoptotic bodies". These apoptotic bodies are almost immediately phagocytosed, preventing exposure of cellular content to the exterior and thereby inflammatory response.

In line with the original concept proposed in "The Copenhagen Model" suggesting that cytokine exposure induces a race between deleterious and protective mechanisms, (Nerup et al., 1994) it follows that cytokine exposure initiates many different responses within the beta-cell. Besides, as indicated from the different signalling pathways activated by IL-1β, INFγ and TNFα it would seem unlikely that only one cytokine mediated effector arm should exist.

Indeed, apoptosis can be demonstrated in beta-cells: in the postpartum pancreas (Scaglia et al., 1995), in the neonatal pancreas (Scaglia et al., 1997), in response to hyperinsulinemia induced by transplantation of an insulinoma (Blume et al., 1995) and in islets where glucose promotes survival of rat pancreatic beta-cells by activating synthesis of proteins which suppress a constitutive apoptotic program (Hoorens et al., 1996).

In the literature, reports suggesting *NO independent* apoptosis exist in islets, as:

– blocking NO-production does not fully inhibit cytokine mediated apoptosis (Eizirik et al., 1994a; Rabinovitch et al., 1994a; Delaney et al., 1997; Hoorens et al., 2001), and
– apoptosis has been detected in cytokine exposed islets from iNOS-/- mice (Liu et al., 2000; Zumsteg et al., 2000)

It has been suggested that cytokines can induce Fas expression upon the cell surface of the beta-cell (Stassi et al., 1997). Consequently, Fas/FasL interaction between the beta-cell and T-cell ($CD4^+$ and $CD8^+$) present in the insulitis infiltrate may activate caspases leading to apoptosis of the beta-cell. This initial finding has subsequently been challenged, and the involvement of Fas/FasL as effector molecules for beta-cell destruction in T1DM remains controversial, for review see (Eizirik et al., 2001b). Furthermore, cytokines have (i) shown to induce MAPK (Larsen et al., 1998) and caspase 1 (ICE) (Karlsen et al., 2000) in beta-cells, and (ii) been suggested to mediate beta-cell apoptosis, which lead to focusing at related signalling pathways and apoptotic effector mechanisms as putative mediators of beta-cells death.

However, apoptosis in islets/beta-cells has been identified following exposure to NO, peroxynitrite or cytokine mediated NO-effects (Mabley et al., 1997; Hadjivassiliou et al., 1998; Saldeen, 2000).

Moreover, other reports suggest *NO influence* on the apoptotic process, as:

– blocking NO synthesis leads to reduced PARP-cleavage (indicator of apoptosis) after 24h exposure of islets to IFN/TNF/IL-1, and reduced number of necrotic and apoptotic cells in the islets significantly (Saldeen, 2000)
– NO/oxidative stress decreased redox function modifying the cytokine-induced apoptotic pathway (Stamler, 1994; Dimatteo et al., 1997; Hampton et al., 1998)
– NO induced DNA strand breaks may induce apoptosis *per se* (Ankarcrona et al., 1994; Kaneto et al., 1995) or through activation of the tumour suppressor protein p53 (Messmer et al., 1994)
– Endoplasmatic reticulum stress (perturbations leading to accumulation of malfolded proteins in that compartment) has been suggested to activate JNK in non-beta-cells, however this coupling is not understood (Urano et al., 2000), and NO induced ER stress could be coupled to the pro-apoptotic JNK pathway,
– NO is needed to induce apoptosis in FACS purified rat beta-cells by combinations of viral products and cytokines (Liu et al., 2001), and finally
– NO induces ER depletion of Ca^{++} leading to ER stress and subsequent induction of apoptosis by the CHOP apoptosis (a C/EBP homologous protein, induced by ER stress and plays a role in growth arrest and cell death) (Oyadomari et al., 2001),

Hence, it seems that beta-cell destruction involves NO as well as non-NO-dependent effector arms leading to both necrosis as well as apoptosis. As both forms of beta-cell destruction have been demonstrated, factors influencing the outcome are of interest. Different study designs may help identify such factors – however, the mode of destruction may be influenced by the study design, e.g. beta-cell single cell suspensions versus islet studies. In rat single cells, both apoptosis and necrosis occur, and full apoptotic effect and necrotic index were observed at relatively low cytokine mix concentrations, possible due to limited numbers of cytokine receptors (Eizirik et al., 2001b). In contrast, in rat islets exposed to cytokine mixture a higher increment in necrosis than in apoptosis was seen (Saldeen, 2000), possibly due to higher intra-islet concentrations of NO produced by both beta-cells and non-beta-cells. A study combining exposure of IL-1 and doubled stranded RNA (dsRNA), imitating virus exposure/infection induced NO-dependent apoptosis in contrast to IFNγ + dsRNA exposure leading to NO-independent death of unknown pathways (Liu et al., 2001), illustrating that different agents can trigger various destructive pathways. Further, the potency of the "hit" versus the defence properties may influence the destructive pathway taken as more severe attack tends to lead to necrosis, as the cell depletes from energy to fulfil the apoptotic program (Lemasters

et al., 1999). This may partly explain the finding of apoptosis being more pronounced in human than in rodent islets (Delaney et al., 1997; Hoorens et al., 1999; Hoorens et al., 2001) as (i) human islets are better protected against oxidative stress (see elsewhere) partly due to higher cellular amounts of HSP70 (Welsh et al., 1995b; Burkart et al., 2000) serving an anti-apoptotic effect (Jaattela, 1999), and (ii) a better capacity to continue glucose oxidation resulting in higher ATP-production (regarding human islets, see (Eizirik et al., 1994a) and mouse islets, see (Cetkovic-Cvrlje et al., 1994)), necessary to fulfil the apoptotic program, despite similar amounts of NO are produced (Eizirik et al., 1997). Heterogeneity within beta-cells may also contribute to the outcome. Heterogeneity has been described in FACS purified rat beta-cells (Pipeleers, 1992) – (i) diverse sub-population of beta-cells differently responding in insulin secretion at identical glucose concentration, (ii) high glucose sensitivity associated to high general protein synthesis – could serve as an example of the impact of beta-cell phenotype or functional state. High glucose sensitivity has been proposed to provide a better anti-apoptotic protein response due to the general induced protein synthesis of glucose in beta-cells (Hoorens et al., 1996). On the other hand, islets exposed to high glucose concentrations and high insulin secretion were more sensitive to the deleterious effects of IL-1 exposure compared to low glucose concentrations – suggesting the beta-cell to be "a moving target" (Helqvist, 1994) – a phenomenon not only related to high insulin secretion, as the IL-1 sensitivity were dependent upon the stimulus leading to insulin secretion (Johannesen et al., 1990). This is in line with higher iNOS expression in "high glucose-responsive FACS-purified beta-cells" indicating intercellular differences of beta-cell responsiveness to IL-1 related to the beta-cell glucose-responsiveness (Ling et al., 1998) supporting the existence of variation in beta-cell phenotype. Finally, IL-1 has been demonstrated to induce beta-cell adaptation shown as a reduced cellular sensitivity to conditions that cause necrosis but not to cytokine induced apoptosis (Ling et al., 2000). This adaptation seemed to be independent of NO production as these findings were confirmed in arginine free conditions as well as independent of heme oxygenase and HSP70 as these proteins were not elevated in arginine free condition (Ling et al., 2000).

Lessons from the BB-rat have demonstrated necrosis being the predominant type of islet cell death during development of insulin-dependent diabetes (Fehsel et al., 2003).

Taken together: In beta-cells, NO and NO-independent induced necrotic and apoptotic destruction takes place following cytokine exposure. Whether the necrotic or the apoptotic process – or both – are effectuated may be influenced by e.g. the potency of the "hit" versus the defence properties. Hence, the beta-cell destructive process is dependent upon e.g. (i) the stimuli exposed to the beta-cell and (ii) the functional state of the beta-cell and (iii) possibly influenced by beta-cell heterogeneity. The mode of beta-cell death is still controversial and hence, further studies into these areas e.g. illumination of intercellular, -species or -individual differences – are needed to develop testable preventional actions to diminish cytokine mediated beta-cell destruction.

4.1.3. Intercellular, -species and -individual differences affecting cytokine cytotoxicity

Differences in cytokine sensitivity have been described between single cells and isolated islets. Moreover, heterogeneity among FACS purified rat beta-cells regarding glucose sensitivity has been suggested to be associated with pro-apoptotic protein response (Hoorens et al., 1996) – suggesting that variation in sensitivity to cytokines in beta-cells may depend upon the beta-cell itself and other cell types in the islet. Only very few phenotypic characteristics are available regarding beta-cell age. In 2001, Bonner-Weir speculated whether the secretory and biosynthetic heterogeneity of FACS purified beta-cells was influenced by the age of the beta-cells (Bonner-Weir, 2001) – hence, the age of the beta-cells could influence cytokine susceptibility. Furthermore, differences in cytokine susceptibility have been demonstrated comparing a beta-cell line to a pre-beta-cell line, the former being the most sensitive (Nielsen et al., 1999). Finally, induction of cardiac iNOS expression increases with age in rats (Rosas et al., 2001). However, no difference in cytokine sensitivity was demonstrated *in vitro* using neonatal versus adult islets (Mandrup-Poulsen et al., 1987).

As previously described, inter-species differences exist between human and rat islets regarding:

- the ability of IL-1 vs cytokine mix capable of leading to beta-cell destruction, initially associated to IL-1 not being able to induce iNOS and NO in human islets, in contrast to human hepatocytes (Geller et al., 1995) where IL-1 can induce iNOS expression indicating tissue differences as well,
- different defence capacities, and
- different capacity to continue glucose oxidation leading to higher ATP concentrations possibly favouring apoptosis being the leading destructive process in human beta-cells in contrast to necrosis in rat beta-cells.

Moreover, different sensitivity towards NO has been proposed as it was previously shown that inhibition of iNOS expression failed to protect human islets against the deleterious effects of cytokine mixture exposure (Eizirik et al., 1994a; Delaney et al., 1997). However, the evidence of the toxic effect of peroxynitrite in human beta-cells argues for a role of NO in human beta-cell destruction, as inhibition of iNOS did not abolish peroxynitrite formation, possible due to an unchanged basal NO production, and did not prevent beta-cell destruction (Lakey et al., 2001). Besides, NO donors are able to destroy beta-cells in human islets (Eizirik et al., 1996a), and as iNOS induction is detected in human beta-cells (Arnush et al., 1998) and by neighboring non-beta-cells (Pavlovic et al., 1999), it might be speculated that cytokines can lead to deleterious local intra-islet concentrations of NO. Furthermore, equal susceptibility towards NO-donors in human islets and rat beta-cells has been demonstrated (Delaney et al., 1996; Hoorens et al., 2001).

Comparing human and bovine islets – bovine islets being less susceptible to damage by human cytokines compared to human islets (Piro et al., 2001) – demonstrates inter-species differences.

Besides inter-species differences in islet and beta-cells, alveolar macrophages from rat, hamster, monkey and man have been examined under identical experimental conditions. Clear differences between rodent and the primate species were demonstrated in iNOS expression and nitrite production after LPS and IFNγ stimulation (Jesch et al., 1997).

The different phenotypic characteristics within species may be influenced by genetic variation, as exemplified by the different iNOS regulation in macrophages from chicken of different genetic backgrounds (Hussain et al., 1998). Finally, the islets from diabetes-resistant BB rats have been shown to mount a HSP70 response after heat stress in contrast to the diabetes-prone BB rat. (Bellmann et al., 1997). The lack of a protective stress response in islet cells from diabetes-prone BB rats could be important for initiation or propagation of the disease process.

In vivo as well as *in vitro*, strain-dependent differences in cytokine responsiveness have been demonstrated between two rat strains, Brown Norway and Wistar Kyoto (Reimers et al., 1996). This difference has been found to be associated to different IRF-1, iNOS and HSP70 expression levels, whereas no difference in IL-1R1 expression could be demonstrated (Johannesen et al., 2001b). The study design, however, did not allow the conclusion that a causal relation between IRF-1 and iNOS exists, but it was speculated that polymorphisms in the IRF-1 gene, as well as quantitative differences in the transcriptional regulation could be involved.

In conclusion: Inter- and intra-individual differences and heterogeneity among beta-cells may potentially influence the cytokine sen-

sitivity and might correlate to defence capacities and the level of the induced oxidative stress. These differences could e.g. be influenced by genetic factors hence, a genetic evaluation of involved proteins could increase our understanding of some of the inter- and intra-individual differences.

