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Characterisation of the Autoimmune Regulator (AIRE) Protein

Autoimmunity on a Molecular Level

■

ACADEMIC DISSERTATION

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the Faculty of Medicine of the University of Tampere,
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YHTEENVETO

Mutaatiot autoimmune regulator (AIRE)- geenissä aiheuttavat harvinaisen APECED-autoimmuunioireyhtymän (**a**utoimmune **p**oly**e**ndocrinopathy **c**andidiasis **e**ctodermal **d**ystrophy). APECED-oireyhtymälle on tyypillistä sisäeritysrauhasten autoimmuunivälitteinen tuhoutuminen. Tavallisia tautikomponentteja ovat Addisonin tauti, lisäkilpirauhasen vajaatoiminta, kilpirauhastulehdus ja tyypin I diabetes. APECEDiin liittyy myös jonkin asteinen immuunipuutos, jonka merkkinä potilailla on krooninen Candidainfektio limakalvoilla ja iholla.

AIRE-geeniä vastaava 57.5 kDa proteiini ilmentyy pääasiassa kateenkorvan ytimen epiteelisoluissa ja dendriittisoluissa. AIRE on transkription säätelijä, joka ohjaa nk. ektooppisten geenien ilmentymistä tyymuksessa, mikä johtaa näille autoantigeeneille reaktiivisten T-solujen tuhoutumiseen. Mutaatioiden aiheuttama AIRE-geenin toiminnanvajaus johtaa siten puutteelliseen autoreaktiivisten T-solujen tuhoamiseen ja siitä seuraavaan autoimmuniteettiin.

Tässä väitöskirjatyössä olemme tutkineet AIRE-proteiinin toimintaa, vuorovaikutuksia ja solunsisäistä jakautumista. AIRE:n proteiinialueiden analysointi osoitti että PHD-tyypin sinkkisormirakenteet proteiinin C-päässä muodostavat aktivaatioalueen, kun taas N-päässä on toimiva tumalokalisaatiosignaali, tumasta uloskuljetusta välittävä signaali, homodimerisaatiosignaali sekä alue, joka vastaa sitoutumisesta soluliman filamentteihin. Tumassa AIRE muodostaa pyöreitä nuclear body- rakenteita, joiden osoitimme olevan osa proteiinien muodostamaa tuman tukirakennetta. Osoitimme myös että AIRE:n sijainti solun sisällä on proteasomi-ubikitiini- järjestelmän säätelyn alaista, millä mahdollisesti säädellään AIREn aktiivisuutta molekyylitasolla.

Tunnistimme myös toisenlaisen solutason säätelymekanismin osoittamalla, että AIRE kilpailee CBP-proteiiniin (CREB-binding protein) sitoutumisesta PML (promyelocytic leukaemia)-proteiinin kanssa. Havaitsimme, että AIRE nuclear bodyt eivät todennäköisesti ole paikkoja, joissa AIREn säätelemien geenien luenta tapahtuu. Näiden rakenteiden kolmiulotteinen tarkastelu osoitti, että AIRE-proteiini muodostaa nuclear bodyn uloimman pinnan, ja CBP-proteiini sijaitsee rakenteen pinnan alla, jossa on myös osa rakenteen sisältämästä AIRE-proteiinista.

Osoitimme, että AIRE on voimakas transkription aktivaattori. Tämä on ensimmäinen kuvattu AIRE:n biokemiallinen funktio. Jatkotutkimuksissa selvitimme, että AIRE sitoutuu CBP-proteiiniin, ja että CBP lisäksi toimii AIREn koaktivaattorina. AIRE ja CBP lisäävät yhdessä interferoni beetan lähetti-RNAn ilmentymistä.

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based upon the following original communications, referred to in the text by their roman numerals (I-IV)

I Pitkänen J, Doucas V, Sternsdorf T, Nakajima T, Aratani S, Jensen K, Will H, Vähämurto P, Ollila J, Vihinen M, Scott HS, Antonarakis SE, Kudoh J, Shimizu N, Krohn K, Peterson P (2000): The Autoimmune Regulator Protein Has Transcriptional Transactivating Properties and Interacts with the Common Coactivator CREB-binding Protein. Journal of Biological Chemistry, 275: 16802-16809.

II Pitkänen J, Vähämurto P, Krohn K, Peterson P (2001): Subcellular Localization of the Autoimmune Regulator Protein: Characterization of Nuclear Targeting and Transcriptional Activation Domain. Journal of Biological Chemistry, 276: 19597-19602.

III Pitkänen J, Rebane, A, Rowell J, Murumägi A, Stroebel P, Möll, K, Saare, M, Heikkilä J, Doucas V, Marx A, Peterson P (2005): Co-operative Activation of Transcription by Autoimmune Regulator AIRE and CBP. Biochemical and Biophysical Research Communications, 333: 944-953

IV Akiyoshi H, Hatakeyama S, Pitkänen J, Mouri Y, Doucas, V, Kudoh J, Tsurugaya K, Uchida D, Matsushima A, Oshikawa K, Nakayama KI, Shimizu N, Peterson P, Matsumoto M (2004): Subcellular Expression of Autoimmune Regulator (AIRE) is Organized in a Spatiotemporal Manner. Journal of Biological Chemistry, 279: 33984-33991

ABBREVIATIONS

AIRE	autoimmune regulator
APECED	autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APC	antigen presenting cell
APL	acute promyelocytic leukaemia
APS	autoimmune polyendocrine syndrome
CAT	chloramphenicol acetyltransferase
CBP	CREB-binding protein
CREB	cAMP response element-binding protein
DC	dendritic cell
GAD	glutamic acid decarboxylase
GFP	green fluorescent protein
H2B	histone H2B
HAT	histone acetyltransferase
HEL	hen egg lysozyme
HSR	homogenously staining region
IFNβ	interferon beta
IL	interleukin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
LMB	leptomycin B
LT	lymphotoxin
LTR	long terminal repeat
MHC	major histocompatibility complex
MMTV	mouse mammary tumour virus
mTEC	medullary thymic epithelial cell
NES	nuclear export signal
NLS	nuclear localisation signal
PCR	polymerase chain reaction
PHD	plant homeodomain
PML	promyelocytic leukaemia
PRR	proline rich region
QPCR	quantitative PCR

RAR	retinoic acid receptor
SAND	Sp100, AIRE, NucP41/75, DEAF-1
SMN	survival of motor neurons protein
snoRNP	small nucleolar ribonucleoprotein
snRNP	small nuclear ribonucleoprotein
T_H	T-helper
T_{reg}	regulatory T-cell
TAP	tandem affinity purification
TEC	thymic epithelial cell
TGFβ	transforming growth factor beta
TNFα	tumour necrosis factor alpha
TRAF	tumour necrosis factor receptor-associated factor

ABSTRACT

Mutations in the autoimmune regulator (AIRE) gene result in the rare autoimmune syndrome APECED (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy). The disease is characterised by autoimmune destruction mainly targeting endocrine organs, including Addison's disease, hypoparathyroidism, thyroiditis, and type I diabetes. APECED also involves immune dysfunction manifesting as chronic mucocutaneous candidiasis.

The AIRE gene encodes a 57.5-kDa protein that is expressed in thymic medullary epithelial cells and dendritic cells. AIRE is a transcription factor that controls the ectopic expression of tissue-specific genes in the thymus, resulting in deletion of T-cells autoreactive to these genes. Deletion of AIRE by mutations thus leads to defective negative selection of a multitude of autoreactive T-cells and subsequent autoimmunity.

In this thesis, the biochemical functions, interactions and subcellular localisation of the AIRE protein are characterised. Dissection of the protein domains of AIRE revealed that the plant homeodomain type zinc fingers in the C terminus form the activation domain, whereas the N terminus harbours a functional nuclear localisation signal, a nuclear export signal, a homodimerisation domain, and a motif mediating binding to cytoplasmic filaments. In the nucleus, AIRE forms nuclear bodies which are attached to the nuclear matrix. It was found that the localisation of AIRE is controlled by the proteasome-ubiquitin pathway, indicating a molecular level mechanism for the regulation of AIRE function.

Another level of regulation of AIRE function was identified by showing that AIRE competes for CBP (CREB-binding protein, a common transcriptional coactivator) colocalisation with the promyelocytic leukaemia protein PML. The AIRE nuclear bodies are probably not sites of active transcription. Analysis of three-dimensional reconstructions of CBP-containing AIRE nuclear bodies visualised by laser confocal microscopy revealed a novel structure where AIRE forms the outer surface of the nuclear body, with CBP being found intermingled with AIRE beneath the surface.

This thesis shows that AIRE is a potent activator of transcription, the first biochemical function described for this protein. It is futher demonstrated that AIRE interacts with the common coactivator CBP, and that CBP functions as an AIRE coactivator in model systems of transactivation. Finally, AIRE and CBP are shown to co-operatively increase the transcription of endogenous interferon beta mRNA.

1. INTRODUCTION

APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, is a unique autoimmune disease in the sense that it is caused by defects in a single gene, the autoimmune regulator (AIRE). This monogenic syndrome follows an autosomal recessive inheritance, and the clincal manifestations are typified by endocrine autoimmune phenomena including hypoparathyroidism, Addison's disease, autoimmune thyroiditis, and type I diabetes. Other sequelae are mucocutaneous candidiasis, autoimmune hepatitis, infertility, and defects of the nails and the dental enamel.

The AIRE protein is a transcriptional regulator carrying several conserved protein domains commonly seen in transcription factors, such as the PHD type zinc fingers and the LXXLL nuclear receptor interaction motifs. The transcriptional regulatory properties of AIRE have been shown in *in vitro* reporter assays as well as in Aire deficient mice. AIRE is expressed mainly in the thymus, in medullary epithelial and dendritic cells. Some expression is also seen in the periphery in the spleen, lymph nodes, and peripheral blood mononuclear cells. Subcellularly AIRE has two typical localisations: intermediate filaments in the cytoplasm and nuclear dots in the nucleus.

It has become apparent that AIRE is essential in the development of tolerance to several endocrine autoantigens. AIRE directs the (promiscuous) expression of peripheral autoantigens in the thymus, thus participating in the negative selection of autoreactive T-cells.

The aims of the current study were to establish the function of the AIRE protein at the cellular and molecular level, as well as to study its cellular localisation, and the protein domains responsible for the various functions, and thus gain insight into the mechanisms of transcriptional regulation in the development of immune tolerance.

2. REVIEW OF THE LITERATURE

2.1 IMMUNE TOLERANCE

2.1.1 Basic mechanisms of immune tolerance

There is an inherent risk in the ability of the adaptive immune system to mount aggressive responses against foreign antigens – the risk of the same response turning against self-antigens in the tissues of the host organism, i.e. autoimmunity. The key step in the pathogenesis of APECED is the breakdown of tolerance to several organ-specific antigens, leading to autoimmune attack against several, mostly endocrine, organs. Thus, a brief look at the basic mechanisms of immune self-tolerance and autoimmunity in general is warranted.

2.1.2 Selection of T-lymphocytes in the thymus

Immunological self-tolerance describes a stable state where the immune system does not react in a destructive manner against self-molecules, cells, or tissues (Pugliese 2004). Tolerance can roughly be divided into two types: central and peripheral tolerance. In central tolerance, the interaction between lymphocytes and antigen presenting cells (APCs) occurs in the thymus, whereas peripheral tolerance is formed in the peripheral lymphoid tissues such as lymph nodes and spleen (Schwartz 1993, Sprent and Webb 1995). The thymus plays a critical role during development and early life in shaping the T-cell repertoire. Two processes occurring in the thymus are needed in order to create a T-cell repertoire that is self-tolerant: positive and negative selection. During the development of lymphocytes, those cells without marked self-reactivity are positively selected in the thymic cortex and enter the circulation as mature lymphocytes, whereas cells showing significant self-reactivity die / go to apoptosis in negative selection (Pugliese 2004). Originally, thymic selection was thought to concern only ubiquitously expressed proteins, largely based on the assumption that tissue-restricted antigens would be unavailable for presentation in the thymus (Pugliese 2004). This, together with accumulating data on the existence of tolerance mechanisms in extrathymic lymphoid tissues led to the discovery of peripheral tolerance. However, it has since been shown that several tissue-specific antigens are expressed in the thymus in a limited manner as will be discussed below (Klein and Kyewski 2000). A more detailed discussion of the mechanisms of positive and negative selection in the thymus is beyond the scope of this review, as are the mechanisms of peripheral tolerance. The promiscuous expression of peripheral antigens in the thymus and some aspects of

peripheral tolerance are covered in more detail below as they appear to be connected with the function of AIRE.

2.1.3 Peripheral tolerance

Although the deletion of autoreactive T-and B- cells during development makes the immune system tolerant of most self-antigens, peripheral tolerance mechanisms are also required to prevent tolerance to self-antigens as well as to limit overexuberant responses to foreign antigens (O'Garra and Vieira 2004). The majority of the autoreactive cells destroyed through negative selection are those with a high avidity to self-antigens, whereas T-cells showing a low avidity for self-antigens (which are therefore less likely to initiate autoimmunity), are spared (Liu et al. 1995, Morgan et al. 1998). The processes responsible for the maintenance of tolerance in peripheral lymphoid tissues include ignorance, deletion, anergy, and regulatory T-cells (Klein and Kyewski 2000). For these processes to function the tissue-specific proteins have to be present in the peripheral lymphoid organs; antigen presenting cells (mainly immature dendritic cells) have been implicated in the capture and presentation of these self-peptides (Steinman 2003). It is likely that the APCs responsible for these processes represent bone marrow derived APCs; the expression of type I diabetes-associated antigens in the spleen and lymph nodes by such cells has been demonstrated by Pugliese et al. (2001).

Immunological ignorance represents a state where T- (or B-) cells coexist with antigen but are not activated by it. This stems from the fact that the threshold for deletion in the thymus is lower than the threshold for activation in the periphery (Pircher et al. 1991). Thus, some T-cells with low avidity for self-antigens are not activated in the periphery and remain ignorant of their specific antigens, and peripheral tolerance is formed simply by avoiding self-protein recognition (Ohashi et al. 1991, Oldstone et al. 1991). However, tolerance by ignorance is unreliable as it can be broken in appropriate stimulatory circumstances, such as viral infections, with the possible consequence of autoimmunity (Ohashi et al. 1991, Oldstone et al. 1991). The effect of the level of antigen expression in the periphery on the fate of autoreactive lymphocytes has been shown by Kurts and co-workers (1999) in transgenic mice expressing high or low levels of ovalbumin in the pancreas: injection of ovalbumin-specific T-cells into mice expressing high ovalbumin levels led to rapid initial proliferation and later deletion of the ovalbumin-specific T-cells. However, in mice expressing low levels of ovalbumin, there was initially no proliferation, but at four weeks, ovalbumin-specific T-cells could be recovered from the spleen that responded to antigen *in vitro*, indicating that with low levels of antigen expression, the T-cells remained in a state of ignorance.

By contrast, when the autoreactive T-cells are not ignorant of antigen, tolerance in the periphery can be induced by one of two mechanisms: deletion or anergy. The recognition of antigen by T-cells in the periphery, either presented by APCs or present on other cells in the tissue, leads to tolerance when appropriate costimulatory signals are lacking (Janeway et al. 2001, Redmond and Sherman 2005). Non-antigen presenting cells are unable to express costimulatory molecules, such as B7.1 and B7.2 (Janeway et al. 2001) and in the absence of infection APCs are quiescent and express very low levels of costimulatory factors (Steinman et al. 2003).

There is evidence that lymphocytes that recognise a self-peptide on a self-MHC (major histocompatibility complex) molecule expressed on peripheral antigen presenting cells are deleted in the periphery via apoptosis (Sprent and Webb 1995, Suss and Shortman 1996, Van Parisj et al. 1996, Ducoroy et al. 1999). T-cells that survive the T-cell – quiescent APC interaction are said to be anergic, and are typically unable to proliferate in further exposures to their specific antigen (Janeway et al. 2001). However, the maintenance of anergy requires that the antigen be continuously present as there is evidence that anergic T-cells allowed to rest in the absence of antigen are then able to mount a response (Rocha et al. 1993, Schwartz 2003). Redmond and Sherman (2005) suggest a model where the strength of the interaction of the T-cell receptor with the peptide-MHC complex defines whether the T-cell undergoes deletion or anergy: in tolerogenic circumstances (lack of costimulatory factors and persistence of antigen) chronic weak TCR-signalling activates pro-apoptotic pathways, leading to deletion, whereas chronic strong TCR-signalling inhibits pro-apoptotic pathways with the consequence of anergy.

2.1.4 Regulatory T-cells

Recent research indicates that active suppression of immune responses mediated by regulatory T-cells is crucial for the maintenance of tolerance in the periphery (Maloy and Powrie 2001, Sakaguchi et al. 2001, Shevach 2002, Cobbold et al. 2003). It is worthy of notice that the term regulatory T-cell (T_{reg}) is nowadays mainly taken to represent the CD4+ CD25+ T-cell population with the ability to inhibit autoimmunity *in vivo*; however, other T-cell populations, including T-helper type 1 (T_H1) and T_H2 cells, show regulatory properties by secreting specific cytokines (O'Garra and Vieira 2004). Recently, a new type of T_{reg} has been described that produces interleukin (IL) 10 and secretes transforming growth factor beta (TGFβ), called IL-10 T_{reg} (Groux et al. 1997, Roncarolo et al. 2001, Barrat et al. 2002, Oida et al. 2003, Sundstedt et al. 2003). In addition, there is a population of CD4+ T-cells with regulatory properties that do not express CD25

(Annacker et al. 2001) but these cells are at the moment poorly characterised. The different types of regulatory T-cells and some of their properties are summarised in Table 1.

Expression of the CD25 surface marker, or IL-10 or TGFβ production by IL-10 T_{reg}s, are not specific markers of regulatory T-cells, making the study and characterisation of these cells difficult (Maloy and Powrie 2001). However, the expression of the forkhead/winged helix transcription factor Foxp3 seems to help distinguish between T_{reg} populations. It is specifically expressed by CD25+ T_{reg}s and by CD25- T-cells with regulatory activity (Fontenot et al. 2003, Hori et al. 2003, Khattri et al. 2003). Foxp3 is indeed proposed to control the function and development of a subset of T_{reg}s. IL-10 T_{reg}s do not express Foxp3, making it the most unambiguous factor known to date that defines the naturally occurring CD4+CD25+ T_{reg} population (IL-10 T_{reg}s are CD4+, and acquire CD25 expression upon activation and *in vitro* culture)(O'Garra and Vieira 2004).

The phenotype of mice carrying loss-of-function mutations in the Foxp3 gene is interesting with regard to APECED and AIRE: the mice suffer from autoimmune endocrinopathy, type I diabetes, thyroiditis and, rarely, atopy and food allergy (O'Garra and Vieira 2003, Ramsdell 2003). The human disease caused by FOXP3 mutations, IPEX (the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), can be treated with bone marrow transplantation (Baud et al. 2001, Eisenbarth and Gottlieb 2004). The immune pathology noted in mouse and man is caused by the complete absence of regulatory T-cells (Baud et al. 2001, O'Garra and Vieira 2003, Ramsdell 2003, Eisenbarth and Gottlieb 2004).

In the model systems used to study T_{reg} functions, there seem to be two important mechanisms of regulatory function: IL-10 secretion and cell-contact-mediated suppression (likely to be mediated by TGFβ). Involvement of one of the two functions is probably defined by the type of autoimmune phenomenon in question (Asseman et al. 1999, Hara et al. 2001, Kingsley et al. 2002, Shevach 2002, Sundstedt et al. 2003, Vieira et al. 2004). Regulatory T-cells seem to require antigen-specific activation to function, but their effects are mediated through antigen-independent mechanisms (O'Garra and Vieira 2004). Moreover, no effector functions (such as those displayed by activated T-cells and T_H1 and T_H2 cells), have so far been reported for the CD4+CD25+ or the IL-10 regulatory T-cells; their sole function seems to be the suppression of immune responses (O'Garra and Vieira 2004).

Table 1. Types of regulatory T-cells. Adapted from O'Garra and Vieira (2004).

T_{reg} type	Origin	Phenotype	Foxp3	Mode of action
CD25- T_{reg}	?	CD4+CD25-	Yes	?
CD4+CD25+ T_{reg}	Thymus (periphery?)	CD4+CD25+ $CD45RB^{low}$	Yes	Cell-contact? Membrane or soluble TGF-beta Secreted IL-10
IL-10 T_{reg}	Periphery	CD4+ (acquire CD25 with activation and *in vitro* culture)	No	Cell-contact? Secreted IL-10

2.1.5. Promiscuous expression of peripheral antigens in thymic medullary epithelial cells

It was long assumed that central tolerance mechanisms were not involved in the development of tolerance to tissue-specific antigens, but that they would only function with regard to antigens expressed ubiquitously, or present in the circulation as soluble antigens. Thus, the concept of peripheral tolerance as the mediator of tolerance to these parenchymal antigens was conceived, admittedly supported by experimental evidence.

