

MAARIT HEINO

Identification of the Autoimmune Regulator Gene,  
its Mutation Pattern in APECED Patients  
and Localisation of the Gene Expression



ACADEMIC DISSERTATION

to be presented, with the permission of  
the Faculty of Medicine of the University of Tampere,  
for public discussion in the auditorium of Finn-Medi,  
Lenkkeilijänkatu 6, Tampere, on June 1st, 2001, at 12 o'clock.

*Acta Universitatis Tamperensis 821*  
*University of Tampere*  
*Tampere 2001*

**ACADEMIC DISSERTATION**  
University of Tampere,  
Institute of Medical Technology  
Finland

**Supervised by**  
Professor Kai Krohn  
University of Tampere  
Docent Pärt Peterson  
University of Tampere

**Reviewed by**  
Docent Olli Lassila  
University of Turku  
Docent Aaro Miettinen  
University of Helsinki

Distribution



University of Tampere  
Sales Office  
P.O. Box 617  
33101 Tampere  
Finland

Tel. +358 3 215 6055  
Fax +358 3 215 7685  
taju@uta.fi  
<http://granum.uta.fi>

Cover design by  
Juha Siro

Printed dissertation  
Acta Universitatis Tamperensis 821  
ISBN 951-44-5109-0  
ISSN 1455-1616

Electronic dissertation  
Acta Electronica Universitatis Tamperensis 112  
ISBN 951-44-5110-4  
ISSN 1456-954X  
<http://acta.uta.fi>

Tampereen yliopistopaino Oy Juvenes Print  
Tampere 2001

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## LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by Roman numerals I-V.

- I Nagamine K, Peterson P, Scott HS, Kudoh J, Minoshima S, Heino M, Krohn KJE, Lalioti MD, Mullis PE, Antonarakis SE, Kawasaki K, Asakawa S, Ito F, and Shimizu N (1997): Positional Cloning of the APECED gene. *Nature Genetics*, 17:393-398.
- II Scott HS, Heino M, Peterson P, Mittaz L, Lalioti M, Betterle C, Cohen A, Seri M, Lerone M, Romeo G, Collin P, Salo PK, Metcalfe R, Weetman A, Papasavvas M-P, Rossier C, Nagamine K, Kudoh J, Shimizu N, Krohn K, Antonarakis SE (1998): Common mutations in APECED patients of different origins. *Molecular Endocrinology*, 12:1112-1119.
- III Heino M, Scott HS, Chen Q, Peterson P, Mäenpää U, Papasavvas MP, Mittaz L, Barras C, Rossier C, Chrousos GP, Stratakis CA, Nagamine K, Kudoh J, Shimizu N, Maclaren N, Antonarakis SE, Krohn KJE (1999): Mutation Analyses of North American APS-1 patients. *Human Mutation*, 13:69-74.
- IV Heino M, Peterson P, Kudoh J, Nagamine K, Lagerstedt A, Ovod V, Ranki A, Rantala I, Nieminen M, Tuukkanen J, Scott HS, Antonarakis SE, Shimizu N and Krohn KJE (1999): Autoimmune regulator (AIRE) is expressed in the cells inducing tolerance in thymus medulla. *Biochemical and Biophysical Research Communications*, 257:821-825.
- V Heino M, Peterson P, Sillanpää N, Guérin S, Naquet P, Anderson G, Scott HS, Antonarakis SE, Jenkinson EJ and Krohn KJE (2000): RNA and protein expression of the murine Autoimmune Regulator gene (Aire) in normal, RelB deficient and in NOD mouse. *European Journal of Immunology* 30:1884-1893.

## ABBREVIATIONS

AIRE	Autoimmune Regulator gene
APC	antigen presenting cell
APECED	autoimmune polyendocrinopathy—candidiasis—ectodermal dystrophy
bp	base pair
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CTLA	cytotoxic lymphocyte antigen
CTL	cytotoxic lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EST	expressed sequence tag
GAD	glutamic acid decarboxylase
GM-CSF	granulocyte-macrophage-colony stimulating factor
HLA	human leukocyte antigen
ICAM	intracellular adhesion molecule
ICOS	inducible co-activator
IFL	immuno fluorescence
kb	kilobase
kD	kilodalton
KO	knock-out
LDC	lymphoid dendritic cell
LFA	lymphocyte functional antigen
mAb	monoclonal antibody
MDC	myeloid dendritic cell
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
mTE	medullary thymic epithelial cells
NOD	non-obese diabetic
ORF	open reading frame
PHD	plant homeo domain
PLP	proteolipid protein
PML	promyelocytic leukemia
RACE	rapid amplification of cDNA ends
RAG	recombination activation gene
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SCID	severe combined immunodeficiency
TCR	T cell receptor
Th1/ Th2	T helper cell subset 1 or 2
UTR	untranslated region

## INTRODUCTION

Efficient immunological defense against foreign structures such as bacterial or viral proteins is based on specific recognition of the antigen and, through recognition, activation of the immune system to defend the body against foreign intruders. The price to be paid for the efficient defense is production of lymphocytes that are capable of recognising the body's own structures and thus are potentially harmful if activated. The breakdown of tolerance mechanisms preventing activation of self reacting lymphocytes can lead to development of autoimmune destruction of self structures. The molecular pathogenesis of autoimmune diseases is poorly understood. The genetic impact in many of the autoimmune diseases has been established and in many cases several different gene loci are involved indicating multifactorial genetic background. Furthermore, environmental factors have been suggested to play an important role in determining the phenotype of several autoimmune diseases.

APECED is one of the autoimmune polyglandular syndromes defined by the presence of two of the classical triad of disorders: hypoparathyroidism, adrenocortical failure and chronic mucocutaneous candidiasis, which is often associated with other endocrine autoimmune diseases. APECED has a monogenic background following recessive form of inheritance.

The monogenic etiology of APECED makes it a unique model for endocrine autoimmunity. The phenotype of APECED patients suggests that the defective gene may have an important role in tolerance mechanisms and therefore identification of the defective gene will give implications for understanding the molecular mechanisms of tolerance induction and the molecular pathogenesis of autoimmunity.

The purpose of the present study was to identify the gene responsible for APECED and to gain insight into the pathogenesis of APECED through characterisation of the mutation pattern of this gene and on the other hand through elucidating the expression pattern of the gene.



# REVIEW OF THE LITERATURE

## 1. Autoimmune Polyglandular Syndromes

Autoimmune endocrinopathies are characterised by presence of autoantibodies directed against endocrine tissues preceding lymphocytic infiltrates and destruction of the affected glands (Wagner et al. 1994). Autoimmune endocrine diseases often associate with each other, which has led to the identification of three types of autoimmune polyglandular syndromes (Neufeld et al. 1980, Riley 1992).

Autoimmune polyglandular syndrome type 1 (APS1) or APECED for autoimmune polyendocrinopathy candidiasis ectodermal dystrophy is defined by the presence of two (or one if sibling has all three) out of the classical three disease entities: adrenocortical failure (Addison's disease), hypoparathyroidism and chronic mucocutaneous candidiasis (Neufeld et al. 1980, Ahonen 1985). The clinical and immunological findings in APECED patients and the genetic background are discussed in more detail in the next chapter.

APS 2 or Schmidt's syndrome is defined by Addison's disease and thyroid autoimmune diseases and/or insulin dependent diabetes mellitus (Schmidt 1926). APS2 shows a strong association with HLA-DR3 and DR4 and secondarily with HLA-B8 (Eisenbarth et al. 1979, Maclaren and Riley 1986). Association with HLA class II markers DRB1\*0301, DQA1\*0501, DQB1\*0201 and DPB1\*0101 has been shown in APS2 (Partanen et al. 1994). The syndrome shows familial aggregation (Butler et al. 1984) and is likely to have a polygenic background (Eisenbarth et al. 1979).

APS3 is defined by autoimmune thyroid disease, and the presence of at least one other autoimmune disorder in the absence of Addison's disease. The heterogeneity of this patient group has challenged the genetic analyses for APS3 (Obermayer-Straub and Manns 1998).

### 1.1 APECED

#### 1.1.1 Epidemiology

APECED cases are accumulated in certain populations such as Finns, Iranian Jews and Sardinians. However, isolated patients have been described in other populations as well (Björnses et al. 1996). In Finland the incidence is 1:25000 and among Iranian Jews 1:9000 and in Sardinians 1:14500 (Ahonen et al. 1990, Zlotogora and Shapiro 1992, Rosatelli et al. 1998).

#### 1.1.2 Genetics

The monogenic etiology of APECED has been confirmed by several studies (Hung et al. 1963, Spinner et al. 1968, Ahonen 1985). The causative gene for APECED was localised in Finnish families to the chromosome 21q22.3 (Aaltonen et al. 1994). By linkage analysis in 14 Finnish families the APECED

locus was mapped to an area ~2.6 cM flanked by markers D21S49 and D21S171 (Aaltonen et al. 1994). Subsequently, by linkage disequilibrium studies the critical region for APECED gene was considered to be approximately 500kb between markers D21D1912 and D21S171 (Björnses et al. 1996). The analysis of 9 Iranian Jewish and 21 other non-Finnish APECED patients showed that the disease in different populations is caused by defects in the same gene (Björnses et al. 1996). The haplotype analysis of the Finnish families suggested that 85-90% of the Finnish APECED cases are due to one major mutation and that some minor mutations of different origins are responsible for the remaining APECED cases in Finland (Björnses et al. 1996). In the Iranian Jewish population one common allele was responsible for 92% of disease alleles whereas in the sporadic APECED families the disease chromosomes differed from the Finnish and Iranian Jews ones indicating that the disease is caused by different mutations in one gene (Björnses et al. 1996).

### *1.1.3 Clinical picture*

In addition to chronic mucocutaneous candidiasis, hypoparathyroidism and Addison's disease APECED patients show variable combinations of other disorders such as hypogonadism, type 1 diabetes, chronic atrophic gastritis and thyroid autoimmune disease (Neufeld et al. 1980, Ahonen 1985). Other manifestations in APECED are chronic active hepatitis, alopecia, vitiligo and keratinopathies such as nail and teeth enamel dystrophy. Variation in the phenotype is seen between different populations and also to a considerable extent between patients of the same ethnic origin (Ahonen 1985). In a Finnish follow-up of 68 patients from 54 families the components varied from one to eight, 63% of patients having three to five of them. All of the 68 patients had candidiasis at some time, usually in early childhood and the endocrine autoimmune diseases later in life, age of appearance of the first endocrine component varying from 19 months to 35 years (Ahonen et al. 1990). Of all patients 79% had hypoparathyroidism, 72% adrenocortical failure, 60% (female) and 14% (male) gonadal failure whereas the other components were present in 4 to 29% of the patients. Among Iranian Jewish patients candidiasis is rare (17%) and 96% have hypoparathyroidism, 22% adrenocortical failure and 26% hypogonadism (Zlotogora and Shapiro 1992). Type 1 diabetes is present in 18% of Finnish patients but only in 2% and 4% of Northern Italian and Iranian Jewish patients, respectively (Ahonen et al. 1990, Zlotogora and Shapiro 1992, Betterle et al. 1998).

### *1.1.4 Immunological findings in APECED patients*

In contrast to the isolated Addison's disease APECED patients have autoantibodies not only against adrenal cortex but also against steroid producing cells in gonads and placenta (Irvine et al. 1969, Sotsiou et al. 1980, Elder et al. 1981). The presence of these antibodies has prognostic value as they predict the appearance of adrenocortical and ovarian failure (Ahonen et al. 1987). The main autoantigens in steroid producing tissues are the steroidogenic enzymes steroid 21-hydroxylase (P450c21), corresponding to the presence of adrenal cortex

antibodies and steroid 17 $\alpha$ -hydroxylase (P450c17) and side-chain cleavage enzyme (P450scc), corresponding to the presence of steroid producing cell antibodies (Krohn et al. 1992, Winqvist et al. 1993, Uibo et al. 1994). In a group of 39 APECED patients with Addison's disease 29 (74%) were shown to react with at least one of these antigens whereas in a group of patients without Addison's disease only 3 out of 14 cases (21%) had antibodies against these enzymes (Uibo et al. 1994).

The insulin producing islets of Langerhans in the pancreas are another target for autoimmunity in APECED patients. Many diabetic APECED patients show a strong reaction to the major type 1 diabetes autoantigen 65-kD isoform of glutamic acid decarboxylase (GAD65) and some also a reaction to GAD67 and islet cells (Björk et al. 1994, Tuomi et al. 1996). However 51% of Finnish APECED patients without clinical diabetes had autoantibodies reactive to GAD65, GAD67 or islet cells (Tuomi et al. 1996). In follow-up only few patients developed diabetes indicating the low risk assessment value for diabetes progression in APECED patients (Tuomi et al. 1996). Antibodies against the IA-2 tyrosine phosphatase like protein or insulin was detected in 4/11 (36%) APECED patients, whereas none of the nondiabetics had insulin antibodies and 4% had IA-2 antibodies. Thus giving better positive predictive value for type 1 diabetes in APECED (Gylling et al. 2000). Reactivity to aromatic L-amino acid decarboxylase, expressed in several tissues including pancreatic beta-cells, has also been shown (Husebye et al. 1997).

Tryptophan hydroxylase has been identified as an intestinal autoantigen in APECED patients and correlates with gastrointestinal dysfunction (Ekwall et al. 1998). Immunoreactivity against tyrosine hydroxylase has been found and the reactivity was found to correlate with the presence of alopecia areata (Hedstrand et al. 2000). Other autoantibodies identified in APECED patients are listed in Table 1.

The chronic candidiasis infection suggests either a specific T cell deficiency or impairment of T cell function in APECED patients (Kirkpatrick et al. 1970, Arulanantham et al. 1979). However, the patients have high levels of antibodies against major candidal antigens (Peterson et al. 1996) indicating intact antibody formation and B cell function.

**Table1.** Autoantibodies identified in sera of APECED patients

<b>Autoantigen</b>	<b>Tissue</b>	<b>Reference</b>
P450c21	Adrenal cortex	(Uibo et al. 1994)
P450c17	Adrenal cortex, testis, ovary	(Krohn et al. 1992)
P450scc	Adrenal cortex, testis, ovary	(Uibo et al. 1994)
Extracellular domain of calcium sensing receptor	Parathyroids	(Li et al. 1996)
Thyroid peroxidase	Thyroid gland	(Perheentupa and Miettinen 1998)
Thyroglobulin	Thyroid gland	(Perheentupa and Miettinen 1998)
GAD65/ GAD67	Pancreas	(Tuomi et al. 1996)
ICA	Pancreas	(Ahonen 1985)
IA-2 tyrosine phosphatase-like protein	Pancreas	(Gylling et al. 2000)
insulin	Pancreas	(Gylling et al. 2000)
3beta-hydroxysteroid dehydrogenase	Pancreas, ovary	(Reimand et al. 2000)
Aromatic L-amino acid decarboxylase	Pancreas, liver	(Husebye et al. 1997)
P450 1A2	Liver	(Clemente et al. 1997)
2A6	Liver	(Clemente et al. 1998)
1A1	Liver	(Clemente et al. 1998)
2B6	Liver	(Clemente et al. 1998)
Tryptophan hydroxylase	Serotonin producing cells in central nervous system and small intestine	(Ekwall et al. 1998)
Tyrosine hydroxylase		(Hedstrand et al. 2000)
Intrinsic factor	Gastric mucosa	(Mirakian and Bottazzo 1994)
?	Gastric parietal cells	(Mirakian and Bottazzo 1994)
?	Melanocytes	(Betterle et al. 1992)

## **2. Positional candidate gene cloning approach in disease gene identification**

### **2.1 Approaches to disease gene identification**

Strategies developed for disease gene identification can be accomplished using two strategies; functional cloning and positional cloning (Collins 1992). The functional cloning approach can be employed in disease gene identification if the biochemical defect in the disease is known. Knowledge of the function of the protein enables identification of the disease gene with no need for knowledge of the chromosomal location of the disease locus. Positional cloning in contrast is based solely on knowledge of the chromosomal location of the disease locus. The candidate gene approach requires some knowledge of the non-functional biochemical pathway facilitating selection of interesting genes to be tested by linkage or association analysis or by mutation analysis. In many cases the two latter methods are employed concurrently. After construction of the gene map of the critical chromosome region for the disease gene “educated guesses” direct the testing of those genes with the most interesting function or predicted function considering the pathogenesis of the disease.

#### *2.1.1 Cloning systems for genomic DNA*

Cloning of the chromosomal region of interest in overlapping contigs, employing the ability of bacterial plasmids and phages to replicate after incorporation of foreign DNA in their genomes is essential for isolating the disease genes in the region and sequencing of the chromosomal DNA. The construction of yeast artificial chromosome (YAC) based maps in the end of 1980's was of special importance for genome mapping (Burke et al. 1987). YAC vectors allowing inserts up to two million basepairs enabled the construction of the first chromosome specific libraries (Dausset et al. 1992) and the first generation physical map of the human genome (Chumakov et al. 1995). The construction of new bacterial vectors has provided systems such as P1 bacteriophage system (Sternberg 1990), bacterial artificial chromosome (Shizuya et al. 1992) and P1 artificial chromosome (Ioannou et al. 1994) with more stable replicons and facilitated pooling methods giving advantages over YAC in transcriptional mapping.

#### *2.1.2 Identifying genes in cloned DNA*

Coding DNA sequences can be distinguished by the capability of mRNA production (expression-dependent). Direct cDNA selection (Lovett et al. 1991, Parimoo et al. 1991), hybridization to a northern blot containing mRNAs isolated from various tissues (Alwine et al. 1977) or to cDNA library representing a certain tissue may allow identification of those clones containing coding sequence (see Table 3 for a description of the methodology). These methods which rely on the expression of the genes in the given tissue or cell type are limited by several factors. The expression of the gene of interest may be restricted to a certain cell population or to a certain developmental stage that is

not represented by the samples selected for screening. The intensity of the hybridization signal may be too low, for example, if the proportion of coding sequence in the probe is very low. Occasional transcribed repeats and pseudogenes may also cause problems and false positive signals.

Alternative expression-independent approaches are based on certain sequence features of coding regions. A zoo blot is a Southern Blot of genomic samples from a wide variety of species. A probe giving positive signals in various species indicates the presence of a conserved sequence, which would be expected to contain biologically important coding sequences (Monaco et al. 1986). Half of the human genes are associated with CpG islands, which are short (~1kb) GC-rich sequences found often at the 5' ends of vertebrate genes (Antequera and Bird 1993, Cross and Bird 1995). Exon trapping takes advantage of the exons' ability to engage in an artificial RNA splicing assay (Duyk et al. 1990, Buckler et al. 1991). A major problem in exon trapping is construction of fragments by use of artificial splice sites which, however, can be reduced using large insert vectors (Datson et al. 1996).

The above methods usually produce only a partial sequence of the gene, which can then be completed by RT-PCR with primers designed to the known sequence. RACE, rapid amplification of cDNA ends, can be employed to construct 5' and 3' ends of the sequence. RACE is based on the amplification between a known cDNA sequence and an anchor cDNA sequence added to 3' and 5' ends of the cDNA clones in a library (Frohman et al. 1988).