4.1.4. Genetic structure of the rat iNOS gene

The remaining part of this chapter will describe the structure and functional regulation of the rat and human iNOS gene. The iNOS gene regulation in insulin producing cells and different iNOS promoter sequences within the BN and WKY rat strains will be in focus. Furthermore, in order to understand the genetic impact of iNOS in T1DM an evaluation of the human iNOS gene sequence becomes mandatory as identification of sequence variations are needed to test for genetic association – here illustrated by transmission disequilibrium within the Danish T1DM family collection.

In 1993, Nunokawa was the first to clone the coding sequence of rat iNOS gene (Nunokawa et al., 1993) and in 1996 the first part of the rat iNOS promoter was cloned first by Eberhardt (Eberhardt et al., 1996). The structure of the rat iNOS gene is outlined in Figure 5.

4.1.4.1. The rat iNOS: promoter region

In the rat iNOS promoter, more than 20 transcription-binding factor (TFB) sites are known and represent (i) LPS-related response elements (NF-IL6 and NFκB (Lowenstein et al., 1993)), (ii) IFNγ-related response elements (IRF-1 and STAT1 (Lowenstein et al., 1993) (Teng et al., 2002)) and (iii) IL-1β-related response elements (NFκB and C/EBP (Teng et al., 2002)). Homology of the rat iNOS promoter from different strains is high: >95% (Johannesen et al., 2003), see Figure 6, but decreasing when comparing the rat iNOS promoter to the iNOS promoter of mouse and human (73% and 55% homology, respectively) (Zhang et al., 1998).

Whether the observed sequence differences represent tissue-dependent or intra-strain-dependent differences or "simple sequence inconsistency" is unknown. Two studies using different tissue sources have compared the iNOS promoter from different rat strains, and independently identified a GT-repeat polymorphism in position –1685 to –1634 from the transcription start site (Deng, 1998; Johannesen et al., 2003) – suggesting the difference to be strain and not tissue-dependent. Comparing the BN-rat to WKY-rat, a polymorphism in position +222 within exon 1 was identified (Johannesen et al., 2003).

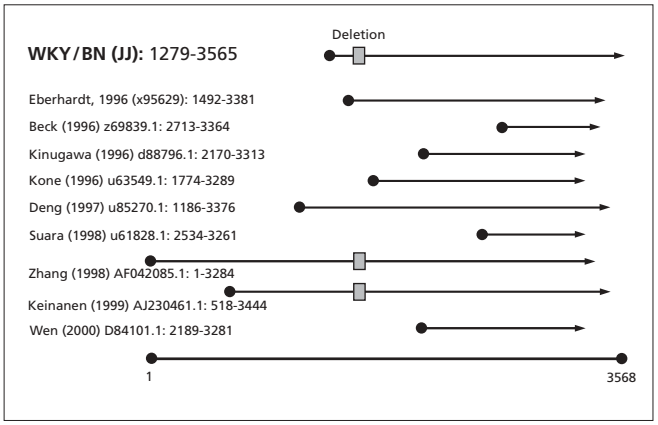

Figure 6. Alignment of cloned rat iNOS promoters.

In Table 5 "*Rat iNOS promoter cloning and function*", the structural and functional findings of different rat iNOS promoters cloned from different tissues and tested in various cell-types are listed.

Expressional control of the iNOS promoter has been shown to be tissue and/or cell as well as species specific. The mouse MØ iNOS promoter organisation are characterised by two distinct regions of importance, Region I and Region II, in the initial 1.2 kb of the promoter conferring full promoter activity (Lowenstein et al., 1993). Within the rat and the human iNOS promoter, 3.2 kb and 16 kb, respectively, are needed for the highest promoter activation (Vera et al., 1996a; Zhang et al., 1998). Further, the JAK/STAT pathway mediates the LPS/IFNγ induced iNOS expression in mouse RAW294.7 cells (Gao et al., 1997), whereas inhibition of the JAK/STAT pathway

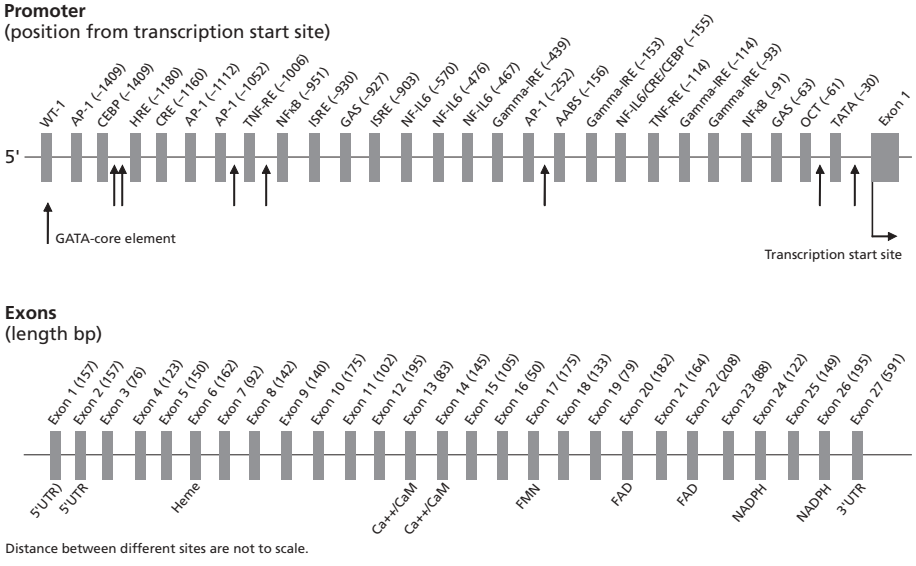

Figure 5. Rat iNOS gene structure. The riNOS gene has been localized to chromosome 10 in rat genome (Deng et al., 1994; Deng et al., 1995), spanning approximately 36kb, containing 27 exons and 26 introns (Keinanen et al., 1999). The promoter region: Homology between the published rat iNOS promoters is >98% (Zhang et al., 1998; Johannesen et al., 2003). The counterparts of the rat promoter to the murine promoter Region I {(position –48 to –209 in mouse Mø) contain LPS-related response elements: NF-IL6 and NFκB (Lowenstein et al., 1993)} and Region II {(–919 to –1029 in mouse Mø) contains IFNγ-related response elements: IRF-1 and STAT1 (Lowenstein et al., 1993)} show 90% identity, despite overall homology of rat and mouse iNOS promoters is 77% (Keinanen et al., 1999). The analogous regions in the rat promoter contain more than 20 putative transcription binding factor sites within the first 2.6 kb of the 5'UTR as in the mouse gene (Keinanen et al., 1999). cDNA size: (ORF) 3441 bp encoding 1147 AA (Iwashina et al., 1996). Inter-species comparisons reveal close structural homology among iNOS cDNA isoforms: the homology between murine or rat and human iNOS cDNAs is approximately 80% (Keinanen et al., 1999).

Table 5. Rat iNOS promoter – cloning and function.

Author/references	Promoter size/position	Structural findings	Functional findings	Functional test tissue
Beck et al., 1996	–497 bp (rat liver) GB: Z69839.1	Identification of NFκB, oct-1 and TATA	+IL-1β/TNF : NFκB and oct-1 binding in EMSA	RMC (Sprague Dawley rats)
Eberhardt et al., 1996	–1.8 kb (rat liver) GB: X95629	More than 30 TFBS (see ref. for details)	+IL-1β: CAT↑ × 2 cAMP↑: CAT↑ × 2	Swiss 3T3 fibroblast
Niwa et al., 1997	–480 bp (rat spleen)	Identification of γ-IRE, NF-IL6, TNF-RE, TATA	Not tested	Not specified
Kinugawa et al., 1997	1111 bp (Neo. rat cardiac myocytes) GB: D88768	Homology to Eberhardt (1996) >99%	Identification of various TFB sites being important for LPS induced CAT activity	Neo. rat cardiac myocytes
Kuo et al., 1997	Not specified		Oxidative stress (BZT) enhances IL-1β stim. CAT activity	Rat hepatocytes
Beck et al., 1998	–1.8 kb (Eberhardt, 1996)		Superoxide (xanthine oxidase/DMNQ) co-stimulate/ enhance IL-1β luc. activity	Rat mesangial cells
Darville et al., 1998	–1514 bp (rat liver) Based upon Eberhardt, 1996	Identification of at least five TFB sites	CM induced luc. activity dependent upon length/ number of various TBF withhin iNOS promoter Cell specific activity	**RINm5F** **FACS sorted alfa- and beta-cells**
Deng, 1998	–2.2 kb (rat liver) GB: U85270	Identification of GT repeat (D10Mco42)	Not tested	
Eberhardt et al., 1998	–1.8 kb		IL-1β and cAMP use distinct as well as overlapping sets of transcriptional activators to modulate CAT activity	Rat mesangial calls
Pahan et al., 1998	1.5 kb (based upon Eberhardt, 1996)		Inhibition of PP1/2A enhance/ abolish LPS mediated CAT activity in astrocytes and rat MØ, respectively	Rat astrocytes Rat MØ
Saura et al., 1998	–388 & –720bp (rat gDNA) GB: U61282	Identification of γ-IRE, NF-IL6, TNF-RE, Oct-1, TATA	DX abrogates the stim. luc. activity of LPS/TNFα DX leads to IkB↑	RMC
Schroeder et al., 1998	Not specified		IL-1β induced CAT activity abolished in the presense of anti-IFNγ or antisense IFNγ	Rat hepatocytes
Zhang et al., 1998	–0.32 to –5.1 kb (rat gDNA) GB: AF042085	More than 30 TFBS (see ref. for details)	LPS or cyt. mix: max. luc. activity for -3.2kb construct	RASMC
Keinanen et al., 1999	–2.6 kb GB: AJ230461	More than 20 TFBS (see ref. for details)	–	
Pahan et al., 1999	1.5 kb (based upon Eberhardt, 1996)		Activation of NF B and inhibition of PI 3-kinase needed for LPS/IL-1β CAT activity	C_6 glial cells Rat primary astrocytes
Punzalan et al., 1999	1.8 kb (based upon Eberhardt, 1996)		Oxidative stress (H_2O_2) enhance IL-1 mediated CAT expression via ARE (–1347)	HepG2
Bellmann et al., 2000	–1002 bp	Not described Promoter gifted from Darville 1998	HSP70 over-expression increases IL-1 induced luc. activity via p38 (MAPK)	**RINm5F** (WEHI)
Inoue et al., 2000	1.4 kb (based upon Eberhardt, 1996)		Hypoxia (not via HRE) and heat abrogates the stim. luc. activity by interfering with NF B/DNA interaction	Hepatocytes
Kuo et al., 2000	–1.8 kb (Eberhardt 1996)	Identification of ARE at position –1347	Enhancing affect of BZT of IL-1β mediated CAT expression Deletion constructs id. ARE	Hepatocytes
Oda et al., 2000	–1042 bp		PAO abrogates the stim. luc. activity p50/p65 (NF B) stimulation in hepatocytes	Hepatocytes
Pahan et al., 2000	1.5 kb (Based upon Eberhardt, 1996)		Mutated $p21^{ras}$ abrogates the stim. luc. activity of LPS and cytokines in astrosytes	Primary astrocytes

Table 5. Continued.