Recent evidence on gene expression, and its regulation, in the thymus has broadened the concept of central tolerance to include the induction of tolerance to tissue-specific antigens; many of them are in fact expressed in the thymus and participate in the selection of the T-cell repertoire (Kyewski and Derbinski 2004). The expression of tissue-specific antigens in the thymus is known as promiscuous or ectopic expression. It seems to be a physiological property of thymic epithelial cells, especially medullary TECs (mTECs) (Derbinski et al. 2001, Gotter et al. 2004).

Beginning from the first reports implying thymic expression of tissue-specific antigens and their possible role in tolerance, accumulating evidence clearly indicates that a significantly diverse selection of tissue-specific genes is expressed by TECs (Heid et al. 1988, Kirchner et al. 1988, Jolicoeur et al. 1994, Pribyl et al. 1996, Geenen and Lefebre 1998, Klein et al. 1998). What is also clear is that the gene pool expressed in a promiscuous manner by the thymic medullary epithelium is large, encompassing genes found in most, if not all, tissues (Derbinski et al. 2001, Kyewski et al. 2002, Gotter et al. 2004). This includes genes expressed in a temporally restricted manner, such as those seen during foetal life, in puberty, or during pregnancy (Kyewski and Derbinski 2004). Furthermore, genes (such as the complement protein C5) that are available in the circulation in abundance, and expressed by thymic dendritic cells, are also promiscuously expressed in mTECs, indicating that the selection of genes transcribed in mTECs is not limited to those (otherwise) not present in the thymus (Kyewski and Derbinski 2004). There is also a number of peripheral restricted genes that appear not to be expressed in mTECs, or at least not at levels

detectable by current methods, one of the most prominent being glutamic acid decarboxylase 65 (GAD65) (Gotter et al. 2004). The expression of such a large diversity of genes in mTECs, a highly differentiated cell type, requires widespread deregulation of gene expression, especially as many of the genes promiscuously expressed belong to terminally differentiated cell types. It has now been shown that AIRE controls the expression of a subset of promiscuously expressed genes (Anderson et al. 2002, Liston et al. 2003). Further, Liston et al have shown, using an experimental (neo)self-antigen, that lack of AIRE leads to significantly decreased tolerance to that antigen (Liston et al. 2003). (These aspects of AIRE function are covered in detail in the *Discussion* section). However, epigenetic mechanisms may also contribute to the (de)regulation of promiscuous gene transcription in mTECs (Kyewski and Derbinski 2004, Derbinski et al. 2005, Johnnidis et al. 2005).

In addition to the deletion of T-cells reactive to tissue-specific antigens expressed by mTECs, regulatory T-cells may also contribute to tolerance to promiscuously expressed antigens. The $CD4^+CD25^+$ T_{reg} cells arise in the thymus, with the expression of MHC class II molecules by cortical TECs being sufficient for their development (Bensinger et al. 2001). Data from transgenic mice ectopically expressing neo-self antigens indicate that regulatory T-cells are also efficiently selected by TECs, in an antigen-dependent manner (Jordan et al. 2001, Apostolou et al. 2002). However, conclusive evidence that promiscuous expression of tissue specific antigens by mTECs is required for the generation of a T_{reg} repertoire specific to these antigens is, so far, lacking (Kyewski and Derbinski 2004). Interestingly, the immune pathology of neonatally thymectomized mice, showing a deficit in their $CD4^+CD25^+$ T_{reg} compartment, remarkably resembles that of AIRE-deficient mice, apparently, but not conclusively, linking promiscuous expression and dominant tolerance mediated by T_{reg} s (Anderson et al. 2002, Ramsey et al. 2002b, Sakaguchi 2004).

2.2 AUTOIMMUNE DISEASES

Transient autoimmune responses are common, but when there is activation of B- or T-cell responses, or both, in the absence of infection or other apparent cause, leading to tissue damage and a clinical syndrome, this response can then be labelled an autoimmune disease (Davidson and Diamond 2001). With the exception of autoimmune thyroiditis and rheumatoid arthritis, autoimmune diseases are relatively uncommon. With these common disorders included, an estimated 5 percent of the population of Western countries are affected by autoimmune diseases (Sinha et al. 1990, Jacobson et al. 1997). As stated in the previous section, some level of autoreactivity is still seen as a by-product of the generation of a broadly reactive lymphocyte repertoire, even after the stringent selection of lymphocytes in the thymus, and mechanisms such as regulatory T-cells have evolved to contain responses against self. Thus, when the adaptive immune

system is concerned, it can be stated that lymphocytes have not evolved to distinguish self from foreign per se, but to respond to antigen only under certain conditions (Davidson and Diamond 2001). However, the events leading to such responses to self as to cause a clinical autoimmune syndrome are still largely unknown.

2.2.1 Classification of autoimmune diseases

From the clinician's point of view, it is practicable to divide autoimmune diseases into systemic and organ-specific, which, although useful clinically, does not necessarily correspond to the pathophysiology underlying the clinical syndromes. Table 2 shows a classification of autoimmune diseases according to the mechanism of tissue damage. Another classification useful for understanding the pathology of autoimmune disorders is to distinguish between conditions where there are general defects in the selection, regulation, or function of T- and B-cells, and those where autoimmunity is caused by (aberrant) responses to specific antigens by an otherwise largely normal immune system (Davidson and Diamond 2001).

Systemic lupus erythematosus is an autoimmune syndrome characterised by a wide scope of clinical manifestations, including glomerulonephritis, vasculitis, and arthritis. High titers of antibodies to several intracellular antigens such as DNA, histones and ribosomes are seen in patients, reflecting a more general change in the activation and survival of autoreactive B-cells (Davidson and Diamond 2001). The anti-ganglioside antibodies seen in Guillain-Barré syndrome, a polyradiculitis syndrome leading to progressive paralysis of skeletal muscles following certain infections, reflect a loss of tolerance to a specific B-cell antigen in the context of an otherwise self-tolerant B-cell repertoire (Yuki 1999). The presentation of disease following thymectomy of neonatal mice depends on the genetic background of the animal, and can lead to an apparently systemic (wasting disease) or organ-specific (autoimmune destruction of the thyroid, gastric parietal cells, or ovaries) disease, underlining that the classification of autoimmune disorders by clinical manifestation alone can be deceptive with regard to the causative defect (Shevach 2000).

In contrast, Sjögren's syndrome (antibodies against ribonucleoprotein antigens) and polymyositis (tRNA synthetases) are good examples of organ-specific autoimmune diseases where the autoantigen is a ubiquitous protein, but the autoimmune attack is only seen in particular organs in a relatively restricted pattern (Targoff 2000, Davidson and Diamond 2001). The local accessibility of antigen and patterns of lymphocyte migration may be factors that determine the sites of autoimmune attack (Austrup et al. 1997). Finally, the expression of some autoantigens is developmentally regulated, and autoimmunity is a risk only at certain stages of development (Davidson and Diamond 2001). Thus, anti-Ro antibodies can bind to the conducting system of the

foetal heart, causing a complete heart block, whereas they have no effect whatsoever on the adult heart (Buyon et al. 1997).

Table 2. Classification of autoimmune diseases by disease mechanism.
Adapted from Janeway et al. (2001)

Syndrome	Autoantigen	Response
Type II antibody to cell surface or matrix antigens		
Autoimmune hemolytic anemia	Rh blood group antigens I antigen	Destruction of red cells by complement and phagocytes, anemia
Autoimmune thrombocytopenic purpura	platelet integrin GpIIb:IIIa	abnormal bleeding, purpura
Goodpasture's syndrome	basement membrane collagen type IV	glomerulonephritis, pulmonary hemorrhage
Pemphigus vulgaris	epidermal cadherin	blistering of skin
Acute rheumatic fever	Streptococcal cell wall antigens cross-react with cardiac muscle	arthritis, myocarditis, scarring of heart valves
Type III immune complex disease		
Mixed essential cryoglobulinemia	rheumatoid factor IgG complexes	systemic vasculitis
Systemic lupus erythematosus	DNA, histones, ribosomes snRNP, scRNP	glomerulonephritis, vasculitis, arthritis
Type IV T-cell mediated disease		
IDDM	pancreatic beta cell antigen	beta cell destruction
rheumatoid arthritis	unknown synovial joint antigen	joint inflammation and destruction
EAE, multiple sclerosis	myelin basic protein, proteolipid protein	brain invasion by CD4 T-cells paralysis

2.2.2 Genetic susceptibility to autoimmune diseases

Some autoimmune diseases are caused by defects in a single gene. These include the IPEX syndrome discussed above (mutations in Foxp3), the autoimmune lymphoproliferative syndrome (Fas) and APECED (AIRE) (Consortium 1997, Nagamine et al. 1997, Baud et al. 2001, Eisenbarth and Gottlieb 2004).

The presentation of human autoimmune lymphoproliferative syndrome, including the Canale-Smith syndrome, resembles that seen in mice with the lymphoproliferation (lpr) and generalized-lymphoproliferative-disease (gld) phenotypes; accordingly, the human disease was

found to be caused by mutations in the Fas gene (Sneller et al. 1992, Fisher et al. 1995, Rieux-Laucat et al. 1995, Drappa et al. 1996). The patients have symptoms of lymphadenopathy and autoimmunity, including hepatosplenomegaly, haemolytic anaemia, and thrombocytopenia. These manifestations are thought to be caused by defective Fas-mediated apoptosis of antigen-primed, activated lymphocytes, leading to an accumulation of these lymphocytes, some of which may be autoreactive (Drappa et al. 1996). However, in their study of Canale-Smith patients and their families, Drappa et al. found that of three family members with the same Fas mutation all had defective Fas-mediated apoptosis *in vitro*, but only one had the clinical syndrome (Drappa et al. 1996). Thus, other factors must exist that modulate Fas deficiency.

Autoimmune diseases with a monogenic background, such as those described above, are rare, and most autoimmune diseases have a multigenic aetiology. A wealth of epidemiologic studies shows that susceptibility to autoimmune disease is dependent on genetic factors (Davidson and Diamond 2001). Familial clustering as well as higher concordance of autoimmune diseases in monozygotic than in dizygotic twins have been established (Gregersen 1997, Kukreja and Maclaren 1999, Ortonne 1999). In addition to autoimmunity, some autoimmune diseases also exhibit signs of immunodeficiency such as the predisposition to mucocutaneous candidiasis in APECED. Other examples include the acquired immunodeficiency syndrome, complement deficiencies, and IgA deficiency (Davidson and Diamond 2001). It is typical of the many autoimmunity susceptibility genes known to date that the polymorphisms also occur in normal people at various frequencies, and contribute to autoimmunity only when other susceptibility genotypes are present (Figure 1) (Becker 1999, Encinas and Kuchroo 2000).

Probably the best known susceptibility genes are the class I and II HLA region molecules. Most autoimmune diseases are in fact associated with a particular class I or II HLA molecule, with the notable exception of APECED, which has no clear HLA association (Consortium 1997, Nagamine et al. 1997, Klein and Sato 2000a,b). Some of the HLA susceptibility genes may require linkage with other genes, such as tumour necrosis factor α (TNF α), but others may predispose to autoimmunity on their own (Klein and Sato 2000a,b). The HLA-DQB1*0301 and HLA-DQB1*0302 alleles cause an increased risk of type I diabetes, but the HLA-DQB1-0602 allele protects from the disease, even when either of the two susceptibility genes are present (Kukreja and Maclaren 1999). Further, there is variation in the association of HLA alleles between populations, exemplified by the findings that the DRB1*0401 and DRB1*0404 alleles associate with rheumatoid arthritis in northern Europeans but not in blacks or Hispanics (Gregersen et al. 1987, McDaniel et al. 1995, Teller et al. 1996).

Accumulating data from animal models with deleted or overexpressed genes leading to an autoimmune phenotype indicates that the ability of a particular mutation to cause disease depends on the genetic background of the host, and that often genetic alterations cause an increased risk of more than one autoimmune disease (Davidson and Diamond 2001). The conclusion is that even when single genes are affected, the disease phenotype depends on the presence and / or activity of other genes (be they susceptibility or protective genes), and that several autoimmune diseases may share common pathogenic pathways. The findings from human studies support these conclusions: a single polymorphism of CTLA-4 is associated with primary biliary cirrhosis, thyroid disease, and type I diabetes (Awata et al. 1998, Agarwal et al. 2000, Kouki et al. 2000). Finally, there is experimental evidence from animal models that the vulnerability of the target organs of autoimmune attack may be genetically determined (Liao et al. 1995, Coelho et al. 1997).

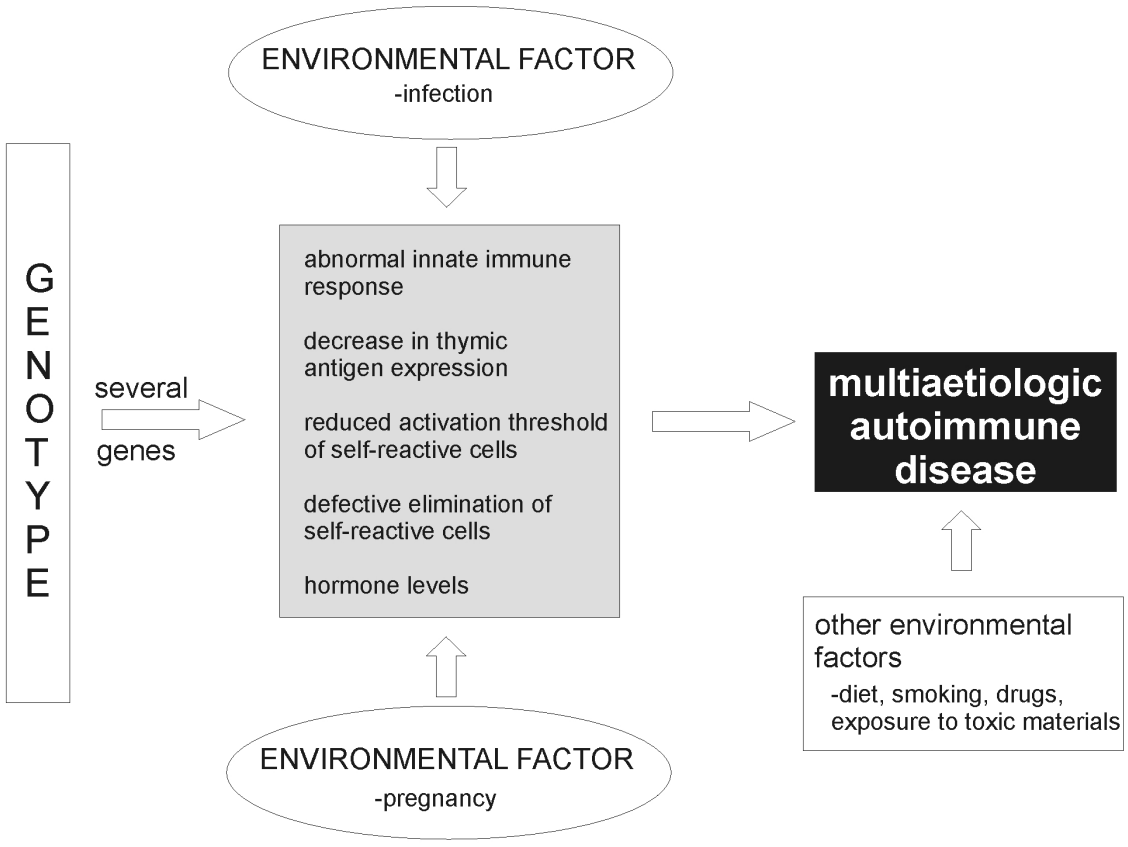

Figure 1. A Schematic representation of the aetiology of multigenic autoimmune diseases where the outcome of autoimmunity is a consequence of several genetic and environmental factors working together. Adapted from Rioux and Abbas (2005).

2.2.3 Triggers of autoimmunity

As discussed above, genetic factors can both predispose to autoimmunity as well as protect against it. However, even in people genetically susceptible to autoimmunity, a trigger, an environmental factor or a change in the internal environment, is often required for the onset of autoimmunity (Davidson and Diamond 2001). Although there is data describing some of these triggers at the population and even individual level, in many autoimmune conditions the initiating factor is unknown (Davidson and Diamond 2001). There is evidence that the incidence of type I diabetes and multiple sclerosis change in a population with the same genetic background when they migrate to a new region (Dahlquist 1998, Noseworthy et al. 2000). The incidence of type I diabetes in the children of Pakistanis who migrated to the United Kingdom is about ten times higher than the incidence in Pakistan, and the same as in non-immigrants living in the UK (Bodansky et al. 1992, Staines et al. 1997). Many of the epidemiologic studies on the effect of environmental factors in autoimmunity in migrants report increases in the incidence of the disease under study (Bach 2002), but the fact that Britons migrating to northern Australia have a decreased frequency of multiple sclerosis offers a negative control (Hammond et al. 2000). These findings, coupled with the fact that the prevalence of autoimmune and allergic diseases has been increasing in developed countries, have led to the formation of the "hygiene hypothesis", that is that infections, especially by parasites, protect against allergy, and possibly autoimmunity (Committee 1998, Holgate 1999, Gale 2002, Thomas et al. 2004). Recent evidence indicates that people with chronic helminthic infections have low levels of allergy (Yazdanbakhsh et al. 2002).

Infections can induce autoimmunity in many experimental systems, and evidence on the clinical significance of some of the observations has become available in recent years (Davidson and Diamond 2001, Bach 2002). Infectious diseases predispose to autoimmunity by several mechanisms including molecular mimicry, polyclonal activation, increases in the immunogenicity of autoantigens (caused by inflammation), and release of sequestered antigens (Davidson and Diamond 2001, Olson et al. 2001, Bach 2002).

The role of molecular mimicry in human autoimmune disease is supported by the findings of cross-reactivity of (auto)antibodies with microbial and host antigens. In rheumatic fever, antibodies against streptococcal myosin cross-react with cardiac myosin (Guilherme et al. 1995, Galvin et al. 2000, Malkiel et al. 2000). In multiple sclerosis, T-cells reactive to the myelin basic protein autoantigen also react against peptides from human papillomavirus, influenzavirus A, and Epstein-Barr virus (Wucherpfennig and Strominger 1995). Similarly, in type I diabetes T-cells specific for a peptide from the GAD autoantigen recognize an analogous peptide from coxsackievirus P2-C protein (Kukreja and Maclaren 2000). In these cases, the infection is probably

responsible for initiating the activation of lymphocytes mediating the disease; the continued presence of (auto)antigen after the eradication of the infectious agent could then facilitate the persistence of autoimmune activation (Davidson and Diamond 2001).

Recent data on a mouse transgenic system offers insight into the role of infection in the initiation of autoimmunity. Using transgenic mice that express lymphocytic choriomeningitis virus glycoprotein under control of the rat insulin promoter, Lang and co-workers (2005) showed that large quantities of autoreactive T-cells can coexist with autoantigen without causing autoimmune tissue destruction unless Toll-like receptors are engaged (as occurs in infections). The initiation of autoimmunity was dependent on MHC I upregulation on pancreatic islet cells caused by Toll-like receptor engagement and the ensuing production of interferon α.

In addition to the examples above of infections initiating autoimmune disease, there is also some experimental evidence to the contrary, although most of it derived from animal studies (Bach 2002). Autoimmune disease in genetically susceptible strains of mice and rats develops earlier when they are bred in specific pathogen-free environments as compared to breeding in a conventional environment (Like et al. 1991, Breban et al. 1993, Moudgil et al. 2001). The development of diabetes in NOD mice can be inhibited by infecting young mice with several pathogens, including mycobacteria, and the same can be achieved by treating the animals with killed bacteria (Bach 2002). Regulatory T-cells are implicated in protection against autoimmunity by the findings that the protection resulting from treatment with mycobacteria can be transferred to uninfected animals by $CD4^+$ T-cells and that it can be averted by giving the recipients cyclophosphamide (Qin et al. 1993). Other possible mechanisms include the downregulation of DC costimulatory activity by pathogens and Th1 / Th2 deviation of the immune response so that a Th2 response to the pathogen would inhibit the development of a Th1 response (which has been linked to autoimmune tissue destruction at least in type I diabetes) (Thomas et al. 2004).