### *2.1.3 Gene identification by computer analysis*

The Human Genome Project (HGP) is an international effort aiming in the characterization of the structure of the human genome and its genes. The first task, the construction of high resolution physical maps of human genome was completed in 1994 (Murray et al. 1994). Thereafter, large clone contigs have been assembled and large-scale sequencing of the whole human genome was to be completed around 2002-2003. However, due to advances in the sequencing technology the final sequence was produced considerably ahead of that schedule. In January 2001 HGP simultaneously with the company Celera Genomics published the completed "working draft" of the human genome covering 97% of the genome with still some gaps and ambiguities (International Human Genome Sequencing Consortium 2001). In order to create a gene map of the human genome random cDNA sequencing has produced about 1 850 000 expressed sequence tags (ESTs), which are usually short stretches of cDNA representing different parts of genes.

The most efficient way to identify putative genes in sequenced genomic DNA relies on computer based analysis of the sequence (Boehm 1998). The algorithms for DNA sequence analysis include homology searches, exon/gene prediction and prediction of regulatory regions. Although at present computer based analysis is a very powerful and efficient method it must be accepted that

computer models still remain theoretical and therefore traditional laboratory work is needed to support them (Mallon and Strivens 1998).

Homology searches are designed to search the databases for the sequences similar to the clone. Any significant homology between test sequence and a known gene cDNA or protein can be considered as suggesting a gene associated sequence (functional or pseudo-gene). The largest available databases for DNA and protein homology sequences are Genbank at NCBI (National Center for Biotechnology Information), the EMBL (European Molecular Biology Laboratory) Nucleotide Sequence Database, DDBJ (GenBank and DNA database of Japan) databases and general protein sequence databases such as SWISS-PROT and PIR (accessible through NCBI). The most popular algorithms used in homology search are BLAST (basic local alignment sequence tool) (Altschul et al. 1990) and FASTA (Pearson 1990), which can be achieved using integrated retrieval systems such as Entrez.

Exon prediction programs are designed to scan large genomic sequences aiming at identification of locations of likely exons relying on conserved sequences at exon/intron junctions and the splice branch site and the presence of comparatively long ORFs etc. GENSCAN and GRAIL2 are considered to be the best of such programs (Burge and Karlin 1997). A variety of computer programs also allow the search of several gene associated motifs such as promoter regions and CpG islands (Mallon and Strivens 1998). More recently exon/gene prediction and homology search programs have been packed into software packages which combine the results from many different analyses and finally produce output in a graphical format. Examples of such packages are NIX (nucleotide identification; UK Human Genome Mapping Project Resources Centre) and Genotator (US Lawrence Berkeley National Laboratory) (Borsani et al. 1998).

**Table 2.** Internet addresses for sites mentioned in Chapter 2.1.3

<b>site</b>	<b>Address</b>
Human Genome Project	<a href="http://www.ncbi.nlm.nih.gov/HUGO">http://www.ncbi.nlm.nih.gov/HUGO</a>
EST data base	<a href="http://www.ncbi.nlm.nih.gov/dbEST">http://www.ncbi.nlm.nih.gov/dbEST</a>
NCBI	<a href="http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html">http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html</a>
EMBL	<a href="http://www.ebi.ac.uk/ebi_docs/embl_db/ebi/topembl.html">http://www.ebi.ac.uk/ebi_docs/embl_db/ebi/topembl.html</a>
DDBJ	<a href="http://www.ddbj.nig.ac.jp/">http://www.ddbj.nig.ac.jp/</a>
Entrez/NCBI	<a href="http://www.ncbi.nlm.nih.gov/Entrez/">http://www.ncbi.nlm.nih.gov/Entrez/</a>
Gene/exon prediction software	<a href="http://igs-server.cnrs.mrs.fr/igs/banbury/">http://igs-server.cnrs.mrs.fr/igs/banbury/</a> <a href="http://gnomic.stanford.edu/GENSCAN.html">http://gnomic.stanford.edu/GENSCAN.html</a> <a href="http://compbio.ornl.gov/Grail-bin/EmptyGrailForm">http://compbio.ornl.gov/Grail-bin/EmptyGrailForm</a>
NIX	<a href="http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix">http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix</a>

**Table 3.** Methods for transcript mapping in the genomic DNA

<b>Method</b>	<b>Description</b>
<b>Expression-dependent methods</b>	
• Northern blotting	Genomic clone hybridized to Northern blots containing mRNAs isolated from a panel of different tissues, cell lines or patients.
• cDNA library screening	Genomic clone hybridized against appropriate cDNA library. Positive plaques can be sequenced.
• cDNA selection	Several rounds of hybridization will lead to the enrichment of the cDNAs representing the genes present in the cloned DNA thus enabling the identification of the corresponding genes.
<b>Expression-independent methods</b>	
• Evolutionary conservation	A DNA clone hybridized to a Southern blot of genomic DNA samples from a variety of animal species
• CpG island detection	<i>Restriction mapping:</i> CpG islands often have restriction sites for rare-cutting enzymes such as SacII, EagI and BssHII. DNA clone is hybridized to Southern blots of genomic DNA cut with these enzymes. <i>Island-rescue PCR:</i> Way to isolate CpG islands from YACs by amplifying sequences between islands and often neighbouring <i>Alu</i> repeats.
• Exon trapping	The genomic clone is subcloned into a suitable expression vector and transfected into an appropriate eucaryotic cell line in which the insert DNA is transcribed into RNA and the RNA transcript undergoes RNA splicing. Novel trapped exons can be PCR amplified and sequenced using known flanking sequences in the resulting mRNA.
<b>Computer analysis</b>	
• Homology searches	Sequence is compared against sequences in the databases. Significant homology to known genes, gene-associated sequences indicate a gene.
• Gene searching algorithms	Several computer programs searching exons or other gene-associated motifs.

## 2.2 Disease causing sequence changes

The ultimate evidence of the nature of a given mutation can be achieved through biochemical studies. Only those changes of the DNA sequence that cause functional disturbance of the protein can be considered as “disease causing” mutations (Cooper and Schmidtke 1992). However, as the elucidating of the function of the novel gene can take years we must rely on the analysis of nature



of the mutation (Table 4) and additional information including family data and population studies.

Deletion of the whole gene, nonsense mutations including a premature stop codon and small deletions and insertions, especially if causing frame shifts, are almost certain to have drastic effects in gene function. Missense mutations in coding sequences can lead to only mild consequences if the amino acid is substituted by another having similar chemical properties. The changes are more likely to be pathogenic if they replace a nonpolar amino acid with a polar or an acidic with a basic one. If a missense mutation occurs in the protein region known or predicted to be functionally important it is more likely to be pathogenic. Single base substitutions which destroy the conserved GT-AG splice sites or activate cryptic splice sites will also often lead to the production of non-functional gene products but the effects of other mutations affecting splice sites are more difficult to predict.

**Table 4.** Disease causing sequence changes

Change
Deletion →the whole gene or part of the gene
Insertion
Coding codon changed to a stop codon
Replacement of an essential amino acid
Translocation or inversion
Promoter mutations
mRNA destabilation →mutations in polyadenylation site →Nonsense mediated RNA decay
Splice site mutations →Inactivate donor or acceptor site →Activate a cryptic splice site

## 2.3 Methods for mutation detection

### 2.3.1 Unknown mutations

Although most of the common mutation detection methods are based on PCR technology, Southern blotting is the only method to detect large genomic deletions or rearrangements (Cooper and Schmidtke 1992). Direct sequencing of coding sequences efficiently reveals and defines sequence changes in the gene. When the mutations detected by other methods must still be confirmed by sequencing and with the power of high throughput automated sequences direct sequencing is the choice, especially for smaller genes. Single strand conformational polymorphism (SSCP) analysis is based on the sequence dependent differences in migration pattern in non-denaturing electrophoresis of single stranded DNA (Orita et al. 1989). SSCP can detect up to 95% of mutations in short fragments (<200bp) but leaves the fragment to be sequenced to detect the nature and position of the mutation. Several methods are based on heteroduplex formation between two sequences. Heteroduplex analysis (HET) detects single

base mismatches between mutated and wild type alleles by gel electrophoresis (Keen et al. 1991). In denaturing gradient gel electrophoresis (DGGE) heteroduplex is resolved from homoduplex by running double stranded DNA through gel containing an increasing gradient of denaturing agent (Myers et al. 1987). Denaturing high-performance liquid chromatography (DHPLC) resolves the heteroduplex by HPLC (Liu et al. 1998). Mismatched bases in heteroduplexes are sensitive to cleavage by chemicals or enzymes. This is exploited for example in the chemical cleavage of mismatch method (CCM). CCM would indicate the precise site of mutation but the need of toxic chemicals and many manipulations are major drawbacks for this method (Cotton 1993).

### *2.3.2 Known mutations*

Rapid and efficient mutation detection methods are needed when screening on population level of carrier frequency for a given mutation. Small deletions or insertions can be detected using primers closely flanking the mutation site and distinguishing the alleles by the size of the PCR fragment on polyacrylamide or agarose gels. A restriction site introduced or abolished by the base substitution allows simple PCR based test for a known mutation, where the normal and mutant alleles can be distinguished by amplifying across the mutant site and digesting with the appropriate restriction enzyme (Cotton 1993). In artificial introduction of restriction sites test (AIRS) a diagnostic restriction site can be introduced by PCR using carefully designed primers with a mismatched nucleotide which together with the sequence of the mutant site creates a restriction site not present in the normal allele.

Allele-specific oligonucleotide blot hybridization (ASO) uses selective hybridization of labelled sample to oligonucleotides specific for the different variant sequences. Oligonucleotide ligation assay (OLA) is based on covalent attachment of the labelled reporter and immobilised target oligonucleotides. Oligonucleotides are constructed to be continuously complementary to the region containing the mutation and in the case of a mismatch no ligation can occur (Nickerson et al. 1990).

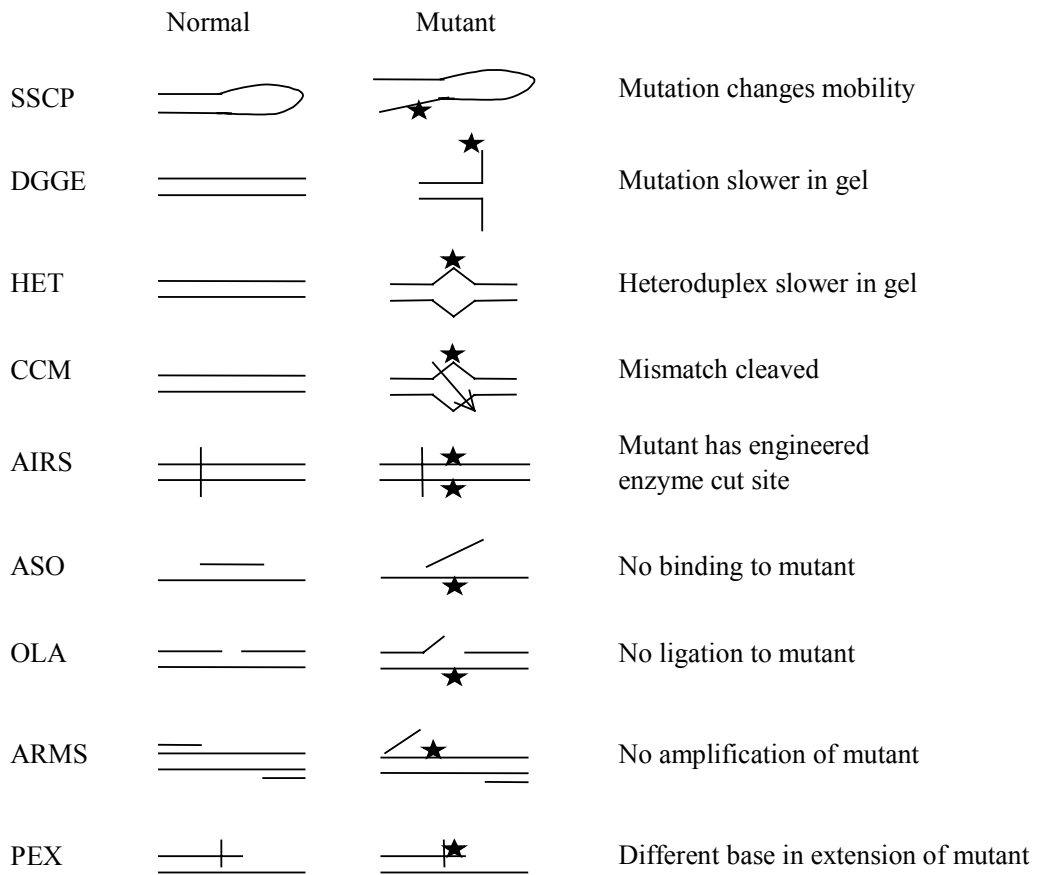
Allele-specific PCR (Amplification refractory mutation system, ARMS test) is based on the use of two different allele specific primers which differ preferably at the most 3' nucleotide, which is of crucial importance to successful PCR reaction (Newton et al. 1989). Using one common primer and several allele specific primers for different mutations in multiplex PCR enables simultaneous screening for several mutations in one reaction. Primer extension (PEX) is based on differential extension of the 3' end of an oligonucleotide by a single-base dependent on the allele of the target.

### *2.3.3. Technologies for large-scale mutation detection*

Traditional mutation detection methods cannot easily meet the demands for rapid and cost effective large-scale sequence and mutational analysis needed for population level genetic risk assessment and diagnostic tests. High density

oligonucleotide arrays (DNA or gene chips) are based either on hybridization or minisequencing (modification of PEX) technology. Examples of the gene chips which have been published include a chip containing complete coding sequence for the cystic fibrosis gene (Cronin et al. 1996), human mitochondrial DNA sequence (Hacia 1999) and a segment of BRCA1 gene (Hacia et al. 1996). Gene chip technologies are thought to be one of the most powerful future technologies for large scale mutation scannings allowing carrier/ genotype screening even at population level (van Ommen et al. 1999, Pastinen et al. 2000).

**Figure 1.** Principles of mutation detection methods described in the text. The abbreviations are given in the text. The asterisk represent the site of the mutation. Adapted from Cotton, 1993.



### 3. Mechanisms of self tolerance

Efficient generation and regulation of immune responses relies on specific antigen recognition by T and B lymphocytes. Diverse specificities are achieved through gene rearrangements during lymphocyte development, which will also produce lymphocytes capable of responding to self structures. In order to prevent the immune system from responding to the body's own structures there must be mechanisms to inactivate potentially self-reacting lymphocytes. Self-specific B and T cells both can be inactivated under appropriate conditions and both are affected independently and differently. However, the ultimate fate of autospecific B cells is determined by the availability of T cell help, as most B cells respond to antigens T cell dependently, i.e. they require T specific help for full activation.

#### 3.1 Activation of T cell response

To recognise antigens T cells use a specific receptor, the T cell antigen receptor (TCR). TCR consists of  $\alpha$  and  $\beta$  polypeptide subunits (95% of T cells) or structurally similar  $\gamma\delta$  polypeptides. These receptors are associated with CD3 complex together forming the T cell receptor complex (Bentley and Mariuzza 1996).  $\alpha\beta$ -T cells can be divided into two distinct populations, a CD4 positive subset, which mainly help and initiate immune responses and a CD8 positive subset, which is responsible for cell-mediated immunity and production of mediators which induce apoptosis in the target cell. CD4<sup>+</sup> cells can further be divided into T helper class 1 and 2 cells (Th1 and Th2) according to differential cytokine secretion profiles (Mosmann et al. 1986). Th1 cells secrete IL-2, IFN- $\gamma$  and TNF- $\beta$  and their principal function has been suggested to be to elicit phagocyte-mediated defence against pathogens and recruit and activate inflammatory leukocytes, whereas Th2 cells secrete IL-4, IL-5, IL-6 and IL-13 and thus stimulate B cells to produce antibodies (Bottomly 1988, Mosmann and Coffman 1989, Abbas et al. 1996).

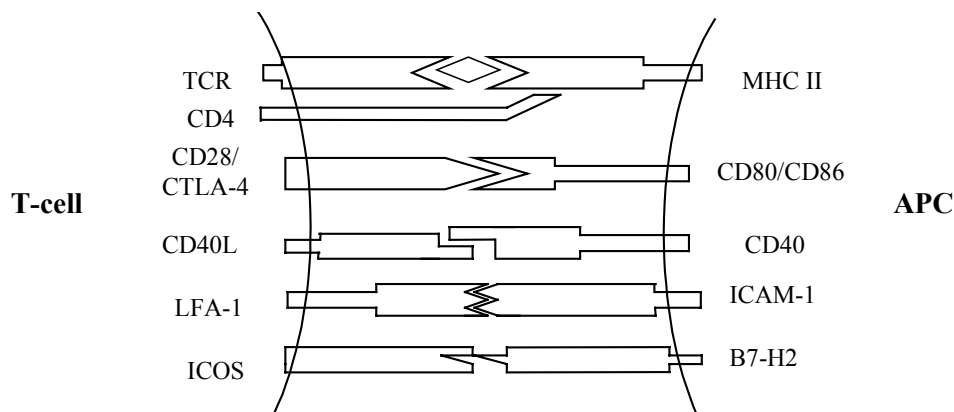
CD8<sup>+</sup> and CD4<sup>+</sup> T cells recognise antigens only when bound to major histocompatibility complex (MHC) class I and class II molecules, respectively (Doherty and Zinkernagel 1975, Germain 1994). MHCs are a group of molecules specialised to the presentation of antigens on the surface of the cell. MHC class I molecules are expressed by nearly all cells in the body. In contrast, MHC II molecules are mostly expressed by specialised antigen presenting cells (APC) including B-lymphocytes, macrophages, dendritic cells and thymic epithelial cells (Brodsky and Guagliardi 1991). Classically MHC I molecules were thought to present only endogenously derived peptides and MHC II molecules exogenously derived peptides. However, it has later been shown that cross-presentation of exogenous antigens on the MHC I molecules on APC can take place (Carbone and Bevan 1990, Huang et al. 1994).

The prerequisite for activation of a naïve T cell upon contact with MHC-peptide complex and TCR and CD4/CD8 is a co-stimulatory signal provided by other cell surface molecules involved in T cell APC interaction (Liu and Janeway, Jr.

1992). The most efficient of co-stimulatory signals is suggested to be mediated through B7-1 (CD80) and B7-2 (CD86) molecules that are constitutively expressed on dendritic cells but can also be upregulated on monocytes, B cells and other APCs (Lenschow et al. 1996). B7-1 and B7-2 are ligands for CD28 cell membrane molecule on T cell and for CD28 homologous CTLA-4 receptor upregulated upon T cell activation. Another well-defined co-stimulatory pathway is through CD40L-CD40 interaction (Grewal and Flavell 1998).