Author/references	Promoter size/position	Structural findings	Functional findings	Functional test tissue
Teng et al., 2000	–1.4 kb (Zhang 1998)		"Reverse NFκB site" in position –901 to –892 binds NFκB Influence upon luc. activity	RASMC
Wen et al., 2000	–1.1 kb (rat liver) GB: D84101	More than 20 TFBS (see ref. for details)	+ IL-1/IFNγ: –1037 to –786 bp (NFκB site) hybridize in EMSA	
Zhang et al., 2000	–3.2 kb (Zhang 1998)		Upstream NF B site has higher effect upon CM induced luc. activity than downstream NFκB site. Non-NFκB sites: –1.0 to –1.37 and –2.0 to –2.5	RASMC
Karlsen et al., 2001	–1.7 kb (based upon Johannesen, 2003)		SOCS-3 abrogates the stim. luc. activity of IL-1β.	**INS-1** ± SOCS-3 over-expression
Liu et al., 2001	–1.0 kb (Darville, 1998)		PIC/IFNγ stim. luc activity to similar levels as IL-1β PIC acts via NFκB	**FACS** primary beta-cells
Syapin et al., 2001	–526 bp (Eberhardt, 1998) –1846 bp (Eberhardt, 1996)		Ethanol abrogates the stim. luc. activity of LPS/IFNγ IFNγ RE is not involved in this inhibitory effect	C_6 glial cells
Zhang et al., 2001	–3.2 kb (Based on Zhang, 1998)		NO abrogates the stim. luc. activity of IL-1β in MØ, but enhance activity in RASMC P50:p65 ratio highest in RASMC	RASMC MØ (NR8383)
Guo et al., 2002	–1.8 kb (Eberhardt, 1996)	Id. of HNF-4α/PC4 protein complex binding to ARE (–1340)	Oxidative stress induced HNF-4α/PC4 augments IL-1β stimulation of iNOS activity	ANA-1 and rat hepatocytes
Teng et al., 2002	–1.4 kb (from Zhang, 1998)	Mutational analysis: ΔGAS:-950	IL-β via NFκB and C/EBP IFNγ via IRF-1 and STAT1	RASMC
Johannesen et al., 2003	–1.8 kb (Leukocytes from BN & WKY rats)	Identification of putative WT-1 (-KTS) site in BN	WT-1 increases the stim. luc. activity of IL-1 leading to a strain dependent difference	**RINm5F**

The origin of the rat promoter characterized is seen in the column "Promoter size and position". E.g.: "–1.8 kb" indicates the 1.8 kb of the promoter 5′ to the transcribed part of the gene has been characterized, in contrast to e.g. "1111 bp" indicating that the specific position within the promoter region has not been specified. Insulin producing cell lines are bolded.

in RASMC enhance iNOS induction by LPS / IFNγ (Marrero et al., 1998). The latter could also be explained by cell-specificity, as IL-1β alone is able to induce iNOS expression in cultured RASMC (Kanno et al., 1993; Koide et al., 1994), but neither in rat pulmonary SMC (Nakayama et al., 1992) nor in the SMC cell-line A7r5 (Spink et al., 1995). Further, testing the same mouse iNOS promoter clone in three different cell-types – RAW264.7, VSMC and RASMC, the two first systems by LPS and the latter by cytokine mix – demonstrated that different TBF sites were of importance. In RAW264.7 and RASMC, the lower NFκB site was indispensable whereas it was the upper NFκB site in VSMC (Xie et al., 1994; Spink et al., 1995; Perrella et al., 1996). Finally, a 1.7 kb rat iNOS promoter in RASMC gave a 13 fold induction by cytokines (Zhang et al., 1998), whereas in Swiss3T3 cells only 3-4 fold induction was observed for the same promoter sequence and stimulation (Eberhardt et al., 1996). In 2001, Zhang demonstrated that the iNOS promoter activity of the initial 3.2 kb rat iNOS promoter was only negative feed-back regulated by NO within the MØ, in contrast to findings within the RASMC (Zhang et al., 2001).

Much work has focused upon cytokine induced iNOS expression and modulation hereof in insulin producing cells (see previous chapters). Only few studies have explored *iNOS promoter gene regulation in insulin producing cells* using promoter activity assays. Early studies using deletional constructs of the rat iNOS promoter to identify significant TFB sites revealed that NF-κB, GAS, ISRE binding-sites were crucial in the cytokine mediated NO-dependent pathway (Darville et al., 1998). Again, cell specificity was observed, as IFNγ (inducing the transcription factors STAT1α and IRF-1, binding to GAS and ISRE, respectively,) enhanced IL-1β mediated iNOS promoter activity in RINm5F cells whereas in primary rat beta-cells, IFNγ neither increased the iNOS promoter activity nor iNOS mRNA expression, but did induce a two-fold increase in NO (Darville et al., 1998). Bellmann showed that over-expression of HSP70 led to enhanced IL-1β induced rat iNOS promoter activity (testing 1 kb of the promoter) in RIN cells through an increased activity of MAPK p38 (Bellmann et al., 2000). Further, based upon the model of viral induced beta-cell destruction, a synthetic dsRNA (PIC) in combination with IFNγ was able to induce NFκB dependent iNOS promoter activity in primary rat beta-cells (Liu et al., 2001). Finally, two studies describe abrogation of cytokine induced iNOS promoter activity: (i) the suppressor of cytokine signalling-3 (SOCS-3) abrogated the rat iNOS promoter activity (1.8 kb) in rat INS-beta-cells (Karlsen et al., 2001) and (ii) the pituitary adenylate cyclase-activating polypeptide (PACAP) abrogated the mouse iNOS promoter activity (1.6 kb) in the mouse beta-cell line, βTC cells (Sekiya et al., 2000).

As depicted above, structural features within the iNOS promoter as well as the cellular environment in which the iNOS promoter operates influence iNOS promoter activity. In an attempt to by-pass the influence of cellular environment, Johannesen et al. tested the rat iNOS promoters from two different rat strains (BN and WKY), in the same test-cell system (Johannesen et al., 2003). Previously, isolated islets from these rat strains were identified as having IL-1 mediated strain-dependent nitrite, iNOS mRNA and protein ex-

pression profiles (Johannesen et al., 2001b). Cloning and sequencing of the iNOS promoters identified two polymorphisms within the promoter region spanning –1744 bp to +333 bp. The upper GT-repeat polymorphism gave rise to a WT-1 (–KTS) TFB site (Bickmore et al., 1992) in the BN rat strain approximately 1650 bp upstream the promoter. For details regarding Wilm's Tumor, please see Table 6).

Strain-dependent and IL-1 dose-response of the tested iNOS promoter sequence spanning –1744 to +267 was demonstrated in a luciferase assay co-expressing the transcription factor WT-1. The promoter activity assay revealed higher iNOS promoter activity of the BN than of the WKY iNOS promoter (Johannesen et al., 2003), whereas data generated *in vitro* from rat islets culture IL-1 dose- and time dependently revealed higher iNOS mRNA and protein expression levels and nitrite production from WKY islets (Johannesen et al., 2001b). This apparent controversy needs to be evaluated in the light of: (i) not full length rat iNOS promoter was tested and hence, additional promoter differences between these two rat strains may exist – indirectly evidenced by the fact that probably due to sequence variations it was not possible to construct a common upper cloning primer further 5' upstream than the one used. (ii) Information regarding the role of the 3' UTR in mRNA stability should be explored, and finally (iii) indeed, the differences in BN and WKY intracellular milieu may influence the respective promoters as illustrated by the findings of Darville (Darville et al., 1998).

4.1.4.2. The rat iNOS: cDNA/gDNA region

In Table 7 "*Rat cDNA/gDNA iNOS cloning*" studies characterizing the iNOS cDNA are listed.

As seen, the homology between the various iNOS clones from different cell-types is very high (>99%), as is the homology of iNOS between rat and mouse MØ, (approximately 92%). The cytokine induced iNOS gene sequence in rat islets is identical to other rat iNOS sequences from other tissues (Karlsen et al., 1995). The translation initiation codon has been located in exon two (Keinanen et al., 1999), as in the mouse and in the human gene (Chartrain et al., 1994). The stop codon is placed in e27 leaving a 3'UTR of 495 bp in length (Keinanen et al., 1999). Sporadic base-pair mutations/mismatches have been identified between the separate clonings when compared to each other, some leading to amino acid changes. None so far have involved known co-factor binding sites. Whether any functional relevance exists for these variations is unknown at present. Co-factor binding sites as depicted in the figure "Rat iNOS gene structure" include CaM, FMN, FAD, NADPH, Heme. Each binding site appears to lie in separate exons, except for CaM, spanning e13 and e14 (Keinanen et al., 1999).

In conclusion: The studies regarding the rat and mouse iNOS promoters have revealed (i) intra- and interspecies differences in genomic sequence, (ii) a complex regulatory mechanism controlling the promoter activity involving various transcription binding sites, and (iii) an intra- and interspecies dependent functional regulation. These differences might influence the different levels of iNOS expression demonstrated within the islets from BN and WKY rats, hence being of importance to the different response of cytokine exposure between these two rat strains.

4.1.5. Genetic structure of the human iNOS gene

The human iNOS cDNA was initially cloned from LPS and cytokine stimulated hepatocytes by Geller at al. (Charles et al., 1993; Geller et al., 1993; Sherman et al., 1993; Hokari et al., 1994). Subsequently, a variety of human cell-lines and cell-types have been shown to express iNOS, including human pancreatic islet cells (Flodstrom et al., 1996a; Corbett et al., 1996b; Flodstroem et al., 1997; Arnush et al., 1998; Scarim et al., 1998; Pavlovic et al., 1999; Karlsen et al., 2000; Chen et al., 2001; Heitmeier et al., 2001) – each of these cDNAs shows >99% homology to the human hepatocyte sequence, for review see (Taylor et al., 2000).

Table 6. Wilm's tumor.

Gene
- Cloned: 11p13 (Call et al., 1990; Gessler et al., 1990). 10 exons.
- Promoter contains sites for: WT1, Egr1 PAX2 PAX8, SP1, SP2, SP3, AP2 and AP4; GAGA and GGAGG motifs (Hofmann et al., 1993).
- Two translation initiation sites leading to two MW's 52-54kDa (Scharnhorst et al., 1997).
- Alternative spliced: exon 5 (17aa) and exon 9 (± KTS), hence multiple isoforms exsist (Haber et al., 1991).
- All four proteins appear to excist in temporally, spatially and evolutionary stable ratio with respect to each other (Haber et al., 1991), predominantly during the development of the urogenital system and WT1 exhibits highly tissue-specific pattern of expression during development (Pritchard-Jones et al., 1990).
- Expressed in urogenital, pericardium, spleen, spinal cord, somites (embryonal) and podocytes, Sertoli cells, granulosa cells and uterus (postnatal) (Pritchard-Jones et al., 1990; Armstrong et al., 1992).

Pathophysiology
- Mutations demonstrated in 10% of all sporadic Wilm's tumor (Little et al., 1997).
- Mutational role in following syndromes: WAGR, Denys-Drash Syndrome, Frasier Syndrome and AML (Little et al., 1997).

Plurifunctional protein (Davies et al., 1999; Little et al., 1999).
- Initially suggested to be a tumour suppressor, subsequently shown to possess pro- and anti-apoptotic properties (Algar et al., 1996; Menke et al., 1997). WT1 can regulate the expression of Bcl2, c-myc and c-myb.
- Cell type dependent transcription activity of WT1 isoforms may explain the bidirectional effects of WT1 on apoptosis (Menke et al., 1998).

Mode of action
- (i) *Transcription factor* due to the similarity of the ZF to EGR1 (Madden et al., 1991): EGR1 as an activator and WT1 as a repressor: proved too simple.
- Review of transcription binding sites and in-vitro reporter assays: (Reddy et al., 1996; Menke et al., 1998):
  - WT1 as an activator and repressor (Maheswaran et al., 1993) – cell type dependent (Little et al., 1999).
- (ii) *RNA metabolism/interaction:* WT1 contains a N-terminal RNA recognition motif (RRM) in all known isoforms (Kennedy et al., 1996). mRNA interaction seems dominant for the WT1 isotypes (+KTS) (Zhai et al., 2001) whereas -KTS appears to co-localise with transcription factors such as Sp1 and Pax6 (Little et al., 1999), but non-overlapping as well as overlapping functions of ± KTS are described (Hammes et al., 2001; Hastie, 2001).
- (iii) *Protein partners:* These may dictate the overall outcome of WT1 action: explaining different roles of WT1 at different times during development, different actions in various cell types and tissues – e.g.: p53, WT1, UBC9, par-4, Ciao 1, Hsp70, SF1 (Little et al., 1999).

WAGR: (Wilm's Tumor, Aniridia, Genitourinary syndrome, mental Retardation): 11p deletion, WAGR-region: contain WT1; (Call et al., 1990), PAX6 (Ton et al., 1991) and reticuloalbin (Kent et al., 1997).
Denys-Drash Syndrome: (i) XY genital anomalities (mild to XY pseudohermaphroditism) (ii) early onset renal failure (mesangial sclerose) and Wilm's tumor (Denys et al., 1967; Drash et al., 1970). Intragenic WT1 point mutations leading to aa substitutions (Pelletier et al., 1991), not able to bind protein (Little et al., 1995).
Frasier Syndrome: (Barbaux et al., 1997) (i) XY pseudohermaphroditism, (ii) end stage renal failure (glomerulonephropathy), (iii) NO Wilm's Tumor. Constitutional intronic mutations of one copy of WT1 that prevents production of the KTS-containing isoform from that allele (Barbaux et al., 1997). Shift in isoform ratio.