Infections are but one environmental factor modulating the outbreak of autoimmune diseases. Several autoimmune diseases are more common in women than men, possibly explained by hormonal differences: in the mouse model of systemic lupus, the disease is exacerbated by estrogens via alterations in the B-cell repertoire (Bynoe et al. 2000). Also, various drugs can influence the immune repertoire. Procainamide not only induces antinuclear antibodies, but also sometimes causes a lupus-like syndrome (Davidson and Diamond 2001). Foreign substances can also act as haptens, creating “novel” antigens, thus making autoantigens immunogenic: antibiotics of the penicillin and cephalosporin classes can bind to the red cell membrane, which ultimately leads to haemolytic anaemia when autoantibodies specific to the neoantigen cause destruction of the red blood cells (Arndt et al. 1999). A novel treatment for inflammatory bowel disease and

rheumatoid arthritis, blockade of TNF-α signalling by a monoclonal antibody or TNF receptor fusion protein (infliximab and etanercept respectively), can also have deleterious consequences as the emergence of antinuclear antibodies, systemic lupus and even multiple sclerosis has been reported in patients treated with these drugs (Charles et al. 2000, Mohan et al. 2000).

2.3 AUTOIMMUNE POLYENDOCRINE SYNDROMES

Autoimmune endocrine diseases include common disorders such as hypothyroidism and type I diabetes, and more rare conditions such as Addison's disease, hypoparathyroidism, hyperthyroidism, autoimmune hepatitis and primary gonadal failure. Any of these may present as a solitary disease, but they often coincide, in various combinations, as autoimmune polyendocrine syndromes (APS). This group of clinical syndromes encompasses three clinical entities: APS type I (also known as APECED), APS type II, and the X-linked polyendocrinopathy, immune dysfunction and diarrhoea syndrome. The key features of these diseases are summarised in Table 3**.**

Table 3. Features of the autoimmune polyendocrine syndromes.
Adapted from Eisenbarth and Gottlieb (2004).

	Autoimmune polyendocrine syndrome type I (APECED)	Autoimmune polyendocrine syndrome type II	IPEX[1]
Prevalence	rare	common	very rare
Time of onset	infancy	infancy - adulthood	neonatal period
Gene	AIRE	polygenic	FOXP3
HLA genotype	diabetes (risk decreased with HLA-DQ6)	HLA-DQ2, HLA-DQ8 HLA-DRB1*0404	no association
Immunodeficiency	susceptibility to candidiasis	none	loss of regulatory T cells overwhelming autoimmunity
Association with diabetes	yes (18%)	yes (20%)	yes (majority)
Common phenotype	candidiasis, hypoparathyroidism Addison's disease	Addison's disease, type 1A diabetes, thyroiditis	neonatal diabetes malabsorption

[1] *Immune dysfunction, polyendocrinopathy, and enteropathy, X-linked*

2.3.1 Autoimmune polyendocrine syndrome type I

Also known as APECED (for autoimmune polyendocrinopathy candidiasis ectodermal dystrophy), autoimmune polyendocrine syndrome type I (hereafter APECED) is a monogenic autoimmune syndrome caused by mutations in the autoimmune regulator (AIRE) gene (Consortium 1997, Nagamine et al. 1997). The inheritance follows an autosomal recessive pattern (Perheentupa 2002).

The various clinical manifestations of APECED are reviewed in this chapter, while a detailed review of the properties of the AIRE gene and corresponding protein is presented below.

The first reports of APECED were published in the 1960s and 1970s, describing a rare disease affecting children (Blizzard and Kyle 1963, Neufeld et al. 1981). Since these early reports, much of what is known of the clinical manifestations and course of the disease comes from studies on Finnish families with APECED (Ahonen et al. 1990, Perheentupa 2002). APECED is a rare entity, but certain populations have a higher prevalence: 1:80000 in Norwegians (Myhre et al. 2001), 1:25000 in Finns (Björses et al. 1996), 1:14400 in Sardinians (Rosatelli et al. 1998), and 1:9000 in Iranian Jews (Zlotogora and Shapiro 1992).

The first sign of APECED is quite often candidal infection; this is usually chronic but in milder cases it is only evident at times of stress (such as other infections or fever) (Ahonen et al. 1990, Perheentupa 2002). The typical patient has candidiasis of the oral mucosa, but some patients also have lesions in the skin, oesophagus, and the lower gastrointestinal tract (Ahonen et al. 1990, Perheentupa 2002). The endocrine components, most often hypoparathyroidism and adrenal insufficiency, then develop, with the age at diagnosis varying from infancy to adulthood (19 months to 44 years and 4.2 to 41 years respectively) (Ahonen et al. 1990, Perheentupa 2002). Severe lesions of the dental enamel and pitted dystrophy of the nails were seen in 77% and 52% of patients in a Finnish series (Ahonen et al. 1990, Perheentupa 2002). The prevalences of the different components of disease in APECED patients are listed in Table 4.

Table 4. Components of APECED.
Adapted from Ahonen et al. (1990)

Disease component	Prevalence (%)
Endocrine	
Hypoparathyroidism	79
Addison's disease	72
Ovarian failure	60[1]
Type I diabetes	12
Hypothyroidism	4
Nonendocrine	
Candidiasis	100
Enamel hypoplasia	77
Alopecia	72
Nail dystrophy	52
Keratopathy	35
Malabsorption	18
Vitiligo	13
Pernicious anaemia	13
Autoimmune hepatitis	12

[1] *of postpubertal female patients*

The presence of autoantibodies to molecules expressed in the target organs of autoimmune destruction in APECED has been established, with the first reports demonstrating antibodies to 3-4 antigens in the cytosomal, mitochondrial, or ribosomal fractions of cells from APECED patients (Goudie et al. 1968, Krohn et al. 1974, Irvine and Barnes 1975). Later, several novel autoantigens have been discovered; the most important of these are detailed in Table 5. The autoantibodies against the steroidogenic enzymes P450c17a, P450scc, and P450c21 (Krohn et al. 1992, Winqvist et al. 1992, Winqvist et al. 1993, Uibo et al. 1994) are especially useful in diagnosing APECED, as well as in follow-up of patients, as the presence of these antibodies may predict the subsequent emergence of adrenal insufficiency (Perheentupa 2002). The same is true for the GAD65 and GAD67 autoantibodies that may herald the development of autoimmune diabetes in asymptomatic subjects (Perheentupa 2002).

The classic triad of conditions required for the diagnosis of APECED includes at least two of the following: chronic or recurring mucocutaneous candidiasis, hypoparathyroidism, and Addison's disease (or adrenocortical / CYP450c21 autoantibodies) (Perheentupa 2002). However, in a Finnish series of patients, only 22% percent met these criteria by the age of five, 67% by the age of ten, and 93.5% by the age of 30 (Perheentupa 2002). Thus, strict adherence to the aforementioned would miss a significant proportion of patients until more disease components emerge with increasing age. Since the resolution of the genetic aetiology of APECED, specialist laboratories offer genetic testing for diagnosis, but the large amount of different disease-causing mutations (see below) makes testing difficult, and exclusion of the syndrome by this means is not possible (Perheentupa 2002). Thus, the presence of chronic or recurrent candidiasis, Addison's disease, hypoparathyroidism, alopecia, non-infectious hepatitis, or vitiligo in a person aged 30 or younger should trigger the suspicion of APECED and further evaluation (Perheentupa 2002). Furthermore, it is also clear that APECED is not solely a paediatric problem – the patients require constant specialist follow-up for the rest of their lives.

Table 5. Autoantigens in APECED. Adapted from Heino et al. (2001) and Meriluoto et al. (2001).

Autoantigens	Tissue affected	Disease component
P450c21, P450c17a, P450scc	Adrenal cortex	Addison's disease
Thyroid peroxidase, thyroglobulin	Thyroid gland	Hypothyroidism
GAD65, GAD67, ICA, IA-2 tyrosine phosphatase type protein	Endocrine pancreas	Type I diabetes
P450CYP1A2, P450CYP2A6, P450CYP1A1, P450CYP2B6, AADC	Liver	Autoimmune hepatitis
SOX9, SOX10	Skin	Vitiligo
Tyrosine hydroxylase	Scalp	Alopecia
Tryptophan hyrdoxylase	Gastrointestinal tract	Malabsorption
H^+K^+ ATPase	Stomach	Autoimmune gastritis
Intrinsic factor	Gastric mucosa	Pernicious anaemia

2.3.2 Autoimmune polyendocrine syndrome type II

Autoimmune polyendocrine syndrome type II is significantly more common than APS type I, and the clinical manifestations appear to be more diverse (Betterle et al. 2002, Schatz and Winter 2002, Betterle and Zanchetta 2003). The typical clinical presentation in APS type II consists of Addison's disease, type IA diabetes, and chronic thyroiditis (Eisenbarth and Gottlieb 2004). The classic first clinical symptom that leads to the diagnosis of APS type II is symptomatic hypotension caused by the adrenal insufficiency of Addison's disease (Eisenbarth and Gottlieb 2004). APS type II is differentiated from APS type I by its polygenic aetiology, its HLA associations, and the absence of the mucocutaneous candidal infection seen in APS type I patients (Betterle and Zanchetta 2003, Eisenbarth and Gottlieb 2004). APS type I presents typically in infancy, whereas APS II may also emerge in adults (Eisenbarth and Gottlieb 2004).

There are, however, different interpretations of the subclassification of APS type II in that Addison's diseases plus type IA diabetes or thyroid autoimmunity would comprise APS type II; thyroid autoimmunity plus another autoimmunity (but not Addison's disease or type IA diabetes) would comprise APS type III; and two or more other organ-specific autoimmune disorders would make up APS type IV. Other authors consider all the above combinations to belong under the heading of APS type II. These differences in opinion probably reflect the fact that APS type II is a genetically, as well as clinically, complex disorder. In APSII families, affected family members typically have multiple yet different autoimmune disorders. (Neufeld et al. 1981, Yu et al. 1999, Betterle et al. 2002, Myhre et al. 2002, Betterle and Zanchetta 2003, Eisenbarth and Gottlieb 2004)

2.3.3 X-linked polyendocrinopathy, immune dysfunction and diarrhoea syndrome

The X-linked polyendocrinopathy, immune dysfunction and diarrhoea syndrome, also called the IPEX syndrome (for immune dysfunction, polyendocrinopathy, and enteropathy, X-linked) is a rare, often fatal disease, that consists of widespread autoimmunity and type IA diabetes and, as stated above, is caused by mutations in the Foxp3 gene, resulting in the absence of regulatory T-cells (Patel 2001, Wildin et al. 2001). The disease usually manifests itself in the neonatal period, and patients may benefit from treatment with bone marrow transplantation (Baud et al. 2001).

2.4. AUTOIMMUNE REGULATOR (AIRE)

In this section, the data that were available when this study was being designed are reviewed; subsequent advances regarding AIRE and APECED are presented in the *Discussion* section. Also, data presented in the *Results* section is not presented here.

2.4.1 Structure of the AIRE gene and protein

The gene defective in APECED, AIRE, was identified by positional cloning (Consortium 1997, Nagamine et al. 1997). The gene is located on chromosome 21q22.3, has 14 exons with a total size of approximately 13kb, and encodes a polypeptide of 545 amino acids (Consortium 1997, Nagamine et al. 1997). Analysis of the predicted polypeptide sequence revealed the presence of several conserved protein domains with properties typical of transcription factors (Consortium 1997, Nagamine et al. 1997, Mittaz et al. 1999). In the C terminus of AIRE, there are two plant homeodomain (PHD) type zinc fingers; between these lies a proline rich region (PRR). The N terminus harbours a nuclear localisation signal (NLS) and a homogenously staining region (HSR) domain. In addition, four LXXLL motifs are found in the AIRE protein, two in the N terminus and two in the C terminus. (Consortium 1997, Nagamine et al. 1997, Mittaz et al. 1999) A schematic of the AIRE protein is shown in Figure 2.

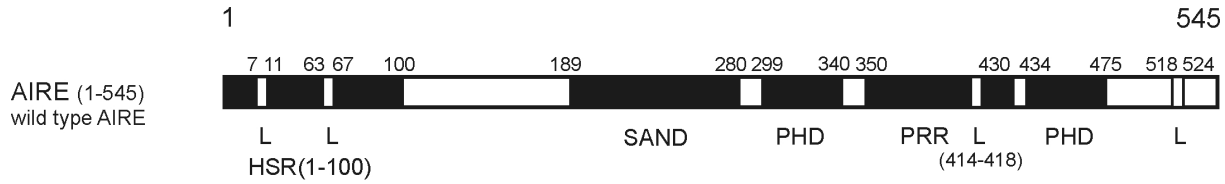

Figure 2. Schematic of the AIRE protein. The protein domains are indicated as follows: PHD, plant homeodomain type zinc fingers; PRR, proline rich region; L, LXXLL nuclear receptor interaction domain; SAND, putative DNA binding domain.

The PHD zinc fingers are characteristic of proteins involved in the regulation of transcription, many of these functioning at the chromatin level (Aasland et al. 1995). More than 400 PHD-containing proteins have been described (Capili et al. 2001). The PHD finger is thought to function as an interaction motif, mediating binding to other proteins (Capili et al. 2001). The solution structures of the PHD fingers from KAP-1 and WSTF support this hypothesis (Pascual et al. 2000, Capili et al. 2001). The function of the PHD fingers of AIRE in this respect remains to be addressed. The HSR domain, first described in Sp100 and Sp140, mediates Sp100 homodimerisation (Sternsdorf et al. 1997, Sternsdorf et al. 1999). LXXLL motifs typically mediate binding of proteins to nuclear receptors, with the proteins then coactivating the nuclear receptors (Heery et al. 1997). The SAND domain (for Sp100, AIRE, NucP41/75, DEAF-1), found in several proteins, is a DNA binding domain that seems to coexist with other protein domains involved in chromatin association and protein interaction (Heery et al. 1997, Gibson et al. 1998, Bottomley et al. 2001). A signature amino acid motif in the SAND domain of the NUDR protein, KDWK, mediates the binding to DNA (Bottomley et al. 2001) The binding of AIRE to specific DNA sequences has also been described. The binding was only demonstrable with reconstituted AIRE homodimers and tetramers, but not with monomers (Kumar et al. 2001).

The murine counterpart of human AIRE (Aire) shows over 70% homology to the human gene, and the predicted polypeptide shares all the same typical functional domains (Blechschmidt et al. 1999, Ruan et al. 1999, Heino et al. 2000, Zuklys et al. 2000).

2.4.2 APECED-causing mutations

At the time of writing this section (June 2005), at least 58 disease-associated mutations in AIRE had been described (Consortium 1997, Nagamine et al. 1997, Pearce et al. 1998, Rosatelli et al. 1998, Scott et al. 1998, Wang et al. 1998, Heino et al. 1999b, Ward et al. 1999, Björses et al. 2000, Ishii et al. 2000, Soderbergh et al. 2000, Cetani et al. 2001, Cihakova et al. 2001, Heino et al. 2001, Myhre et al. 2001, Saugier-Veber et al. 2001, Halonen et al. 2002, Meloni et al. 2002, Sato et al. 2002, Vogel et al. 2003, Sato et al. 2004, Meloni et al. 2005, Podkrajsek et al. 2005). The most recently reported mutations include intronic mutations leading to improper splicing of the protein, and compound heterozygous mutations (Sato et al. 2004, Meloni et al. 2005, Podkrajsek et al. 2005).Two main types of mutations can be distinguished: nonsense or frame-shift mutations leading to a truncated polypeptide and missense mutations resulting in the change of a single amino acid (Peterson et al. 2004). Most of the mutations affect the different AIRE protein domains mentioned above, with a significant clustering of the mutations in the HSR domain of the N terminus, and in the PHD fingers in the C terminus of the protein (Peterson et al. 2004, Su and Anderson 2004). Of

the twenty missense mutations described to date, 17 appear either in the HSR or PHD finger domains, strongly suggesting that these domains are needed for proper AIRE function.

Predominant mutations in the populations with the highest prevalence of APECED have been identified. In Finnish patients, the R257X mutation, leading to a truncated protein lacking most of the C terminus including the PHD fingers, is found in 83% of patients (Consortium 1997, Nagamine et al. 1997, Björses et al. 2000). The same mutation is also seen at a relatively high frequency in Northern Italian and Eastern European populations (Scott et al. 1998, Cihakova et al. 2001). The 967-979del13bp mutation is the most common mutation in British and North American APECED patients (Pearce et al. 1998, Wang et al. 1998, Heino et al. 1999b), the Y85C mutation is dominant in Iranian Jews (Zlotogora and Shapiro 1992, Björses et al. 2000), and the R139X in Sardinian subjects (Rosatelli et al. 1998).

Certain APECED component diseases seem to have a correlation with specific HLA haplotypes. In their series of APECED patients, Halonen and co-workers (2000) found that HLA-DRB1*03 associated with Addison's disease, and DRB1*04- DQB1*0302 with alopecia; HLA-DRB1*15-DQB1*0602 had a negative correlation with type I diabetes. Gylling et al. found a similar protective effect (against type I diabetes) of the DQB1*0602 allele in their study of Finnish APECED patients (Gylling et al. 2000). With these exceptions, no other HLA haplotype correlations have been reported, making APECED stand out from most autoimmune endocrine diseases with their numerous and complex HLA associations (Peterson et al. 2004).

Despite the abundant variation in APECED phenotype and combinations of disease components in individual patients, no evident genotype-phenotype correlations have emerged. There are, however, some differences in the prevalence of mucocutaneous candidal infection: it is very rare in Iranian Jews sharing the Y85C mutation, and in Finnish patients carrying the K83E mutation (Zlotogora and Shapiro 1992, Nagamine et al. 1997, Björses et al. 2000).

2.4.3 AIRE expression pattern

The expression patterns of both human AIRE and mouse Aire have been extensively studied. The most prominent site of human AIRE expression is the thymus. Lesser AIRE expression is seen in other immune tissues including lymph nodes, spleen and foetal liver (Consortium 1997, Nagamine et al. 1997, Björses et al. 1999). Expression of AIRE in human differentiated dendritic cells and peripheral blood mononuclear cells has also been noted (Kogawa et al. 2002, Sillanpää et al. 2004). Notably, no expression of AIRE has been detected in the organs targeted by autoimmune destruction in APECED (Björses et al. 1999, Heino et al. 1999a). The same expression pattern is also seen in the case of mouse Aire, but in addition, expression in a wide variety of tissues has been

reported: bone marrow, the urinary tract, the genitals, the alimentary tract, the respiratory tract, the brain, and endocrine organs including the adrenals and thyroid are Aire-positive (Blechschmidt et al. 1999, Ruan et al. 1999, Heino et al. 2000, Halonen et al. 2001, Kogawa et al. 2002).

In the thymus, AIRE is only seen in a minor subset of cells, which have been identified as mTECs by immunofluorescence microscopy (Heino et al. 1999a). These findings have been elaborated in studies of mouse Aire expression. In immunofluorescence stainings, Aire was seen in cells with cytokeratin markers, with some colocalisation also with the costimulatory markers CD80, CD86, and CD40 (Heino et al. 2000). Interestingly, some Aire expression is also seen in cells of the monocytic-dendritic lineage (Heino et al. 1999a, Heino et al. 2000). RT-PCR analyses of Aire expression revealed the presence of transcripts in CD11c- and MHC class II-positive cells isolated from the thymus, as well as in two FACS-sorted thymic and splenic dendritic cell subsets (Heino et al. 2000). Thus, the main cell subsets of AIRE / Aire expression are medullary epithelial cells and dendritic cells.

The analysis of Aire expression in the thymus during mouse development indicates that Aire is first seen at day E14, a time at which the medullary and cortical epithelial cell populations can already be distinguished and T-cell progenitors have arrived to the thymus (Backstrom et al. 1998, Blechschmidt et al. 1999, Zuklys et al. 2000). The amount of Aire expression increases significantly by day E16, when CD4+CD8+ cells have already appeared, but TCR-mediated selection has not begun (Zuklys et al. 2000). Aire expression is absent in the thymus of the Tgε26 mouse which never develops a normal three-dimensional thymic architecture, due to a block in early thymocyte development at E14.5, when Aire expression should already be emerging (Wang et al. 1995, Zuklys et al. 2000). Then again, in the RAG-/- mouse, which has a block in thymocyte development at a later stage, E15.5, when the thymic epithelial environment has already been formed, there is Aire expression at levels comparable to wild type mice (Zuklys et al. 2000). These data indicate that a normal medullary architecture and/or presence of differentiating thymocytes are required for Aire expression. Further confirmatory data comes from the RelB-deficient mouse in which the medullary epithelium is disorganised and there is a lack of myeloid dendritic cells: no Aire expression is seen in this mouse model (Heino et al. 2000).