Several other interacting molecules between T cell and APC have been reported (Figure 2), for example intracellular adhesion molecules (ICAM-1, -2 and -3) interacting with lymphocyte functional antigen-1 (LFA-1) (Marlin and Springer 1987, de Fougerolles and Springer 1992) and the CD2 molecule on T cell and lymphocyte functional antigen 3 (LFA-3) widely expressed on all APCs (Dustin and Springer 1991). Recently, a third member of the CD28/CTLA-4 family, the inducible co-activator (ICOS) was identified (Hutloff et al. 1999). Signalling through ICOS has been shown to enhance proliferation of T cells, secretion of lymphokines and help for antibody production but is not needed for CTL responses (Hutloff et al. 1999, Kopf et al. 2000). Novel members of the B7 family have also been identified; B7-H2 is a ligand for ICOS and is expressed on dendritic cells (Wang et al. 2000). Another novel member of the B7 family, B7-H1 does not bind to CD28, CTLA-4 or ICOS and has been suggested to be involved in negative regulation of cell-mediated immune responses (Dong et al. 1999).

**Figure 2.** Examples of the molecules involved in T cell-antigen presenting cell interaction.



### 3.2 T cell tolerance

#### 3.2.1 T cell development in thymus

Thymus is an organ essential for normal  $\alpha\beta$  T cell maturation. The thymus is composed of admixed epithelial and lymphoid elements and also other cellular constituents such as a variety of mesenchymal elements and neuroendocrine cells (Boyd et al. 1993). Histologically, thymus is composed of lobules, each of which can be divided into cortex containing sparse epithelial cells and numerous closely packed thymocytes and medulla, which in contrast contains a larger number of

epithelial cells and fewer lymphocytes. The medulla contains circular bodies called Hassal's corpuscles which are isolated masses of concentric layers of flattened epithelial cells (Suster and Rosai 1990).

During embryogenesis the thymic medullary epithelium is derived from the endoderm of the third pharyngeal pouch and the cortical epithelium from ectoderm of the third pharyngeal cleft (Haynes et al. 1984, Boyd et al. 1993). Embryonic mesoderm originating in the neural crest region forms the thymic capsule, vessels and interlobular septae (Suster and Rosai 1990). In human embryo at 4 to 5 weeks of gestation the third pharyngeal cleft ectoderm proliferates to cover the third pharyngeal endoderm to form the paired thymic rudiments. The first wave of lymphoid cells arrives to the thymus between 8 to 9 weeks of gestation (Papiernik 1970). In mouse the thymic rudiment is populated by lymphoid precursor cells from fetal day 11 onwards. The expansion of the medullary epithelial cells in mouse thymus occurs only beyond fetal day 17, when the first mature double positive and single positive thymocytes appear (Huiskamp et al. 1985). The findings that medullary and cortical epithelium share some antigens on embryonic rudiments and identification of small clusters of undifferentiated epithelial cells in corticomedullary junction has cast doubt on the dual origin on thymic epithelium and the exact contribution of each embryonic layer remains controversial (Boyd et al. 1993).

Thymic stromal cells play a critical role in the differentiation and selection of the precursor T cells arriving in the thymus, but on the other hand the organization and development of the thymus epithelium is dependent on the signals provided by the developing T cells. This phenomenon of mutual regulation of differentiation is designated as "*thymic crosstalk*" and is best understood by studies on mouse models (van Ewijk et al. 1994). In transgenic mice where the T lymphocyte maturation is arrested at the early prethymic CD44+CD25- stage cortical epithelium parallel to the medullary epithelium is disorganised (Hollander et al. 1995). Whereas if the T cell development is blocked at the thymic CD44-CD25+ stage mice show normal cortex but fail to develop normal medulla (Shores et al. 1991, van Ewijk et al. 1999).

### 3.2.2 *Thymocyte selection*

Development of thymocytes into immunocompetent T cells involves translocation and recombination of the minigenes coding for the V, D and J segments for the TCR chains and expression of TCR together with CD3 complexes and acquisition of the CD8 and CD4 expression. In thymus cortex the approval takes place of those thymocytes that have acquired a TCR affinity for the MHC-peptide complexes in the local microenvironment; a process referred to as positive selection (Figure 3) (von Boehmer 1994). The thymocytes that are CD4+CD8+ (double positive) and express TCR with low but measurable affinity for MHC-self peptide complex are allowed to differentiate further into mature CD4+ or CD8+ (single positive) T cells. In contrast the thymocytes, which have no avidity for the selfpeptide-MHC complexes, will not survive and will die by

apoptosis. The positively selected thymocytes still include cells that have strong avidity for selfantigen-MHC complexes (Laufer et al. 1996) and thus will have to be deleted to eliminate potentially autoreactive lymphocytes, a process referred as negative selection (Nossal 1994).

The cell type responsible for negative selection has been studied in several different mouse models but so far the matter is controversial and the roles for both cortical and medullary epithelial cells and bone marrow derived thymic dendritic cells and macrophages and thymic B cells have been implicated (Nossal 1994, Viret et al. 2001). It has been shown that clonal deletion may take place already in the double positive stage in the cortex (Kisielow et al. 1988, Baldwin et al. 1999) or later in the single positive stage in the medulla (Surh and Sprent 1994, Zal et al. 1994, Baldwin et al. 1999). Cortical deletion has been suggested mainly to involve antigens that are produced by the cortical cells themselves e.g. MHC and housekeeping proteins as the blood-borne macromolecules have poor access to cortex (Nieuwenhuis et al. 1988). In contrast the medullary APC have been suggested, in addition to presenting intracellular antigens, to pick up antigens from serum and process them further to be presented to the thymocytes (Zal et al. 1994). TCR affinity and thymocyte avidity can also play a role in timing the deletion in such a way that those thymocytes with high affinity for the antigen-MHC complexes and thus most likely to initiate autoimmune responses are deleted earlier (Baldwin et al. 1999). T cells with low TCR affinity, but still high enough for positive selection, would be deleted later in thymic development and have been shown to be allowed to escape the central selection (Miller 1996).

Negative selection of thymocytes requires costimulatory signal in addition to TCR-MHC ligation (Page et al. 1993). Several different costimulatory molecules have been shown to mediate negative selection including CD40/gp39 (CD40L) interaction (Foy et al. 1995, Dautigny et al. 1999). Gp39 deficient mice show reduced deletion of self-specific thymocytes confirming the role of CD40-gp39 signalling in negative selection (Foy et al. 1995). The costimulation can also be mediated through CD28/ B7 interaction (Punt et al. 1994, Punt et al. 1997). The other B7 receptor, CTLA-4, has also been suggested to have a role in thymic selection (Cilio et al. 1998). A role for B7 molecules in negative selection is supported by the correlation between expression of B7 molecules and deletion of self-reactive thymocytes in thymus medulla (Degermann et al. 1994). However, the findings that blocking B7 molecules with CTLA-4Ig fusion protein does not block negative selection mediated by superantigens (Jones et al. 1993) and that CD28 deficient mice undergo negative selection mediated by superantigens (Shahinian et al. 1993) have argued their role in negative selection.

Although interaction of Fas and Fas ligand (FasL) plays an important role in the regulation of peripheral immune responses (Nagata and Golstein 1995, Mouliau and Berrih-Aknin 1998), the role of Fas in negative selection in thymus is somewhat controversial. The Fas dependent pathway may only be functional with high concentrations of antigen (Kishimoto et al. 1998). Or as suggested by

normal negative selection in FasL defective mice but affected negative selection in mice with defective Fas there might be an unknown ligand for Fas or there is not necessarily any need for ligand interaction (Dautigny et al. 1999). Another member of the tumor necrosis family of receptors (TNFR), CD30, has also been suggested to act as a costimulatory molecule in negative selection as CD30<sup>-/-</sup> mice show impaired negative selection (Amakawa et al. 1996, Chiarle et al. 1999). Further evidence for the role of CD30 in negative selection has been provided by a transgenic (tg) mouse model overexpressing CD30 in T lymphocytes which has enhanced deletion of specific thymocytes by bacterial superantigens and by peptide antigens (Chiarle et al. 1999).

### *3.2.3 Role of medullary epithelial cells in tolerance induction*

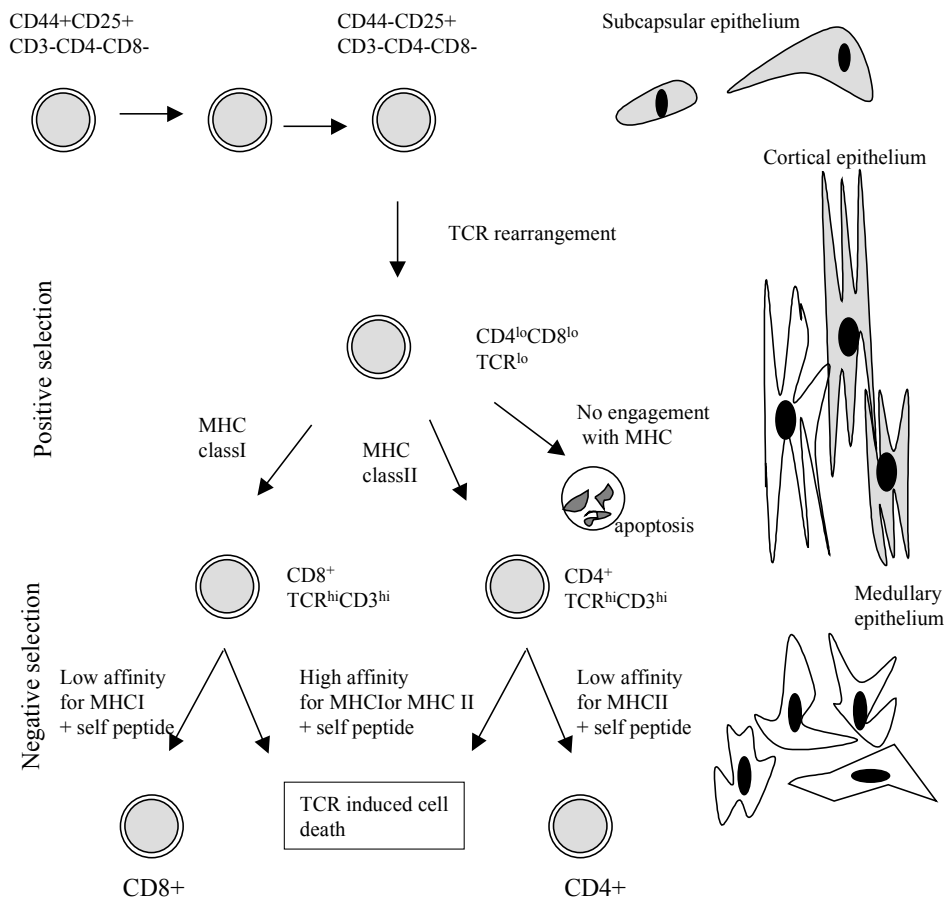
Thymic epithelial cells can be divided according to intrathymic localisation into perivascular/subcapsular, cortical and medullary subsets. Morphological studies by electronmicroscopy and immunohistochemistry with several monoclonal antibodies has allowed discrimination of at least six distinct epithelial cell subtypes (Boyd et al. 1993). In the medulla the most abundant cell type is composed of reticulated cells expressing ER-TR5, MTS10 and 95 epitopes in mouse (Naquet et al. 1999). These cells are considered to be “resting” in contrast to another medullary subtype showing features of “activated” cells. These cells bind the fucose-specific lectin UEA-1 and express high levels of MHC, ICAM-1, CD40 and B7 molecules and share markers with thymic dendritic cells (Naquet et al. 1999).

Although several different cell types in thymus are able to mediate negative selection the essential role for medullary epithelial cells (mTE) has been implicated in several experiments. RelB transcription factor has been shown to be expressed in the UEA-1<sup>+</sup> medullary epithelial cells and in medullary dendritic cells (Carrasco et al. 1993, Weih et al. 1995, Burkly et al. 1995). In RelB knockout mice these cell types are absent and medulla is poorly organized. Furthermore, the mice have indications of affected negative selection as they develop severe inflammatory syndrome of multiple organs indicating the role for UEA-1<sup>+</sup> medullary epithelial cells in negative selection (Weih et al. 1995, Burkly et al. 1995).

Experiments with chimeric transgenic mice with a restricted expression of MHC ligand in thymic medulla have confirmed tolerance induction by both deletional and nondeletional mechanisms by mTE (Schonrich et al. 1992, Heath et al. 1992, Hoffmann et al. 1995). The specialised ability of mTE to induce both CD4 and CD8 tolerance to intranuclear antigens was suggested in a model expressing  $\beta$ -gal antigen restricted to mTE nucleus. Both high and low avidity T cells were shown to be tolerised to class I restricted epitopes (Oukka et al. 1996a) and later tolerance was shown to be induced to MHC II restricted antigens (Oukka et al. 1996b). In contrast if the transgene was expressed only in the nucleus of dendritic cell the tolerance was only induced to MHC class I epitopes (Oukka et al. 1996b).



**Figure 3.** T cell development and selection in thymus. Prethymic CD3-CD8-CD4-CD25+CD44+ T cell precursors enter the thymus and proliferate below the subcapsular region. Here the cells acquire CD8 and CD4 expression at low density and rearrange their TCR genes. Maturing cells move deeper to the cortex where the TCR of the thymocytes is exposed to epithelial MHC molecules. Recognition of selfpeptide-MHC complex leads into positive selection whereas those thymocytes with no avidity for the MHC-peptide complex will undergo apoptosis. Positively selected cells acquire increased expression of TCR and CD4 or CD8 during migration to the corticomedullary junction and the medulla, where those thymocytes with high affinity TCR for selfpeptide-MHC complex are negatively selected i.e. undergo TCR mediated cell death.



#### 3.2.4 Expression of “tissue specific” antigens in thymus medulla

The sensitivity of negative selection has been suggested to be relatively high. In the hen egg lysozyme (HEL) transgenic mice expressing different amounts of the HEL gene product, serum levels of less than  $10^{-10}$  M were seen to induce tolerance within T cell population (Adelstein et al. 1991). In another example the expression of a T antigen under rat insulin promoter was not revealed in thymus when looking at mRNA by Northern blot or protein by immunohistochemistry but RT-PCR revealed low level expression in thymus, sufficient thus for negative selection indicated by systemic tolerance towards the T antigen (Jolicoeur et al. 1994).

Several endogenous peripheral antigens have been shown to be expressed in the thymus including pancreas specific genes (Jolicoeur et al. 1994, Smith et al. 1997), components of myelin sheath (Fritz and Zhao 1996, Pribyl et al. 1996), acetylcholine receptor (Kirchner et al. 1988), retinal proteins (Egwuagu et al. 1997) and neuro-endocrine hormones (Martens et al. 1996). Histochemical analyses have localised tissue-restricted antigens to thymus medulla, suggesting a particular role for thymus medulla and presumably mTE in tolerance induction (Farr and Rudensky 1998). Correlation between allelic variation at the susceptibility locus for type 1 diabetes, INS VNTR-IDD3, and expression levels of insulin and proinsulin in thymus suggests that higher levels of antigen expression in thymus would lead to more efficient thymic negative selection and protection against autoimmunity (Pugliese et al. 1997, Vafiadis et al. 1997).

Intrathymic expression of a specific immunodominant splice variant may be sufficient for tolerance induction as indicated by studies of Klein et al on experimental autoimmune encephalomyelitis (EAE), a model disease to multiple sclerosis in humans. Upon immunization with the proteolipid protein (PLP), the main protein of the myelin sheath, SJL but not BL/6 mice are susceptible to induction of EAE. Intrathymic expression of PLP is restricted to the shorter splice variant, DM20 in mouse and human (Klein et al. 2000). The major T cell epitopes (i.e. epitopes selected by their MHC molecules) of the EAE resistant BL/6 mouse are encoded by the DM20 splice variant thus confirming tolerance to all epitopes of PLP. In contrast, in SJL mouse strain that is susceptible to EAE, the major T cell epitope is encoded by the central nervous specific exon of PLP not expressed in thymus thus offering an explanation for the susceptibility to EAE (Klein et al. 2000).

Inducible acute phase proteins, which oscillate between tolerogenic and nontolerogenic concentrations would be one of the challenges for self-tolerance. A transgenic model showed that tolerance to transgenic hCRP was mediated by intrathymic deletion and that expression of transgene on medullary epithelium without induction was sufficient to induce tolerance (Klein et al. 1998). Furthermore, ectopic expression of CRP in mouse and human thymus medullary epithelial cells was revealed and was suggested to be responsible for the physiological tolerance for this inducible acute phase protein (Klein et al. 1998).

### 3.2.5 *Peripheral T cell tolerance*

Negative selection in thymus is not complete and potentially autoreactive cells are present in the peripheral pool of T cells even in healthy individuals. Therefore, self-tolerance must be established by alternative mechanisms of peripheral tolerance induction or by down-regulation of the immune response.

Deletion as a mechanism for extrathymic tolerance has been suggested for several antigens including superantigens (Webb et al. 1990), proteins (Critchfield et al. 1994) and transgenic self-antigens (Fields and Loh 1992, Forster et al. 1995, Bertolino et al. 1995). Peripheral deletion of T cells involves signalling through Fas (CD95) or TNF receptor which induces apoptosis in T cell. Mutations in Fas or Fas ligand result in lupus like syndrome due to abnormal survival of helper T cells and inability to eliminate autoreactive B cells by apoptosis (Rathmell et al. 1995, Van Parijs and Abbas 1998). CD30 molecule is also suggested to have a role in preventing extensive proliferation of primed effector cells in parenchymal tissues upon second contact with the antigen (Kurts et al. 1999).

Anergy is defined as a state of functional unresponsiveness, which can be induced by foreign antigens if administered without adjuvant because they do not induce inflammation and thus activate APCs to provide the needed secondary signals (Morahan et al. 1989, Lenschow et al. 1992, Matzinger 1994). Some studies have also suggested that the administration of alternative receptor for B7 recognition, the CTLA-4 receptor instead of CD28 would lead to unresponsive state of the lymphocyte (Waterhouse et al. 1995, Perez et al. 1997).

Some self-antigens are simply ignored by the potentially auto-reactive lymphocytes because of being anatomically hidden from lymphocytes. Such sequestered antigens are those localising only inside the cell or antigens of sperm or lens which are protected by the inability of the lymphocytes to cross the endothelial barrier. Another explanation for ignorance is the level of antigen expression. An antigen expressed on extremely low levels would not be presented by professional APC in regional lymph nodes and would thus not induce specific tolerance in T cell population, whereas for highly expressed antigens the autoreactive T cells would be deleted (Kurts et al. 1998, Kurts et al. 1999).