The genetic structure, chromosomal localisation including the promoter region are outlined in Figure 7.

4.1.5.1. The human iNOS: promoter region

A number of different groups have cloned and functionally tested various parts of the human promoter region. Promoter activity has been identified as far as 16kb upstream of the transcription start site (Vera et al., 1996a).

The existence of several *transcription-binding factor* (TBF) sites (cytokine-response elements (CRE)) have been shown (Vera et al., 1996a). More than 30 putative TBF sites are identified within the first 1.5 kb, although this region does not exhibit any significant activity in promoter activity analysis probably due to nucleotide ex-

Table 7. Rat cDNA/gDNA iNOS cloning.

Author/references	GenBank	Cloning size	Source	Structural findings
Wood et al., 1993	Not specified	3610 bp 1147 AA / 131 kDa	Rat hapatocytes	94% identical to mouse MØ cell line, RAW264.7: sporadic AA substitutions
Nunokawa et al., 1993	D14051	3441 bp 1147 AA/131 kDa	Rat, VSMC	93% homology for AA sequence to MØ
Galea et al., 1994	Not specified	3444 bp ORF 1147 AA	Rat Astrocytes	92% homology at DNA level and 93% homology at AA level to mouse MØ iNOS 99% homology to rat VSMC and hepatocyte iNOS
Geng et al., 1994	X76881	3440 ORF 1147 AA	Rat aortic SMC	92% homology at DNA level and 93% homology at AA level to mouse MØ iNOS 80% homology to human hepatocyte iNOS
Karlsen et al., 1995	U26686	131 kDa	Cytokine exposed rat islets and RIN cells	>99% homology to rat hepatocyte and VSMC iNOS at both bp and AA level
Iwashina et al., 1996	Not specified	3441 bp 1147 AA	Rat aortic endothelial cells	92% homology at DNA level to mouse MØ iNOS
Garban et al., 1997	Not specified	4.1 kb construct	Rat, penile iNOS	13 bp differences and 6 AA differences comparing Nunocawa (1993), Geng (1994) and Garban (1997).
Deng, 1998	Not specified	cDNA	Rat liver (Dahl salt-resistant)	92% homology at DNA level to mouse MØ iNOS Variation in gDNA at e25; in kidney only one form (identical) is expressed Gene localized to rat chromosome 10
Keinanen et al., 1999	Not specified	gDNA spanning 36 kb	Rat genomic cosmic library (No 961502, Stratagene)	27 exons 99.7% homology to astrocyte iNOS-DNA: 12 bp and 8 AA differences

changes within the LPS/IFNγ-responsive region leading to hypo-responsiveness of LPS/IFNγ (Zhang et al., 1996; Spitsin et al., 1997). However, putative cytokine response elements are identified within the interval –3.8 kb to –16 kb: AP-1, NFκB, γIRE, NF-IL6, GAS, IRF-E, ISRE, TNF-RE Oct-1 and STAT1 (Vera et al., 1996a; Linn et al., 1997; Chu et al., 1998).

Functional analysis of the promoter region: The initial studies elucidating the transcriptional control of iNOS used murine MØ. Two regions of importance were identified within the first 1.0 kb upstream from transcription start site (Lowenstein et al., 1993; Xie et al., 1993): an NF-KB site at –85 to –76 (Xie et al., 1994a) and an IRF-E/ISRE at –923 to –913 (Martin et al., 1994), the latter serving as an enhancer site, as IFNγ or LPS exposure to this site alone could not induce promoter activity (Lowenstein et al., 1993). In contrast, the proximal 16kb from transcription start site of human NOS2 cloned from hepatocytes was needed to show maximal activity when tested in the human hepatocyte cell line AKN-1 (Vera et al., 1996a).

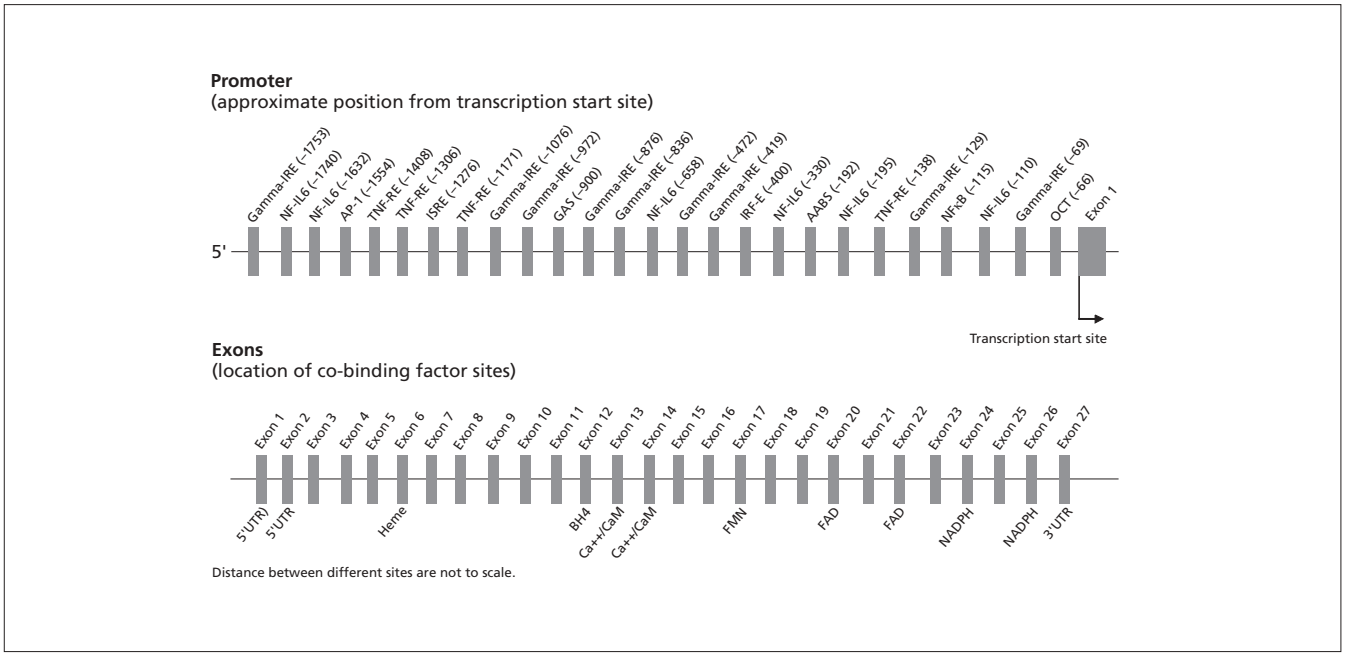

Figure 7. The human iNOS gene maps to chromosome 17q11.2 (Marsden et al., 1994), spanning 37 kb (Chartrain et al., 1994) and comprises 27 exons and 26 introns (Xu et al., 1996). The promoter region functionally divides into a basic region: 0-1.5 kb and an enhancer region: –8.8 to –10.6 kb comprising various potential transcription factor binding sites – activated in response to either IFNγ (IRF-1, STAT1) or IL-1β (AP-1, IRF-1) (Vera et al., 1996a). At Figure 7, only the TBF sites within in the initial 1.7 kb of the human iNOS promoter region are illustrated. For localisation of the remaining TBF sites further upstream, see (Spitsin et al., 1996; Linn et al., 1997). Alternative transcription start sites (OPF) have been identified at pos.: –221, –36, +191 (Chu et al., 1995). cDNA size 4145 bp; homology to human constitutive NOS' approximately 50% (Janssens et al., 1992; Marsden et al., 1992; Nakane et al., 1993) and 80% to murine iNOS (Geller et al., 1993). The hiNOS protein has a MW of 131 kDa, function as a homodimer and contains recognition sites for co-factors: FMN, FAD, NADPH, heme, biopterin and calmodulin.

Based on several different promoter activity assays (luciferase and CAT assays) various central areas have been identified:

- –3.8 kb to –16 kb, especially –7.0 kb to -16 kb: no identification of specific elements (Vera et al., 1996a)
- NFκB site at position –115 to –106 (Nunokawa et al., 1996)
- –10.7 to –8.7 kb, potential transcription factor binding sites identified: 2xOct-1, 3xIRF-1, 4xSTAT1, 3xAP-1 and 2NF-κB (Linn et al., 1997)
- 5xNF-KB sites important in –7.2 to –4.7 kb, especially at –5.8kb verified by site-directed mutagenesis (Taylor et al., 1998)
- an area of inhibition at –7.3 to –6.8 kb (Chu et al., 1998), and between –351 and –632 (Pance et al., 2002)
- importance of the AP-1 sites (-5301 and –5115) and the NF-KB site (–115 and –8283) (Marks-Konczalik et al., 1998), and
- synergistic regulation of promoter activity of NFκB (–115 to –106) and of the A-activator-binding site (AABS: an CCAAT/enhancer binding protein (C/EBP)-binding site)(–192 to –184) (Sakitani et al., 1998). C/EBP is also found to be important in murine iNOS promoter regulation (–153 to –142bp upstream: NF-IL6 binding site) (Dlaska et al., 1999) as well as in human iNOS promoter regulation (–205/+88 bp region) along with the NFκB site (Kolyada et al., 2001).
- Finally, two repeat polymorphisms $(CCTTT)_n$ and $(TAAA)_n$ have been demonstrated to influence the human iNOS promoter activity (Warpeha et al., 1999; Morris et al., 2002). The functional mechanism of these findings is presently unknown.

The latter two repeat polymorphisms influencing the human promoter activity have been tested in diseases, where iNOS have been proposed to influence the pathogenetic process, see Table 8. Additionally, an iNOS promoter sequence variation search might identify nucleotide substitutions involving known or unknown TFB sites of functional relevance.

4.1.5.2. The human iNOS: exon organisation

cDNA has been cloned from hepatocytes (Geller et al., 1993), chrondrocytes (Charles et al., 1993; Maier et al., 1994), DLD-1 cells (Sherman et al., 1993), fibroblast (Chartrain et al., 1994), a human glioblastome cell line (A-172) (Hokari et al., 1994) two human cosmid DNA libraries (Xu et al., 1996), heart and skeletal muscle (Adams et al., 1998). The cDNA sequence obtained from hepatocytes (Geller et al., 1993) reveals 4145 bp, an open reading frame of 3459 bp encoding 1153 amino acids and has an estimated mass of 131kDa. The gene spans approximately 38kb and comprises 27 exons. Unprocessed pseudogenes (Park et al., 1997) have been described. iNOS is distinct from the other human NOS genes nNOS (Kishimoto et al., 1992) and eNOS (Marsden et al., 1993) located at chromosomes 12q24.2 and 7q35-36, respectively.

Taken together: The human iNOS gene possesses a long promoter region comprising several transcription binding factor sites and promoter activity has been identified as long as 16kb upstream of the transcription start site. Repeat sequences within the human iNOS promoter influencing the promoter activity have been demonstrated. Furthermore, the coding region spans approximately 38 kb and comprises 27 exons.

4.1.6. Expressional control of iNOS

Initially, it was believed that the iNOS activity was regulated predominantly at the transcriptional level (Cho et al., 1992), which

Table 8. iNOS promoter polymorphisms in human diseases.