The intracellular expression of AIRE follows two distinctive patterns: a punctate nuclear pattern, and a fibrillar cytoplasmic pattern (Heino et al. 1999a). Notably, the fibrillar cytoplasmic staining has only been demonstrated in cultured cells into which AIRE has been transfected. The nuclear dot type pattern is also evident in tissue sections and peripheral blood cells. The fibrillar staining resembles intermediate filaments and / or microtubules, varying slightly

depending on the cell type used. Colocalisation of cytoplasmic AIRE with vimentin and α tubulin has been reported. (Björses et al. 1999, Heino et al. 1999a, Rinderle et al. 1999)

2.5 NUCLEAR BODIES

2.5.1 The different types of nuclear bodies

The nucleus of the mammalian cell is a highly organised and dynamic structure that contains, in addition to chromatin, a large number of subnuclear organelles. Many of these are in fact collections of various nuclear factors localised in distinct structures, made visible by immunofluorescence and / or electron microscopy (Lamond and Spector 2003). Unlike cytoplasmic organelles, they are not surrounded by a lipid bilayer (Boisvert et al. 2001). These subnuclear structures have been originally classified on the basis of their appearance under microscopy and, later, according to the nuclear factors found in them, and their function. They traditionally include Cajal bodies, speckles, Gems, and promyelocytic leukaemia (PML) bodies (Lamond and Spector 2003). In addition, recent reports have indicated the presence of novel nuclear bodies such as the Sam68/SLM nuclear body, the cleavage body, the polycomb or Pc-g body, and the clastosome (Zimber et al. 2004). Cajal bodies, Gems, speckles, and PML bodies are discussed in more detail below as they have been extensively studied.

Cajal bodies are subnuclear bodies first described in 1903 in neurons (Ogg and Lamond 2002). They are also known as coiled bodies (based on their appearance on electron microscopy) and nucleolar accessory bodies. Cajal bodies are seen in almost all cell types, with the exception of some primary cells and tissues. Typically, up to 10 Cajal bodies with a diameter of 0.1-0.2 μm are seen in a cell nucleus. Cells with higher transcriptional activity display more prominent Cajal bodies. (Ogg and Lamond 2002) Various nuclear transcription factors colocalise in Cajal bodies, including polymerase II transcription factors, nucleolar proteins, small nuclear ribonucleoproteins (snRNPs), and small nucleolar ribonucleoproteins (snoRNPs). They do not contain DNA or other (non-snRNP) splicing factors, indicating that they are not likely to be sites of transcription or pre-mRNA splicing. (Raska et al. 1991, Carmo-Fonseca et al. 1992, Thiry 1994, Ogg and Lamond 2002) Coilin is the protein predominantly used to identify coiled bodies in immunofluorescence microscopy; it was originally identified by staining cells with human autoimmune sera (Raska et al. 1991). Only a minority of coilin is present in Cajal bodies at a given time, most of it is found diffusely in the nucleus (Carmo-Fonseca et al. 1993, Platani et al. 2000). Data from coilin-deficient mice show reduced viability, and cells from these mice show Cajal-body like subnuclear domains lacking several of the nuclear factors found in wild type cells, indicating

that coilin is a key component of Cajal bodies (Hebert et al. 2001, Tucker et al. 2001). Experiments using time-lapse analysis of green fluorescent protein (GFP)-tagged coilin in living mammalian cells have shown that Cajal bodies are dynamic structures that move in the nucleus. The rate of movement detected has been slower than that in the known ATP-dependent cytoplasmic transport systems driven by motor proteins, and the movement probably depends on simple diffusion (Platani et al. 2002). Despite this, some Cajal bodies appear to be restricted in their movement, indicating some form of tethering to adjacent structures (Platani et al. 2002). Accordingly, there is evidence that Cajal bodies associate with chromatin and specific gene loci, suggesting that they may have a role in the transport of factors involved in transcription (Jacobs et al. 1999, Shopland et al. 2001, Platani et al. 2002). Recent studies indicate that the Cajal bodies harbour a minority of total nuclear snRNPs and snoRNPs, corresponding to newly assembled RNPs that are subsequently seen to colocalise respectively in speckles and the nucleolus (Carvalho et al. 1999, Gall et al. 1999, Naranyan et al. 1999, Sleeman and Lamond 1999, Sleeman et al. 2001). Thus, the current hypothesis is that the Cajal body functions as a site of snRNA modification, an important process for stable RNA function (Ogg and Lamond 2002). Other functions, such as co-ordinating the preassembly of transcription factors, and feedback regulation of gene expression have been proposed (Matera 1998, Gall 2000).

Gems (for Gemini of Cajal bodies) are nuclear bodies that are associated with the survival of motor neurons (SMN) protein, deleted in over 98% of patients with spinal muscular atrophy, a hereditary neurodegenerative disease (Zimber et al. 2004). SMN is ubiquitously expressed protein that is seen both in the cytoplasm and nucleus of mammalian cells; in the nucleus it is seen in nuclear bodies that are similar to Cajal bodies in appearance (Zimber et al. 2004). Depending on the cell type, Cajal bodies and Gems can show either significant overlapping, or separate nuclear bodies. The function of Gems is currently thought to reflect that of Cajal bodies in that the SMN protein would be involved in the assembly and metabolism of snRNPs, snoRNPs, and splicing factors (Meister et al. 2002, Massenet et al. 2002, Pellizoni et al. 1998, Paushkin et al. 2002, Zimber et al. 2004).

Speckles are another type of nuclear body with distinct functions. In immunofluorescence microscopy they are usually identified by staining with antibodies against the splicing factor SC-35 (Lamond and Spector 2003). The equivalent of the speckles seen in immunofluorescence microscopy is the interchromatin granule cluster seen in electron microscopy (Lamond and Spector 2003). Speckles are seen as small, numerous, punctate structures throughout the nucleoplasm, at sites containing little or no DNA (Thiry 1995). There is a large body of evidence that links speckles to storage / assembly / modification of transcription factors (Lamond

and Spector 2003). Imaging of live cells has shown that splicing factors are transported from speckles to sites of active transcription, and that the inhibition of transcription or pre-mRNA splicing leads to the accumulation of splicing factors in enlarged speckles (Spector et al. 1983, O'Keefe et al. 1994, Misteli et al. 1997). The main constituents of speckles include several pre-mRNA splicing factors, including snRNPs and SR proteins (Fu 1995). Several kinases and phosphatases with the ability to phosphorylate or dephosphorylate splicing factors have also been identified in speckles (Colwill 1996, Trinkle-Mulcahy 1999, Ko et al. 2001, Kojima et al. 2001, Trinkle-Mulcahy et al. 2001, Brede et al. 2002, Sacco-Bubulya and Spector 2002). As is the case with Cajal bodies, speckles also move within the nucleus, although in a relatively small area surrounding each speckle (Misteli et al. 1997). However, experiments with GFP-tagged speckle components have shown very rapid movement of the component factors into and out of the speckles (Kruhlak 2000, Phair and Misteli 2000). Lamond and Spector (2003) have suggested that speckles are formed via self-assembly, independent of an underlying scaffold or matrix component. The current hypothesis of speckle function thus is that they function as depositories and modification sites of splicing factors so that at any given time, there are sufficient numbers of these factors in a properly modified state, and that inactive or unnecessary factors are sequestered away from the "active" nucleoplasmic pool (Lamond and Spector 2003).

2.5.2 The PML bodies

The PML nuclear bodies are spherical intranuclear particles that are currently defined by the presence of the PML protein (Borden 2002) and are, in fact, similar to nuclear bodies identified by electron microscopy already in the 1960s (Melnick and Licht 1999). PML bodies are also known as PODs (PML oncogenic domains), ND10 (nuclear domain 10), and Kr bodies (Kramer bodies) (Borden 2002). PML bodies represent multiprotein complexes with more than 50 reported protein components (Maul et al. 2000, Borden 2002, Dellaire and Bazett-Jones 2004). PML bodies exist in almost all cell lines studied to date, and they are present at all stages of the cell cycle (Chan et al. 1997, Borden 2002). Typically, 10-30 bodies, 0.2 – 1 μm in diameter, are seen per nucleus. Some PML is also seen in the cytoplasm, at least in some cell types (Melnick and Licht 1999). PML bodies are associated with the nuclear matrix and remain unchanged after treatment of cells with RNAse and DNAse (Stuurman et al. 1992, Sahlas et al. 1993, Nayler et al. 1998). The expression of PML is inducible by interferon, and treatment leads to increases in the number and size of PML bodies (Chelbi-Alix et al. 1995, Lavau et al. 1995, Maul et al. 1995). Furthermore, the structural integrity of PML bodies is altered by various stresses, including viral infections and other environmental stress factors (Maul et al. 1995, Borden 2002, Dellaire and Bazett-Jones 2004).

There is no DNA, RNA, TFIIH, or polymerase II in the PML bodies, which makes direct participation in transcription highly unlikely (Ascoli and Maul 1991, Grande et al. 1996, Melnick and Licht 1999).

The association of PML bodies with acute promyelocytic leukaemia (APL) has been established recently. APL is a myelogenous leukaemia characterised by a block in myeloid cell development in the promyelocyte stage (Grimwade and Solomon 1997, Melnick and Licht 1999). The disease is caused in over 90% of cases by a chromosomal translocation t(15:17) leading to the generation of a fusion protein joining PML and retinoic acid receptor α (PML-RAR α) (Melnick and Licht 1999). The PML body staining pattern (visualized by staining against PML) is disrupted in APL patients carrying the translocation, resulting in a diffuse speckled pattern in both the nucleus and cytoplasm (Kastner et al. 1992, Dyck et al. 1994, Weis et al. 1994). Treatment of patients with all-trans retinoic acid reverses this by facilitating the proteolysis of the fusion protein (not affecting the endogenous production of wild type PML and RARα), leading to reformation of the PML bodies and clinical remission in the patients, thus linking the PML bodies and their integrity to disease in humans (Grimwade and Solomon 1997, Melnick and Licht 1999, Jing 2004). Currently the opinion is that loss of RAR α leads to a block in differentiation, and loss of PML to loss of growth control (Grimwade and Solomon 1997, Melnick and Licht 1999). It must be stated, however, that PML $^{-/-}$ mice are completely viable, develop normally, and do not seem to develop cancer at rates higher than wild type mice (Wang et al. 1998).

In addition to PML, several proteins have been described to localise in PML bodies. These include the transcriptional coactivator CBP, Sp100 and Sp140, Daxx, p53, Rb, Ataxin-1, Huntingtin, BLM, and WRN proteins (Skinner et al. 1997, Sternsdorf et al. 1997, Shen and Loeb 2000, Yankiwski et al. 2000, Pearson and Pelicci 2001, Regad and Chelbi-Alix 2001, Kegel et al. 2002). These different proteins participate in a wide range of cellular functions and some are involved in the pathogenesis of neurodegenerative disorders (Ataxin-1, Huntingtin, BLM, and WRN in spinocerebellar ataxia, Huntington's diseases, Bloom syndrome, and Werner's syndrome respectively) (Zimber et al. 2004).

The subnuclear localisation of PML bodies may offer some clue as to their function. Interestingly, they seem to have a preference for certain locations. They seem to cluster in the presence of other functional nuclear compartments, such as the speckles or Cajal bodies discussed above, as well as cleavage bodies (Grande et al. 1996, Melnick and Licht 1999). It has also been reported that of all the PML bodies per nucleus, one is always found adjacent to an MHC locus on chromosome 6, independent of transcriptional activity or phase of cell cycle (Shiels et al. 2001). As U2-snRNP and U1-snRNP are absent from PML bodies, it is possible that they are involved in the

processing of RNA (Grande et al. 1996, Borden 2002). Like the other nuclear bodies discussed so far, PML bodies also move in the nucleus. Three separate populations of bodies could be separated based on the range and rate of motion in analyses of PML bodies in live cells: 25% were stationary, 63% showed minimal motion, and 12% moved very rapidly, in an ATP-dependent fashion (Muratani et al. 2002). It has been suggested that PML bodies may act as depositories of nuclear proteins, and possibly form a catalytic surface on which specific biochemical reactions could occur (Borden 2002).

Although the exact nature of the biochemical processes PML bodies are involved in remains largely unknown, there is some recent data elucidating the function of PML and PML bodies. PML interacts with the eIF4E (eukaryotic initiation factor 4E) protein that binds an m^7G cap to mRNA, a rate-limiting step in the initiation of translation (Sonenberg and Gingras 1998, Lai and Borden 2000, Kentsis et al. 2001) A small proportion of total cellular eIF4E is found in the nucleus, where it probably participates in nuclear translation of proteins, as part of the nonsense-mediated decay pathway (Iborra et al. 2001). Furthermore, it has been shown that overexpression of eIF4E leads to increased transport of mRNAs (notably including cyclin D1) to the cytoplasm, although the amount of the specific mRNA remains the same; thus the level of the different proteins increases without an increase in transcription (Rosenwald et al. 1995, Rousseau et al. 1996). When this is combined with the data showing that PML can inhibit the 5' capping activity of eIF4E more than 100 fold (being the first biochemical function demonstrated for PML) (Kentsis et al. 2001), it seems that PML and the PML bodies could participate in the posttranscriptional regulation of gene expression (Borden 2002). PML bodies have also been suggested to function in apoptosis (Takahashi et al. 2004) and DNA damage repair (Dellaire and Bazett-Jones 2004).

2.6 CREB-BINDING PROTEIN (CBP)

The CREB (cyclic AMP response element-binding protein) binding protein (CBP) is a transcriptional coactivator possessing intrinsic histone acetyl transferase (HAT) activity, and shares significant homology with p300, a highly related protein sharing many of the functions of CBP (Vo and Goodman 2001, Kalkhoven 2004). The current opinion on coactivator function implies that the coactivators bind to transcription factors, positioning HATs near specific target gene promoter regions to enable the chromatin modifications required for transcription to proceed (Sterner and Berger 2000, Vo and Goodman 2001). CBP is a ubiquitously expressed protein that is required for viability: loss of a single allele in humans is associated with the Rubinstein-Taybi syndrome showing severe developmental defects (Petrij et al. 1995). A deletion of both alleles (of CBP or p300) in mouse leads to embryonic lethality (Yao et al. 1998, Kung et al. 2000). Loss of CBP

function has also been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, amytrophic lateral sclerosis, and the polyglutamine tract diseases. These are, however, preliminary data on animal models and confirmatory data from humans remains to be shown (Rouaux et al. 2004). CBP has been shown to associate directly with over forty transcription factors, including nuclear hormone receptors, and to affect the activity of several more. It also interacts with components of the basal transcription machinery, including TBP, RNA helicase A, and TFIIB and thus functions as a coactivator to many of the transcription factors it is associated with (Chrivia et al. 1993, Arias et al. 1994, Kwok et al. 1994, Blobel 2000, Goodman and Smolik 2000, Shikama et al. 2000, Vo and Goodman 2001, Kalkhoven 2004).

The CBP protein (Figure 3) contains several conserved protein domains including a bromo domain, three cysteine and histidine rich domains (CH1-CH3), and a large domain responsible for the histone acetyl transferase activity (HAT domain). The bromo domain, found in several other proteins, is thought to mediate binding to histones. The CH1 and CH3 account for interaction with most of the transcription factors reported to bind CBP. (Vo and Goodman 2001, Kalkhoven et al. 2002) Recently, the CH2 domain, which is located within the HAT domain, was shown to contain a PHD type zinc finger that is essential for the HAT activity of CBP (Kalkhoven et al. 2002). In this context, the HAT activity was required for full coactivation of target promoters (Kalkhoven et al. 2002). It must be noted, however, that while CBP possesses intrinsic HAT activity, it also interacts with other HAT proteins, such as pCAF and SRC1. It is probable that CBP exerts its effects through several mechanisms, and that the relative importance of these depends on the transcription factor that is being coactivated. (Vo and Goodman 2001, Kalkhoven et al. 2002)

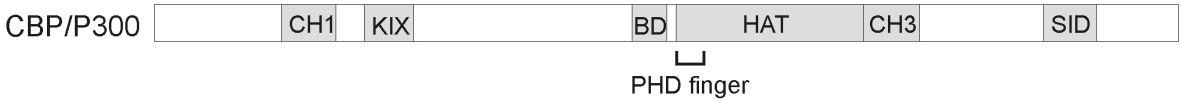

Figure 3. A schematic of the wild type CBP/p300 protein. The protein domains are indicated as follows: CH1 and CH3, cysteine-histidine rich domains; HAT, histone acetyl transferase domain; BD, bromo domain; KIX, binding site for CREB; SID, steroid receptor coactivator I interaction domain. The location of the PHD type zinc finger within the HAT domain is indicated.

The regulation of transcription at the single gene level is a complex process involving several steps of interaction of multiple, large, protein complexes, with the final step being the assembly of the

basal transcription complex on the promoter (Vo and Goodman 2001). Regulation is achieved through the participation of additional components such as coactivators, enhanceosomes, and mediator complexes, which seem to integrate the various intracellular and extracellular signals that affect transcription (Vo and Goodman 2001). CBP has been shown to be involved in the interferon β (IFNβ) enhanceosome complex, where NFκB, ATF-2/c-Jun, regulatory factors, and HMG1(Y) are recruited to the IFNβ promoter region. CBP appears to mediate the interaction of these factors with components of the basal transcriptional machinery (Kim et al. 1998, Wathelet et al. 1998). Although the detailed mode of action of CBP remains unknown, it is clear that it is a key regulator of assembly and modification of the basal transcription machinery, probably integrating various intracellular and extracellular signals (Vo and Goodman 2001).

The subcellular localisation of the CBP protein shows similarities to that of AIRE: in some cell lines, it is seen as spherical nuclear dots in immunofluorescence stainings (LaMorte et al. 1998, Boisvert et al. 2000, Boisvert et al. 2001). In cells where the nuclear pattern is seen (SK-N-SH, COS-1, CHO), CBP resides in PML bodies, completely colocalising with PML (LaMorte et al. 1998, Boisvert et al. 2000, Boisvert et al. 2001). Even in cells where CBP normally is not seen in PML bodies, it can be induced to translocate to them by overexpression of PML or CBP itself, and upon the treatment of cells with interferon α (Boisvert et al. 2001). It has been shown that CBP can move very rapidly into and out of the PML bodies (Boisvert et al. 2001). Taken together, these data imply a mechanism of regulation of transcription related to PML bodies: it is possible that they account for the regulation of the concentration of CBP in specific areas in the nucleus that correspond to sites in close proximity of transcriptionally active chromatin (Boisvert et al. 2001).

3. AIMS OF THE STUDY

Since the inception of this study in 1998, a wealth of data concerning the molecular pathogenesis of APECED and the function of the AIRE protein has emerged, both from the work described here and from several researchers in the same field. As initial analysis of the (predicted) protein structure of AIRE indicated a role in the regulation of transcription, and functional data concerning the protein were scarce, the specific aims of the present study were:

- to see whether AIRE could regulate transcription in model systems of transcriptional activation / repression
- to find AIRE-interacting proteins and the functional significance of such interactions
- to examine more closely the subcellular localisation of AIRE, and its regulation and impact on function
- to determine the protein domains responsible for the different functions and subcellular localisations of AIRE
- to identify target genes for AIRE-mediated transcriptional regulation

4. MATERIALS AND METHODS

An overview of the experimental procedures is presented here. The detailed reaction conditions can be found in the original communications.

4.1 CLONING OF EXPRESSION VECTORS AND MUTAGENESIS (Studies I-IV)

No cell lines to date have been found or established that naturally express the endogenous AIRE protein. Thus, transient and stable transfections of AIRE were used to introduce AIRE into mammalian cells. The expression vectors containing the full length or truncated AIRE cDNA were constructed either by amplifying the desired cDNA fragment by PCR, or by cutting it from existing constructs using restriction enzymes, and by then cloning the fragment in question into an appropriate vector. The identities of the cloned inserts were confirmed by sequencing. Table 6 lists all the vectors constructed for this study.

When mutations in the AIRE cDNA needed to be introduced, two techniques were employed: PCR mutagenesis or the GeneEditor mutagenesis kit (Promega). The PCR mutagenesis followed a standard technique, where two slightly overlapping fragments together covering the full length target cDNA were amplified by PCR using two sets of primers. The primers in the overlapping stretch of DNA contained the nucleotide changes that yield the mutation. The two fragments produced were then used as templates for another PCR that yields the full length cDNA, with the desired mutation. This was then cloned into the appropriate target vector using standard techniques. The GeneEditor (Promega) system was used to generate point mutations according to the instruction manual. Again, the presence of the mutations was confirmed by sequencing.