T suppressor/ regulatory T cell subset has the ability to regulate the responses of B cells and other T cell subsets. Several experiments have indicated the participation of T cells in preventing autoimmunity (Modigliani et al. 1996). Mice thymectomised before the age of 3 days develop several organ specific autoimmune diseases (Asano et al. 1996) due to elimination of CD4<sup>+</sup>CD25<sup>+</sup> T cells which have been shown to be mediating self-tolerance by down regulating immune responses (Sakaguchi et al. 1995). These CD4<sup>+</sup>CD25<sup>+</sup> T cells constitutively express CTLA-4 and blocking of CTLA-4 abrogates the

suppression (Takahashi et al. 2000). Skewing of T cell response to Th2 derived cytokines has been found to be associated with tolerant stages in NOD and EAE susceptible mice whereas Th1 response is associated with disease (Kruisbeek and Amsen 1996). The factors determining this immune deviation is not known but the type of APC has been implicated as one possible parameter through different cytokine profiles and accessory molecules (Kruisbeek and Amsen 1996).

### **3.3 Dendritic Cells and their role in immune response and tolerance**

#### *3.3.1 Ontogeny and features of dendritic cells*

Dendritic cells (DC) are leukocytes specialised in presenting antigens to T cells. DCs were first visualised by Langerhans in the skin in 1868. But only in 1973 did Steinmann and Cohn describe a novel cell type in murine spleen and called it dendritic cell as the cells displayed processes including highly motile cytoplasmic sheets (Steinman and Cohn 1973). DCs are found in every organ of the body and have been classified into several subgroups including interstitial DC found in heart, kidney, gut and lung; Langerhans cells in the skin and mucous membranes; DCs in the thymus medulla and secondary lymphoid tissues and blood and lymph DCs (Caux et al. 1995). Mature murine DCs can be recognised by their expression of the integrin CD11c or marker DEC-205 (which is also expressed by activated macrophages and thymic cortical epithelium), together with their characteristic morphology and high class II MHC expression (Hart 1997). In humans the CD83 antibody stains cultured DC, LC and some interdigitating cells in lymph nodes (Hart 1997).

DCs can be cultured from proliferating hematopoietic precursors in the presence of GM-CSF and IL-4 and are therefore classically considered to be of myeloid origin (Reid et al. 1992, Inaba et al. 1993). However, there is evidence for another DC population of lymphoid origin mainly present in thymic medulla and T cell zones of the spleen and lymph nodes. In mouse these DCs were shown to develop from a common early T cell precursor which could still form T and B cells, NK cells and DCs but not erythroid or myeloid cells (Ardavin et al. 1993). In contrast to myeloid dendritic cells (MDCs) IL-3 drives the development of these lymphoid DCs (LDC), which lack myeloid markers (Saunders et al. 1996). Murine CD8 $\alpha$ <sup>+</sup> DC appear to represent a lymphoid related DC lineage whereas the MDC are CD8 $\alpha$ <sup>-</sup> (Vremec and Shortman 1997). No CD8 expression has been detected on human DC, but human DCs can be grown in the presence of the same mixture of growth factors that stimulate proliferation of mouse LDCs (Grouard et al. 1997), and in human thymus the CD34<sup>+</sup>CD38<sup>dim</sup> thymocytes have been shown to be able to differentiate into T, NK and DCs (Res et al. 1996).

#### *3.3.2 Dendritic cells in immune response and tolerance*

In most peripheral tissues DCs are present as “immature” i.e. unable to stimulate T cells (Banchereau and Steinman 1998) but these cells, however, are highly capable of capturing antigens as they can take up particles and microbes by phagocytosis (Reis et al. 1993, Sallusto et al. 1995). The captured antigens induce maturation of the DCs and upregulate the migratory and adhesive

properties enabling migration and homing to the T cell areas of lymphoid organs (Cella et al. 1997, Brown et al. 1997). DCs efficiently produce MHC class II-peptide complexes and as the cells mature they discharge the MHC-peptide complexes to the surface of the cell where these remain stable for days (Nijman et al. 1995, Cella et al. 1997, Pierre et al. 1997). Under certain conditions DCs are able to load peptides derived from exogenous antigens such as non-replicating microbes or dying infected cells onto the MHC class I molecules and cross-present these to CD8 T cells (Bhardwaj et al. 1995, Albert et al. 1998).

The general function of DCs is considered to be the initiation of immune responses to foreign antigens in peripheral lymphoid organs (Macatonia et al. 1989, Inaba et al. 1990). The maturing DCs upregulate the expression of costimulatory receptors CD40, CD54 and CD80 (B7-1) CD86 (B7-2) and other accessory molecules such as ICAM-1, LFA-1 and LFA-3. At the same time they retain high levels of MHC-peptide complexes, which makes them excellent in stimulating naïve T cells. DCs are considered the most effective APC for peripheral T cell activation; the capacity exceeding 100-1000 fold when compared to other APCs, macrophages and B cells (Steinman and Inaba 1989, Inaba et al. 1990, Liu and MacPherson 1993).

In addition to inducing immune responses DCs appear to be able to induce immune tolerance. DCs can capture and present self-antigens that are tissue-specific, for example peptides derived from insulin producing  $\beta$ -cells of the pancreas (Kurts et al. 1996). Presenting of these endogenous antigens in the draining lymph nodes has been shown to induce tolerance presumably through T cell anergy or deletion (Kurts et al. 1997). Thymic DCs present self-antigens on MHC molecules and have been shown to be capable of inducing self-tolerance through thymic negative selection (Anderson et al. 1998). This ability seems not to be restricted to thymic DCs as DCs isolated from spleen are able to mediate negative selection as well (Matzinger and Guerder 1989). DCs isolated from pancreatic lymph nodes of NOD mice and transferred to pre-diabetic NOD can limit the progression to diabetes suggesting regulatory function for DCs in autoimmunity progression (Clare-Salzler et al. 1992).

### **3.4 Mechanisms for tolerance breakdown and autoimmunity**

#### *3.4.1 Genetics of autoimmune diseases*

Susceptibility genes for autoimmunity can be considered to be those either directing the response to particular autoantigens or on the other hand those influencing tolerance, apoptosis or inflammatory responses. Many autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and type 1 diabetes show strong association to certain haplotypes of MHC complexes. Multiple other genes have been shown to be involved in autoimmune disease including cytokines, adhesion molecules, immunoglobulins and genes encoding T cell receptors (Theofilopoulos 1995). For example CTLA-4 polymorphism has been implicated in susceptibility to type 1 diabetes (Marron et al. 1997), Graves disease (Yanagawa et al. 1995), Addison's disease (Kemp et al. 1998) and

multiple sclerosis (Ligers et al. 1999). Familial clustering of different autoimmune diseases and coassociation of multiple diseases is often seen (Vyse and Todd 1996). Genome wide scanning has proved that some susceptibility loci are shared between different autoimmune diseases and conserved regions from experimental disease models indicating that a common set of susceptibility genes may control distinct autoimmune diseases (Becker et al. 1998). In most cases autoimmunity seems to be a result of complex interaction between different genetic and environmental factors and only a few examples of autoimmune diseases caused by a defect in a single gene are known. Defective apoptosis due to mutations in either Fas or FasL genes is responsible for autoimmune lymphoproliferative syndrome type 1 (ALPS-1) (Fisher et al. 1995, Wu et al. 1996) and mutations in caspase 10 gene for ALPS-2 (Wang et al. 1999).

#### 3.4.2 MHC and autoimmunity

It was thought that disease associated MHC molecules would efficiently bind autoantigens and thus result in immune response leading to autoimmune diseases (Vaysburd et al. 1995). However, recent results suggest that the autoimmunity is rather a result of poor peptide binding properties (Fairchild et al. 1993, Carrasco-Marin et al. 1996). Weak binding of self peptide in the thymus would allow high affinity self-reactive T cells to escape negative selection (Boyton et al. 1998, Ridgway et al. 1998). These self-reactive T cells would then, in collaboration with other disease related genes, mediate autoimmunity in the periphery (Ridgway and Fathman 1999). Another explanation is provided by the EAE model where differential expression of self-antigen in the thymus and in the periphery together with permissive MHC haplotype predisposes to autoimmunity (see chapter 3.2.3) (Klein et al. 2000). Some MHC haplotypes have been found to be protective for a disease. The protection has been shown to be mediated either by efficient negative selection of the self recognising T cells (Verdaguer et al. 1997) or through positive selection of protective T cells (Luhder et al. 1998).

#### 3.4.3 The “cryptic self” hypothesis

The “cryptic self” hypothesis for autoimmunity induction is based on the finding that each self protein is presented by a minority of dominant epitopes against which the immune system is driven tolerant through central tolerance induction mechanisms. The majority of subdominant/cryptic epitopes are poorly displayed and would therefore leave a large number of potentially self reacting T cells to exist (Theofilopoulos 1995). “Determinant spreading” would occur after the release of large amounts of intramolecular and intermolecular determinants after the initial response against the major self determinant (Lehmann et al. 1993). NOD mouse model has provided evidence for cryptic determinants in the induction of autoimmunity (Kaufman et al. 1993, Tisch et al. 1993). GAD65 is suggested to be the essential autoantigen initiating autoimmunity against  $\beta$ -cells in NOD mouse (Yoon et al. 1999) and after the autoimmune reaction against GAD65 has destroyed  $\beta$ -cells other antigens are released and presented by APC in local lymph nodes to activate the subdominant epitope specific T cells (Yoon et al. 1999).

#### *3.4.4 Molecular mimicry hypothesis*

Molecular mimicry is suggested to induce autoimmunity through cross recognition of conserved epitopes shared between self-antigens and proteins from infective agents such as viruses and cytokines. Infected cells present viral antigens by MHC I molecules to CD8 cells. APC which have phagocytosed the antigen present it to CD4 cells which then mediate help to CD8 cells. Once activated to react against foreign epitope these effector cells would also recognise similar self-epitopes presented in tissues on MHC I molecules. Some T cells may carry two TCR due to incomplete allelic exclusion (Padovan et al. 1995). These dual receptor T cells can be activated through recognition of an unrelated environmental antigen by the second receptor and then allow mediation of autoimmune reaction through the other putative self-reacting TCR (Padovan et al. 1993, Zal et al. 1996).

#### *3.4.5 Danger hypothesis*

Release of previously sequestered antigens within tissue damage can induce autoimmune disease as indicated by sympathetic ophthalmia after eye injury or orchitis following vasectomy, anticardiomuscular antibodies following infarct etc. According to second signal theory, tissue injury itself cannot be the cause of autoimmune reaction. The induction of immunity instead of anergy in self-specific T cells would need a “danger” message to be provided by APC, which is in turn previously activated by antiseif CD4<sup>+</sup> T cell or viral infection of the APC (Matzinger 1994). Some virus infections have been shown to upregulate expression of MHC class II molecules thus enabling presentation of antigens to T helper cells. Viral infection can increase release of cytokines, which may augment CTL and expression of adhesion molecules, which then may lead to increased recruitment of CD4 T cells to the lesion (Theofilopoulos 1995). DC can also become conditioned by certain exogenous mechanisms such as viral infection of the APC which may circumvent then the need of CD4<sup>+</sup> help in cytotoxic T cell activation (Buller et al. 1987).

## **AIMS OF THE STUDY**

At present the molecular mechanisms leading to tolerance and on the other hand into breaking of tolerance and to autoimmune diseases are largely unknown. Most autoimmune diseases occur as a result of complex interplay between genetic susceptibility and environmental factors. The monogenic etiology of APECED makes it an intriguing model for human autoimmunity. Identification of the defective gene will make genetic diagnosis of the disease possible. In future defining the molecular pathogenesis of APECED will provide opportunities for novel treatment strategies and can also be considered to give insights into the mechanisms of immune tolerance and pathogenesis of autoimmunity in general. As a part of this pursuit the specific aims of this study were:

1. To isolate the gene defective in APECED localised previously to chromosome 21q22.3.
2. To characterize the mutation pattern of the identified gene in APECED patients to enhance genetic diagnosis for the disease and to obtain insights into the functional domains in the gene.
3. To examine the spatial expression pattern of the identified gene on mRNA and protein level.
4. In order to assess the validity of a genetically engineered mouse model for APECED to examine the spatial and temporal expression of the mouse homologue for the identified gene.



## MATERIALS AND METHODS

### 1. Genomic sequencing and isolation of cDNA fragments

Genomic sequencing of cosmid DNAs was performed by the shotgun method (Kawasaki et al. 1997). The phage DNAs prepared from a human thymus cDNA library (Clontech) were used as a PCR template (Nagamine et al. 1997). 20 ng of phage DNA, representing approximately  $4 \times 10^8$  phages, was added to a reaction mixture containing 16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 50 mM Tris-HCl, pH 9.2, 1.75 mM  $\text{MgCl}_2$ , 0.001% (w/v) gelatin, 0.2 mM of each dNTPs, 1 M Betaine, 0.35U *Taq* and *Pwo* DNA polymerase (EXpanding Long Template PCR System, Boeringer Mannheim) and 0.5 M of each of the primers, 2F and c33R, 2F and 4R, 2F' and 2R', 3F and c33R, respectively. PCR conditions used were 94 °C for 3 min, 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec with 2F/c33R and 2F/4R and 3F/c33R or 65 °C with 2F'/2R' and 68 °C for 90 sec. 3'- and 5'-RACE were carried out by Marathon cDNA Amplification Kit (human thymus, Clontech). 3' RACE of AIRE cDNAs and 5'-RACE of AIRE-2 and AIRE-3 were amplified with primers c33F/AP1 and AP1/4R, respectively. 5' RACE of AIRE-1 was amplified first with AP1/3R' and further with nested primers AP2/1R. The entire AIRE-1 ORF containing cDNA was amplified with primers EnF/ExR from Marathon Thymus cDNA. Dye-deoxy terminator cycle sequencing was carried out according to the standard protocols on ABI377 automated sequencers (Perkin Elmer Applied Biosystems). Primer sequences: 2F 5'-GGA TTC AGA CCA TGT CAG CTT CA-3', C33R 5'-CTC GCT CAG AAG GGA CTC CA-3', 4R 5'- AGG GGA CAG GCA GGC CAG GT-3', 2F' GTG CTG TTC AAG GAC TAC AAC-3', 2R' 5'- TGG ATG AGG ATC CCC TCC ACG-3', 3F 5'-ATG GGC GTC TCT TGC CTG TGC CA-3', AP1 5'- CCA TCC TAA TAC GAC TCA CTA TAG GGC-3', AP2 5'- ACT CAC TAT AGG GCT CGA GCG GC-3', C33F 5'- GAT GAC ACT GCC AGT CAC GA-3', 3R' 5'- AGG CTT CGG GCC ACT GCT GCT-3', 1R 5'- CGT TCC CGA GTG GAA GGC GCT GCT-3', ExF 5'-CCA CCC CAT GGC GAC GGA CG-3', ExR 5'- GGA ATT CTC CAC AGG GGC TGG GGT CA-3'.

### 2. Patients selection and DNA isolation

In Study I one Swiss and six apparently unrelated Finnish APECED families and as control material 30 healthy Finnish blood donors, 20 healthy Japanese volunteers at Keio University and 50 healthy Swiss were studied. In Study II a total of 15 apparently unrelated patients were studied. Nine were of North-Italian origin, 2 were from Britain, 1 was from New Zealand and 3 were of Finnish origin. The diagnosis (I and II) was based on typical clinical findings, and in some cases in the presence of autoantibodies against steroidogenic enzymes P450c17 and/or P450scc. In Study III a total of 16 unrelated North American APECED patients were studied. These patients included 13 females and 3 males. The diagnosis of APECED was based on the presence of at least two of the three diseases, i.e. hypoparathyroidism, mucocutaneous candidiasis and Addison's disease as defined by Neufeld et al. (1980). None of the patients in Study III was affected by ectodermal dystrophies. Thirteen of the patients were American

Caucasian of Northern or Western European origin, one was of Hispanic, one of Asian and one of Arabic origin. No consanguinity was documented in any of the families.

### 3. Mutation detection and haplotype analyses

The 14 exons of AIRE gene (GenBank accession no. AB006684) were PCR amplified from genomic DNA using the primers and conditions shown in Table 5. Amplification was carried out in a 30 cycle PCR with initial 5 min denaturation of template DNA at 94 °C followed by touch-down PCR program for 10 cycles: 94 °C/20sec, 65 °C/20sec(-1 °C/cycle), 72 °C/ 1 min and 20 cycles 94 °C/20sec, 55 °C/20sec(-1 C/cycle), 72 °C/ 1 min, in a volume of 20µl containing 10mM Tris-HCl pH 8.3, 50mM KCl, 0.2 mM of each dNTP, 1.5 mM MgCl<sub>2</sub>, 0.5 µM of each primer, 10% DMSO and 0.5 U of *Taq* polymerase. For some exons Pfu polymerase and 50% deazaGTP in place of dGTP and a buffer containing 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 67 mM Tris-HCl, pH8.8, 6.7 MgCl<sub>2</sub>, 10 mM β-mercaptoethanol, 1.25 mM of each dNTPs was used (Table 5). For exon 9 Kit Taq Polymerase (Qiagen) with Q-solution and 5% DMSO was used. PCR products were purified either using Qiaquick PCR purification columns (Qiagen) or prepared by Bandprep kit (Pharmacia, Sweden).

Dye-terminator sequencing was carried out using the primers listed in Table 5. according to the standard protocols on ABI310 or 377 automated sequencers (Perkin Elmer Applied Biosystems). The fragment containing the R257X mutation was amplified with primers 5IF (5'-GCG GCT CCA AGA AGT GCA TCC AGG-3') and 5IR (5'- CTC CAC CCT GCA AGG AGG AGG GGC-3'). The DNA product was digested with restriction enzyme *TaqI* and run on a 1.5% agarose gel. The 1094-1106del was detected by analysing the length of the PCR fragment amplified with the primers 5'-GCG GCT CCA AGA AGT GCA TCC AGG-3' and 5'AGC GCC CTG GGC TCC CTT GGC TCG-3'. Haplotype analyses for the markers JA-I, *D21S1912* and *PFKL* were performed as described earlier (Björnses et al. 1996).

**Table 5. Oligonucleotides and PCR Conditions for mutation analysis**

Exon	Forward Primer	Reverse Primer	Product size (bp)	conditions
1	gctgccagtgtcccgggaccacc	gggcgggtcctcctggaactcc	276	1, 2, 4
2	caccctctagtcgatggaatg	ccactccggtccagtcagctgg	342	5
3	gcttgccaaggtgtccagtctg	gggaagactggagaccctggctgg	289	5
4	ccactgagaggggagccagcctg	ctcagagggcaggecctgctctga	203	
5	ctgcttctgcatagagtatgtgc	gtggtcctcctccatcttgagc	256	
6	ggcctacacgactgccaaggcagg	ccagaggcaacgctgttctctgg	286	5
7	acagccatgtgcaccctcgtgct	cccaggtaaaggcagaggcagcag	225	5
8	agttcaggtaccagagatgctgc	gtggcttccctcagggtcagtg	308	5
9	aaagacatggtcggagccctggag	gggcgtggccatgtggacaggagg	255	1, 6
10	ccagcagtcactgactcctgggtg	ctcctgtttcaggtgaattcatcc	338	3, 5
11	gtgaggctcctcactgctgctag	tgtggttgtggctgtatgatgtg	354	2
12	catacccggaggtggcactcctg	agggtctgcctgagatgtgtcc	260	
13	ccaccagggtgtggagttgg	agcctgggtgccggctgaggcagg	182	
14	tgacttcttgaacgatggccatg	cactgacaagaggtggcctgtcc	278	

Conditions: 1)1.5% DMSO; 2)Pfu polymerase; 3) buffer 2; 4) 50% deaza G; 5) annealing at 60 C; 6)Qiagen Kit Taq polymerase with Q-solution.