				Association	
Disease	**Author/references**	**Population**	**Polymorphism**	**case/control**	**TDT**
Astma	(Gao et al., 2000)	British	$(TAAA)_n$	No	
Atopy	(Konno et al., 2001)	Japanese	$(CCTTT)_n$	Yes	
Chagas disease	(Calzada et al., 2002)	Peruvian	$(CCTTT)_n$	No	
CAD	(Morris et al., 2001)	Anglo-Celtic/ Northern European	$(TAAA)_n$	No	
Dementia (DLB)	(Xu et al., 2000)	Caucasian	$(CCTTT)_n$	Yes	
T1DM	(Johannesen et al., 2000[b])	Danish	$(TAAA)_n$		No
		Caucasian	$(CCTTT)_n$		No
T1DM retinopathy/ nephropathy	(Johannesen et al., 2000[a])	Danish Caucasian	$(CCTTT)_n$	Yes	
	(Warpeha et al., 1999)	Nothern Ireland	$(CCTTT)_n$	Yes, functional testing[1]	
T2DM, retinopathy	(Kumaramanickavel et al., 2002)	Indian	$(CCTTT)_n$	Yes	
	(Morris et al., 2002)	Caucasian (British)	$(TAAA)_n$	Yes, functional testing[2]	
Arterial hypertension	(Glenn et al., 1999)	Anglo-Australian	$(TAAA)_n$	No	
		Caucasian	$(CCTTT)_n$		
	(Rutherford et al., 2001)	Caucasian (British)	$(TAAA)_n$	Yes	
Malaria	(Kun et al., 1998)	Gabon	–954 G/C	Yes	
	(Kun et al., 2001)	Gabon	–954 G/C	Yes	
	(Levesque et al., 1999)	Tanzanian	–954G/C	No	
			$(CCTTT)_n$	No	
	(Ohashi et al., 2002)	Thai	–954 G/C	Not tested	
			$(CCTTT)_n$	Yes	
	(Hobbs et al., 2002)	Tanzanian	–1173 C/T	Yes, functional implications[3]	
Migraine	(Lea et al., 2001)	Caucasian	$(TAAA)_n$	No	
RA	(Pascual et al., 2002)	Spanish	–954 G/C	No	
			$(TAAA)_n$	No	
			$(CCTTT)_n$	No	No
Parasitic diseases	(Martin et al., 1999)	Peruvian	–954 G/C	No, only wild type occurred	

Regarding the functional testing: 1) Promoter activity was most effective in constructs carrying the 14-repeat allele. 2) The longest repeat conferred the highest iNOS expression in a promoter assay, and finally 3) The genotype C/T was associated with increased fasting urine and plasma NO metabolite concentrations.

partly were based upon the high number of transcription binding sites within the promoter region (Vera et al., 1996a). Subsequently, increasing evidence points towards the importance of both 5' and 3' UTRs being implicated in the regulation of gene expression (Kozak, 1992; Altmann et al., 1993). Vodovotz was the first to show that post-transcriptional mechanisms such as decreasing mRNA stability, reducing mRNA translation and increasing degradation of iNOS protein influenced NO production in TGFβ exposed mouse peritoneal MØ (Vodovotz et al., 1993; Vodovotz, 1997).

In summary:

- Various stimuli increase promoter activity by different TBF (see Figures in promoter sections). The intensifying effect by using cytokine mix compared to single cytokines suggests interaction of signal transduction pathways (Taylor et al., 1998). HSR attenuated the iNOS promoter activity (De-Vera et al., 1996; Vera et al., 1996)
- Structural diversity in the 5'UTR in mRNA isolated from stimulated cells (freshly isolated alveolar MØ, bronchial epithelial cells and several types of cultured cells) has suggested alternative splicing as an additional way of regulating the expression of the gene (Chu et al., 1995)
- Tissue specificity exists: expressed transcription binding factors vary in different cells types (Chu et al., 1995; Kolyada et al., 1996), and the transcription of the human iNOS gene has shown tissue specific regulation using human cell-lines from pulmonary and hepatic biliary epithel (Mellott et al., 2001).
- Alternative splicing at the mRNA level (Eissa et al., 1996; Park et al., 2000), in exon 8 and exon 9 (Park et al., 1996; Eissa, 1998). This could explain the finding of Adams and co-workers (Adams et al., 1998) only showing 79% homology both at the protein and nucleotide level when cloning iNOS cDNA and protein from cardiac and skeletal muscle.
- Effect of 3'UTR region at expression: 1.1 kb of the iNOS promoter and approximately 1.5 kb of the 3'UTR inserted in luciferase constructs showed lower basal activity and hence relatively higher stimulated activity compared to the construct without 3'UTR (Nunokawa et al., 1997), suggesting that the 3'UTR region may alter the mRNA stability (Geng et al., 1995; Belin et al., 2000).

Several promoter activity studies have shown low levels of promoter activity in the absence of cytokine stimulation (Vera et al., 1996a), and *in vivo*, Kobzik et al. have demonstrated iNOS expression without cytokine stimulation in epithelial cells and alveolar MØ lining the larger airways of humans by immunochemistry (Kobzik et al., 1993). It is speculated that low grade basal expression of iNOS mRNA takes place in many tissues, but these transcrips are highly unstable in the absence of cytokines – a putative effect of iNOS 3'UTR. Cytokines may stabilize iNOS mRNA, hence transcription increases (Nunokawa et al., 1996). Finally:

- Post-translatory events: Cytokine stimulation of DLD-1 cells indicates a >20 fold steady-state of iNOS mRNA (Salzman et al., 1996), which is in contrast to iNOS promoter activity (luciferase activity of 13.1 kb) where only 2-4 fold increase was observed (Linn et al., 1997) – could be due to 3'UTR effects
- The activity of the iNOS enzyme requires binding of many cofactors (FAD, FMN, NADPH, tetrahydrobiopterin and calmodulin) (Marletta, 1993; Fossetta et al., 1996)

In conclusion: iNOS seems to be under tight expressional control at various levels which seems adequate as iNOS possess many different beneficial functions in various cellular systems in normal physiology, however leads to detrimental effects when expressed inadequately. Hence, an understanding of the various ways the expression of iNOS is controlled becomes essential, when searching for and evaluating genetic variation within the gene that might influence its expressional control.

4.1.7. Genetic variations in the human iNOS gene

Within the human iNOS *promoter region*, four polymorphisms have been described (i) G/C (position –969, subsequently corrected to position –954) (Kun et al., 1998; Kun et al., 2001), (ii) $(TAAA)_n$ (position –754 to –739) (Bellamy et al., 1997), (iii) $(CCTTT)_n$ (position –2662 to –2608) (Xu et al., 1997) and (iv) C/T (position –1173) (Hobbs et al., 2002). The $(CCTTT)_n$ repeat polymorphism has been functionally tested *in vitro*, associating the 14 repeat allele to high promoter activity (Warpeha et al., 1999).

Different allelic frequency of the $(CCTTT)_n$ repeat polymorphism has been observed between ethnically diverse populations (Africa, Europe, Asia and Caribbean) (Xu et al., 2000) and China (Lu et al., 2002). The G/C (position –966) (Kun et al., 1998) has not been identified in any Caucasians tested so far (Kun et al., 1998) (Johannesen et al., 2000b).

In Table 8 "*iNOS promoter polymorphisms in human diseases*" publications are listed examining the above polymorphisms within various diseases, in which iNOS mediated NO production has been suggested to have a possible pathogenical role.

It appears from the table that in most diseases tested no association has been found, though some inconsistent findings within hypertension (Glenn et al., 1999) (Rutherford et al., 2001) and malaria (Levesque et al., 1999; Kun et al., 2001; Ohashi et al., 2002) are seen. Regarding T1DM, no genetic association/linkage was identified (Johannesen et al., 2000b). However, association to the iNOS promoter has been reported in a subset of T1DM patients suffering from nephropathy/retinopathy (Warpeha et al., 1999; Johannesen et al., 2000a; Kumaramanickavel et al., 2002; Morris et al., 2002).

The *coding region* of the human iNOS gene has been characterised by (Xu et al., 1996) identifying intron/exon splice sites. Three papers have identified polymorphisms within the exons (Johannesen et al., 2001a; Shen et al., 2002; Levecque et al., 2003). The paper of Johannesen et al. tested the identified polymorphisms for linkage to T1DM. In total, 10 polymorphisms were identified from a complete iNOS gene scan of all exons. The four most common polymorphisms (in exon 1, 8, 16 and 20) were tested for linkage using the TDT analysis. Linkage was identified for T1DM among HLA DR3/4 positive individuals having a T at the C/T polymorphisms in exon 16. Furthermore, haplotypes were constructed and tested by ETDT although no increase in genetic information of disease susceptibility could be demonstrated. However, the C/T polymorphism in exon 16 gave rise to an amino acid shift $Ser^{608}Leu$ only six amino acids from a region identified as being of importance to the Ca^{++} independency of iNOS (Daff et al., 1999; Johannesen et al., 2001a). As this polymorphism may have functional implications it would be interesting to test this polymorphism in other autoimmune diseases, in which iNOS mediated NO-production has been proposed in the pathogenesis (Singh et al., 2000).

In the genome scans of T1DM, the region in which human iNOS is located (17q11) has not been demonstrated to be in linkage with T1DM (see Chapter 2) with the exception of Vaessen demonstrating linkage of 17q24 to T1DM in a small genetically isolated Dutch population (Vaessen et al., 2002). Furthermore, a genome scan of Crohns Disease in a Jewish population demonstrated linkage to the chromosomal regions 17q21-23 (Ma et al., 1999). The distances between 17q11 (NOS2, position 50.6 cM at http://research.marshfieldclinic.org) and 17q24 (D17S2059, position 93.3 cM at http://research.marshfieldclinic.org) and 17q21 (D17S787, position 75.0 cM at http://research.marshfieldclinic.org) are 42.7 cM and 24.4 cM, respectively. Hence, these distances do not support the iNOS gene being an obvious candidate gene within these regions in the respective populations.

In conclusion: Polymorphisms within the iNOS gene promoter region have been tested for association to several different diseases. However, only the studies within diabetic retinopathy/nephropathy,

arterial hypertension and malaria have been replicated, and the association has only been reproduced for diabetic retinopathy/nephropathy. No association to T1DM of the iNOS promoter polymorphisms has been shown; however linkage for the exon 16 polymorphism was demonstrated in high risk HLA T1DM individuals. Despite recent findings from a genome scan of possible T1DM linkage to the iNOS gene region in a genetic isolate, testing of association and linkage of the iNOS gene to T1DM should be replicated in other populations to confirm or reject the present findings.

4.1.8. Critical transcription factors for iNOS transcription

Only the genetics of IRF-1 and NFκB in T1DM will be briefly reviewed, as these genes have been examined in relation to T1DM. Furthermore, interaction of IRF-1 and NFκB during activation of iNOS transcription has been illustrated (Saura et al., 1999), as well as a NFκB binding motif in the IRF-1 gene has been demonstrated (Miyamoto et al., 1988)

4.1.8.1. IRF-1

Interferons involved in antiviral defence, cell growth regulation and immune activation, elicit their effects through transcriptional activation of the target genes, e.g. iNOS which possesses specific consensus DNA-binding recognition sites for IRF-1 in their promoters. These interferon-regulated genes are regulated through the JAK-STAT pathway and the interferon regulatory factors (IRFs). Additionally, the IRFs also act as transcription factors for the IFNs. The IRF family is rapidly expanding in number and covers a broad range of activities, for review see (Mamane et al., 1999).

In IRF-1-/- mice, the gene has been shown to be involved in T-cell selection and maturation, as these mice are 90% deficient of mature $CD8^+$ T-cells (Matsuyama et al., 1993). In disease models of autoimmunity in mice lacking IRF-1-/- was shown to be protected against the mortality mediated by TNF and IFN-γ, possibly due to the impaired production of TNF and IFNγ, as IRF-1-/- mice have similar mortality to coinjections of TNF and IFNγ as wild type mice (Senaldi et al., 1999). Furthermore, mice lacking IRF-1 in a model of EAE demonstrate higher Th2-type cytokine responses thereby protected from severe autoimmune brain inflammation (Buch et al., 2003). This observation is in line with the previous finding of IRF-1 deficient mice having an impaired Th1 and enhanced Th2 response (Lohoff et al., 1997). Finally, IRF-1 along with TGF-β and STAT-1 have been implicated in refining the regulation of class II MHC genes through differential control of class II transactivator (CIITA) promoters (Piskurich et al., 1999).

Indeed, IRF-1 may possess an important regulatory role regarding cytokine mediated iNOS expression: IFN-γ induced binding of IRF-1 to the ISRE sequence of the RAW264.7 iNOS promoter – this binding activity was reduced in cells pre-treated with IL-4. Moreover, IL-4 down-regulated the IFN-γ induced IRF-1 mRNA expression (Coccia et al., 2000). Finally, IL-4 has also been shown to suppress IFN-γ stimulated iNOS transcription by elevating the level of IRF-2 which, through competition, prevents IRF-1 from binding to ISRE in the iNOS promoter (Paludan et al., 1999).

The role of IRF-1 and NFκB in IL-1 mediated beta-cell destruction has been discussed in a previous chapter.