Table 6. Expression constructs used in this thesis.

name	insert (aa / mut.)	backbone	usage	note
pGST-AIRE (1-545)	AIRE 1-545	pGEX1λT	GST pull-down	full length AIRE
pGST-AIRE (86-545)	AIRE 86-545	pGEX1λT	GST pull-down	truncation
pGST-AIRE (175-545)	AIRE 175-545	pGEX1λT	GST pull-down	truncation
pGST-AIRE (292-545)	AIRE 292-545	pGEX1λT	GST pull-down	truncation
pGST-AIRE (1-207)	AIRE 1-207	pGEX1λT	GST pull-down	truncation
pJEX-AIRE (1-207)	AIRE 1-207	pJEX1	GST pull-down	truncation
pGST-AIRE (1-138)	AIRE 1-545 / R139X mut.	pGEX1λT	GST pull-down	truncation; patient mutation
pGST-AIRE (1-256)	AIRE 1-545 / R257X mut.	pGEX1λT	GST pull-down	truncation; patient mutation
pGST-AIRE (1-293)	AIRE 1-545 / K294X mut.	pGEX1λT	GST pull-down	truncation
pGST-AIRE (1-348)	AIRE 1-545 / Q349X mut.	pGEX1λT	GST pull-down	truncation
pGST-AIRE L28P/K83E	AIRE 1-545 / L28P, K83E	pGEX1λT	GST pull-down	HSR domain mutation
pGST-AIRE L28P	AIRE 1-545 / L28P	pGEX1λT	GST pull-down	HSR domain (patient) mutation
pGST-AIRE C437P	AIRE 1-545 / C437P	pGEX1λT	GST pull-down	PHD2 domain mutation
pCAIRE	AIRE 1-545	pCDNA3.1B	GST pull-down	full length AIRE
pJEX-AIRE (86-545)	AIRE 86-545	pJEX1	GST pull-down	truncation
pJEX-AIRE (175-545)	AIRE 175-545	pJEX2	GST pull-down	truncation
pJEX-AIRE (292-545)	AIRE 292-545	pJEX3	GST pull-down	truncation
pJG-AIRE	AIRE 1-545	pJG-45	yeast two-hybrid	full length AIRE
pLEX-AIRE	AIRE 1-545	pLEXA	yeast two-hybrid	full length AIRE
pM-AIRE (1-545)	AIRE 1-545	pM	mammalian two-hybrid	full length AIRE GAL4 DNA binding domain fusion
pVP16-AIRE (1-545)	AIRE 1-545	pVP16	mammalian two-hybrid	full length AIRE activation domain fusion
pM-AIRE (175-545)	AIRE 175-545	pM	mammalian two-hybrid	truncation
pM-AIRE (1-216)	AIRE 1-216	pM	mammalian two-hybrid	truncation
pM-AIRE (1-138)	AIRE 1-545 / R139X mut.	pM	mammalian two-hybrid	truncation; patient mutation
pM-AIRE (1-256)	AIRE 1-545 / R257X mut.	pM	mammalian two-hybrid	truncation; patient mutation
pM-AIRE (1-293)	AIRE 1-545 / K294X mut.	pM	mammalian two-hybrid	truncation
pM-AIRE (1-348)	AIRE 1-545 / Q349X mut.	pM	mammalian two-hybrid	truncation
pM-AIRE L28P/K83E	AIRE 1-545 / L28P, K83E	pM	mammalian two-hybrid	HSR domain mutation
pM-AIRE L28P	AIRE 1-545 / L28P	pM	mammalian two-hybrid	HSR domain (patient) mutation
pVP16-AIRE L28P/K83E	AIRE 1-545 / L28P, K83E	pVP16	mammalian two-hybrid	HSR domain mutation
pVP16-AIRE L28P/K83E	AIRE 1-545 / L28P	pVP16	mammalian two-hybrid	HSR domain (patient) mutation

pGAD424-AIRE	AIRE 1-545	pGAD424	yeast two-hybrid	full length AIRE GAL4 activation domain fusion
pGBT9CBP	CBP 1-2442	pGBT9	yeast two-hybrid	full length CBP GAL4 DNA binding domain fusion
pGEXCBP	CBP 1-2442	pGEX2T	GST pull-down	full length CBP
pSI-AIRE (1-545)	AIRE 1-545	pSI	mammalian expression	full length AIRE
pSI-AIRE (86-545)	AIRE 86-545	pSI	mammalian expression	truncation
pSI-AIRE (175-545)	AIRE 175-545	pSI	mammalian expression	truncation
pSI-AIRE (292-545)	AIRE 292-545	pSI	mammalian expression	truncation
pSI-AIRE (1-138)	AIRE 1-545 / R139X mut.	pSI	mammalian expression	truncation; patient mutation
pSI-AIRE (1-256)	AIRE 1-545 / R257X mut.	pSI	mammalian expression	truncation; patient mutation
pSI-AIRE (1-293)	AIRE 1-545 / K294X mut.	pSI	mammalian expression	truncation
pSI-AIRE (1-348)	AIRE 1-545 / Q349X mut.	pSI	mammalian expression	truncation
pSI-AIRE L28P/K83E	AIRE 1-545 / L28P, K83E	pSI	mammalian expression	HSR domain mutation
pSI-AIRE L28P	AIRE 1-545 / L28P	pSI	mammalian expression	HSR domain (patient) mutation
pSI-AIRE K83E	AIRE 1-545 / K83E	pSI	mammalian expression	HSR domain (patient) mutation
pSI-AIRE C302P	AIRE 1-545 / C302P	pSI	mammalian expression	PHD1 domain mutation
pSI-AIRE C437P	AIRE 1-545 / C437P	pSI	mammalian expression	PHD2 domain mutation
pSI-AIRE C302P/C437P	AIRE 1-545 / C302P, C437P	pSI	mammalian expression	PHD1/PHD2 domain mutation
pGST-AIRE C302P	AIRE 1-545 / C302P	GST	GST pull-down	PHD1 domain mutation
pGFP-AIRE NLS	AIRE 101-141	pEGFP-C3	immunofluorescence	green fluorescent protein fusion
pGFP-H2B	Histone H2b 1-126	pEGFP-C2	immunofluorescence	full length histone 2B
pEGFP-AIRE	AIRE 1-545	pEGFP-C1	immunofluorescence	full length AIRE
pFLAG-AIRE	AIRE 1-545	pCR3	mammalian expression	full length AIRE, FLAG tag
pFLAG-AIRE C299A	AIRE 1-545 / C299A	pCR3	mammalian expression	full length AIRE, PHD1 mutation
pFLAG-AIRE C434A	AIRE 1-545 / C434A	pCR3	mammalian expression	full length AIRE, PHD2 mutation
pMX-puro-FLAG-AIRE	AIRE 1-545	pMX-puro-FLAG	retroviral system	full length AIRE
CMX-CBP	CBP 1-2442	CMX	mammalian expression	full length CBP
CMX-PML	PML 1-641	CMX	mammalian expression	full length PML
pRc/RSV-mCBP-HA-RK	CBP 1-2442	pRc	mammalian expression	full length CBP, HA tag
CMX-GAL4-CBP	CBP 1-2442	CMX	GAL4 promoter assays	full length CBP GAL4 DNA binding domain fusion

Table 6 (continued)

4.2 REPORTER SYSTEMS

The ability of AIRE to regulate transcription was studied in transient transfection experiments where wild type AIRE as well as various truncation and missense mutants was cotransfected with a reporter construct. In assays of coactivation of AIRE by CBP, both CBP and AIRE were cotransfected with the reporter. The amounts of DNA, the method of transfection, and the amount of cells used per assay were optimised for each cell line and application as detailed in the original communications. Two reporter systems: the GAL4 system and the interferon beta minimal promoter system were used.

4.2.1 GAL4 system (Studies I, III)

The ability of AIRE to regulate transcription was first tested in a GAL4 based reporter system, using the Mammalian Matchmaker kit (Clontech). The genes of interest (AIRE and various mutants thereof) were cloned into the pM vector as in-frame fusions with the GAL4 DNA binding domain. The reporter construct pG5-CAT contains three GAL4 response elements upstream of the E1b minimal promoter driving the expression of chloramphenicol acetyl transferase (CAT). The pM construct and the pG5-CAT reporter were cotransfected into mammalian cells (HUH-7 or COS cells) using calcium phosphate precipitation according to the manufacturer's instructions. The total amount of DNA was brought up to 10 μg using an empty vector (pBlueScript KS). The cells were grown on six well plates. All transfections were performed in triplicate. The CAT activity was measured from cell lysates 48 hours after transfection using an ELISA assay (CAT ELISA, Roche Molecular Biochemicals). The CAT activity of the empty pM vector was used as the baseline value to which the values yielded from the pM-AIRE constructs were compared.

In Study III, the reporter construct $(GAL4)_3$TK-luc was used, and the reporter gene product (luciferase) was assayed using the Luciferase Assay System (Promega) according to the manufacturer's protocol. Cells were grown and transfected on 24-well culture plates.

4.2.2 Interferon beta system (Studies II, III)

In the interferon beta system the reporter plasmid (pIFNβ-LUC) contains nucleotides -55 to +19 of the interferon beta minimal promoter, upstream of a luciferase gene. The AIRE constructs were introduced into the pSI mammalian expression vector (Promega) that merely expresses the gene of interest (without a DNA binding domain as in the GAL4 system). HEK293, COS-1 and COS-7 cell lines were used.

In Study III HEK293 and COS-7 cells were transfected using the ExGen transfection reagent according to the manufacturer's instructions. Cells were transfected and grown on 24-well

culture plates. 0.1 µg of the reporter and 0.3 µg each of the other constructs were transfected. The total amount of DNA was maintained constant by adding necessary amounts of empty vector (pSI). In Study II, COS-1 cells were transfected using electroporation. $1x10^6$ cells were transfected with 2 µg of the reporter and 2 µg of the pSI-AIRE construct.

For all cell lines, luciferase activity was measured from cell lysates 48 (Study II) or 24 (Study III) hours after transfection using the Luciferase Assay System (Promega). Again, the activity from the reporter alone was used as the baseline to which the activities from the pSI-AIRE (and CMX-CBP where applicable) transfections were compared.

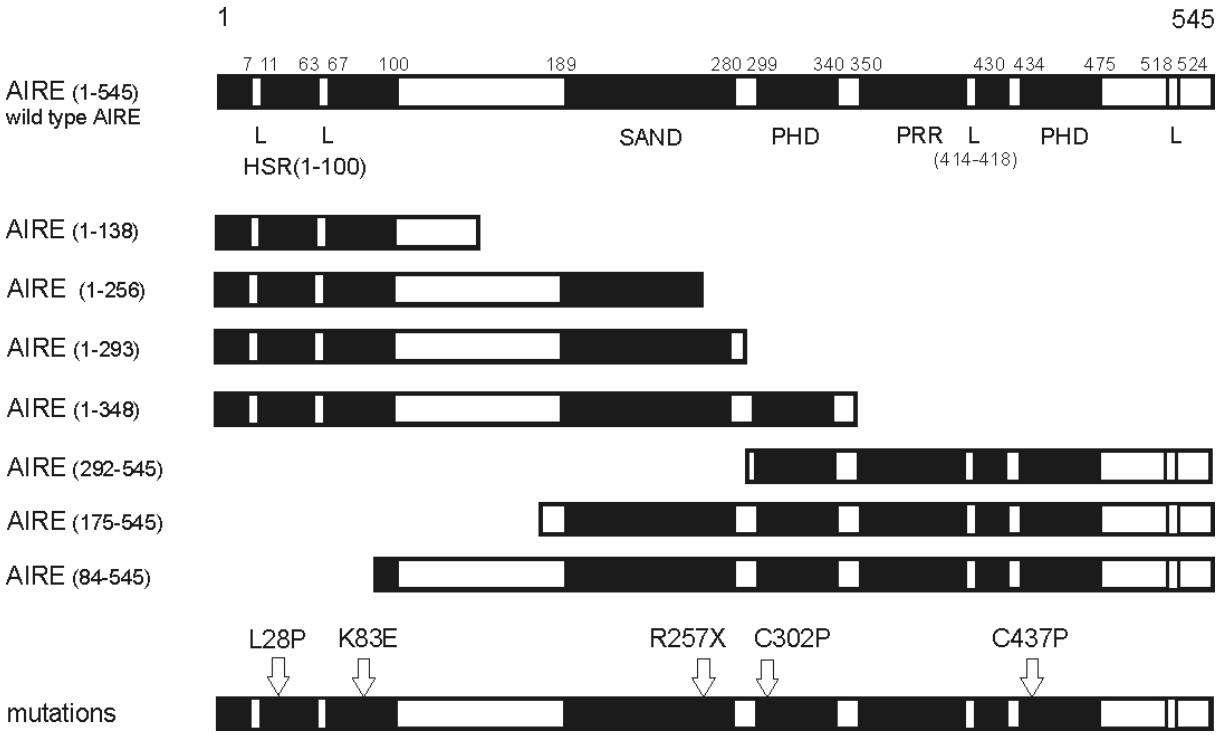

Figure 4. Schematic of the AIRE protein and deletion constructs. The deletion constructs and mutations analysed in this thesis are shown. L28P, K83E, and R257X are APECED patient mutations. The C302P and C437P mutations were designed to disrupt the structure of the PHD fingers

4.3 PROTEIN – PROTEIN INTERACTION ASSAYS

4.3.1 Mammalian Matchmaker (Study I)

The Mammalian Matchmaker System was used to study AIRE-AIRE homodimerisation. The system allows the user to study the interaction of two proteins of interest in the physiological setting of mammalian cells. The system relies on the GAL4 transcription factor and a reporter responsive to it, as detailed above. The proteins of interest are cloned into two vectors: one as an in-frame fusion with the GAL4 DNA binding domain in the pM vector, and the other as an in-frame fusion with the GAL4 activation domain in the pVP16 vector. The DNA binding domain of pM directs the construct to the reporter promoter (via the GAL4 response elements). If the proteins of interest interact, the activation domain is also brought to the proximity of the reporter, and expression of the reporter gene, CAT, ensues. Wild type AIRE and a series of deletion mutants were cloned into the pM and pVP16 vectors to study homodimerisation and to delineate the domain responsible for the interaction. The transfections and CAT assays were performed as detailed above, and the CAT activity yielded by the empty pM and pVP16 vectors was used as the baseline value.

4.3.2 Yeast two-hybrid system (Study I)

Yeast two hybrid assays were used to study AIRE-AIRE homodimerisation and AIRE-CBP interaction, with essentially the same principle of action as that in the Mammalian Matchmaker system described above. The reporter gene used for the detection of interaction is beta-galactosidase. For the homodimerisation experiments, the Matchmaker LexA (Clontech) system was used according to the manufacturer's instructions. Wild type AIRE was cloned into the pLEXA (DNA binding domain fusion) and pJG-45 (activation domain fusion) and cotransformed into the CG-1945 yeast strain. The yeast were grown on leucine, histidine and uracil deficient selection medium and assayed for beta-galactosidase expression by a colony-lift assay.

For the AIRE-CBP interaction studies, a series of pGBT9-CBP (DNA binding domain fusion) plasmids (wild type and deletion mutants) and pGAD424-AIRE (activation domain fusion) were cotransformed into the Y190 yeast strain. The transformants were grown on tryptophan and leucine deficient plates, and selected colonies were inoculated into 1 ml liquid cultures and assayed for beta-galactosidase expression as described (Anderson et al. 1998). All experiments were performed in triplicate.

4.3.3 GST pull-down assays (Study I)

In vitro studies of AIRE-AIRE homodimerisation and domain mapping were done using the GST pull-down assay. Wild type AIRE and a series of deletion mutants were labelled with [^{35}S]methionine on the TNT T7-coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. The *in vitro* translation was performed with constructs cloned into the pJEX vector. The GST fusion proteins were expressed and purified as described (Frangioni and Neel 1993). The wild type AIRE and deletion mutants were cloned into the pGEX1λT vector (from Kalle Saksela, University of Tampere) as in frame fusions with glutathione S transferase (GST). In brief, the GST fusion proteins were expressed and purified in bacterial cultures and bound to a glutathione Sepharose matrix (Pharmacia). These were then incubated with the radiolabelled AIRE polypeptides, washed, eluted in SDS loading buffer and run on 10% SGS-PAGE gels. Detection of binding of the radiolabelled proteins was performed by exposing the SDS gels to radiography film. GST alone was used to control for non-specific binding to GST.

4.3.4 Far western assays (Study I)

The far western assay was used to study AIRE-CBP interaction, and to map the interaction domain in CBP. The method closely resembles the GST pull-down assay described above. In this technique, the purified GST fusion proteins were first run on SDS-PAGE gels, and the proteins were then transferred to nitrocellulose membranes. The membranes were then incubated with radiolabelled polypeptides, washed, and analysed after exposure to radiography film, as above. CBP and deletion mutants were expressed and purified from pGEX-CBP, and wild type AIRE was labelled with [^{35}S]methionine as described above.

4.4 MOLECULAR MODELLING (Study I)

The amino terminus of AIRE, containing the HSR domain, was modelled on the basis of the known structure of CBL, a proto-oncogene that has a four-helix bundle structure (Meng et al. 1999). The sequences of the two proteins were aligned based on the features of amino acids in the four-helix bundle structure. The programs Insight II and Discover (Molecular Simulations, Inc.) were used to create the model.

4.5 CELL CULTURE AND TRANSFECTIONS (Studies I-IV)

The following mammalian cell lines were used in the studies included in this thesis: COS-1 and COS-7 (SV40-transformed green monkey kidney cells), RD (human rhabdomyosarcoma), U937 (human monocyte), HUH-7 (human hepatocyte), HeLa (human cervical epithelium), mTEC 1C6

(human thymic medullary epithelium), NIH-3T3 (mouse fibroblast), HEK-293 (human kidney), and NB4 (human promyelocyte).

COS-1, HUH-7, RD, HeLa, NIH-3T3, HEK293, and mTEC 1C6 cells were maintained as monolayers, and U937 and NB4 cells were maintained in suspension, both in appropriate media supplemented with 100 units/ml penicillin/streptomycin and 10% foetal calf serum.

COS-1 and HUH-7 cells were transfected using the calcium phosphate precipitation method in Study I (Ausubel et al. 1994); U937 and NB4 cells were transfected by electroporation. RD cells (and in later experiments, COS-1 and HEK293 cells) were transfected using the ExGen transfection reagent (MBI), according to the manufacturer's instructions. HeLa, mTEC 1C6, and NIH-3T3 cells were transfected with the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions.

The number of cells, the amount of DNA per transfection, and the appropriate method were optimised for each experiment as detailed in the original communications.

4.6 IMMUNOFLUORESCENCE STAINING AND MICROSCOPY (Studies II, III, IV)

Immunofluorescence staining of mammalian cells and human thymus sections was used to study the subcellular localisation of AIRE, CBP, and PML. The same techniques were used to stain endogenously expressed proteins and cells transiently or stably transfected to express the proteins of interest.

COS-1, COS-7, HEK293, and RD cells were grown on coverslips on the bottoms of the wells in a six-well plate; U937 and NB4 cells were grown in suspension, and transferred to microscopy slides using a Cytospin centrifuge. The cells were transfected as detailed above, and 24 to 48 hours after transfection, fixed and stained using antibodies specific for AIRE, CBP, PML, or acetyl-histone H3 as described (Heino et al. 1999a). In Studies II and IV FITC-labelled secondary antibodies were used when single stainings were performed. In dual stainings, Texas Red-labelled secondary antibodies were included. DAPI staining was used in all instances to stain the nuclei. In Study III, Alexa-594 and Alexa-488 were used as secondary fluorescent antibodies.

Both regular immunofluorescence and laser confocal microscopy were used to detect the subcellular staining pattern of AIRE, CBP, or PML. Confocal microscopy is described below. Immunofluorescence analysis was performed on an Olympus BX-50 microscope, and images were captured using a Photometrics Imagepoint cooled CCD video camera and IPLab Spectrum 3.1a software.

4.7 QUANTITATIVE PCR (Study III)

For the analysis of endogenous IFNβ mRNA, HEK293 cells were transfected as described above, and 24 h post transfection trichostatin A (TSA, a histone deacetylase inhibitor) was added where indicated. TSA was used to prevent the suppressive activity of histone deacetylases in the assay. After a further 24 h incubation, the cytoplasmic fraction of the cells was isolated as described (Lykke-Andersen et al. 2000), then cytoplasmic RNA was purified, followed by cDNA synthesis. The cDNA was then subjected to quantitative PCR to measure the amounts of IFNβ mRNA using the ABI Prism 7000 SDS instrument (Applied Biosystems) and the qPCR SYBR Green Core Kit (Eurogentec).