#### **4. Northern-blot analysis**

The fragment of AIRE cDNA spanning nucleotides 1545-2000 containing exons 12 (partly), 13 and 14 was amplified from fetal liver mRNA with primers 8IF (5'-GAT CCT GCT CAG GAG ACG TGA CCC-3') and GR1R (5'-CAC CAG GCA AGG AGA GGC TCC CGG-3') and cloned into pCRII plasmid vector (Invitrogen). The DNA probe was labelled with  $\alpha^{32}$ -P dCTP using the random primer extension system (NEN Research). The probe was hybridized to Northern blots containing 2 $\mu$ g of poly(A)<sup>+</sup>RNA from various human tissues (Clontech) according to the manufacturer's protocol. Filters were exposed to an Image plate (Fuji Photo Film) for 36h and then analysed with bio-image analyser BAS2000 (Fuji Photo Film).

#### **5. Human tissue samples**

Human tissue samples were obtained, with informed consent, from young cardiac patients undergoing corrective surgery (thymus), from surgical biopsies or from autopsies. Tissue collection was approved by the Hospital Ethics Committees.

#### **6. Mouse tissue samples**

6-12 week BALB/c, 8 week female NOD mice and RelB knockout mice were used as a source of mouse tissues. Mice were sacrificed by decapitation and the organ samples were embedded into Tissue-Tec and snap-frozen in liquid nitrogen or fixed in 4 % paraformaldehyde and embedded in paraffin. Seven  $\mu$ m sections were cut and mounted on glass slides and stored at -70 °C until use.

#### **7. mRNA *in situ* hybridization**

Three cDNA fragments spanning nucleotides 127-804, 728-1176 and 1545-2000 of the AIRE transcript (GenBank Acc AB006682) were amplified by RT-PCR from thymus mRNA (Clontech) to generate riboprobes with primers: 5'-ATG GCG ACG GAC GCG GCG CTA CGC-3' and 5'-CCT GGA TGT ACT TCT TGG AGC CGC-3', 5'-GAG CCC GAG GGG CCG TGG AGG GGA-3' and 5'-GGC TGC ACC TCC TGG ACT GTT GCC-3', 8IF and GR1R, respectively, and were subcloned into a pCRII-TOPO vector (Invitrogen). For *in vitro* transcription the plasmids were linearised, and sense and antisense probes were synthesised with digoxigenin-UTP as described in Boehringer Mannheim Nonradioactive Hybridization Application manual pp. 44-45. Labelled probes were purified with MicroSpinG-50 columns (Pharmacia Biotech). Pre-treatment and hybridization of formaldehyde fixed, paraffin embedded tissue sections was performed as described in Boehringer Mannheim Nonradioactive Hybridization Application manual pp. 136-138.

#### **8. AIRE/Aire antibodies**

The full-coding region of AIRE was amplified from Marathon human thymus cDNA (Clontech) with primers ExF and ExR2 cloned into pET32a (Novogen) vector with His tag to produce pHPAIRE which was expressed in *E. coli*. Balb/c mice were immunised with column purified AIRE protein. Rabbit anti-AIRE

serum was prepared by immunising white rabbits with a synthetic peptide representing the 20 C-terminal amino acids of AIRE (GenBank accession no: O43918).

Eleven peptides corresponding to the predicted Aire sequence amino acids 42-55, 102-115, 128-141, 159-172, 231-244, 239-252, 287-300, 345-358, 375-388, 420-433 and 466-479 (GenBank accession no:AJ007715) were chosen according to the antigenicity prediction by the Pepsort program (GCG package, University of Wisconsin, USA) and the peptides were synthesized as described earlier (Krohn et al. 1997). Twelve-week old New Zealand White rabbits were immunized with the mixture of the peptides with an intradermal injection of 100 µg of each peptide in 0.25 ml of a 1:1 mixture of Freund's complete adjuvant (Difco Laboratories) and PBS. Two weeks later animals were boosted with intradermal injection of 100 µg of each peptide in 1:1 mixture of Freund's incomplete adjuvant (Difco Laboratories) followed by an intramuscular injection of 50 µg of each peptide four weeks later and finally the sera was collected 10 days later.

### **9. Immunohistochemistry**

For immunoperoxidase staining frozen sections and cytospin slides were fixed 20 min in 4% paraformaldehyde. Endogenous peroxidase activity was blocked by treating the slides with 0.3% H<sub>2</sub>O<sub>2</sub> in MeOH for 5 min. Appropriate dilution of the AIRE or Aire antiserum (rabbit or mouse) in PBS/1%BSA was incubated for 30 min at 37°C, biotin conjugated anti-mouse or anti-rabbit secondary antibody (Vector) further 30 min at 37°C and finally peroxidase conjugated avidin for 30 min at 37°C. The antibody binding was revealed with DAB staining kit (Vector).

For double immunofluorescence detection AIRE staining was revealed with incubating Texas Red-avidin (Vector) 30 min at 37°C. The second primary antibody in appropriate dilution was then incubated for 30 min at 37°C and the reaction was revealed with incubating FITC conjugated secondary anti-mouse antibody (Vector) 30 min at 37°C. Antibodies used were: CD11c, CD83 (Immunotech), CD40, CD80, CD86, CD13, CD 34, CD64 (Pharmingen), HAM56, CD68, CD163 (DAKO A/S) AE-1 and AE-3 (kindly provided by Dr. Barton Haynes).

For costainings on mouse tissues anti-Aire serum in 1:1000 dilution in PBS was incubated for 45 minutes at 37 °C following incubation with FITC-conjugated anti-rabbit IgG (Vector laboratories) in dilution 1:1000 for 30 minutes. For double immunostainings rat anti mouse thymic pan-epithelium Ab, MTS5, Rat anti mouse thymic medullary epithelium Ab, MTS10 (Pharmingen), mAb29 or mAb95 (Naspetti et al. 1997) were applied in 1:500 dilution for 45 minutes at 37 °C following biotinylated anti-rat IgM or anti-rat Ig secondary antibody (Pharmingen) in dilution 1:500 for 30 minutes at 37 °C and incubation with Texas Red conjugated avidin D (Vector laboratories) for 30 minutes at 37 °C. The stained preparates were mounted with Vectashield mounting medium

(Vector Laboratories) containing DAPI (4',6-diamine-2-phenylindole, Sigma) for nuclear staining, and viewed with a Zeiss Axioplan 2 epifluorescence microscope. Digital images were captured using Hamamatsu model C5985 "cooled CCD video camera". For double immunofluorescence with CD11c the anti-AIRE or preimmune serum were incubated on acetone-fixed thymic cryosections, presaturated with blocking reagent (NEN Life Science Products, Inc.), followed by a biotinylated F(ab')<sub>2</sub> sheep anti-rabbit serum and revealed using streptavidin-peroxydase. The Texas Red-tyramide substrate was then added following the manufacturer's instructions (NEN Life Science Products, Inc.). Subsequently, FITC-coupled anti-CD11c Ab (Pharmingen) was used. Pictures corresponding to 0.5 µm thick optical sections were taken on a Leica confocal microscope. Data acquisition on the FITC and the Texas Red channels was simultaneously performed after setting the photomultipliers on single colour stained sections, without further compensation.

#### **10. Transfection of AIRE into mammalian cells**

The full-coding region of AIRE was amplified from pHPAIRE construct (see above) with primers AIRE5Eco (5'-TTT TGA ATT CAC CAT GGC GAC GGA CGC GGC GCT-3') and AIRE3Hind (5'-GGG AAG CTT TCG GAG GGG AAG GGG GCC G-3'), digested with EcoRI and HindIII enzymes and cloned into pCDNA3.1-Myc-His vector (Invitrogen) to give pCAIRE. The full-length Aire cDNA was amplified with primers 5'EcoF (5'TTT TCT AGA TGG CAG GTG GGG ATG GA-3') and 3'HindRStop (5'-AAA AAG CTT TCA GGA AGA GAA GGG TGG-3'), digested with EcoRI and HindIII restriction enzymes and cloned into pCDNA3.1 Myc-His vector (Invitrogen). The correct sequence was verified by sequencing, and expression of protein was tested by Western blotting. The AIRE construct was transfected into HeLa and U937 cells by electroporation (BioRad, 960mF, 220V). Subsequently, transfected HeLa cells were grown attached to coverslips, and U937 cells were cytopinned to microscopy slides for observation in immunofluorescence using the immunocytochemistry protocols above. For colchicine and cytochalasin B treatment (both ICN Biochemicals), the cells were incubated with 1µg/ml for 17 hours. Transient transfections of AIRE construct were performed by SuperFect reagent (Qiagen) as instructed by manufacturer into Cos-1 cells and after 48 hours the cells were harvested for cell lysate and cytospin sample preparation.

#### **11. Mouse cell sorting**

Fetal thymic stromal cells depleted of lymphoid and dendritic cell precursors were obtained by organ culture of 14-day gestation fetal thymus lobe for 5-6 days in the presence of 1.35mM deoxyguanosine and purified cortical epithelial cells were obtained by immunomagnetic selection of MHC class II<sup>+</sup> cells from trypsinised suspension of these lobes as described earlier (Anderson et al. 1994). CD3<sup>+</sup> mature T cells were similarly prepared by positive selection using anti-CD-3 coated magnetic beads (Dynal). Stromal cells were prepared by enzymatic digestion of whole thymuses as described (Saunders et al. 1996). After enrichment of low-density cells by Percoll gradient, cell subsets were sorted with

a combination of PE-conjugated CD11c (Pharmingen) and FITC-conjugated 29 mAbs. Thymocyte and peripheral T cell subsets were sorted using a FACSstarPlus and FITC- or PE- conjugated antibodies (Pharmingen) directed against CD4, CD8, Thy-1, Mac1, B220, and Gr1. Thymic and splenic dendritic cells were purified by FACS sorting as described (Vremec et al. 2000).

### **12. RNA isolation from mouse tissues and isolated cells**

Total RNA was isolated from snap frozen tissue samples with Promega RNAgents<sup>®</sup> Total RNA Isolation System (Promega) according to manufacturer's instructions. RNA samples were treated with RNase free DNase to eliminate contaminating genomic DNA. First strand cDNA synthesis was performed with first strand cDNA synthesis Kit (Pharmacia Biotech) as instructed by the manufacturer. From sorted cells total RNA was purified using TRIzol reagent (GibcoBRL). One µg of total RNA isolated from sorted cell populations was reverse-transcribed with random hexamers using the SuperScript II reverse transcriptase (GibcoBRL).

### **13. Western blotting**

Transfected cell and tissue samples were homogenized and boiled for 10 minutes in 2x sample buffer containing 2% SDS, 100mM DTT, 60mM Tris-HCl pH 6.8, 10% glycerol and 0.01% bromphenol blue. Proteins were then resolved by 12 % SDS-PAGE, using Tris-glycine as running buffer and, subsequently, transferred onto nitrocellulose filter. For immunodetection non-specific binding was saturated with 5% milk in TBS for 1 hour, primary antisera was applied in 1:1000 dilution in TBS for 1 hour, biotinylated anti-rabbit in 1:500 dilution in TBS for 30 minutes following peroxidase conjugated avidin D for 30 minutes. Colour detection was performed with cloronaphtol reagent.

### **14. Mouse Aire RT-PCR amplifications**

From tissue RNA samples mouse Aire cDNA was amplified with primers D: 5'-TCG TGG GAC TGA GGT CAG CTT CAG-3' and F: 5'-TCA GGA AGA GAA GGG TGG TGT CTC-3' designed to amplify the exons 10-14 in first round and further with primers E: 5'-GCG ATG CAG TGT GTG TGG CGA TGG-3' and G: 5'-TCT GGA TGG CCC ACT GCA GGA TGC-3' corresponding to exons 11-14 in nested PCR experiment. The amplification was carried out by denaturation at 94 °C followed by 35 cycles at 94 °C 30 sec, 62 °C 30 sec and 72 °C 1 min, and a final extension at 72 °C for 5 min in a volume of 25µl containing 0.2 mM of each primer, 0.1mM of each dNTP, 10mM Tris-HCl, pH 8.8, 1.5 mM MgCl<sub>2</sub>, 50mM KCl and 0.1% Triton X-100. Control amplification was performed with G3PDH primers: 5' TCC ACC ACC CTG TTG CTG TAG and 3' GAC CAC AGT CCA TGC CAT CAC T.

For RT-PCR on sorted cells the amount of cDNA was standardized with reference β-actin-primers (forward primer: 5'-GTG GGC CGC TCT AGG CAC CAA-3' and reverse primer: 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3') using condition as above except using 30 cycles and annealing at 57°C. The Aire-

specific primers used in these PCR experiments are as above and in addition primers B: 5'-GGG AGC CCA GGT CAC TAT ACC TGG-3', and C: 5'-TGA GCT CCC TGG ATG GGC CCC TGG-3' corresponding to sequences from Aire exons 6-10. For PCR on the purified DC populations, cDNA from equivalent to 1000 purified DC and control cells were used. The primers covering exons 11 to 14 were: 5'-TCT GCA GAC TCG ACT CCC ACG CCA-3' and primer G.

## RESULTS

### 1. Identification of the AIRE gene (I)

#### 1.1 Cloning strategy

To identify the gene responsible for APECED we used genomic sequence information and previously trapped exons from a cosmid/BAC contig covering markers D21S1460/D21S1912-PFKL-D21S154 (Kudoh et al. 1997). Computer programs such as GRAIL and GENSCAN predicted several putative genes to the critical region for the APECED gene. One of the predicted genes localised just proximal to PFKL gene and contained previously trapped exons HC21Exc33 and MDL04M06. We screened several cDNA libraries with primers designed according to the predicted gene sequence. A cDNA containing the trapped exons HC21Exc33 and MDL04M06 was amplified from thymus cDNA library. The cDNA was completed using 5' and 3' race from the same thymus cDNA library. The overlapping PCR product contained a sequence of 2027 bp with an open reading frame of 1635 bp encoding a predicted protein which was named AIRE, for Autoimmune regulator. The AIRE gene is approximately 13 kb in length and has 14 exons all following the consensus AG-GT splice site rule.

RACE and RT-PCR of cDNA libraries and library screenings failed to produce additional 5' or 3' sequences. However, 5'-RACE from thymus cDNA library produced two alternatively spliced cDNAs encoding for proteins of 348 and 254 amino acids and were named AIRE-2 and AIRE-3, respectively.

#### 1.2 Structure of the predicted AIRE protein

The open reading frame coded for a predicted protein of 545 amino acids with a predicted isoelectric point of 7.32 and a calculated molecular weight of 57,723 kDa. Protein sequence analysis revealed that AIRE-1 protein has a proline rich region (aa 350-407), two PHD zinc finger motifs (aa 299-340 and 434-475), which is a cysteine rich structure containing a consensus of C4HC3 distinguished from RING finger (C3HC4) and LIM domain (C2HC5) (Aasland et al. 1995). Furthermore AIRE-1 contains four LXXLL motifs, which has been shown to be a signature motif binding to nuclear receptors (Heery et al. 1997) (Figure 9). The AIRE PHD finger motifs have strong homology to nuclear proteins such as MI-2, TIF1, Sp140 and LYSP100-B.

### 2. Mutation analysis in APECED patients (I, II and III)

In Studies I, II and III we have analysed a total of 37 apparently unrelated APECED families including 8 Finnish, 1 Swiss, 9 Northern Italians, 2 British, 1 New Zealander, 13 American caucasians of Northern or Western European origin, one of Hispanic, one of Asian and one family of Arabic origin.



One common mutation was supposed to be responsible for approximately 90% of Finnish APECED cases (Björnses et al. 1996). In our material 12/16 (75%) of Finnish APECED alleles R257X mutation was seen to be associated with the common APECED haplotype (3-5) (Björnses et al. 1996). The other Finnish APECED haplotype (7-5) was seen in one allele which has K83E mutation. The X546C seen in two alleles and 1094-1106del account presumably for the remaining Finnish APECED alleles with varying haplotypes (Björnses et al. 1996).

R257X is also the common Northern Italian APECED mutation, as in this material it accounts for 10/18 independent alleles. The other Northern Italian common mutation is 1094-1106del detected in 5/18 alleles. In patient material from North America representing the Caucasian population the most common mutation is 1094-1106del accounting for 15/26 of the APECED alleles whereas the R257X was seen in 4/26 alleles. In addition one Swiss family, was homozygous for R257X and one British and one family of Asian origin were homozygous for the 1094-1106del and one New Zealander and one British patient were heterozygous for both of these mutations.

In addition we have identified 11 mutations which are only detected in individual families either in hetero- or homozygosity (Table 6). These mutations are located through the gene coding region. However, the missense mutations changing an amino acid are mainly localised to the N-terminus whereas most of the mutations localising to exons 5-14 are either stop or frame shift mutations.