The IRF-1 gene has been assigned to chromosome 5q31.1 by fluorescent in situ hybridisation (Willman et al., 1993). The gene is 7.72 kb in length and comprises 10 exons (Cha et al., 1992). Several genetic polymorphisms within the gene have been identified:

- *promoter –300G/T, 4396 A/G, 6355 G/A* (Noguchi et al., 2000), were identified by SSCP in order to test for association to asthma using TDT. The 6355G/A polymorphism was very rare. The –300G/T polymorphism was in nearly complete linkage disequilibrium with the 4396A/G which by TDT did not show significant transmission to atopy- or asthma-affected children. Recent studies from patients with chronic hepatitis C have identified association to the –300A allele (Promrat et al., 2002), and Saito and colleagues demonstrated that in chronic hepatitis C patients being –300A/A the Th1-type $CD4^+$ cell population was significantly increased by IFNβ administration (Saito et al., 2002). Promoter assay studies of the IRF-1 promoter (Saito et al., 2001) suggest that the single nucleotide polymorphisms identified contribute to determining responses to interferons.
- *GT-repeat in intron 7* (Kroef et al., 1993). This polymorphism has been tested by Johannesen et al. without finding any association to T1DM (Johannesen et al., 1997), but has been demonstrated to associate to childhood atopic asthma in a Japanese population (Nakao et al., 2001).
- *A C/T polymorphism in intron 6* of the IRF-1 gene has in a gene-gene (to p21 and p53) and gene-environmental testing been associated to cervical cancer susceptibility in Korean women (Park et al., 2003).
- *HinfI* polymorphism in the 3'UTR, position 1688 with reference to EMBL sequence HSIRF1 (Donn et al., 2001) showed association to juvenile idiopathic arthritis. Seegers et al studied this polymorphism in Celiac Disease by use of TDT without finding any distorted transmission from parents to affected offspring (Seegers et al., 2003).

Finally, genetic variations within the IRF-2 gene have been examined in atopic dermatitis with contradictory results (Nishio et al., 2001) and (Hosomi et al., 2002).

4.1.8.2. NFκB

Only two studies have tested a polymorphism within the NFκB in different T1DM populations. Hegazy et al demonstrated association of alleles to T1DM (Hegazy et al., 2001) which could not be confirmed in a Danish T1DM collection (Gylvin et al., 2002).

Recently, a new gene (SUMO4, a IκBα modifier) has been identified in the IDDM5 region at chromosome 6q25 being associated to T1DM (Guo et al., 2004). This study demonstrates that fine mapping of a chromosomal region linked to T1DM can successfully lead to identification of new genes possibly modifying the genetic risk of T1DM.

In conclusion: Obviously, genes encoding transcription factors being of importance to a candidate gene may themselves be candidate genes. However, only a limited number of studies have been performed testing association of iNOS related genes in T1DM – hence, no genetic predisposition to T1DM for IRF-1 and NFκB can be confirmed or rejected and further studies are needed.

4.2. MORTALIN

Mortalin was initially identified as a 66-kDa protein of pI 5.9 in *mouse* embryonic fibroblasts (MEF) (Wadhwa et al., 1991), later shown to be a member of the mouse HSP70 family (Wadhwa et al., 1993a). Its presence in the cytosol was correlated to the normal mortal phenotype, in contrast to its absence in the cytosolic fraction of immortal cells (Wadhwa et al., 1993a). Microinjection of anti-mortalin antibodies into senescent mouse cells led to transient stimulation of cell division, suggesting an anti-proliferative function of the protein (Wadhwa et al., 1993a), hence the name mortalin. Subsequently, an isoform of mortalin in mouse was identified in immortalized cells as well as in the perinuclear space (Wadhwa et al., 1993b). The isoform associated to the normal mortal phenotype has a uniform pancytosolic distribution (mot-1) and the immortal phenotype located perinuclarly (mot-2) only differs at two amino acids. It was shown later that in the mouse the mot-1 and mot-2 genes segregated in two mouse generations (Kaul et al., 2000a), which illustrates that the mot-1 and mot-2 genes are allelic in mice, and were assigned to mouse chromosome 18 (Kaul et al., 1995; Ohashi et al., 1995). Transfection of mouse mot-1 cDNA (pancytosolic form) induced cellular senescence in NIH 3T3 cells, whereas mot-2 cDNA (perinuclear form) did not impart any equivalent effect (Wadhwa et al., 1993c).

In the *rat*, a homologue protein named Grp75 (glucose regulated protein, 75kDa) was identified (Mizzen et al., 1989; Massa et al., 1995), a resident mitochondrial matrix protein, mediating the import of translocation-competent proteins into the mitochondria and subsequent assembly of proteins within this organelle (Mizzen et al., 1991). In normal rat tissue, expression studies on mortalin have revealed functional *in vivo* characteristics: non-dividing tissues and cells are observed to have higher levels of expression than the ones with division potential, supporting an anti-proliferative function of mortalin in normal tissue. However, in samples of brain tumour tissue the expression was dysregulated and non-pancytosolic distributed, suggesting its involvement in pathways leading to malignant transformation (Kaul et al., 1997). Hence: mouse mot-1 cDNA and pancytosolic distribution of mortalin are associated to mortality in normal cells ("mot-1 effect") – contrasting perinuclear localisation associated to immortality/malignancy ("mot-2 effect").

In 1993, the *human* counterpart to the mouse mortalin gene was cloned from B-lymphoblastomas under the name PBP74, a new member of the HSP70 family, suggested to be involved in antigen processing, however not inducible by heat (Domanico et al., 1993). In 1995 it was cloned under the name mitochondrial-HSP75 (mthsp75) due to its subcellular fraction (Bhattacharyya et al., 1995). Mortalin cDNA isolates from normal and immortalized human cells showed differential localisation patterns by staining (Wadhwa et al., 1995a) but identical sequences, implying that (i) cellular distribution rather than the presence or absence of the protein marks cellular mortal and immortal phenotypes, and (ii) the differential distribution of the protein in human cells is due to e.g. protein modifications and does not originate from distinct cDNA's as in mouse cells. Similar to the mouse mot-2 cDNA, human mortalin induced malignant transformation of NIH 3T3 cells (Kaul et al., 1998a), and stable transfected human lung fibroblast with human mortalin underwent extended population doublings *in vitro* (Kaul et al., 2003). Further, it has been shown that differentiation of HL-60 promyelocytic leukemia cells was accompanied by a decreased level of human mortalin expression (Xu et al., 1999), whereas over-expression of mortalin impaired the growth advantage of the leukemia cells and attenuated their differentiation (Xu et al., 1999). Recently, targeting mortalin using RNA-helicase-linked hybrid ribozymes successfully suppressed the expression of mortalin in transformed human cells, which resulted in growth arrest (Wadhwa et al., 2003). Transient transfection of cells with human mortalin cDNA led to a delay in the development of apoptosis after serum deprivation (Taurin et al., 2002), and finally, over-expression of mot-2 resulted in reduced level of Ras and phosphorylated ERK2, involved in the apoptotic pathway (Wadhwa et al., 2003). All these studies support a "mot-2" effect of human mortalin in various experimental settings.

4.2.1. Mortalin expression

Mortalin has been shown to be expressed in all cell types and tissues studied so far, including pancreas and islets of Langerhans (Wadhwa et al., 1995a; Kaul et al., 1997; John et al., 2000; Mose-Larsen et al., 2001; Johannesen et al., 2004). Expression levels of mortalin have been correlated to muscle activity (Ornatsky et al., 1995), mitochondrial activity (Ibi et al., 1996) and biogenesis (the accepted theory that life can originate only from pre-existing life and never from non-living material) (Takahashi et al., 1998). Various stimuli can induce mortalin expression:

- glucose deprivation (Mizzen et al., 1989)
- calcium ionophores (Resendez-E. et al., 1985)
- ischemia (Massa et al., 1995)
- hyperthyroidism (Craig et al., 1998)
- ozone (Wu et al., 1999)
- IL-1/nitric oxide (John et al., 2000; Mose-Larsen et al., 2001; Johannesen et al., 2004)

Furthermore, mortalin has been demonstrated to interact with and inhibit the function of the tumour suppressor p53 (Wadhwa et al., 1998; Wadhwa et al., 1999; Kaul et al., 2001; Wadhwa et al., 2002d), which partly can explain why mortalin is able to induce immortality. The mortalin – p53 interaction can be abrogated by MKT-077 (a lipophilic cationic dye possessing anti-tumour effect) which binds to mot-2 and lead p53 translocate to the nucleus, followed by growth arrest of the tumour cells (Wadhwa et al., 2000a). Mortalin-p53 complexes have also been detected in mitochondria during p53–induced apoptosis, implicating a role of mortalin in apoptosis (Marchenko et al., 2000). This indicates that mortalin may possess a role in cell fate determination (Rivolta et al., 2002). Besides binding to p53, mortalin has also been shown to bind (i) fibroblast growth factor-1 and aiding in its intracellular trafficking (Mizukoshi et al., 1999; Mizukoshi et al., 2001), (ii) and the IL-1RI and mortalin have been suggested to take part in IL-1RI internalisation (Sacht et al., 1999).

Increasing evidence supports a role of mortalin in mitochondrial function. Previously, it has been shown in yeast that the distribution of mitochondria changes in response to heat shock treatment (Collier et al., 1993) and recently, mortalin has been proposed to be essential for optimizing the functions of as-yet-unidentified heat-labile proteins in the mitochondrial matrix in controlling the mitochondrial morphology (Kawai et al., 2001). These studies suggest that mortalin may play an important role regarding mitochondrial function and that the differentiated distributions of mortalin in immortal versus mortal cells, at least in part, may be related to altered mitochondria morphology and function. That mortalin is involved in mitochondrial function can also explain the associations of mortalin to cellular energy supply, regulation of calcium levels, apoptosis, cellular localisation and cellular immortality (Wadhwa et al., 2002a).

In line with these observations are the findings of Johannesen et al of inter-individual expression of mortalin in isolated islets of Langerhans from two rat strains, the strain being most susceptible to the cytotoxic effect of IL-1 having the highest expressing of mortalin (Johannesen et al., 2004).

In summary: Being a protein involved in cell fate determination possibly by its involvement in mitochondrial functioning mortalin has been demonstrated to be upregulated in cytokine exposed islets of Langerhans. Hence, mortalin is a relevant protein/gene to study further in cytokine mediated beta-cell destruction.

4.2.2. Human mortalin gene

The human mortalin is encoded as a large protein containing a 46 residue pre-sequence which is not present in the mature protein purified from cells (Domanico et al., 1993). This pre-sequence shares features common to other mitochondrial targeting sequences (Bhattacharyya et al., 1995). Mitochondrial targeting proteins serve as facilitators for mitochondrial proteins to target and enter the mitochondria (Hartl et al., 1989). In accordance, the mitochondria are a central localisation of mortalin – but not unique – in human immortalised cell lines (Ran et al., 2000).

As described above, mortalin can be induced by various forms of cellular stress and is associated to the determination of cell fate. Furthermore, rat mortalin expression was:

- identified in IL-1 exposed/NO-treated islets of Langerhans (John et al., 2000; Mose-Larsen et al., 2001), and
- associated to different IL-1 sensitivity in two rat strains (Johannesen et al., 2004). Moreover,
- NIH3T3 cells over-expressing rat mortalin induced decreased cellular survival (Johannesen et al., 2004), and finally
- human mortalin has been localised to chromosome 5q31 (Kaul et al., 1995) – see Table 9
- thus, the human mortalin gene qualify as a T1DM candidate gene.

Table 9: "*Diseases associated to chromosome 5q31*", reflects papers reporting genome scan data that positively identifies 5q31 as a genomic region of interest. It is not the aim of this review to specifically compare to other studies within each of the diseases that either can or cannot confirm the findings listed in the table. The list simply illustrates that this genomic region possibly may enhance susceptibility of several immune mediated diseases.

The study of Johannesen et al (Johannesen et al., 2004) is the first paper to identify polymorphisms in the human mortalin gene. Three nucleotide polymorphisms were identified within the coding region, however none of them led to amino acid substitutions. Neither the tested polymorphisms, the D5S500 dinucleotide marker located close to the gene nor constructed haplotypes were identified to be linked to T1DM in this Danish Caucasoid collection (Johannesen et al., 2004). These identified polymorphisms are obvious SNPs to be tested within other of the diseases listed in the Table 9, *Diseases associated to chromosome 5q31*.