4.8 CONFOCAL MICROSCOPY AND THREE-DIMENSIONAL RECONSTRUCTION (Study III)

In later experiments studying the subcellular localisation of AIRE, CBP, PML, and histone H2B in more detail, laser confocal microscopy on an Ultraview Confocal Imaging System (Perkin Elmer Life Sciences), mounted on an Olympus IX70 microscope, was used. 20 to 40 z-plane sections of individual cells were acquired, and subjected to three-dimensional rendering using Volocity v. 1.4.4 (Improvision) software. The Volocity software enables exact measurement of the dimensions of image elements, including the volume. This feature was used to calculate the volumes of AIRE and PML nuclear dots under AIRE, CBP or PML staining. The volumes were measured as voxels (three-dimensional image elements) contained in a given nuclear dot. The mean dot voxel counts for AIRE-CBP and PML-CBP nuclear bodies were calculated, and compared using a two-tailed t-test in Microsoft Excel 2000 (Microsoft).

4.9 CREATION OF A CELL LINE EXPRESSING A GFP-AIRE FUSION GENE (Study IV)

No cell line known to date expresses endogenous AIRE. Thus, a cell line stably expressing AIRE was created. To facilitate analysis of subcellular localisation under different conditions, a fusion gene expressing GFP-AIRE was used. HeLa cells were transfected with the GFP-AIRE vector, and 48 h post transfection, culturing in selective medium containing G418 (Gibco BRL) was started. After 14 days of selection, fluorescent clones were detected by immunofluorescence microscopy, and clones positive for the fusion gene were further expanded to give a cell line expressing GFP-AIRE that was named GFP-AIRE/HeLa.

4.10 ISOLATION OF CELLULAR PROTEINS (NUCLEAR MATRIX ISOLATION) (Study IV)

In order to study whether AIRE is localised subcellularly in the nuclear matrix fraction, GFP-AIRE/HeLa cells grown on coverslips were subjected to sequential extraction *in situ*, using detergent lysis, DNase I treatment, ammonium sulphate and high salt, to leave only the nuclear matrix fraction (He et al. 1990). Disappearance of DAPI staining on immunofluorescence microscopy was used as a measure of the efficiency of chromatin digestion and removal, and the presence of the nuclear matrix after the treatments was assayed by the detection of lamin A/C using immunofluorescence staining.

4.11 ASSESSMENT OF UBIQUITINATION OF AIRE (Study IV)

The modification of AIRE by ubiquitin was assessed using an immunoprecipitation approach. Vectors expressing Myc-tagged AIRE and HA-tagged ubiquitin were cotransfected into COS-7 cells. Immunoprecipitation of cell lysates was performed using a monoclonal anti-Myc antibody (Santa Cruz Biotechnology), and the precipitates were subjected to immunoblotting using a polyclonal anti-HA antibody (Santa Cruz Biotechnology).

AIRE protein expressed in 293T cells was also subjected to peptide mass fingerprinting: AIRE was expressed using a modified tandem affinity purification (TAP) system (Oshikawa et al. 2003). Purified proteins were separated on an SDS-PAGE gel, and Coomassie blue-stained bands were excised from the gel. The excised bands were digested with trypsin, and the molecular masses of the digested peptides were determined using a MALDI-TOF mass spectrometer (Bruker Daltonics). Protein identification was done using the MASCOT search engine (Matrix Science).

The possible self-ubiquitination activity of AIRE was studied using an *in vitro* ubiquitination assay. Recombinant full length AIRE protein, Ub, recombinant E1 and Ubc4 (E2) were mixed in the presence of ATP. Immunoprecipitation of the reaction with anti-AIRE antibodies was performed, with subsequent immunoblotting of the precipitate with anti-Ub antibodies to detect ubiquitinated proteins.

5. RESULTS

5.1 AIRE IS A TRANSCRIPTIONAL ACTIVATOR (Studies I, II)

5.1.1 AIRE activates transcription when fused with a heterologous DNA binding domain (Study I)

Initial characterisation of the AIRE gene and protein revealed several conserved protein domains whose presence in the predicted polypeptide strongly suggested a role in the regulation of transcription (Consortium 1997, Nagamine et al. 1997). Particularly interesting in this respect were the plant homeodomain (PHD) type zinc fingers in the carboxy terminus of AIRE and the SAND domain, a putative DNA binding motif (Gibson et al. 1998); the PHD finger is a structural motif seen in a variety of proteins mediating transcriptional control, often at the chromatin level (Aasland et al. 1995), and DNA binding would be a typical feature of a transcription factor.

First, the full length AIRE cDNA was fused in frame with the GAL4 DNA binding domain (DBD. The transcriptional influence of AIRE was assayed using a reporter construct. Wild type AIRE was found to activate transcription in the GAL4 system, causing a significant increase, measuring approximately 30 fold over baseline activation (Figure 5A). Neither an N terminal (AIRE 1-216) nor a C terminal (AIRE 175-545) deletion construct was able to activate transcription, indicating that, in this system, the full length protein is required.

5.1.2 AIRE activates transcription from the interferon beta minimal promoter (Studies II, III)

The GAL4 system demonstrated that AIRE possesses transcriptional regulatory properties. However, the GAL4 system does not mimic the natural setting very efficiently as the fusion protein is forced to the vicinity of the minimal promoter by the GAL4 DNA binding domain. The activity of AIRE was therefore tested on the HIV long terminal repeat (LTR), the mouse mammary tumour virus promoter (MMTV), and the interferon beta (IFNβ) minimal promoter to see whether AIRE could activate any of these without artificial DNA binding. In each case, the luciferase gene was used as the reporter with the corresponding promoter upstream.

AIRE activated all of the promoters tested, at levels comparable to that seen in the GAL4 system (Study II, Figure 5B and our unpublished data). The interferon beta minimal promoter, containing nucleotides -55 to +19 of the promoter region, was selected for further analysis as the other two are viral promoters.

5.1.3 The PHD type zinc fingers form the activation domain (Study II)

In order to clarify the roles of the conserved protein domains of AIRE in its function, N and C terminal deletion constructs were tested in the interferon beta minimal promoter system. None of the N terminal deletions showed significant activation of the interferon beta minimal promoter, whereas all the C terminal constructs tested showed activation comparable to wild type. The construct lacking the first 291 amino acids of the N terminus, AIRE (292-545), was sufficient for activation. AIRE (292-545) contains both the PHD zinc fingers and the proline rich region lying between these. Thus, the PHD zinc fingers are implicated as the activation domain. To further confirm the assumption, the missense mutants AIRE C302P, AIRE C437P and AIRE C302P/C437P were generated, carrying cysteine to proline changes in the first, second, or both PHD domains respectively. The mutants target conserved cysteines in the PHD fingers, with the likely result of severe disruption of their structure. All mutants had reduced activity in the IFN beta system, approximately 30% of wild type (Figure 5B). It is also of note that two of the C terminal deletions tested do not contain the bipartite nuclear localisation signal (NLS), and still maintain the capacity to activate transcription.

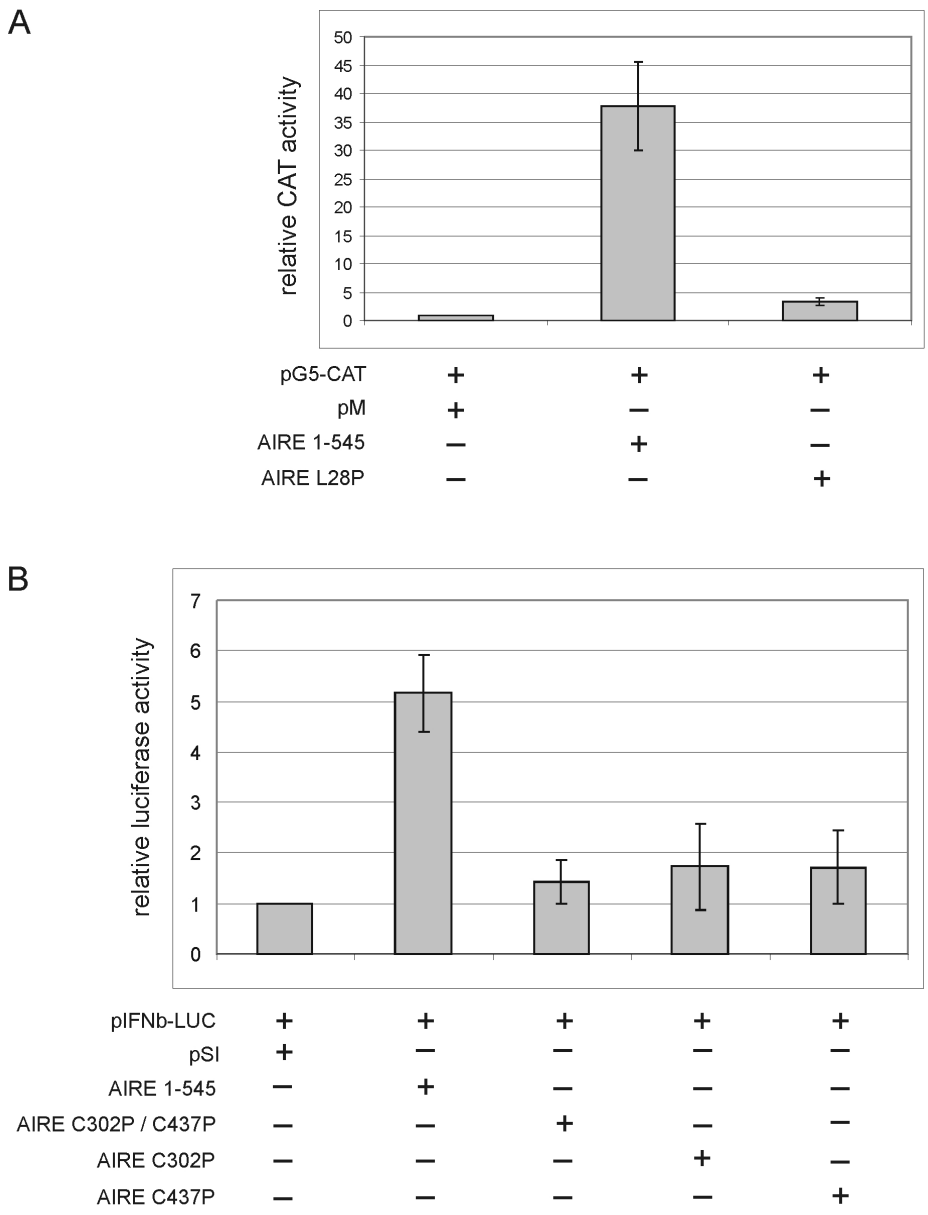

Figure 5. AIRE activates transcription from the GAL4 and IFNβ promoters. A, AIRE (fused to the GAL4 DNA binding domain) activates transcription. The L28P patient mutation significantly reduces activation. B, AIRE activates transcription in the IFNβ minimal promoter system. The PHD fingers are required as missense mutations designed to alter their structure significantly reduce activation. Plasmids were transfected into COS-1 (A) and HeLa (B) cells as indicated. Reporter activity relative to an empty expression vector is shown. The results are pooled from at least three independent transfections. The error bars are ±1SD. Empty pBS-KS vector added where needed to keep the amount of DNA transfected constant.

5.1.4 APECED-causing mutations interfere with AIRE activity (Studies I, II, III)

In order to study the role of APECED-causing patient mutations on the transactivation capacity of AIRE the following mutations were tested: AIRE L28P, K83E, R139X, and R257X. These represent the two common types of APECED-causing mutations: nonsense mutations that truncate the protein so that it lacks the PHD fingers (R139X, R257X); and missense mutations that change single amino acids in the N terminal HSR domain. The R257X mutation is also the predominant Finnish mutation, seen in 83% of patients (Consortium 1997, Nagamine et al. 1997).

As could be expected of mutations leading to such severe structural alterations, AIRE R139X (Study I) and R257X (Study III) were unable to activate transcription in our reporter systems. The same was true for the AIRE L28P mutant (Figure 5A and Study III) which was only marginally active. Surprisingly, the other mutation affecting the HSR domain, AIRE K83E, showed transactivation at levels indistinguishable from the wild type protein (Study II). This hints at the existence of other functions of AIRE no less critical than the transcriptional activation. It also demonstrates that the alteration of different amino acids in a given domain, albeit in close proximity to each other, can have dramatically varying consequences on the function of the protein. The fact that patient mutations interfere with AIRE transactivation does, however, underline its importance in the physiological function of the molecule.

5.2 CBP COACTIVATES AIRE ON THE INTERFERON BETA MINIMAL PROMOTER (Studies I, III)

5.2.1 CBP physically interacts with AIRE through the CH1 and CH3 domains (Study I)

As AIRE showed strong transactivation properties, it was considered interesting to examine what other proteins might be involved in mediating or regulating that function. Initial experiments were carried out with a common coactivator of transcription, the CREB-binding protein (CBP). Using far Western assays it was shown that CBP interacts with AIRE via the minimal CH1 and CH3 interaction domains. This *in vitro* binding was then confirmed using the yeast two-hybrid system where AIRE interacted with the minimal CH1 domain. Binding with the minimal CH3 domain in yeast cells could not be shown; however, a deletion construct lacking the CH1 domain (ΔCH1) also bound wild type AIRE.

5.2.2 CBP coactivates AIRE in a dose-dependent manner (Study III)

The physical interaction of AIRE and CBP, a known coactivator protein, naturally prompted the question whether this interaction might also have a functional dimension. Thus, the GAL4 system

and the interferon beta minimal promoter system were used to see if CBP coactivates AIRE. The assays were performed in two cell lines: HEK293 and COS-7. Both cell lines express CBP but not AIRE.

Cotransfection of increasing doses of CBP together with GAL4-AIRE led to a significant dose-dependent enhancement in activation (up to 120 fold) compared to GAL4-AIRE alone. CBP alone also caused some activation in this system, around 4 to 5 fold compared to GAL4 alone. When GAL4-CBP was used, a very strong activation was seen (up to 200 fold), as could be expected. When AIRE was cotransfected, the activation was again enhanced, up to 3 fold. The results were essentially the same for both cell lines studied: synergistic coactivation of the GAL4 promoter by AIRE and CBP. As the introduction of these proteins to close proximity of the promoter as GAL4-fusions was required for the full activation, indirect cellular pathways are unlikely to contribute significantly. The common Finnish APECED-causing mutation R257X was also tested and, consistent with previous results, there was no activation when GAL4-AIRE R257X was transfected alone. Further, there was no significant coactivation of the mutant by CBP.

Next, the coactivation was tested on the IFNβ minimal promoter in HEK293 cells. Both AIRE and CBP expressed alone caused a small increase in promoter activity (1.5- 2 fold). The activation seen when the two proteins were expressed together was up to 6 fold, again indicating co-operative activation. The R257X and L28P patient mutations were also tested in this system, as well as the C437P and C302P/C437P PHD finger-disrupting mutations. There was practically no coactivation of AIRE R257X or AIRE L28P, and the slight coactivation seen with C437P and C302P/C437P was reduced to less than half of that seen with wild type AIRE. Although the absolute coactivation of the IFNβ minimal promoter was lower than in the GAL4 system, the deleterious effect of APECED patient mutations indicates the specificity of the data.

5.2.3 AIRE and CBP enhance the expression of endogenous interferon beta mRNA (Study III)

As AIRE and CBP could co-operatively activate the transfected IFNβ minimal promoter, their influence on endogenous IFNβ expression was next examined. The expression of IFNβ mRNA was measured using quantitative RT-PCR in HEK293 cells. TSA was used to counteract suppressive histone deacetylase activity. In untreated cells, transfection of AIRE increased IFNβ mRNA expression 2 fold; CBP had no effect. In TSA-treated cells, AIRE and CBP alone showed 4 fold and 1.5 fold increases in the amount of IFNβ mRNA respectively. When the two were cotransfected, the IFNβ mRNA level increased up to 10 fold, suggesting that AIRE and CBP can act together to coactivate the endogenous IFNβ promoter.

5.3 SUBCELLULAR LOCALISATION OF AIRE (Studies II, III)

Early papers on the characterisation of AIRE expression noted that AIRE is seen in two distinct subcellular patterns: small, spherical nuclear dots, and filamentous structures in the cytoplasm, later identified as intermediate filaments (Heino et al. 1999a, Rinderle et al. 1999, Björses et al. 2000). Using various mutants and deletion constructs, it was undertaken to determine the various protein domains responsible for the different localisations, and their relation to AIRE function.

5.3.1 The amino terminal HSR domain is responsible for localisation in cytoplasmic filaments and for homodimerisation (Studies I, II)

Testing various AIRE deletion constructs, showed that only polypeptides containing an intact N terminus could be observed in the cytoplasmic filaments; constructs lacking the N terminus exhibited a diffuse cytoplasmic staining pattern (Table 7). The minimal construct able to form filaments was AIRE (1-207), containing the HSR domain. Full length polypeptides carrying missense mutations in the HSR domain were then tested: AIRE L28P and AIRE L28P/K83E. Neither was able to localise in cytoplasmic filaments, displaying a diffuse staining instead. Thus, the HSR domain confers the localisation of AIRE into filamentous structures in the cytoplasm. It is noteworthy that the L28P patient mutation was also unable to activate transcription in the reporter assays (Study I).

Existing data on Sp100, a nuclear dot protein which, like AIRE, has an N terminal HSR domain, indicated that Sp100 homodimerisation occurs through the HSR domain (Sternsdorf et al. 1999). It was thus tested whether the same might also apply for AIRE. The homodimerisation of the full length AIRE protein, AIRE (1-545), was first tested in a GST pull-down assay. *In vitro* translated, radiolabelled AIRE (1-545) bound GST-AIRE (1-545) but not GST alone, confirming that AIRE can form homodimers *in vitro*. The interaction was then tested in the mammalian two-hybrid system. In that context, AIRE (1-545) was also able to form homodimers. Moreover, the N terminal deletion constructs tested were able to bind full-length AIRE, implicating the N terminus of the protein in dimerisation. The same was seen in GST pull-down assays and, in addition, labelled AIRE (1-207) bound GST-AIRE (1-207), indicating that the N terminus is sufficient for AIRE-AIRE interaction. The N terminus is also required for homodimerisation as C terminal deletion constructs (AIRE 84-545, 175-545, and 292-545) were unable to interact with full-length GST-AIRE (1-545).

To further map the dimerisation domain, point mutations were introduced into the HSR domain: AIRE L28P and AIRE L28P/K83E, mimicking patient mutations. Neither mutant was

able to form homodimers as tested in the mammalian two-hybrid system. Thus, AIRE is able to homodimerise through the N terminal HSR domain.

Computer homology modelling was used to build a three-dimensional model of the N-terminal HSR domain, based on a structural prediction. Secondary structure prediction strongly suggested that the HSR region of AIRE is alpha helical; the proto-oncogene CBL was found to form a four-helix bundle containing alpha helices of similar length to those in AIRE. Based on this information, the HelicalWheel and GCG program packages were used to refine the structural prediction with the result that the HSR domain forms a four helix bundle, similar to that of CBL. Most of the APECED patient mutations in the HSR domain are missense mutations, indicating that this region is susceptible to conformational changes. The L28P patient mutation, which abrogates AIRE homodimerisation by changing the leucine residue at position 28 to proline would cause a bend to the alpha helix it resides in, thereby affecting the loop between the first two helices. This would weaken the packaging of the entire domain structure, with the demonstrated consequence of rendering AIRE-AIRE binding dysfunctional.

Table 7. Transactivation capacity and subcellular localisation of AIRE deletion constructs and mutations.
CBP coact., coactivation by CBP; cytopl., cytoplasmic; CBP coloc., colocalisation with CBP
nuc. dot., loclisation in nuclear dots; fibr., localisation in cytoplasmic fibrils

			subcellular localisation				
Deletion construct or mutant	activation	CBP coact.	cytopl.	nuclear	nuc. dot	fibr.	CBP coloc.
1-545 (wild type)	+	+	+	+	+	+	+
1-256 (R257X)	–	–	+	+	–	+	N.D.
1-293	–	N.D.[1]	+	+	–	+	N.D.
1-348	–	N.D.	+	+	–	+	N.D.
84-545	+	N.D.	(+)	+	–	–	N.D.
175-545	+	N.D.	+	+	–	–	N.D.
292-545	+	N.D.	+	+	–	–	N.D.
L28P	–	–	+	+	–	–	–
L28P/K83E	–	N.D.	+	+	–	–	N.D.
K83E	+	N.D.	+	+	+	+	+
C302P	–	N.D.	+	+	+	+	N.D.
C437P	–	–	+	+	+	+	N.D.
C302P/C437P	–	–	+	+	+	+	N.D.