**Table 6.** Mutations and haplotypes detected in this study

<b>Patient</b>	<b>Origin</b>	<b>D21S1912</b>	<b>Mutation</b>	<b>PFKL</b>
MP	Finnish	3/3	R257X / R257X	5/5
RP	Finnish	3/3	R257X / R257X	5/5
TP	Finnish	3/3	R257X / R257X	5/5
AP	Finnish	3/3	R257X / R257X	5/5
NP	Finnish	3/7	R257X / K83E	5p/5m
VP	Finnish	3/5	R257X / X546C	5m/6p
PP	Finnish	3/5	R257X / X546C	5m/6p
KP	Finnish	3/5	R257X / 1094-1106del	5/5
SwP	Swiss	4/4	R257X / R257X	3/3
FamA	N. Italian	4/10	R257X / R203X	6/3
FamB	N. Italian	3/3	1096^1097ins CCTG / 1096^1097ins CCTG	5/5
NITA	N. Italian	4/5	R257X / R257X	5/5
NITB	N. Italian	3/3	R257X / R257X	5/5
NITC	N. Italian	3/5	R257X / R257X	5/3
NITD	N. Italian	5/5	1094-1106del / 1094-1106del	5/5

NITE	N. Italian	3/3	R257X / R257X	5/5
NIT-H	N. Italian	3/5	R257X / 1094-1106del	5/5
NIT-I	N. Italian	3/5	1094-1106del / 1094-1106del	5/5
CS	British	5/5	1094-1106del / 1094-1106del	5/5
AL	British	2 <sup>a</sup> /4 <sup>a</sup>	R257X <sup>a</sup> / 1094-1106del <sup>a</sup>	5 <sup>a</sup> /3 <sup>a</sup>
MAR	New Zealander	4 <sup>a</sup> /5 <sup>a</sup>	R257X <sup>a</sup> / 1094-1106del <sup>a</sup>	5 <sup>a</sup> /3 <sup>a</sup>
101	AmCauc	5/5	1094-1106del / 1094-1106del	5/5
105	AmCauc	2/5	1094-1106del / 1094-1106del	3p/5m
106	AmCauc	5/10	1094-1106del / 1094-1106del	5p/5m
116	AmCauc	10/10	1094-1106del / 1094-1106del	5/5
119	AmCauc	5 <sup>a</sup> /6 <sup>a</sup>	1094-1106del <sup>a</sup> / 1094-1106del <sup>a</sup>	3 <sup>a</sup> /5 <sup>a</sup>
117	AmCauc	7 <sup>a</sup> /6 <sup>a</sup>	1094-1106del <sup>a</sup> / NI <sup>a</sup>	5 <sup>a</sup> /5 <sup>a</sup>
114	AmCauc	3 <sup>a</sup> /6 <sup>a</sup>	1094-1106del <sup>a</sup> / 1423G->AC <sup>a</sup>	4 <sup>a</sup> /5 <sup>a</sup>
MEA01.03	AmCauc	5/3	1094-1106del / 318-353del	5/5
MEA02.03	AmCauc	3/5	R257X / 1094-1106del	1/5
109	AmCauc	4/4	1094-1106del / R257X	3p/5m
113	AmCauc	4/4	R257X / R257X	3p/3m
124	AmCauc	4/5	1391delC/ NI	7p/5m
118	AmCauc	4 <sup>a</sup> /6 <sup>a</sup>	L28P <sup>a</sup> / NI <sup>a</sup>	3 <sup>a</sup> /5 <sup>a</sup>
128	Hispanic	2m/10p	Q173X / IVS9-1G->A	5m/5p
104	Arabian	6/6	335 <sup>^</sup> 336insCAGG / 335 <sup>^</sup> 336insCAGG	5/5
130	Asian	6/5	1094-1106del / 1094-1106del	3/5

<sup>a</sup>)Uncertain haplotypes

NI) not identified

Nucleotide numbering according to GenBank accession no. AB006682

### 3. AIRE expression in human tissues (I, IV)

Northern blot analysis with a probe from 3' region of the AIRE transcript revealed transcripts of 2, 3, 4 and 4.5 kb in thymus, transcripts of 2, 3 and 4 kb in lymph node. Weak bands were also seen in fetal liver (2.6 kb) and appendix (4 kb). We did not detect any transcripts in other tissues, including the target tissues in APECED such as adrenal gland, adult liver and pancreas. At the time of gene identification no EST sequences representing AIRE could be found in databases presumably corresponding to the low level and tissue restricted expression of the gene. The probe specific for AIRE-2 and AIRE-3 did not reveal transcription in any of the tissues examined indicating that AIRE-1 is the predominantly expressed mRNA.

Three probes corresponding to AIRE nucleotides 127-804, 728-1176 and 1545-2000 (according to GenBank accession no. AB006684) were used to examine AIRE expression by mRNA *in situ* hybridization. In thymus AIRE mRNA expressing cells were revealed scattered in the thymus medulla and occasionally adjacent to or in the Hassal's corpuscles (Figure 4a). AIRE expressing cells were often seen to possess long cytoplasmic processes extending to adjacent small lymphocytes. Some AIRE positive cells were also detected in spleen, lymph node paracortex and fetal liver. No AIRE mRNA expression was detected in other tissues examined including the target tissues for autoimmune destruction in APECED such as adrenal cortex, thyroid glands, gonads or pancreas nor other tissues including kidney, liver, skin, lung and heart.

AIRE antisera was produced by immunising Balb/c mice with full length AIRE protein or by immunising white rabbits with a synthetic peptide corresponding to the 20 C-terminal amino acids of the AIRE protein. Immunohistochemistry with AIRE antisera revealed a staining pattern corresponding to *in situ* hybridization results. AIRE staining was seen to localise inside the nuclei of scattered medullary cells (Figure 4b). By immunofluorescence (IFL) the AIRE staining was localised to distinct dots, giving a dot like staining pattern. In the few cells expressing AIRE in lymph nodes and fetal liver AIRE protein was also shown to localise to the cytoplasm.

Double stainings with antibodies defining different cell types in thymus were analysed to characterize the cell types expressing AIRE. 90% of AIRE positive cells costained with cytokeratin antibodies AE1 and AE3 indicating that the AIRE expressing cells mainly were medullary epithelial cells. AE1 and AE3 stain low and high molecular weight cytokeratins respectively, expressed by thymus epithelial cells (Laster et al. 1986). A proportion (~20%) of AIRE cells expressed costimulatory molecules CD80 and CD86 and the CD40 antigen. Costaining of 5-10% of AIRE positive cells with the dendritic cell markers CD11c and CD83 suggested that AIRE is also expressed in thymic dendritic cells. Some AIRE cells also co-stained with CD13 and CD34 expressed in hematogenic progenitor cells. None of the macrophage markers used (CD68, CD168, HAM68 and CD64) stained AIRE cells. Some (10-20%) AIRE positive cells in lymph nodes were also positive for CD83 marker.

The *in vitro* localisation pattern was characterised by transfecting HeLa and U937 cell lines with a full-length AIRE expressing plasmid (pCAIRE). In transfected cells AIRE protein showed dual localisation in some cells into nuclear dot-like structures and in some cells into cytoplasm along fibrillar structures. Treatment with colchicine but not with cytochalasin B disrupted the fibrillar network indicating localisation with microtubular cytoskeleton or intermediate filaments.

## 6. Murine Aire expression pattern (V)

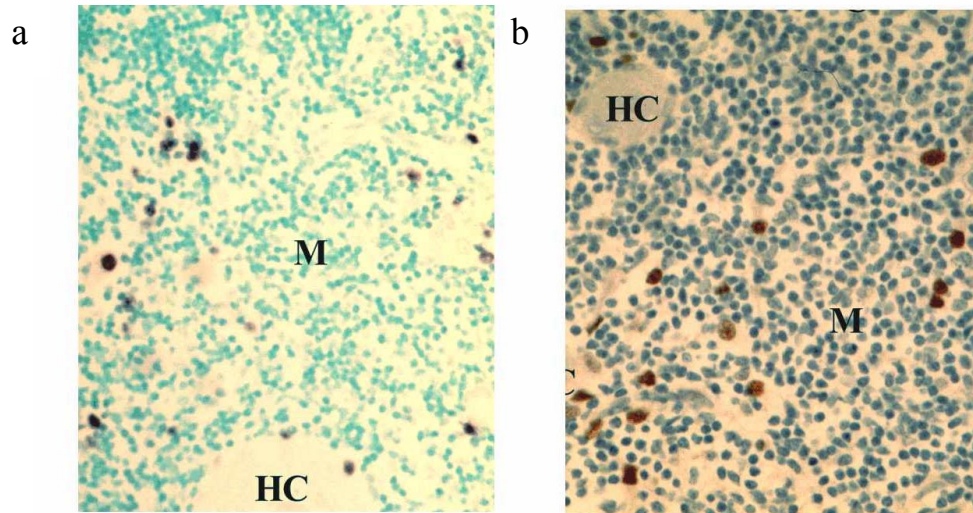
The isolation of mouse Aire gene has revealed that the mouse Aire protein shares 71% identity with the human protein and is suggested to have all the protein motifs identified in the human protein (Mittaz et al. 1999). To further characterize the expression pattern of Aire/AIRE genes we studied the expression pattern of Aire in normal adult and fetal mice.

Aire specific antiserum was produced by immunizing New Zealand White rabbits with a mixture of 11 peptides along the predicted Aire protein chosen according to antigenicity prediction by the pepsort program (GCG package, university of Wisconsin, WI). In Western Blot analysis a 60 kDa Aire-specific band was detected only from thymus but could not be detected in any other tissues examined, including skeletal muscle, testis, ovaries, kidneys, adrenal gland, lung, lymph nodes, spleen and liver. The staining was abolished by preabsorption of the serum with the peptides used to immunise the rabbits indicating specificity.

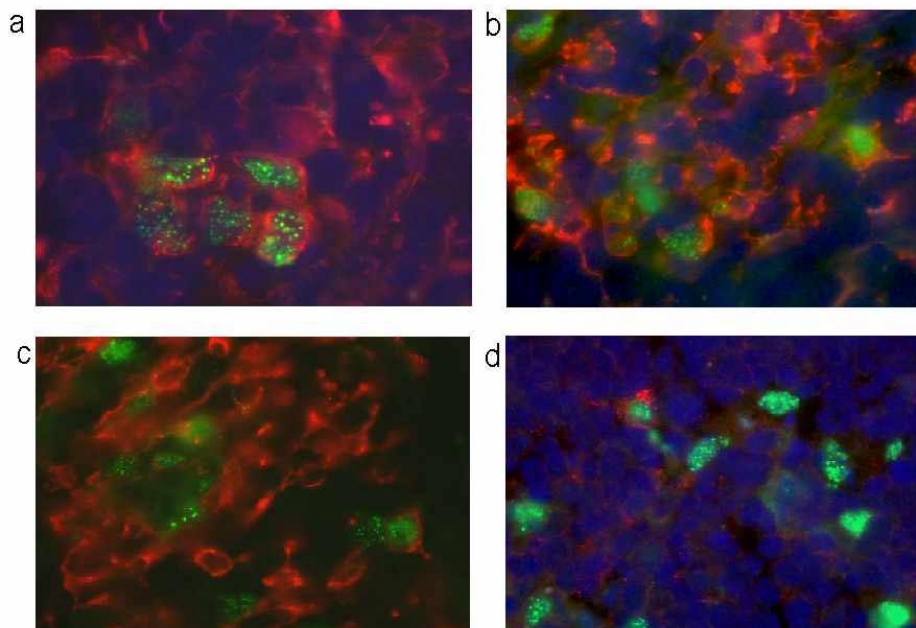
Immunohistochemistry with Aire antiserum detected Aire positive cells in the thymus medulla corresponding to the staining pattern seen in human thymus. Subcellularly Aire protein localised into the nuclei concentrated into the nuclear dot-like structures like human AIRE protein. To characterize the Aire expressing cell type in mouse thymus we used epithelial cell markers MTS5 and MTS10 which recognise all epithelial cells and medullary epithelial cells respectively. Moreover, we used monoclonal antibodies mAb29 and mAb95 which stain two previously described subpopulations of medullary epithelial cells defined as activated and resting epithelial cells by the expression of costimulatory molecules (Naspetti et al. 1997). Costainings with epithelial cell markers MTS5 and MTS10 consequently revealed that mouse Aire expressing cells were also mostly epithelial (Figure 5a and 5b). Approximately 60% of Aire-positive cells costained with mAb95 and 30% with mAb29 indicating that Aire is expressed by both “resting” and “activated” medullary epithelial cells (Figure 5c and 5d).

The expression patterns of mAb95 and mAb29 vary during thymic ontogeny (Naspetti et al. 1997). MAb95 expressing cells are seen already at fetal day 14 whereas mAb29 appear at day 15 on both stromal cells and most thymocytes. From day 15 until birth thymocyte mAb29 staining decreases and stromal mAb29+ cells become more apparent after day 16. We examined costaining of Aire and these mAbs in fetal thymuses at fetal days 14,16,18 and newborn. At these developmental stages Aire positive cells were distributed into mAb29 and mAb95 subclasses according to the adult thymus.

**Figure 4.** AIRE expression in thymus. a) AIRE mRNA in situ hybridization (AIRE positive cells stained blue) and b) AIRE protein expression detected by immunohistochemistry (AIRE stained brown). M: thymus medulla, HC: Hassal's corpuscle.



**Figure 5.** Double staining of the Aire-positive cells (green) with anti-epithelial markers (red). A) MTS10 staining MEC, b) MTS5 pan-epithelial marker, c) mAb95 and d) mAb29.

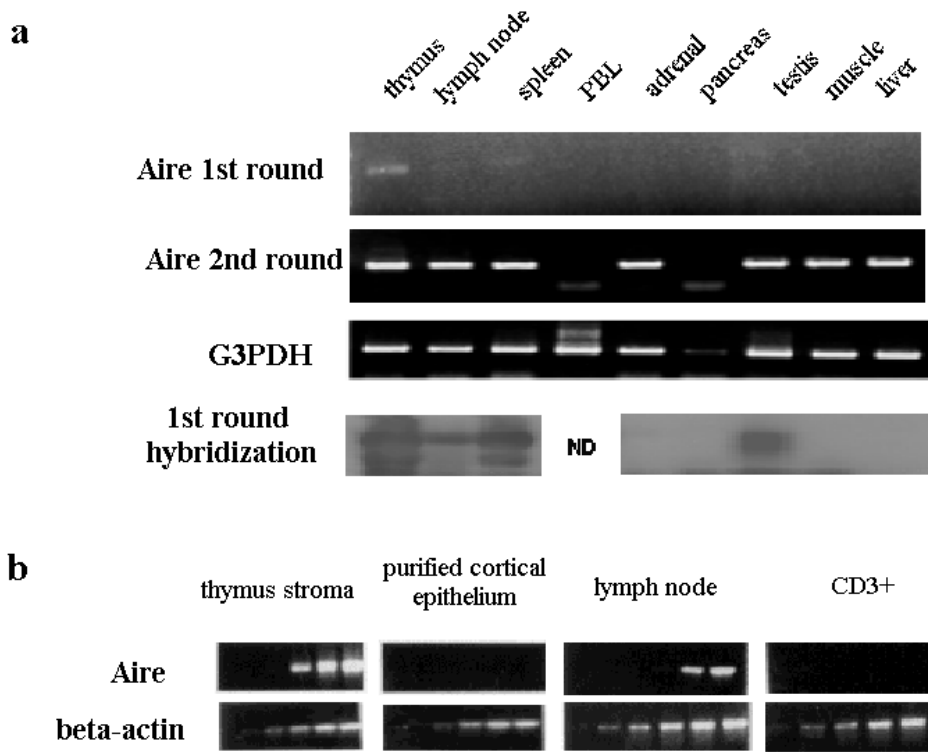


The expression pattern of Aire mRNA was examined with RT-PCR using primers spanning exons 10 to 14 following second round PCR with primers to exons 11 to 14. The first round amplification revealed Aire mRNA only from thymus, lymph node and spleen (Figure 6a). Southern hybridization of the amplification products with a digoxigenin-labeled Aire probe also revealed amplification product in testis. In second round PCR Aire mRNA could be amplified from most tissues analysed including adrenal gland, skeletal muscle and liver (Figure 6a). Aire mRNA expression was further analysed in a panel of cells isolated from fetal day 14 thymus and treated with deoxyguanoside to eliminate precursors for lymphoid and dendritic cells (Farr and Anderson 1985). Stromal cells containing cortical and medullary epithelial cells, macrophages and fibroblasts were clearly Aire positive whereas purified cortical epithelium was Aire negative. Isolated lymph node cells were positive in Aire amplification but purified CD3<sup>+</sup> T cells from lymph nodes remained negative (Figure 6b).

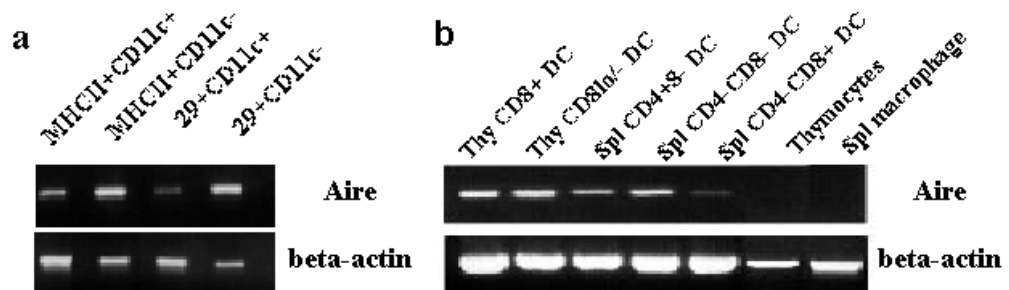
According to findings in human, in mouse thymus some Aire positive cells contained CD11c<sup>+</sup> suggesting dendritic cell expression of Aire. To further study the possibility of Aire expression in dendritic cells we employed RT-PCR analysis on isolated cells from thymus and spleen. Thymic stromal cells isolated using a combination of CD11c marker and mAb29 which distinguish the mAb29<sup>+</sup>CD11c<sup>-</sup> epithelial cells and mAb29<sup>+</sup>CD11c<sup>+</sup> dendritic cells by flow cytometry. The Aire transcript was clearly detected in the epithelial cell population but also weakly within the CD11c<sup>+</sup> dendritic cells (Figure 7a). Identical results were obtained by sorting cells with combination of MHC class II-specific antibody and CD11c antibody.

Two thymic DC populations CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>low/-</sup> and three splenic CD4<sup>+</sup>8 $\alpha$ <sup>-</sup>, CD4<sup>-</sup>8 $\alpha$ <sup>-</sup> and CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> have been identified (Vremec et al. 2000). The expression of CD8 $\alpha$  distinguishes the myeloid derived and lymphoid derived DC populations as discussed in Chapter 3.3.1. These DC populations were purified by FACS sorting (Vremec et al. 2000). RT-PCR amplification was performed on these sorted DCs with primers covering exons 11-14 of the Aire gene. Here Aire mRNA was detected in all of the mentioned DC populations with a slightly stronger expression in thymic DC populations. Thymocytes and splenic macrophages remained negative for Aire expression (Figure 7b).

**Figure 6.** a) Aire mRNA detection by RT-PCR. B) Aire mRNA is amplified from fetal thymic stromal cells but not from sorted cortical epithelium, Aire mRNA is expressed in lymph nodes but not in sorted CD3+ T cells from lymph nodes.

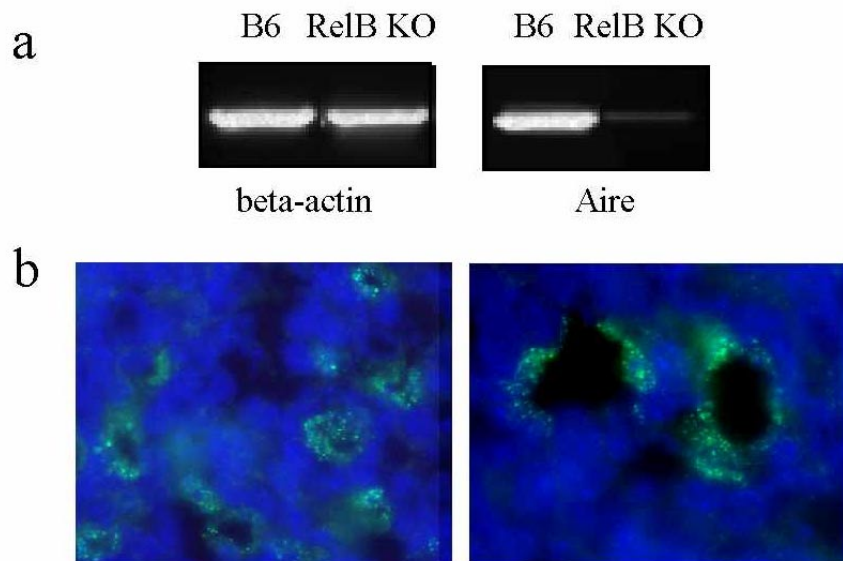


**Figure 7.** a) Aire mRNA is detected in sorted thymic mAb29+CD11c- and MHC II+CD11c- MEC, but also in mAb29+ CD11c+ and MHC II+CD11c+ DCs. b) Aire mRNA is expressed in all thymic and splenic DC populations but not in thymocytes and splenic macrophages.