In the study of Johannesen et al (Johannesen et al., 2004) overlapping PCR-products based on cDNA sequence were used to screen the coding sequence for polymorphisms. Our cDNA sequencing data were 100% identical to the published mRNA-based sequences except for the identified SNP's. When initially establishing typing assays for the identified polymorphisms, the use of the cDNA designed primer pairs in genomic DNA revealed only 97 to 99 percent identity between the cDNA and genomic DNA sequences within the same individual, the variation depending upon the primer set used. This inconsistency led us to initiate a NCBI BLAST search for genomic sequences that could possibly explain the deviating sequence results using cDNA versus genomic DNA material (pseudogenes?). At www.ncbi.nlm.nih.gov/LocusLink/ mortalin has been given the Locus ID 3313 and symbol HSPA9B. Two loci links are given: 5q31.1 and 2q36.1 – corresponding to the mRNA sequences L11066 and L15189, respectively. These mRNA sequences show 99% identity. A BLAST search in NCBI of L15189 (chromosome 2) identified a BAC clone, RP11-71J24 (GenBank accession number AC009302) located at the human chromosome 2 (227M, GenBank) where a part of the BAC clone showed 94% homology to the full length published L15189 mortalin cDNA sequence. The nucleotide sequence we obtained in genomic DNA material using the cDNA based primers showed 100% similarity to this specific BAC clone and hence, was not identical to the published cDNA sequences or the sequences we obtained in cDNA material. Subsequently, the gDNA material based typing assays of the polymorphisms were based upon the human sequence of chromosome 5 (AC011385) at the time it was available to the public. Using this sequence to design primers, a 100% sequence homology was obtained between cDNA and gDNA sequence for each tested individual, see Figure 8.

However, should the mortalin gene be located at chromosome 2, then the marker D2S339 is located less than 1.3 cM (230.1-228.8 cM) from the putative localisation of HSPA9B at chromosome 2. We have previously tested the D2S126 marker (a marker of the IDDM13 locus at 2q33) (Larsen et al., 1999) without finding any evidence of linkage or association to T1DM in the Danish population, and since no recombination between D2S339 and D2S126 has been demonstrated the lack of linkage of mortalin to T1DM in the Danish population seemed substantiated.

In 2000, Xie et al published (Xie et al., 2000) the exon/intron organisation of the HSPA9 (human Mortalin) gene as part of their search for variations in the human mortalin gene. This was performed using a BAC-clone (15L17) as template, not available to the public. They used intron-based primer sequences to amplify the 17 identified exons followed by direct sequencing of amplified PCR products. They identified a C to T substitution in the BAC-clone 15L17 corresponding to position 1933 in the human mortalin gene. As Xie et al used a BAC clone as template in contrast to full genomic DNA in our design, they were not in a position to identify putative pseudogenes. In future studies it should be of no difference whether to use the primer pairs designed by Xie et al or by Johannesen et al.

In conclusion: Mortalin (i) is a protein induced by various forms of cellular stress, associated to determination of cell fate, and functionally involved in e.g. mitochondrial function; (ii) has been located to chromosome 5q31, a region of putative interest in immune mediated diseases and, (iii) has been identified and demonstrated to be up-regulated in cytokine exposed rat islets of Langerhans – used as a model for beta-cell destruction in T1DM. Hence, mortalin was considered a candidate gene in the pathogenesis of T1DM. Furthermore, the inter-individual expression of mortalin was associated to different IL-1 sensitivity in two rat strains suggesting inter-individual expressional control of this candidate gene being of importance in cytokine mediated beta-cell destruction. However, the precise pathogenitical involvement of mortalin needs further exploration. In a Danish national wide collected T1DM family collection, the mortalin gene could not be demonstrated to be in linkage to T1DM. In order to finally exclude the mortalin gene as a susceptibility gene in T1DM, additional screenings for polymorphisms in the 5' UTR and the 3'UTRs are requested, as well as the identified polymorphisms in the gene should be tested in other T1DM collections.

Conclusion from Chapter 4

By means of a combined candidate gene approach based upon an experimentally testable pathogenetic model of cytokine mediated beta-cell destruction, three genes were selected: the iNOS, the IRF-1 and the mortalin genes. These genes were examined for sequence

Table 9. Diseases associated to chromosome 5q31.

Disease	Author/references	Population	Putative gene	Chromosome localization	Association	Linkage
ADLD	(Coffeen et al., 2000)	American-Irish family	Not specified	5q31		Yes
Asthma	(Los et al., 1999) (review) (Heinzmann et al., 2000) (Yokouchi et al., 2000)	– British and Japanese Japanese	β-adrenergic R IL-13 (Gln110Arg)	5q31-33 5q31 5q31-33	Yes/(No) Yes Yes	Yes
Celiac disease	(Naluai et al., 2001)	Swedish and Norwegian		5q31-33	Yes	
Crohn's disease	(Ma et al., 1999) (Rioux et al., 2000)	Jewish Toronto area (including Jewish families)		5q33-35 5q31-33	Yes Yes	
Schizophrenia	(Crowe et al., 1999)	Workshop data worldwide		5q23.3-31.1 5q31.3-35.1	 (Yes)*	
Rheumatoid arthrisis	(Cantagrel et al., 1999)	French	IL-4 (RP1 allele)	5q31-33	Yes	
Schistosoma Manisoni	(Marquet et al., 1999)	Brazilian	Not specified	5q31-33	Yes	

ADLD: Adult-onset autosomal dominant leukodystrophy.
*) "(Yes)" indicates that concensus has not been achived in all populations studied.

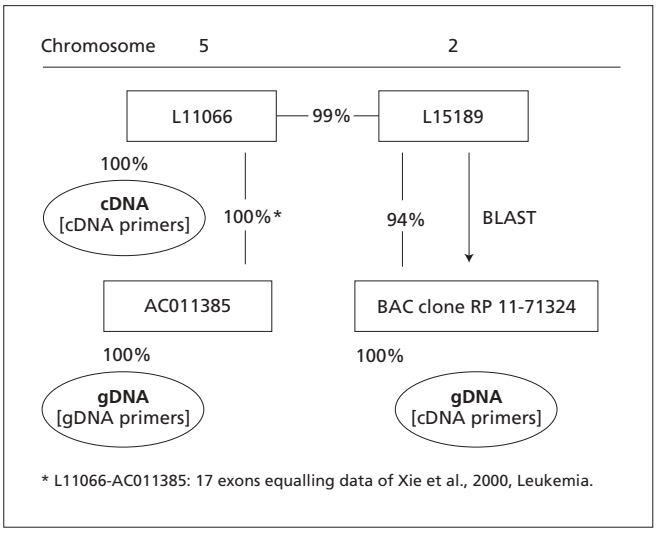

Figure 8. Mortalin at chromosome 5 and/or chromosome 2? Boxed illustrations represent published sequences. The oval illustrations represent sequences obtained in our hands using various combinations of cDNA or gDNA as template and cDNA or gDNA based primers. Similarity from alignment is expressed in percentages. As seen using cDNA based primers in gDNA identified 100% similarity to the BAC clone RP 11-71324 – a putative pseudogene as this genomic sequence contains no introns in contrast to AC011385.

variation and tested for association to diabetes in a population based nationwide Danish T1DM collection. The expression patterns of the selected genes were examined in a rat model using two different rat strains showing different sensitivity to cytokine exposure as defined by different insulin release from cytokine exposed islets *in vitro.*

In beta-cells, NO and NO-independent induced necrotic and apoptotic destruction takes place following cytokine exposure. Whether the necrotic or the apoptotic processes – or both – are effectuated may be influenced by e.g. the potency of the "cytokine hit" versus the defence properties of the beta-cell. iNOS is expressed in cytokine exposed human beta-cells/islets and the evidence of the toxic effect of peroxynitrite and NO donors argues in favour of a role of NO in human beta-cell destruction. Furthermore, inter-individual expression levels of iNOS in the rat model and its association to riNOS promoter polymorphism, and the genetic association of the human iNOS gene to T1DM is a further substantiation of a role of NO in T1DM pathogenesis. However, a polymorphism in the IRF-1 gene (a transcription factor of the iNOS gene) was not demonstrated to be associated to T1DM in a Danish collection.

Mortalin expression – associated to determination of cell fate as demonstrated by decreased cellular survival when over-expressed in NIH-3T3 cells – was differentially expressed in the rat model suggesting an inter-individual expressional control of this candidate gene. However, in a Danish nation-wide T1DM family collection the mortalin gene could not be demonstrated to be in linkage to T1DM.

5. CONCLUDING REMARKS AND FUTURE ASPECTS

5.1. SUMMARY

This thesis has aimed at identifying predisposing T1DM genes with special reference to those genes selected upon a functional basis of the target organ in accordance with the hypothesis of this thesis:

Target organ candidate genes are identified from an experimentally testable pathogenetic model of cytokine mediated beta-cell destruction. Such candidate genes may show inter-individual sequence variation, conferring a genetic risk of or protection against T1DM – alone or in combination. Functional characterisation of such gene variants might show correlation between genetic risk of or protection against T1DM development and beta-cell function.

By means of a combined candidate gene approach based upon an experimentally testable pathogenetic model of cytokine mediated beta-cell destruction, three genes were selected: the iNOS, the IRF-1 and the mortalin genes. These genes were examined for sequence variation and tested for association to diabetes in a population based nationwide Danish T1DM collection. The expression patterns of the selected genes were examined in a rat model using two different rat strains showing different sensitivity to cytokine exposure as defined by different insulin release from cytokine exposed islets *in vitro.*

In beta-cells, NO and NO-independent induced necrotic and apoptotic cell death takes place following cytokine exposure. Whether the necrotic or the apoptotic processes – or both – are effectuated may be influenced by e.g. the potency of the "cytokine hit" versus the defence properties of the beta-cell. iNOS is expressed in cytokine exposed human beta-cells/islets and the evidence of the toxic effect of peroxynitrite and NO donors argue in favour of a role of NO in human beta-cell destruction. Furthermore, inter-individual expression levels of iNOS in the rat model and its association to riNOS promoter polymorphism, and the genetic association of the human iNOS gene to T1DM development among HLA DR3/4 positive individuals further substantiate a role of NO in T1DM pathogenesis.

The expression patterns of the selected genes were examined in a rat model using two different rat strains showing different sensitivity to cytokine exposure, and strain dependent differences were demonstrated for iNOS expression in the pancreatic islets correlating with IRF-1 and HSP70 expression. Hence, high cytokine sensitivity of the islets, as defined by inhibited insulin release in response to cytokine exposure, correlated to high iNOS, IRF-1 and HSP70 expressions in both dose – and time responses, hypothesize a role of IRF-1 in cytokine mediated iNOS expression. However, no association to the tested IRF-1 polymorphism was demonstrated in the present Danish T1DM collection.

Mortalin (i) is a protein induced by various forms of cellular stress, functionally involved in e.g. mitochondrial function, and associated to determination of cell fate, and; (ii) has been located to chromosome 5q31, a region of putative interest in immune mediated diseases and, (iii) has been identified and demonstrated to be up-regulated in cytokine exposed rat islets of Langerhans. In the rat model, high mortalin expression correlated with increased cytokine sensitivity. Furthermore, over-expression of mortalin in the NIH3T3 cell-line was performed demonstrating decreased cellular viability suggesting a senescence effect of rat mortalin, indicating a pathogenetical role of mortalin in T1DM, despite no association to the mortalin gene was demonstrated in the present Danish T1DM collection.

5.2. FUTURE ASPECTS

Genetics of T1DM

Of the three selected candidate genes, only the iNOS gene demonstrated association to T1DM the tested Danish T1DM collection. Replication studies in independent and large population based collections are needed to substantiate these findings. However, an extension of the examined regions of the gene could be of relevance, if the screening for genetic variation within the genes has been incomplete, e.g. only a previous identified gene polymorphism in the IRF-1 gene has been tested in the present study. Naturally, not all selected candidate genes will influence the genetic risk of T1DM, however the encoded protein may still be of pathogenical relevance in T1DM.