[1] *N.D. indicates not done*

5.3.2 AIRE is subject to nuclear export (Study II)

Upon analysing the subcellular localisation of the different deletion constructs, it was noted that all the polypeptides containing the bipartite nuclear localisation signal (NLS) in the N terminus were also seen in the nucleus of transiently transfected cells (Table 7). To see if the NLS of AIRE on its

own is sufficient for nuclear transport, it (AIRE amino acids 101-141) was cloned as an in-frame fusion with the green fluorescent protein, into the pEGFP-C3 expression vector (Clontech). The AIRE NLS could efficiently transport GFP into the nucleus, demonstrating the functionality of the domain.

The localisation of AIRE both in the cytoplasm and nucleus suggested that it might also be shuttled between the nucleus and cytoplasm. Nuclear import is obvious, and confirmed by the functionality of the NLS. The possible active export of AIRE from the nucleus was then tested for. Initial experiments performed with leptomycin B (LMB), a specific inhibitor of CRM-1-mediated nuclear export (Nishi et al. 1994, Fukuda et al. 1997, Kudo et al. 1999), showed that AIRE accumulates in the nucleus upon LMB treatment, indicating that it is subject to nuclear export, most likely mediated by the CRM-1 pathway. Putative nuclear export signals (NESs) were found in both the C and N terminus of AIRE. On testing deletion constructs, it was found that the N terminus harbours the AIRE NES, as AIRE (1-207) behaved in a manner identical to wild type upon LMB treatment. More credence to the hypothesis is offered by the fact that AIRE (84-545), lacking a potential NES, is localised almost exclusively in the nucleus (Table 7).

5.3.3 The localisation of AIRE is dependent on cell cycle and the proteasome pathway (Study IV)

As there is no cell line known to express (endogenous) AIRE, a HeLa cell line stably transfected with GFP-AIRE was established, expressing a fusion of AIRE and the green fluorescent protein. Existing data on PML nuclear bodies indicated that their formation is dependent on the stage of the cell cycle (Seeler and Dejean 1999, Zhong et al. 2000). Time-lapse analysis of the stably transfected GFP-AIRE/HeLa cells revealed that the AIRE nuclear dots largely dissolve before mitosis, and are then reformed almost immediately after cell division, much like PML bodies, indicating that the subcellular localisation of AIRE is dependent on the cell cycle.

Recent evidence on the regulation of the cellular dynamics of PML suggested that it is under the control of the proteasome pathway (Mattsson et al. 2001). The subcellular localisation of AIRE was therefore studied under proteasome pathway inhibition with MG132 and ALLN (proteasome inhibitors), in GFP-AIRE/HeLa cells, and also in mouse medullary thymic epithelial cells (mTECs) and NIH3T3 cells. Upon proteasome pathway inhibition, the total level of AIRE protein per cell was increased, as well as the staining intensity and size of the AIRE nuclear dots. The AIRE nuclear dots were also translocated to the immediate proximity of the nucleoli, but not inside them, as there was no colocalisation with nucleolin. Thus, the intracellular amount of AIRE protein seems to be controlled by the proteasome pathway.

5.3.4 AIRE is subject to ubiquitination (Study IV)

To confirm that AIRE is under the regulation of the ubiquitin-proteasome pathway, the possible subjection of AIRE to modification by ubiquitin was examined. Coimmunoprecipitation experiments showed that AIRE is ubiquitinated. A recent paper by Uchida et al (2004) showed that AIRE itself functions as a Ubiquitin (Ub) E3 ligase. Many E3 ligases exhibit self-ubiquitination activity; therefore the hypothesis that AIRE may also ubiquitinate itself was tested. AIRE was immunoprecipitated from an *in vitro* ubiquitination reaction, and ubiquitination was analysed by immunoblotting with an anti-Ub antibody. The total reaction mixture contained an abundance of ubiquitinated proteins, but none were observed in the anti-AIRE immunoprecipitate, indicating that AIRE is unlikely to be a self-ubiquitinating protein. This rather suggests that another, currently unknown Ub E3 ligase is responsible for the modification of AIRE with ubiquitin.

5.3.5 AIRE is a component of the nuclear matrix (Studies III, IV)

The nuclear matrix is an insoluble, skeletal framework in the nucleus, directing specific sites of the chromatin for access to factors regulating gene transcription (Nickerson 2001, Uchida et al. 2004). As AIRE is found in distinct nuclear dots, it seemed interesting to see whether AIRE is associated with the nuclear matrix, which would certainly be consistent with its function as a transcriptional regulator. Stably transfected GFP-AIRE/HeLa cells grown on coverslips were sequentially extracted to leave the nuclear matrix. Most of nuclear AIRE, in the form of nuclear dots, was retained with this fraction. The finding was confirmed by costainings against lamin A/C, which showed that the nuclear matrix was present.

As AIRE was shown to be associated with the nuclear matrix, the colocalisation of AIRE with chromatin was tested for, which might indicate that the AIRE nuclear dots are actual sites of transcription. To achieve this, the human histone H2B (H2B) was cloned in-frame with the green fluorescent protein in a mammalian expression vector. The staining pattern was taken to represent chromatin in the nucleus; an AIRE-expressing vector was cotransfected with the GFP-H2B vector, and the relative localisations were analysed by immunofluorescence and confocal microscopy with three-dimensional image reconstruction.

When AIRE and GFP-H2B were costained, it was evident that the stainings did not overlap. AIRE nuclear dots were seen interspersed inside the histone H2B staining (representing chromatin) covering the entire area (and volume) of the nucleus. The AIRE dots resided in small pockets devoid of chromatin. Consistent with this observation, AIRE did not colocalise with acetylated histone H3 (indicating sites of active transcription). Thus, AIRE nuclear bodies are unlikely to be sites of active transcription.

5.3.6 AIRE colocalises with CBP in cultured cells and in rare cells in the human thymus (Study III)

In analysing the AIRE-CBP interaction described above coimmunostainings were performed in RD, HEK293, and NB4 promyelocytic leukaemia cells (carrying the typical PML-RARα fusion protein arising from a chromosomal translocation). The same costainings were also done in human thymus sections from healthy donors, against endogenous AIRE, CBP, or PML. PML was included in the experiments as CBP has been shown to be associated with PML nuclear bodies (Doucas 2000, Maul et al. 2000, Zhong et al. 2000).

There is no detectable PML or AIRE expression in RD cells (our unpublished data); accordingly, CBP showed a diffuse nuclear staining (Figure 6). When AIRE was introduced by transient transfection, CBP accumulated in AIRE nuclear dots. The same was observed when PML was transfected: CBP was seen in PML nuclear bodies. However, when AIRE and PML were cotransfected and costained with CBP (AIRE-CBP and PML-CBP and AIRE-PML costainings), CBP was still seen in PML bodies. There was no colocalisation of AIRE and CBP or AIRE and PML, indicating that AIRE and PML form discrete nuclear dots, and that CBP preferentially colocalises with PML when both proteins are present. The results were somewhat different in NB4 leukaemia cells that contain the PML-RARα fusion protein (and no detectable AIRE expression): the staining pattern with anti-PML antibodies showed a more diffuse, speckled pattern reflecting the aberration in PML body structure caused by the PML-RARα fusion (Figure 7). In costainings, CBP was still seen to colocalise with PML. When AIRE was transfected, CBP still mainly colocalised with PML, although partial overlapping with AIRE nuclear bodies was seen in rare cells. Thus, the PML part of the fusion protein can still compete with AIRE for CBP binding.

In HEK293 cells, endogenous CBP was partially seen in a small minority of AIRE nuclear bodies, with most of it still localising in PML bodies. When HA-tagged CBP was overexpressed, there was near complete localisation of the HA-tagged CBP in AIRE nuclear bodies, indicating that endogenous CBP may be present at limiting conditions, and that overexpression of CBP or lack of PML allows more CBP to associate with AIRE.

The colocalisation of AIRE carrying APECED-causing mutations L28P and K83E with CBP was analysed in RD cells (our unpublished results). The K83E mutation had no discernible effect on the staining pattern of AIRE, and showed a complete colocalisation with CBP (Table 7). AIRE L28P, on the other hand, was unable to form AIRE nuclear bodies, and there was no colocalisation with CBP.

The relative localisations of AIRE, CBP and PML were then studied in human thymus sections. AIRE expression was seen in a subset of cells in the vicinity of Hassal's corpuscles in the

thymus medulla. All AIRE-positive cells also expressed CBP, and in most cells, both proteins formed separate nuclear dots. However, in a small minority of CBP- and AIRE- positive cells (approximately 5%) CBP was seen in AIRE nuclear bodies. When CBP and PML were costained, CBP was seen in PML nuclear bodies. There was no colocalisation between AIRE and PML. Thus, in the human thymus, the natural environment of AIRE expression, there is a partial colocalisation of CBP in AIRE nuclear bodies.

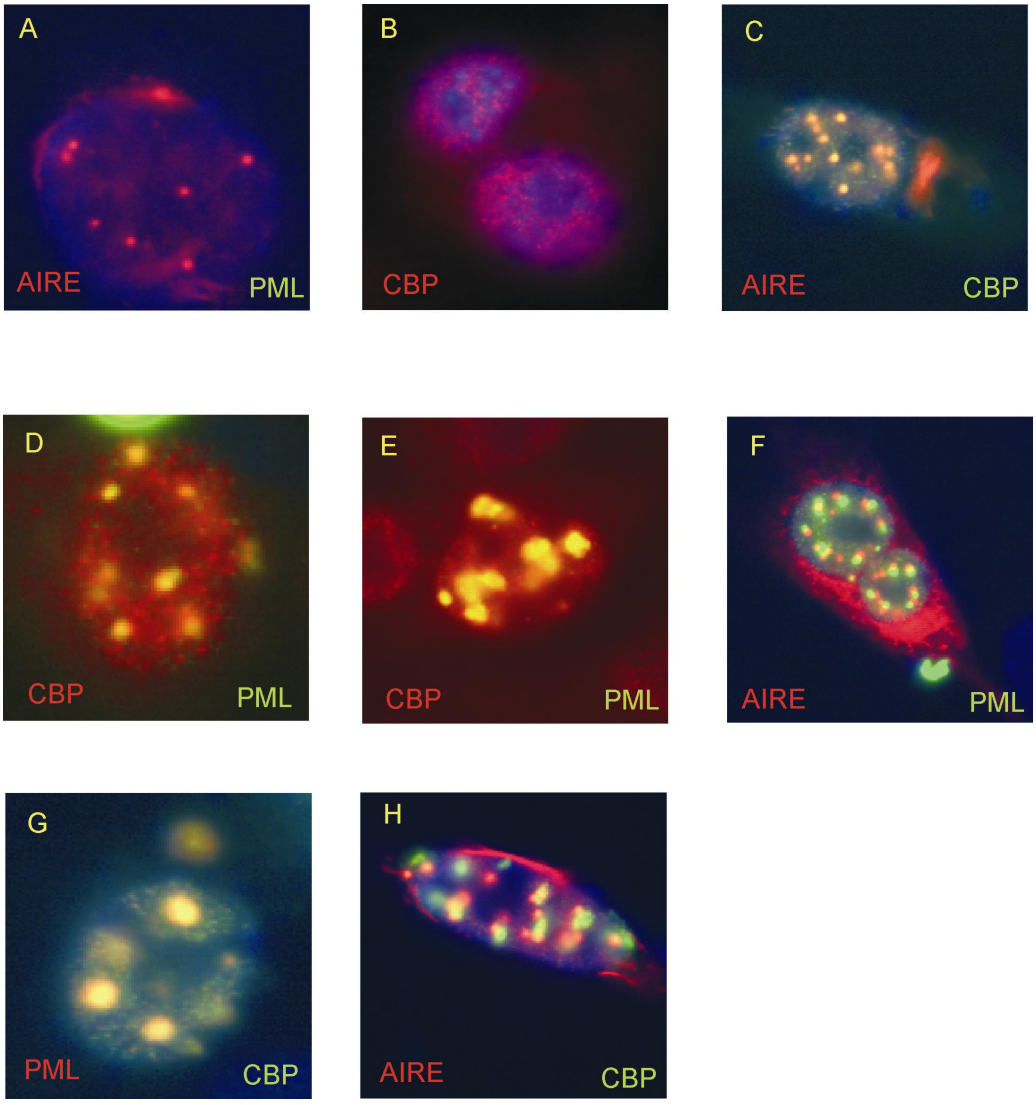

Figure 6. AIRE, CBP, and PML in RD cells. AIRE forms distinct nuclear dots. There is no detectable endogenous PML staining in RD cells (A). Endogenous CBP in these cells does not form nuclear dots but is found in a diffuse / speckled pattern (B). Transiently transfected AIRE recruits CBP into AIRE nuclear dots, although some diffuse staining remains (C). Transient transfection of PML alone leads to the recruitment of CBP to PML dots (D). In some cells, PML overexpression forms large aggregates. Even then CBP still colocalises with PML (E). When AIRE and PML are cotransfected, both form discrete, separate nuclear dots with partial overlap of staining but clearly no colocalisation (F). In these conditions, CBP colocalises with PML (G). Thus, in cotransfected cells (with AIRE and PML) AIRE does not colocalise with CBP (H). CBP therefore preferentially colocalises with PML. Merged images where blue colour indicates DAPI staining (the nucleus), green FITC, and red Texas Red secondary antibody staining (the text in the bottom corners indicates the primary antibody target). Yellow colour indicates colocalisation.

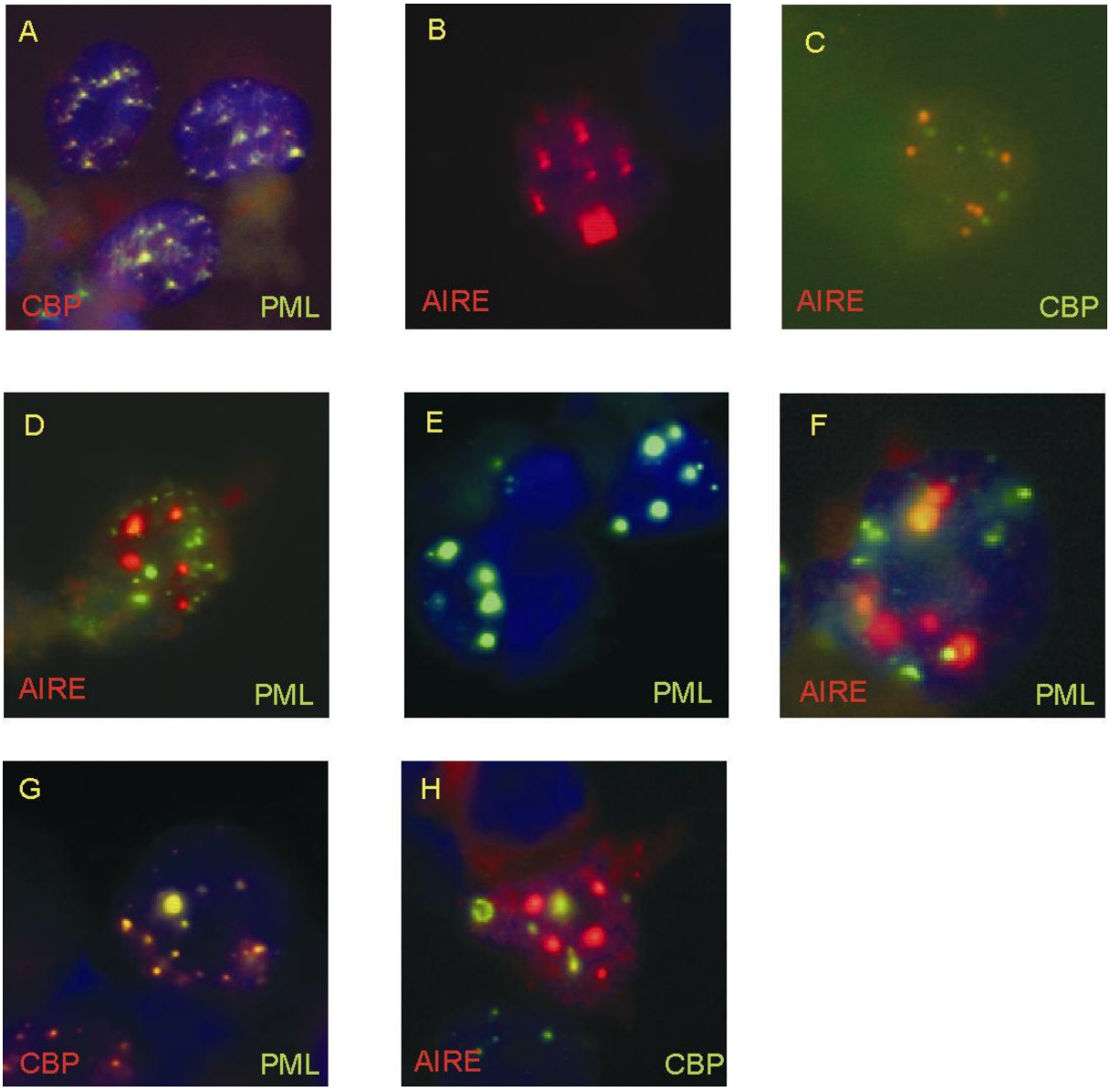

Figure 7. AIRE, CBP and PML in NB4 promyelocytic leukaemia cells. In these cells the PML staining (reflecting the localisation of the PML-RAR fusion protein) is diffuse / speckled, and colocalises with endogenous CBP (A). Transiently transfected AIRE forms discrete nuclear dots (B). In most cells, AIRE does not colocalise with endogenous CBP (C), but in a minority of cells there is a partial overlap (not shown). AIRE clearly does not colocalise with endogenous PML-RARα (D). When wild type PML is overexpressed, distinct PML bodies are formed (E). In cotransfection AIRE and PML form separate nuclear dots (F). Endogenous CBP then colocalises with PML (G) but not with AIRE (H). Merged images where blue colour indicates DAPI staining (the nucleus), green FITC, and red Texas Red secondary antibody staining (the text in the bottom corners indicates the primary antibody target). Yellow colour indicates colocalisation.

5.3.7 Three-dimensional analysis of AIRE nuclear dot structure (Study III)

Laser confocal microscopy was used to analyse in detail the structure of AIRE nuclear dots, in RD cells costained with anti-AIRE and anti-CBP antibodies. Interestingly, in some dual immunofluorescence images of AIRE and CBP colocalisation, the AIRE staining appeared larger. Three-dimensional reconstruction of confocal image stacks was thus employed to scrutinise the structure of the AIRE nuclear dots. The AIRE nuclear dots themselves, when visualised with this technique, took the shape of spherical nuclear dots (Figure 8) as could be expected based on the two-dimensional immunofluorescence results (Björses et al. 1999, Heino et al. 1999a, Rinderle et al. 1999, Björses et al. 2000).

The three-dimensional structure of AIRE dots in RD cells transfected with AIRE, and stained against AIRE and CBP, where AIRE and CBP colocalise in the same nuclear dots (see above) was next examined. The AIRE staining of a particular nuclear body formed a larger dot than when the same dot was visualised by CBP staining, with CBP lying inside, and AIRE forming the outer surface. The AIRE nuclear dots, when imaged alone without anti-CBP staining were not hollow; thus AIRE and CBP are likely to be intermingled inside the AIRE dots, whereas the outer surface seems to be formed by AIRE alone. To exclude artefacts the secondary fluorochromes were switched and costainings with GFP-AIRE and endogenous CBP were also done. The results still showed the same dual substructure of the AIRE nuclear dots (Study III and unpublished data).

PML nuclear bodies in RD cells transfected with a PML-expressing vector, where CBP is seen in PML bodies, were then examined to see whether the structure described above is a more common feature of nuclear bodies. In PML nuclear bodies, however, CBP and PML were intermingled, with both proteins apparently occupying the same volume.

The volumes of the individual nuclear dots were then measured under AIRE – CBP and PML-CBP staining respectively. If there really was a difference in the spaces occupied by AIRE and CBP, the volume of the dots under AIRE staining would have to be larger. A total of 31 AIRE nuclear dots and a total of 62 PML nuclear dots were analysed. The AIRE stained nuclear dots were on average two times larger than the same dots stained against CBP. The difference was statistically significant. There was no statistically significant difference in the volumes of the PML dots under PML or CBP staining respectively.