RelB-deficient and NOD mouse strains both show abnormalities in thymic structure and function and have autoimmune reactions in their phenotypes (Kolb 1987, Weih et al. 1995), which prompted us to study Aire expression in these mouse models. RT-PCR amplification using primers to exons 6-10 or exons 10-14 from RelB KO mouse thymus amplified only faint Aire product even when using nested PCR (Figure 8a). In immunohistochemistry no Aire positive cells were detected in the thymus of RelB KO mice indicating that Aire protein expression is virtually absent in RelB deficient thymus. In Western blot of NOD mouse thymus Aire specific band corresponding to that seen in normal mouse was detected. In immunohistochemistry Aire protein was accordingly predominantly seen in medullary epithelial cells. However, most of Aire positive nuclei showed a strikingly abnormal morphology where the nuclei were large and vacuolated and Aire staining localised to the periphery of the nuclei (Figure 8b).

**Figure 8.** a) By RT-PCR Aire mRNA is amplified on a significantly lower level in RelB KO thymus than in normal B6 thymus. b) Abnormal morphology of the Aire positive cells (green) in NOD thymus.





## DISCUSSION

### 1. AIRE has features indicative for a transcriptional regulator

The protein motifs in the AIRE protein strongly suggest its role in transcription regulation. At the PHD zinc finger domains AIRE shows homology with several nuclear proteins including Sp100, Sp140, and the CBP and p300 proteins which activate transcription through the cAMP-responding transcription factor CREB (Chrivia et al. 1993). PHD motifs are also present in several proteins involved in transcriptional chromatin modulation including Mi-2 (Ge et al. 1995), TIF1 (transcription intermediary factor1) (Thenot et al. 1999), ALL-1 (Yano et al. 1997), ATRX (Gibbons et al. 1997) and KRIP-1 (Kim et al. 1996). For Sp100 and Sp140 protein the homology with AIRE is shared beyond the PHD motifs. The 100 N-terminal amino acids of AIRE show significant homology to the HSR domain of Sp100 and Sp140. Furthermore, a novel putatively DNA binding domain, called the SAND domain (Gibson et al. 1998) was identified in AIRE, Sp100 and Sp140 proteins. Recently it has been shown that AIRE can mediate transcriptional activity (Pitkänen et al. 2000, Björnses et al. 2000, Pitkänen et al. 2001) and can bind to the CBP protein (Pitkänen et al. 2000) further strengthening the idea of AIRE involved in the regulation of transcription. The HSR domain in AIRE mediates AIRE-AIRE homodimerization, according to Sp100 and Sp140 proteins, and the dimerization is suggested to be necessary for the transcriptional transactivation function (Pitkänen et al. 2000).

AIRE harbours four LXXLL motifs, which are known to mediate interaction with the nuclear steroid receptors in other proteins (Heery et al. 1997). Nuclear receptors regulate gene expression during development and differentiation in response to several hormone ligands such as steroid and thyroid hormones and retinoids (Laudet 1997). Whether AIRE can bind to nuclear receptors remains to be tested. However, in this respect the finding that steroid hormones can regulate immune regulation and tolerance induction in thymus is of special interest (Vacchio et al. 1998).

As expected from the putative transcription regulator pattern of the AIRE protein and the nuclear localisation signal, AIRE protein in thymus is localised to the nuclei of the cells. The speckled nuclear localisation of the AIRE protein resembles the promyelocytic leukemia (PML) nuclear bodies, also known as ND10, nuclear dots, or potential oncogenic domains (PODs) (Doucas and Evans 1996, Sternsdorf et al. 1997). Often the nuclear dots have been described to be specifying functional organization such as sites for transcription or pre-mRNA splicing (Lamond and Earnshaw 1998). The nuclear bodies are suggested to regulate gene expression by repressing transcription rather than by activating it (Lamond and Earnshaw 1998). Although AIRE dot pattern resembles that seen with PML and Sp100 AIRE seems to be localising other nuclear compartments as non-overlapping co-staining has been reported (Rinderle et al. 1999, Björnses et al. 1999).

The cytoplasmic localisation *in vitro* in transfected cells and in rare lymph node and fetal liver cells indicate that AIRE also binds to cytoplasmic microtubular structures. Cytoplasmic staining has been confirmed by others and it has been shown to be co-localising with vimentin (Rinderle et al. 1999, Björnses et al. 1999). The AIRE localisation both in the nucleus and cytoplasm may indicate that AIRE is shuttled between these two compartments. Shuttling may mediate regulation of AIRE activity through binding to cytoplasmic proteins, similar to the regulation of NF- $\kappa$ B/RelA family of transcription factors (Crepieux et al. 1997).

## 2. Mutations in APECED patients

### 2.1 Mutational hotspots

R257X mutation was first detected in 10/12 (83%) Finnish APECED alleles associated with the common APECED haplotype (4-3-5) of markers JA-1, D21S1912 and PFKL, suggesting that this is the common Finnish APECED mutation. In Study II the same mutation was also shown to be the common mutation in Northern Italian APECED patients. The same mutation was moreover detected in several patients of different origin and associated with several different haplotypes of the markers in this work and by others (I, II and III), (The Finnish-German APECED Consortium 1997, Wang et al. 1998, Pearce et al. 1998, Rosatelli et al. 1998, Ward et al. 1999, Björnses et al. 2000). Association of this mutation to several different haplotypes reflects the occurrence of a mutational hotspot at the CpG dinucleotide known to be susceptible to C  $\rightarrow$  T transitions (Yousoufian et al. 1986, Cooper et al. 1995).

A 13 bp deletion in exon 10, 1094-1106del, was shown to be the other common APECED mutation as it was observed in several patients of different origin (I, II and III). This mutation is also seen to be associated with several different haplotypes, 9 in our data, indicating another hotspot for mutations, most likely by the formation of hairpin structures from the imperfect inverted repeat near the ends of the mutation site (GGCCTGCCTGTC-**CCCTCCGCTCCGG**), the deletion in bold and the imperfect inverted repeat underlined), which is also a known mutational mechanism (Scott et al. 1992). Pearce et al. reported that the same mutation is responsible for 17/24 of British APECED alleles and associated with one haplotype indicates founder effect in the British population (Pearce et al. 1998).

In addition to the common mutations we identified 11 mutations in APECED patients including nonsense mutations, missense mutations, insertions, deletions and splice site mutation. By now, several other reports of APECED mutations have been published giving the total number of identified mutations 34 (The Finnish-German APECED Consortium 1997, Wang et al. 1998, Pearce et al. 1998, Rosatelli et al. 1998, Myhre et al. 1998, Ward et al. 1999, Björnses et al.

2000, Söderbergh et al. 2000, Ishii et al. 2000) (Table 7). In contrast to the two common mutations, most of these mutations have only been found in isolated APECED patients. Although the mutations are spread throughout the coding region, two other regions susceptible to mutations appear to localise to exons 2 and 10 with so far reported seven and nine mutations respectively (Figure 9). None of the exon 2 mutations are due to GpC dinucleotides while 6 of the 8 exon 10 mutations are due to errors in replication of repetitive strings of nucleotides 2 or more.

## **2.2 Implications for functional domains**

Of special interest in respect to the function of the gene are the disease causing missense mutations which in published material so far are mostly concentrated on the HSR region, the first 100 amino acids of the AIRE protein. The HSR domain has a predicted  $\alpha$ -helical four-helix bundle structure, which is suggested to be vulnerable to conformational changes which may account for the disease causing nature of missense mutations in this region (Pitkänen et al. 2000). In contrast to the Sp100 and Sp140 proteins where the HSR domain is responsible for targeting these proteins into the nuclear bodies the HSR region in AIRE mediates microtubular localisation (Sternsdorf et al. 1999, Pitkänen et al. 2001). APECED mutation L28P abolishes the dimerization and transactivation properties of AIRE and fails to localise the protein to the microtubular structures (Pitkänen et al. 2000, Pitkänen et al. 2001).

Most of the APECED mutations are either nonsense or frame shift mutations which would abolish at least second of the two PHD motifs, indicating a functional role for these in the AIRE protein. Mutations in PHD domains have been reported to be responsible for genetic disorders. In ATR-X syndrome, caused by mutation in the ATR gene mutations, mutations in the PHD domain lead to classical form of the syndrome (Gibbons 1997). Björnses et al. have identified two missense mutations in the first PHD domain of AIRE protein (Björnses et al. 2000) further supporting the importance of this structure to the function of the protein. Mutated AIRE protein with disruption of the first of the zinc fingers (C311Y) has been shown have reduced transcriptional activity and to localise mainly in the cytoplasm in small granules indicating the role of PHD domain in nuclear targeting of the protein (Björnses et al. 2000). Pitkänen et al suggest that the AIRE protein contains a nuclear transport signal in its C-terminus but outside the PHD domains (Pitkänen et al. 2001).

One of the missense mutations destroys the stop codon of normal AIRE protein and leads to the addition of 60 amino acids to the protein. The patients heterozygous for this and the common Finnish mutation show typical APECED phenotype with early onset of candidiasis, hypoparathyroidism and Addison's disease indistinguishable from the R257X homozygotes (II). This mutation has been reported in three additional patients by others (Björnses et al. 2000). Although the protein produced by this allele has all the coding motifs the

additional amino acids probably affect the three-dimensional folding of the protein and thus interaction with other proteins.

### **2.3 Clinical relevance**

Despite the growing number of different APECED causing mutations the phenotypic variation between APECED patients cannot be explained by allelic variation in the AIRE gene. The considerable phenotypic variation of APECED in Finnish patients who are known to have homozygous common Finnish mutation and variation in phenotype between siblings indicates that there are other genetic or environmental factors determining the phenotype of the disease. Genotype-phenotype correlation may only be possible after long-term follow-up and division of patients into subgroups such as different HLA genotypes or other genetic components modifying autoimmunity. Although the risk associated HLA genotypes for APECED are not known, HLA alleles mediating protection against certain autoimmune diseases may still be functional. Protection by certain HLA alleles is indicated in a study where out of 67 APECED patients non of the 11 diabetics but 15/56 nondiabetics had the DQB1\*0602 allele considered to be protective for type 1 diabetes (Gylling et al. 2000).

In the Iranian Jewish population the phenotype of APECED seems to be milder than in Finnish population. Iranian Jewish patients rarely have mucocutaneous candidiasis and ectodermal dystrophies and often hypoparathyroidism is the only clinical entity of the disease (Zlotogora and Shapiro 1992). The Iranian Jewish common mutation Y85C does not affect the subcellular localisation of the protein and this mutation was also shown to retain transcriptional activity (Björnses et al. 2000). Therefore it can be speculated that the milder phenotype might be appreciated by functional properties carried by this mildly affected AIRE protein (Björnses et al. 2000).

For APECED patients and their families the possibility for genetic diagnosis is of great importance when it allows organisation of necessary follow-up for those carrying mutated alleles and avoiding of unnecessary follow-up of those family members not having risk for APECED. At present, when diagnosing a new possible APECED patient the detection of the two common mutations is relatively simple. The R257X mutation destroys a *TaqI* restriction site and can thus be demonstrated easily by *TaqI* digestion (I). The 1094-1106 del can also be easily detected by fragment length difference in agarose gel electrophoresis (III). If these common mutations cannot be detected sequencing of the coding region for the gene can be performed, as the gene is relatively short, composed of only 14 exons. Efficient mutation screening of the AIRE gene by SSCP has also been reported (Björnses et al. 2000). However, it must be kept in mind that there may be disease causing mutations in the promoter or intronic region of the gene as there has been reported so far several APECED alleles where mutations could not be detected in the coding region.

**Table 7.** Mutations detected in APECED patients

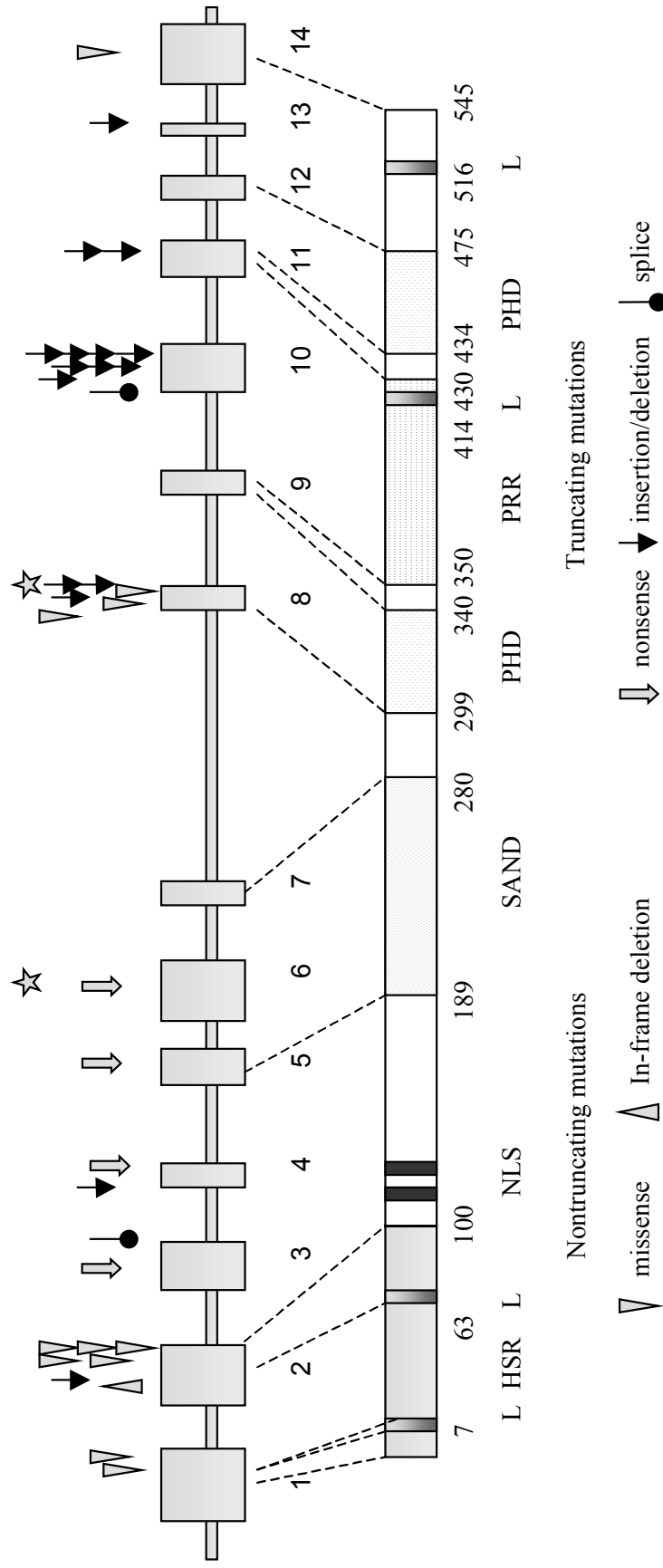
CDNA change (AB006682)	CDNA change (AIRE ORF)	exon/ intron	Effect on coding sequence	Population	No of alleles	Ref.
171 G>T	44G>T	E1	R15L	British	1	3
210 T>C	83T>C	E1	L28P	Am Cauc, British	2	3, III
365 G>T	238G>T	E2	V80L	Italian	1	7
374 A>G	247A>G	E2	K83E	Finnish	1	I
381 A>G	254A>G	E2	Y85C	Iranian Jewish	26	7
396 A>G	269A>G	E2	Y90C	British	1	3
318-353del	191-226del136bp	E2	del64- 75&D76Y	Am Cauc	1	III
335^6insCAGG	208^9insCAGG	E2	D70fsX216	Arabian	2	III
405 T>G	278T>G	E2	L93R	French-Canadian	1	6
542 C>T	415C>T	E3	R139X	Sardinian	19	2
IVS3+2 T>C	IVS3+2 T>C	I3	GT>GC	USA	1	4
635^636ins13bp	508^509ins13bp	E4	A170fsX219	German	1	7
644 C>T	517C>T	E4	Q173X	Hispanic, Italian	2	III, 7
734 C>T	607C>T	E5	R203X	North Italian, Italian	6	II, 7
896 C>T	769C>T	E6	R257X	Finnish, North Italian, Am Cauc, Swiss, British, New Zealand, Swedish, Dutch, German, Norwegian	170 (of these Finnish 129)	I, 1, II, 4, III, 5, 6, 7
1028G>A	901G>A	E8	V301M		1	9
1058delT	931delT	E8	C311fsX376	French	2	7
1059G>A	932G>A	E8	C311Y	Finnish	2	7
1094-1106del	967-979del13bp	E8	C322fsX372	Finnish, North Italian, Am Cauc, British, New Zealand, Dutch, German, Canadian, Swedish	81	1, II, 2, 3, 4, III, 6, 7
1096insCCTG	969^970ins CCTG	E8	L323fsX372	North Italian, Italian	6	II, 7
1101C>A	977C>A	E8	P326Q	Finnish	1	7
1230^1231insC	1103^1104insC	E10	P370fsX370	Japan	1	8

1290 <sup>^</sup> 1291ins A	1163 <sup>^</sup> 1164insA	E10	M388fsX422	Finnish	2	7
1316delC	1189delC	E10	L397fsX478	French	2	7
1320delC	1193delC	E10	P398fsX478	French, Italian	4	1, 7
1371 <sup>^</sup> 2insC	1244 <sup>^</sup> 1245insC	E10	L417fsX422	German	2	7
1369 <sup>^</sup> 70insA	1242 <sup>^</sup> 1243 insA	E10	H415fsX422	Norwegian	1	5
1376delC	1249delC	E10	L417fsX478	British	1	3
1391delC	1264delC	E10	P422fsX478	Am Cauc	2	III
IVS9-1G>A	IVS9-1G>A	I9	AG>AA skip exon 10/60aa	Hispanic	1	III
1422 <sup>^</sup> 3insAC	1295insAC	E11	C434fsX479	USA	1	4
1423G-->AC	1296delG insAC	E11	R433fsX502	Am Cauc	1	III
1640delG	1513delG	E13	A502fsX519	Japan	1	8
1765A>T	1638A>T	E14	X546C + 59aa	Finnish	5	II, 7

1) The Finnish-German APECED Consortium 1997, 2) Rosatelli et al. 1998, 3) Pearce et al. 1998, 4) Wang et al. 1998, 5) Myhre et al. 1998, 6) Ward et al. 1999, 7) Björnses et al. 2000 8) Ishii et al. 2000, 9) Söderbergh et al. 2000

Mutation nomenclature according to (den Dunnen and Antonarakis 2000)

**Figure 9.** Mutations in APECED patients detected in the AIRE gene and schematic representation of the AIRE protein. The two common mutations are highlighted with an asterisk. HSR domain (HSR), PHD zinc finger motif (PHD), proline-rich region (PRR), the LXXLL motif (L), SAND domain (SAND).