Linkage-designed studies are efficient in the case of rare variants with major effect whereas association designed studies are more efficient in the case of common variants with modest effect (Hirschhorn, 2003). Hence, an approach to select robust candidate genes seems essential – here demonstrated as a multiple string based candidate gene selection including a functional selection bias. Furthermore, the presented strategy for the evaluation of the selected candi-

date gene and protein has been demonstrated to be plausible. However, in order to further characterise a pathogenetic role of the selected candidate genes in cytokine mediated beta-cell destruction, a functional characterisation of the human gene variants should be examined – e.g. the iNOS E16 variants in expressional studies. In addition, the effect of rat mortalin expression should be replicated in insulin-producing cells with and without cytokine stimulation.

Besides testing susceptibility genes in T1DM, the non-model based, intra-familiar association designed studies provide the possibility of identifying T1DM protective genes when including data from non-affected offspring. The approach of identifying protective genetic association to T1DM is in accordance with the idea of a cytokine induced race between deleterious and protective mechanisms in the beta-cell and is strongly recommended for future studies.

Despite the limited power of linkage-designed studies to detect common variants with modest effect and the sparse outcome from these studies so far, the search for genomic areas being linked to T1DM should be continued. A genom scan using SNP's or haplotype Tag SNP's in order to reduce the number of SNP's tested in the entire genome could be an innovative approach. A putative benefit from such studies is the possibility of excluding low-risk susceptibility genomic regions.

Furthermore, the undertaken analytical methods for examining the genetic data should be optimised and new analytical methods including gene to gene and gene to environmental interaction should be introduced.

Finally, as described below, an increasing focus on the T1DM phenotype should be encouraged, as non-stratified phenotypic mixing naturally will blur the genetic picture.

Pathogenesis of T1DM

The rat model has demonstrated that destructive as well as protective mechanisms are activated in cytokine exposed islets. This race between protective and destructive processes needs to be further explored in order to develop intervention strategies that may lead to the favour of the protective responses.

An important aspect of studying these destructive and protective mechanisms is the understanding of inter-individual differences in cytokine response and responsiveness. Such differences may even lead to different phenotypic characteristics, e.g. a strong destructive capacity combined to a weak protective response may be seen in patients characterised by an absent remission phase. Furthermore, it seems that different cytokine mediated pathways can lead to beta-cell death. The determination of which pathway is taken could reside within the target organ itself as beta-cell heterogeneity has been described for e.g. glucose sensitivity as well as cytokine sensitivity. These parameters may be influenced by age of the beta-cell as well as the genetic make-up. Hence, the development of individual prevention and/or curative strategies may be future aspects.

Such prevention strategies may include anti-cytokine therapy (Prud'homme et al., 2001; Sharma et al., 2003), anti CD3mAb as immunogenic modelling (Herold et al., 2003), over-expression gene therapy altering e.g. the Th1/Th2 response, blockage of encoded mRNA's or disease prevention with islet autoantigens, (see (Eisenbarth et al., 2004) for review of the latter). This concept has been proven successful in animal studies, however not in DPT1; possibly a matter of dose of the antigen.

However, in order to monitor such interventions it may be needed to detect pathogenic T-lymphocytes in humans in order to evaluate the influence of immunologic therapies on T-lymphocytes causing beta-cell destruction as well as imaging of beta-cell mass *in vivo*.

Finally, curative initiatives may include improved islet transplantation protocols and pancreas transplantations, however both, are limitied because of the lack of available tissue. Genetic engineered insulin secreting hepatocytes being more resistant than pancreatic beta-cells to adverse effects of cytokines (Tabiin et al., 2001) or insulinoma cell lines with resistance to IL-1β and IFNγ induced toxicity (Giannoukakis et al., 2002) could be attractive alternatives in the future.

ABBREVIATIONS

A20:	TNFα induced protein 3 (inhibits NFκB activity)
AA:	Amino acid
AABS:	A-activator-binding site
ADLD:	Adult-onset autosomal dominant leukodystrophy (mimicking chronic progressive MS)
AIR-1:	Activator immune response gene 1 (encoding MHC class II transactivator factor)
AIRE:	Autoimmune Regulator
AG:	Aminoguanine
AGER:	Advanced glucosylation end product receptor
AKN-1:	Human hepatocyte cell line
AML:	Acute myeloid leukaemia
ANA-1:	Murine MØ
AP:	Activating transcription factor(s)
ARE:	Antioxidant-responsive element
AS:	Arginino succinate synthase
ASP:	Affected sib pair
ATP:	Adenosine triphosphate
BAC:	Bacterial artificial chromosome
BAT2:	HLA-B-asociated-transcript
BB rat:	BioBreeding rat
Bcl2:	Member of a family of oncogenes involved in tumor suppression
BF:	Properdin factor B
BH4:	Tetrahydrobiopterin
BN:	Brown Norway
BZT:	Benzenetriol (autocatalytic source of superoxide)
C4:	Compliment C4
CIITA:	Class II transactivator
CAD:	Coronary artery disease
CaM:	Calmodulin
CAT:	Chloramphenicol acetyltransferase (promoter activity assay)
CCR:	CC-chemokine receptor
CD:	Cluster of differentiation
C/EBP:	CCAAT/enhencer binding protein
CFA:	Complete Freuds adjuvance
CHOP:	C/EBP homologous protein
Chr:	Chromosome
cM:	Centimorgan
CM:	Cytokine mixture
cNOS:	Constitutive nitric oxide synthase
CRE:	Cytokine response element
CTLA4:	Cytotoxic T lymphocyte-associated antigen 4
DAG:	Diacylglycerol
DD:	Death domaine
DEX/DX:	Dexamethason
DIEGG:	Danish Insulin-Dependent Diabetes Mellitus Epidemiology and Genetics Group
DLB:	Dementia with Lewis Bodies
DLD-1 cells:	A human colorectal adenocarcinoma cell line
DMB:	HLA gene encoding class II-like α- and β-chains
DMNQ:	2,3 dimethoxy-1,4-naphthoquinone
DNA:	Deoxyribonucleic acid
DSBD:	Danish Society for Childhood and Adolescent Diabetes
dsRNA:	Double stranded RNA
E16:	Exon 16
EBP:	Enhancer binding protein
Egr1:	Estrogen receptor 1
EMSA:	Electrophoretic mobility shift assay
eNOS	Endothelial nitric oxide synthase
ER:	Endoplasmatic reticulum
ERK:	Extracellular regulated signal kinase
ESR1:	Estrogen receptor 1
EST:	Expressed sequence tag
ETDT:	Extended transmission disequelibrium test

FACS: Flourescence-activated cell sorting
FAD: Flavin adenine nucleotide
FADD: Fas-associated death domain protein
Fas: Human Fas gene (tumor necrosis factor receptor superfamily, member 6)
FasL: Fas ligand
FMN: Flavin mononucleotide
FSK: Forskolin
GAD: Glutamic acid decarboxylase
GADD: Growth arrest and DNA-damage inducible
GALN: Galanin
GALNT3: N-acetyl-galactosaminyltransferase-T3
Gas: Growth arrest specific
GAS: Gamma activated site
GB: GenBank
GC: Vit D binding protein
GCGR: Glycagon receptor
GCK: Glucokinase
Grp75: Glucose regulated protein, 75kDa
GSH: Glutathione peroxidase
Herb: Herbimycin
HERV-K(C4): A variable endogenous human endogenous retroviral element
HIT-cells: Hamster insulin producing tumor cell
HLA: Human leukocyte antigen
HOX: Homeobox gene(s)
HSP: Heat shock protein
HSR: Heat shock response
HRE: Hypoxia response element
HVA: High voltage activate
IA-2: Protein tyrosine phosphatase-2
ICAM: Intercellular adhesion molecule
ICE: Interleukin-1 converting enzyme
ICOS: Inducible co-stimulator
IDDMK$_{1,2}$22: The product of HERV-K18, possible a superantigen
IFNγ: Interferon gamma
IGFBP: Insulin like-growth factor binding protein
IGH: Immun-globulin heavy chain
IkB: Inhibitor kB
IL: Interleukin
IL-1AcP: Interleukin-1 accessory protein
IL-1Ra: IL-1 receptor antagonist
IL-1RI: Interleukin-1 type 1 receptor
IL-1RN: IL-1 receptor anatagonist
iNOS: Inducible nitrogen oxide synthase
INS: Insulin gene
INS-1: Insulin producing cell line
IRE: Interferon response element
IRF: Interferon regulating factor
IRS-1: Insulin receptor substrate-1
ISRE: Interferon-stimulated response element
JAK: Janus tyrosine kinase
Kidd: Kidd blood group system
LCK: A lymphoid T-cell protein tyrosine kinase
LD: Linkage Disequelibrium
LMP: Large multifunctional protease
LOD: Logarithm of odds
LPS: Lipopolysaccharide
LST1: Leucocyte specific transcript-1
LUC: Luciferase
MAPK: Mitogen activated protein kinase
MHC: Major histcompatibility complex
MICA: MHC class I chain-related gene A
MLD-STZ: Multiple low dose streptozotocin
MLS: Maximum lod score
MnSOD: Mangan SOD
mot-1 and 2: Mouse mortalin gene 1 and 2
mRNA: Messenger ribonucleotide acid
myb: Oncogene, found to be rearranged in human colon and bone marrow tumors
myc: Oncogene, involved in the chromosome translocation found in Burkitt's lymphoma
MZ: Monozygotic
MX 1: Myxovirus resistance
MØ: Macrophage
NAD: Nicotinamide adenine nucleotide
NADPH: Nicotinamide adenine nucleotide phosphate hydrogen
NAT2: N-acetyltransferase
NCBI: National Center for Biotechnology Information
NeuroD/ BETA2: β-cell E-box transactivator 2 (a transcription factor af the insulin gene)
NF B: Nuclear factor kappa beta
NHE1: Sodium/hydrogen exchanger
NHI-glu: Cell line derived from the glucagon-producing MSL-G2 culture
NHI-ins: Insulin-producing phenotype of the NHI-Glu after maturation in syngeneic NEDH rats
NIH-3T3: Mouse fibroblast cell line
NIK: NFκB inducing kinase
NO: Nitric oxide
NOD mouse: Non obese diabetic mouse
NOS: NO synthase
NQO1: NAD(P)H quinone oxidoreductase
NRAMP1: Natural resistance associated macrophage protein 1
OAS: 2',5' oligoadenylate synthetase
OCT-1: Octamer binding transcription factor-1
ORF: Open reading frame
p53: Tumor suppressor
PAI1: Plasminogen Activitor Inhibitor-1
PACAP: Pituitary adenylate cyclase-activating polypeptide
PARPγ: Poly(ADP-ribose) polymerase
PAX: Transcription activation domain-interacting protein 1
PDTC: Pyrrolidine dithiocarbamate
PAO: Phenylarsine oxide
PIC: Polyinosinic-polycytidylic acid (synthetic dsRNA)
PKC: Protein kinase C
PP: Protein Phosphatase
PPARγ: Peroxisome proliferator activated receptor gamma
PTPRN: The gene encoding for IA2, a transmembrane protein tyrosine phosphatase
RA: Rheumatoid Arthritis
RAW264.7: Murine macrophage-like cell line
RASMC: Rat aortic smooth muscle cell
RIP: Rat insulin promoter
RINm5F: Rat insulinoma cell line
RMC: Rat mesangial cells
ROS: Reactive oxygen species
SEL1L: The human homolog of *C-elegans sel-1*SNP:Single nucleotide polymorphism
SOCS-3: Suppressor of cytokine signalling 3
SOD: Super oxide dismutase
SOX13: The ICA12 autoantigen gene
SSCP: Single stranded conformation polymorphism
STAT: Signal transducer and activator kinase
T1DM: Type 1 Diabetes Mellitus
TAP: Transporter associated with antigen processing
TCF7: Transcription factor 7
TCR: T-cell receptor
TDT: Transmission disequelibrium test
TFBS: Transcription factor binding factor sites
Th: T-lymphocyte, helper
TNF: Tumor necrosis factor
TNFR: TNF receptor
TRAF: TNF receptor assocated factor
UTR: Untranslated region
VDR: Vit D receptor
VNTR: Variable number of tandem repeats
VSMC: Vascular smooth muscle cell
WAGR: Wilm's Tumor, Aniridia, Genitourinary syndrome, mental Retardation
WEHI: Mouse fibrosarcoma cell line
WFS1: The Wolframin gene

WHO:	World Health Organisation
WT:	Wilms Tumor
ZF:	Zink finger
y+CAT:	Cationic amino acid transporter system

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