### **3. AIRE/ Aire gene is strongly expressed in thymic medullary epithelial cells and at low level in peripheral lymphoid organs and in dendritic cells**

Northern blot analysis with a probe from 3' region of the AIRE cDNA (exons 12-14) revealed transcripts of 2, 3, 4 and 4.5 kb in thymus; transcripts of 2,3 and 4kb in lymph node and 2,6 and 4 kb in fetal liver and a weak 4 kb signal in the appendix. No AIRE signal was detected in APECED target tissues such as adrenal cortex or pancreas. Another group reporting the identification of AIRE gene in contrast reported expression of the same gene as 2.0 kb transcript in several tissues including thymus, pancreas and adrenal cortex with full-length AIRE cDNA as a probe (The Finnish-German APECED Consortium 1997). We were also able to detect ubiquitous expression of a transcript of 2kb with a probe from 5' end of the AIRE cDNA (exons 1-2) and suggest that this is non-specific detection with the probe from 5' having high CG content (not published). This interpretation has also been supported by others (Blechsmidt et al. 1999).

By mRNA *in situ* hybridisation and by immunohistochemistry AIRE expression was limited to lymphoid tissues. AIRE positive cells were seen in thymus medulla and rare AIRE positive cells were also detected in lymph nodes and spleen. Inconsistent results have been published by others (Rinderle et al. 1999, Björnses et al. 1999). In addition to strong thymic staining, staining was reported in spleen and lymph nodes in a large number of lymphocytes and neutrophilic granulocytes. In PBL smears a large population of lymphocytes, neutrophilic granulocytes and monocytes was reported to show strong staining as well (Björnses et al. 1999). In contrast, another group reported expression in very few cells in PBL smears (Rinderle et al. 1999). The discrepancy in the findings can partly be explained by the use of different antibodies and differences in their affinity and epitope recognition. However, we obtained similar results by using two different antisera one of which was targeted at the 20 N-terminal amino acids of the protein and other was polyclonal serum against the whole AIRE protein. Later we have also confirmed our results with monoclonal antibodies (not published).

Characterisation of the murine homologue for AIRE gene and its expression pattern is essential for determining whether the genetically engineered Aire deficient mouse could be considered as a model for APECED pathogenesis. The mouse Aire gene has been isolated by several groups (Blechsmidt et al. 1999, Wang et al. 1999, Mittaz et al. 1999). The gene is very similar to the human gene, sharing 71% homology at the protein level, and all the protein motifs present in AIRE gene. Furthermore isolation of Aire gene allowed identification of the conserved nuclear localisation signal shared with these proteins (Mittaz et al. 1999). Several groups reported that Aire expression was not detected by Northern Blot analysis (Blechsmidt et al. 1999, Wang et al. 1999, Mittaz et al. 1999). However, recently a group reported detection in Northern Blot analysis in thymus, spleen and lymph node (Zuklys et al. 2000). Aire amplification by RT-



PCR from various tissues has been reported (Blehschmidt et al. 1999, Ruan et al. 1999, Halonen et al. 2001). We detected amplification from thymus, spleen and lymph nodes and using nested PCR Aire mRNA could be amplified from most tissues analysed including adrenal gland, skeletal muscle and liver. This may indicate that in these tissues Aire gene is expressed at low levels, for example by a rare migrating cell type such as dendritic cells. Another possibility is that the low-level expression is a result of illegitimate transcription due to a “leaky promoter”.

The expression pattern of Aire protein by immunohistochemistry proved to be consistent with human AIRE. Strong expression was detected in thymus medullary epithelial cells localising to nuclear dot-like structures. No Aire protein expression could be detected outside thymus either by Western blotting or by immunohistochemistry. However recently Halonen et al. reported Aire protein also outside the immune system. They detected Aire protein in brain, liver, kidney, pancreas, intestines, gonads, pituitary, thyroid and adrenal glands. The cells expressing AIRE in these tissues represented diverse cell types from epithelial cells in several organs to neurons and glial cells in the central nervous system (Halonen et al. 2001). In these studies an antisera against a synthetic polypeptide corresponding to Aire amino acids 160-176 was used (Halonen et al. 2001).

In *in vitro* transfections Aire protein has dual localisation properties similar to human protein. Occasionally Aire protein was seen in nuclear dots but in most cells protein localised along fibrillar structures in the cytoplasm. High similarity in the gene coding region and also in the promoter region would suggest similar function of AIRE and Aire proteins. This is further supported by the similar expression pattern and subcellular localisation pattern of these proteins.

Co-staining with some cellular markers suggested that in human thymus a minority of AIRE expressing cells is not epithelial but represent hematopoietic derived dendritic cells. Therefore we analysed Aire expression by RT-PCR in sorted dendritic cells from mouse lymphoid tissues. Indeed, various dendritic cell populations isolated from thymus and spleen appeared to express Aire mRNA. Comparison of promoter regions for AIRE and Aire genes revealed conserved transcription factor binding sites for the essential components of the basal transcriptional complex and in addition binding sites for thymus specific transcription factors and factors important in hematopoiesis (Mittaz et al. 1999). This finding supports our finding that monocyte derived DC express the Aire gene at least in lymphoid tissues and probably also in other peripheral tissues. In addition to the classical myeloid dendritic cells Aire expression was detected in the CD8<sup>+</sup> DC representing the lymphoid related DCs (see Chapter 3.3.2). In this study Aire expression was not detected in thymocytes or CD3 positive lymphocytes from lymph nodes, further supporting a limited cellular expression pattern for AIRE and Aire genes.

#### 4. Aberrant Aire expression in NOD and RelB knock-out thymus

RelB is a transcription factor that belongs to the NF- $\kappa$ B family of transcription factors. These transcription factors are characterised by interaction with each other and are regulated by I $\kappa$ B inhibitor (Baldwin, Jr. 1996). RelB expression in thymus is restricted to medulla and is responsible for the development of myeloid DC and UEA1<sup>+</sup>mAb29<sup>+</sup> activated epithelial cells (Carrasco et al. 1993, Burkly et al. 1995, Wu et al. 1998). In RelB deficient mouse the thymus architecture is disrupted with the mAb95<sup>+</sup> MEC scattered in the cortex and disorganised corticomedullary junction and the mAb29<sup>+</sup> cells are absent. Although T lymphocyte development in these mice is completed, negative selection is affected (Laufer et al. 1996, Wu et al. 1998). The lack of Aire expression in RelB-KO thymus may be caused by the lack of the mAb29<sup>+</sup> cells, however the Mab95<sup>+</sup> cells expressing Aire in normal thymus are also absent in these mice. Lack of Aire expression has also been reported by others (Zuklys et al. 2000). They also found that Aire is not expressed in thymi of Tg $\epsilon$ 26 mice where thymocyte development is arrested at the prethymic CD44<sup>+</sup>CD25<sup>-</sup> stage but is expressed in Rag<sup>null</sup> mice thymi where thymocyte development is blocked at a later stage. This indicates that Aire expression is associated with the correct establishment of a regular thymic microenvironment (Zuklys et al. 2000). A direct transcriptional regulation of Aire by RelB is unlikely, since the promoter region of Aire does not contain conserved NF- $\kappa$ B elements (Mittaz et al. 1999).

The NOD mouse is a model for type 1 diabetes due to several similarities to human diabetes pathogenesis and genetics (Atkinson and Leiter 1999). The thymic microenvironment in NOD mouse shows premature involution where cells seem to lose their limiting membranes and become crossed by cystic cavities bordered by epithelial cells containing granulations (Nabarra and Andrianarison 1991). The size of the medulla in NOD thymus is slightly reduced and similar to RelB-deficient mice, the mAb95<sup>+</sup> MEC are scattered into the cortex (Atlan-Gepner et al. 1999). This altered phenotype is already seen before autoimmune manifestations and is shared with other autoimmune prone mice, for example NZB mice (Watanabe et al. 1993, Thomas-Vaslin et al. 1997). These microenvironmental changes have been suggested to be linked to impaired thymocyte maturation, which in turn predisposes to autoimmune disease (Watanabe et al. 1993). Thymus epithelium apparently has a determining role in the development of autoimmunity. Grafting of NOD thymus epithelium to athymic non-autoimmune prone mouse strain has been shown to result in tissue-specific autoimmunity (Georgiou and Mandel 1995, Thomas-Vaslin et al. 1997). Sequence analysis of the coding region for Aire in B6, NOD and SJL mice has not revealed any sequence variations (Shi et al. 1999). If Aire is related to the pathogenesis of autoimmunity in NOD mouse remains to be studied. However, it can be speculated that nuclear disorganisation of Aire expressing cells detected in this study (V) is likely to affect the function and may thus lead, for example, to impaired negative selection of the diabetogenic thymocytes.

## 5. Role of AIRE in immune/tolerance regulation

Besides the missense mutations in APECED patients localising to the HSR and first of the PHD motifs, most of the APECED mutations are either frame shift or nonsense mutations. Whether other kind of mutations in AIRE gene can contribute to development of other diseases remains to be investigated. The lack of phenotype-genotype correlation and the fact that mutations in AIRE can cause different phenotypes indicate that AIRE may contribute to other genetic diseases. For a number of genetic disorders different mutations in different domains of the gene have been shown to be responsible for distinctive phenotypes (Romeo and McKusick 1994, Horton 1997, Radhakrishna et al. 1997). Due to its expression pattern in thymus medulla AIRE may also be involved in other autoimmune diseases, possibly contributory to polygenic diseases. In this respect, interestingly, the prevalence of type 1 diabetes is highest in Finland and Sardinia, two populations where the occurrence of APECED is most common. Analysis of 90 cases of sporadic Addison's disease for the 1094-1106del suggested that this mutation does not have a major role in Addison's disease (Vaidya et al. 2000). In a study of 726 patients with Addison's disease, IDDM, Graves' disease and Hashimoto's thyroiditis for the mutation R257X the mutation was found only in one subject with Hashimoto's thyroiditis in heterozygous form suggesting that this mutation does not contribute to susceptibility for isolated autoimmune diseases (Meyer et al. 2001). However, future association studies with additional AIRE polymorphisms, including promoter polymorphisms, may show a link between AIRE and other organ specific autoimmune diseases.

The phenotype of APECED suggests a defect in tissue specific tolerance indicated by the presence of tissue specific autoantibodies and autoimmune diseases. Due to the expression of AIRE in mTE the lack of tolerance can be considered as a result of impaired negative selection of autoreactive T cells as the elimination of potentially self-reacting lymphocytes is mainly believed to take place in the thymic medulla (Nossal 1994). Although the molecular mechanisms leading to tolerance induction in thymus are largely unknown, the importance of medullary epithelial cells has been established (see Chapter 3.2.3). Studies by Zuklys et al. indicate that Aire expression in thymus can be modulated by thymocytes undergoing negative selection and is associated with TCR-mediated apoptosis. They reported that in thymi undergoing only positive selection, Aire positive mTE cells were scattered throughout the thymus. In contrast, in thymi undergoing only negative selection a large number of Aire expressing epithelial cells were seen in the medulla (Zuklys et al. 2000).

The predicted functional domains and some experimental data (Pitkänen et al. 2000, Björnses et al. 2000) indicate that AIRE may mediate its function by regulating the expression of other genes. Some candidates for this regulation might be the co-receptors known to be involved in negative selection, such as B7-molecules, CD30 or Fas-FasL (see Chapter 3.2.2). Another interesting

regulatory pathway is through the expression of tissue specific antigens by mTE. Apparently expression of specific T-cell dominating epitope can be crucial for tolerance assurance such as the DM20 epitope of PMP in EAE C57Bl/6 mouse (Klein et al. 2000)(see Chapter 3.2.4). Also, the expression level of a certain antigen in mTE can affect the efficiency of negative selection as indicated by the correlation between allelic variation at the promoter region of insulin gene and susceptibility to type 1 diabetes (Pugliese et al. 1997, Vafiadis et al. 1997). Of the autoantigens in APECED at least mRNA expression of steroidogenic enzymes P450SCC and P450c21 in thymus have been detected (Pazirandeh et al. 1999). In future straightening out the genes up- and down- regulated by AIRE will elucidate whether the pathogenesis of APECED is related to one of these mechanisms and presumably will also elucidate the mechanisms of negative selection and tolerance induction in general.

## SUMMARY AND CONCLUSIONS

In this thesis a novel gene, mutations of which are responsible for a rare autoimmune disease Autoimmune Polyendocrinopathy—Candidiasis—Ectodermal Dystrophy (APECED) has been identified. The gene had been localised by linkage studies to chromosomal region 21q22.3. Using sequencing data provided from this region in terms of the Human Genome Project and artificially trapped exons from this critical region we attempted to identify candidate genes for APECED. One of the genes identified proved to be mutated in APECED patients. This gene codes for a protein having several predicted functional motifs indicative of a transcriptional regulator and several homologous proteins known to be involved in the transcription regulation and was thus named AIRE for Autoimmune Regulator.

In this thesis 13 mutations in 37 APECED patients are described. One of them is the common Finnish APECED mutation R257X, which is also reported to be the common Northern Italian mutation. Several other patients carrying the R257X mutation with different haplotypes of closely linked markers are described, indicating a hotspot for mutations. The other common mutation and also a hotspot for mutations is the 13bp deletion 1094-1106del. In this study this mutation was the most common in a patient group representing North American Caucasians and was also identified in several patients of different geo-ethnic origins.

In the fourth article of this study AIRE expression is revealed by mRNA *in situ* hybridization in thymus medulla and in rare cells in spleen, lymph nodes and fetal liver but not in other tissues. By immunohistochemistry the expression is predominantly seen in thymic medullary epithelial cells but also in a few cells presumably representing hematopoietic derived cells. In the medullary epithelial cells AIRE protein localises inside the nucleus concentrated into dot-like structures resembling the PML nuclear bodies. *In vitro* in transfected cells AIRE shows dual localisation pattern. In the nucleus AIRE is seen in dot like structures and in the cytoplasm fibrillar staining pattern resembling intermediate filament or microtubules is seen. Localisation to nuclear dots is compatible with the predicted nuclear localisation signal in the AIRE protein and further supports the role in transcription for AIRE.

AIRE gene has a murine homologue Aire, sharing 71% identity with the human gene and protein. In the fifth article of this study spatial and temporal expression pattern of Aire was studied. Aire protein expression is also predominantly seen in thymus medullary epithelial cells. Subcellular localisation in thymus and *in vitro* in transiently transfected cells resembled that seen with human AIRE. Expression of Aire mRNA in dendritic cell subpopulations isolated from thymus and spleen representing both lymphoid and myeloid dendritic cell lineages was detected by RT-PCR. No Aire mRNA expression could be detected in thymocytes or in T cells isolated from lymph nodes.

Aire expression was also studied in the thymi of RelB-deficient mice, known to show abnormal thymus development leading to inefficient negative selection and in thymi of NOD mouse, which is considered as a model for human type 1 diabetes. In RelB-deficient mice Aire expression could not be detected and in NOD mice Aire expressing cells showed abnormal morphology. These findings suggest that Aire may contribute to the autoimmune phenomena seen in these two mouse models.

The identification of the gene defective in APECED and the characterization of the mutation pattern enable direct DNA diagnosis for APECED patients. Predicted functional domains in the AIRE protein and subcellular localisation into nuclear dots suggest that AIRE may act as transcriptional regulator. In future identification of the molecules interacting with AIRE and the genes regulating AIRE or regulated by AIRE will presumably clarify the pathogenesis of autoimmunity in APECED patients and enable the development of new therapeutic strategies. The clinical picture in APECED and expression of AIRE in thymic medullary epithelial cells and also in dendritic cells, both known to be involved in negative selection of thymocytes, indicates that AIRE may be regulating tolerance induction in thymus medulla. These studies provide tools for further study of the role of AIRE in the molecular mechanisms involved in negative selection in thymus.

## ACKNOWLEDGEMENTS

This study was carried out at the department of Pathology, Institute of Medical Technology, University of Tampere during the years 1996-2000.

I would like to express my deepest gratitude to my supervisors Professor Kai Krohn M.D., Ph.D. and Docent Pärt Peterson Ph.D. for introducing me to the fascinating world of autoimmunity and giving me the chance to work with them at this most interesting project. Their devotion to science has been the base and driving force for these studies.

My sincere thanks are due to Docet Aaro Miettinen M.D., Ph.D. and Docent Olli Lassila M.D., Ph.D. for their careful review and constructive criticism of the manuscript.

I am most grateful to Professor Stylianos Antonarakis M.D., Sc.D., Dr. Hamish Scott Ph.D., Professor Nobuyoshi Shimizu M.D., Ph.D., Dr. Jun Kudoh Ph.D., and Dr. Kentaro Nagamine Ph.D., for collaboration which has been crucially important for this study.

Professor Eric Jenkinson M.D., Ph.D., Professor Philippe Naquet M.D., Ph.D., Professor Noel Maclaren M.D., Ph.D., Professor Annmari Ranki M.D., Ph.D., Dr. Li Wu M.D., Ph.D., Dr. Sandrine Guerin Ph.D., Dr. Graham Anderson Ph.D. and all the other co-authors are acknowledged for fruitful collaboration.

I wish to thank my co-authors Ulla Aapola M.Sc., Anssi Lagededt M.D., Niko Sillanpää B.M and Vladimir Ovod M.Sc. Special thanks belong to them along with Auni Collings M.D., Helena Miettinen M.D., Ph.D., Heli Pehrlman M.Sc., Jukka Pitkänen B.M., Mari Strengell M.Sc., Marja Tähtinen Ph.D. and Perttu Vähämurto B.M. for sharing everyday joys and miseries of making science.

Ms. Ulla Kiiskinen, Mrs. Marianne Karlsberg, Mrs. Aulikki Lehmus, Mrs. Merja Jokela, Mrs. Tuula Myllymäki, Mrs. Satu Ruisniemi and Mrs. Suvi Heinämäki are acknowledged for excellent technical assistance and for sharing many joyful moments at our coffee room.

My warm thanks go to my family, to my mother and father and to my brother Ari for their support and interest in my work. My friends, especially the "sparkling sisters" Riika, Tea, Riina, Annukka and Riikka and my dear colleagues Eppu, Mari and Minna are acknowledged for bringing joy and laughter to my life during these years. Finally, I want to thank my dear fiancé Alekski for his loving care and encouragement throughout the study.

This study was financially supported by personal grants from the Emil Aaltonen Foundation, The Finnish Medical Foundation, the Research Fund of the Tampere University Hospital and the University of Tampere. The research project also received financial support from the Academy of Finland, EU biomed 2 Program and Novo Nordisk Foundation.

Tampere, April 2001

Maarit Heino



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