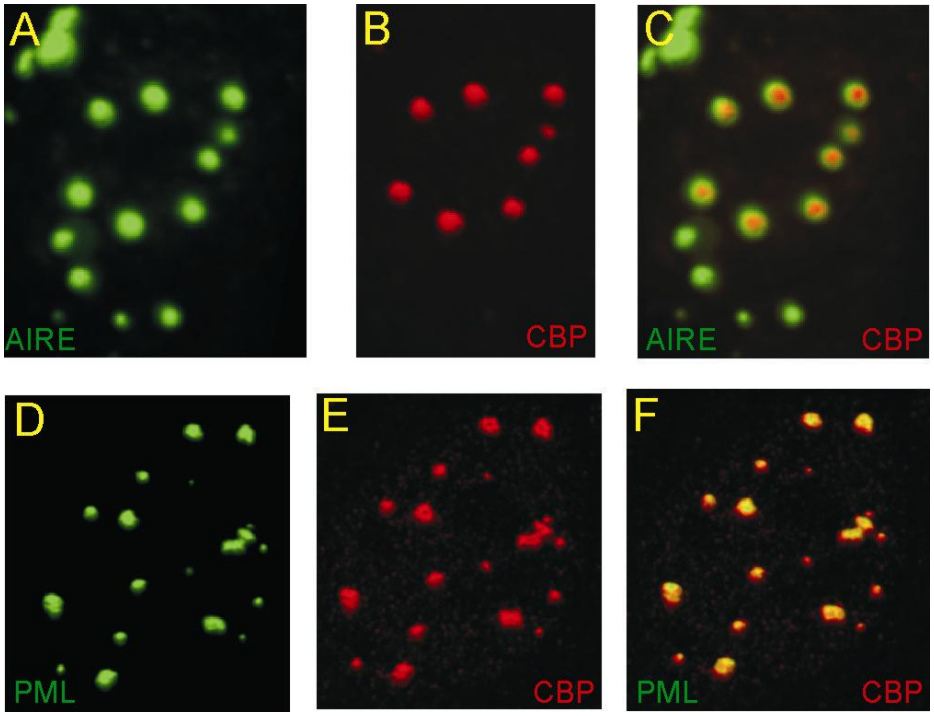

Figure 8. Three-dimensional structure of AIRE nuclear bodies. Three-dimensional reconstructions of two-dimensional image stacks of RD cells stained with anti-CBP, anti-PML, and anti-AIRE antibodies. FITC (green) and Texas Red (red) secondary antibodies used. Panels A-C show AIRE nuclear bodies in cross-section with approximately half the volume of the nuclear bodies cut away from the top. In the single stainings, the bodies in the AIRE staining (A) appear larger than in the CBP staining (B). When the two are merged, the CBP staining (red) is seen in the inside, surrounded by the AIRE (green) staining (C). Panels D-F show PML nuclear bodies viewed from the outside (i.e. not in cross-section). The individual stainings are similar in size (D and E), and in the merged image, the stainings are intermingled, with the surface apparently containing both proteins (F).

6. DISCUSSION

A general discussion of the results of this thesis and those in the current literature on the function of AIRE and the pathogenesis of APECED is presented here. More detailed discussions on the results of this thesis can be found in the original communications.

6.1 AIRE is a transcriptional activator

Consistent with the presence of protein domains in AIRE that are shared by proteins controlling or modulating transcription, it was shown that AIRE functions as a potent activator of transcription, the first biochemical function defined for this protein (Studies I, II, III). The capacity of AIRE to activate transcription in the GAL4 system (while fused to a heterologous DNA binding domain) has also been demonstrated by Björses and co-workers (Björses et al. 2000). That mouse Aire exhibits similar transcriptional activation in the same system underlines the importance of this function with regard to the physiological role of AIRE (Halonen 2003). Also, APECED-causing patient mutations significantly inhibited transactivation both in the GAL4 and the IFNβ systems (Studies I, II, III)

It was found that AIRE can also activate transcription from various mammalian promoters in cotransfections with reporter plasmids carrying the promoters upstream of a reporter gene. The MMTV, HIV LTR, and IFNβ minimal promoter were tested in an effort to find a model system for the study of AIRE activity without having to express it as a fusion protein with a DNA binding domain. As the first two are viral promoters, the IFNβ minimal promoter was selected for further experiments. The fact that a C-terminal deletion construct (AIRE 175-545) showed nearly wild type transcription activation in the IFNβ system, and none in the GAL4 system indicates that tethering of the protein of interest with the GAL4 DBD may alter its function, possibly by interfering with protein folding.

The exact mechanism by which AIRE activates transcription in these model systems is not known. AIRE carries the SAND domain, shown to mediate DNA binding (Gibson et al. 1998, Bottomley et al. 2001). There is also evidence that AIRE can directly bind DNA, at least in the *in vitro* setting (Kumar et al. 2001). The same group mapped the DNA binding to the SAND and PHD finger domains, each recognizing a specific DNA sequence in electrophoretic mobility shift assays (Purohit et al. 2005). Interestingly, an APECED-causing missense mutation targeting the SAND domain (G228W) completely abolishes the transcriptional activation by AIRE (Halonen et al. 2004). It is thus possible that AIRE binds to specific DNA sequences to activate transcription. As most transcription factors are involved in multiprotein complexes, it is not surprising that AIRE has

been reported to form large complexes, presumably also consisting of several proteins (Halonen et al. 2004). Thus, AIRE may participate in large protein complexes involved in the regulation of transcription, possibly mediating DNA binding in this setting. Whether either of these biochemical properties (i.e. DNA binding and complex formation) is indeed involved in the transcriptional activation imposed by AIRE remains to be shown. The computational analysis of the localisation of Aire-controlled genes (derived from microarray analyses of gene expression in Aire-positive and Aire-negative MECs) along the mouse chromosomes revealed siginifcant clustering of Aire-controlled genes (Johnnidis et al. 2005). However, it was noted that Aire-controlled genes were often interspersed with Aire-independent genes in a punctate pattern, indicating that the mechanisms Aire operates are unlikely to be explainable simply by chromatin remodelling events (Johnnidis et al. 2005). That taken together with the results presented in this thesis, I would suggest that AIRE exerts its regulatory function on its targets as part of a multiprotein complex that most likely binds to cis-acting elements within the genes. Recent data on Aire-deficient mice indicate that in thymic medullary epithelial cells, Aire not only up-regulates the expression of more than 100 genes, but also seems to repress the expression of a significant number of others (Anderson et al. 2002, Johnnidis et al. 2005). As no evidence of transcriptional repression was seen in our experiments, or those of others (Björses et al. 2000, Halonen et al. 2004), it is possible that downstream genes, activated by AIRE, would mediate such repression.

6.2 The protein domains of AIRE are responsible for distinct functions and subcellular localisation patterns

Drawing together the data from transactivation assays and immunofluorescence microscopy using AIRE deletion constructs and APECED patient mutations, distinct roles for the different AIRE protein domains can be distinguished. Table 7 summarises the data on various AIRE deletion constructs and APECED-causing patient mutations analysed for subcellular localisation and function in this thesis.

Using deletion constructs and PHD finger-targeting missense mutations it was shown that the PHD fingers form the activation domain of AIRE (Study II). These results have been confirmed by Halonen (2003), who observed that a polypeptide containing the PHD fingers was sufficient to activate transcription. However, when a construct ontaining the PHD fingers and the SAND domain was used, a decrease in activation was observed (Halonen 2003). These authors also showed that a missense mutation in PHD1 (P326Q) only slightly impaired the transactivation capacity of AIRE (75% of wild type) (Halonen et al. 2004). It is thus possible that the other protein domains of AIRE modulate the activation imposed by the PHD fingers.

In Study II it was shown that the N-terminal HSR domain mediates AIRE homodimerisation. The minimal deletion construct that showed self-interaction was the AIRE (1-207) fragment, HSR being the only intact protein domain in the polypeptide. Although the PHD fingers have been implicated in mediating protein-protein interactions (Aasland et al. 1995, Pascual et al. 2000, Capili et al. 2001), the PHD fingers of AIRE were not able to mediate homodimerisation. APECED-causing mutations in the HSR domain inhibited homodimerisation (Study II), and two recently described mutations identified in Italian APECED patients also abolished AIRE-AIRE interaction (Meloni et al. 2005). Again, the homodimerisation of AIRE has been corroborated in independent experiments (Halonen et al. 2004) where, in addition, it was noted that a patient mutation in the SAND domain (G228W) inhibited AIRE-AIRE interaction. It may be that while the HSR domain in itself is sufficient for homodimerisation, other domains may regulate this in the *in vivo* setting.

Apart from homodimerisation, the HSR domain is also responsible for the subcellular localisation of AIRE in intermediate filaments in the cytoplasm (Study II), which has been confirmed by Ramsey et al (Ramsey et al. 2002a). Some APECED-causing missense mutations affecting the HSR domain abrogate the localisation of AIRE into cytoplasmic filaments, and others have no effect on it (Study II and (Halonen et al. 2004)). It must also be noted that the fibrillar cytoplasmic localisation of AIRE has only been shown in cultured cells transfected with AIRE, but not in cells that express endogenous AIRE (mTECs and peripheral blood cells) (Björses et al. 1999, Heino et al. 1999a, Rinderle et al. 1999, Björses et al. 2000). Thus, based on these data it is unclear whether the localisation of AIRE in cytoplasmic filaments has any bearing on the function of the protein.

In addition to the N-terminal conserved nuclear localisation signal, which in itself is functional and sufficient to translocate GFP to the nucleus (Study II), there appears to be another domain in the C terminus able to mediate nuclear import: deletion constructs containing the SAND domain plus the PHD fingers or just the PHD fingers were all present in the nucleus (Study II). Ramsey et al. (2002a) found in their systematic dissection of AIRE protein domain function that deletion of the SAND domain or point mutations therein inhibited nuclear targeting. It was also shown that AIRE is subject to nuclear export (Study II), probably mediated by the CRM-1 mediated pathway (Nishi et al. 1994, Fukuda et al. 1997, Kudo et al. 1999). Nuclear export is involved in the regulation of function of several proteins, including transcription factors (Komeili and O'Shea 2000). In the case of p53, for instance, masking of the nuclear export signal leads to inhibition of nuclear export enabling the active form of the protein to be retained in the nucleus (Stommel et al. 1999).

Regarding the targeting of AIRE to nuclear dots, no single domain seems to be responsible. In our experiments (Table 7) none of the deletion constructs formed nuclear dots, although several were located in the nucleus. The L28P patient mutation inhibited targeting to nuclear dots, while the K83E mutation had no effect. In their analysis of APECED-causing mutations, Halonen and co-workers found that all mutations inhibiting nuclear dot formation also significantly reduced the transactivation capacity of AIRE (Halonen et al. 2004).

In summary, the N terminus of AIRE contributes homodimerisation, localisation to cytoplasmic filaments, and trafficking into and out of the nucleus. The bidirectional nuclear transport of the protein and its tethering to cytoplasmic filaments may be involved in the regulation of its function, as it is obvious that the nuclear pool of AIRE is the active one, at least for the activation of transcription. The C-terminal PHD fingers form the activation domain, and the C terminus also contains elements sufficient for nuclear targeting even in the absence of the NLS. A recent report showed that AIRE can function as an E3 ubiquitin ligase, a function also mediated by the PHD fingers (Uchida et al. 2004). The only clear correlation between localisation and function seems to be that the presence of AIRE nuclear dots is required for transactivation. It is of significant interest that, while most APECED patient mutations interfere with the properties of AIRE described above, there is at least one that shows no apparent defect: the K83E mutation (Study II, Table 7). This suggests that there may still be aspects of AIRE function at the molecular level that need to be resolved, although the effect of this mutation on AIRE Ub ligase activity has not been studied.

6.3 CBP and AIRE interact, colocalise subcellularly, and coactivate transcription

The CREB-binding protein CBP is the only protein partner of AIRE to have been described so far (Study I). It is an essential, ubiquitously expressed protein that functions as a coactivator to several transcription factors (see Chapter 2.6). The results of this study demonstrate that the AIRE-CBP interaction has a functional dimension: CBP functions as a coactivator to AIRE in the GAL4 and IFNβ systems (Study III). The coactivation was inhibited by APECED-causing patient mutations, as well as by (artificial) mutations targeting the PHD fingers, implicating the involvement of CBP in the function of the AIRE activation domain. In addition to the model systems for transactivation, it was shown that AIRE and CBP can co-operatively increase the expression of endogenous IFNβ mRNA (Study III). The effect was shown in HEK293 cells, which do not express endogenous AIRE and do not represent the natural cellular environment of AIRE. Notwithstanding, this is the first description at the molecular level of AIRE target gene activation. It also suggests that the effect of AIRE on the expression of autoantigens (and other promiscuous transcripts in mTECs) may involve regulation of transcription *per se* and not posttranscriptional events, including mRNA processing or

trafficking, although contribution of the latter cannot be excluded. It must also be stated that other coactivators or corepressors might be involved in the function of AIRE. No such interactions have been published so far, however.

As discussed above, the subcellular localisation of AIRE in the nuclear dots that we call AIRE nuclear bodies is likely to be important for its function. Identifying them as AIRE nuclear bodies seems warranted as they do not correspond to the major types of nuclear bodies (such as speckles, Cajal bodies, or PML bodies). It was shown in Study IV that AIRE and PML form discrete nuclear bodies in cultured cells (also shown by others (Björses et al. 1999, Rinderle et al. 1999)) and in the thymus. We have also found that AIRE does not colocalise with SC-35, the defining element of speckles (our unpublished results). In most cases in cells expressing AIRE, CBP colocalises with PML in PML bodies, and the AIRE nuclear bodies are seen as separate structures. In a small minority of HEK 293 cells (around 5%), CBP was seen in AIRE nuclear bodies (Study III). When CBP is overexpressed (Study III) or PML is not present (Study IV), CBP is predominantly targeted to AIRE nuclear bodies. This indicates that competition for CBP may be involved in the regulation of AIRE function. There is evidence that such a mechanism for the regulation of transcription exists: STAT-2 and the p65 subunit of NF-κB compete for a binding site in CBP, depending on the type of extracellular stimulus applied, as a means of regulating human immunodeficiency virus gene expression (Hottiger et al. 1998). Most importantly, CBP is seen in AIRE nuclear bodies in a small subset of AIRE-positive cells in human thymus sections, the natural environment of AIRE function (Study III).

In further analyses of AIRE nuclear bodies it was found that they most likely do not represent sites of active transcription (Study III). This is in keeping with the known functions of the other types of nuclear bodies that include various posttranscriptional modifications and / or sequestration of nuclear (transcription) factors, but not transcription itself (see Chapter 2.5). This hypothesis is further corroborated by the data that the AIRE nuclear bodies are a component of the protein-containing nuclear matrix (Study IV).

In Study III analysis of the relative localisations of AIRE and CBP in AIRE nuclear bodies using three-dimensional reconstruction of laser confocal microscopy images revealed the surprising finding that AIRE seems to form the outer surface of AIRE nuclear bodies, with CBP and AIRE occupying the inner core. The functional significance of this observation is open to discussion, but a mechanism for the regulation of AIRE-CBP function can be envisaged. If the hypothesis that PML bodies form a catalytic surface on which biochemical reactions mediated by PML body components occurs (Borden 2002) is extended to AIRE nuclear bodies, sequestration of

CBP beneath such a catalytic surface could be used to regulate the amount of "active" CBP available at the surface.

As stated above, homozygous deletion of CBP in mice is embryonic lethal (Yao et al. 1998, Kung et al. 2000), and deletion of a single allele by heterozygous mutation causes the Rubinstein-Taybi syndrome in humans (Petrij et al. 1995). If the CBP coactivation of AIRE shown here is in fact critical to the *in vivo* function of AIRE, it could be assumed that some defect in AIRE function might be evident with partial loss of function of CBP. CBP $^{+/-}$ mice have not been specifically studied with regard to immune system function, but they do not seem to show overt autoimmune disease (Tanaka et al. 1997, Yao et al. 1998, Oike et al. 1999). A small subset of patients with the Rubinstein-Taybi syndrome have been reported to have diabetes but no other endocrine or autoimmune diseases have been found (Hennekam et al. 1990, Rubinstein 1990). Hennekam and co-workers (1990) also state that several of their patients have had extensive immune work-up showing no major abnormalities with the exception of one case of immunoglobulin A deficiency. Thus, a partial loss of CBP function does not seem to lead to such a lack of AIRE function as to cause an autoimmune phenotype. However, more subtle disturbances of the immune system, such as autoantibodies without overt disease, may be present. Alternatively, other coactivators such as p300 may compensate for the decrease in CBP activity in this context.

6.4 Control of AIRE targeting by the proteasome – ubiquitin pathway

In Study IV it was demonstrated that AIRE is subject to ubiquitination by a so far unknown Ub E3 ligase. Although AIRE itself can function as an E3 ligase (Uchida et al. 2004) it does not show self-ubiquitination (Study IV). Upon treatment of AIRE-expressing cells with proteasome inhibitors, the subcellular localisation of AIRE was altered in that relatively more protein was seen in the nucleus than in the cytoplasm. The size and number of AIRE nuclear bodies also increased, as did their localisation: they were translocated to the perinucleolar region. These data indicate that the subcellular localisation of AIRE is controlled by the proteasome-ubiquitin pathway. The results also indicate that not all nuclear AIRE is present in the AIRE nuclear bodies, but that the protein is also present diffusely in the nucleoplasm. Further studies are needed to ascertain which nuclear pool of AIRE is the active one.

The solution structure of the first PHD finger of AIRE was reported recently (Bottomley et al. 2005). The structure is indeed a typical PHD finger structure, and clearly distinct from the RING finger structure which has been shown to mediate Ub E3 ligase activity (Joazeiro and Hunter 2000, Pascual et al. 2000, Capili et al. 2001). Bottomley et al. (2005) show that in an *in vitro* substrate-free assay very similar to ours (Study IV), their AIRE PHD1 or full-length AIRE

constructs show no self-ubiquitination, and therefore no Ub E3 ligase activity. They conclude that rather than mediate Ub E3 ligase activity, the AIRE PHD fingers mediate protein interactions. These results do not exclude the ability of AIRE to ubiquitinate other substrates, which Uchida et al. (2004) have in fact already shown.

6.5 AIRE controls the promiscuous expression of peripheral antigens in the thymus and participates in the negative selection of autoreactive T-cells

Significant advances in understanding to the role of AIRE in the development of tolerance and the pathogenesis of APECED have become available recently with the publication of several papers describing Aire-deficient mice (Anderson et al. 2002, Ramsey et al. 2002b, Kuroda et al. 2005). The fact that the mice develop spontaneous organ-specific autoimmunity is direct evidence that AIRE has a critical role in the prevention of autoimmunity. Analysis of the general parameters of the immune system in Aire $^{-/-}$ mice has revealed only a few aberrations: an increase in the number of mTECs in the thymus and in the frequency of activated or memory T-cells was noted (Anderson et al. 2002), likewise an increased proliferative response to hen egg lysozyme (HEL) in an antigen recall experiment (Ramsey et al. 2002b). In these animals, the autoimmune phenotype correlated with radioresistant cells of the thymic stroma, but not with haematopoietic cells (Anderson et al. 2002).

Using cDNA microarray analysis of the relative transcription levels of 12 000 transcripts in mTECs from Aire $^{-/-}$ and Aire $^{+/+}$ mice, Anderson et al. (2002) showed that the expression of more than 200 organ-specific transcripts was repressed in Aire-deficient mTECs. These included APECED autoantigens preproinsulin and cytochrome P4501A2. Notably, several organ-specific genes were not repressed in Aire $^{-/-}$ mice, including the C-reactive protein and GAD67, indicating that Aire controls the promiscuous expression of a subset of organ-specific genes in mTECs. When the expression data from this study were re-evaluated, it was found that the expression of nearly 100 genes was up-regulated in Aire-deficient mice, demonstrating that *in vivo*, Aire can both activate and repress the expression of organ-specific genes (Johnnidis et al. 2005).

The direct participation of Aire in the negative selection of T-cells has been demonstrated in two sets of experiments where Aire $^{-/-}$, Aire $^{+/-}$, and Aire $^{+/+}$ mice were crossed with transgenic mice expressing HEL under different promoters (Liston et al. 2003, Liston et al. 2004). First, it was shown in mice expressing both a HEL-specific TCR transgene and HEL under the rat insulin promoter that in double transgenic, Aire-deficient mice the negative selection of the HEL-specific T-cells was defective, and HEL-reactive T-cells were detected in the periphery and in the thymus, whereas in Aire-sufficient mice, the negative selection was unaffected (Liston et al. 2003).

The same group went on to show that the expression of HEL mRNA under the rat insulin promoter in thymic stroma was completely undetectable in Aire $^{-/-}$ mice, high in Aire $^{+/+}$ mice, and in Aire $^{+/-}$ mice, intermediate levels of HEL mRNA expression were observed (Liston et al. 2004). This correlated with the frequency of HEL-specific T-cells: in Aire $^{-/-}$ mice most of the HEL-specific T-cells escaped deletion, and in Aire $^{+/-}$ mice, reduced levels of these T-cells were observed. Again, in the Aire $^{+/+}$ mice, efficient negative selection took place. In the thymus, the number of Aire-positive cells was the same in Aire $^{+/+}$ and Aire $^{+/-}$ mice, but the amount of Aire per cell was lower in the latter. (Liston et al. 2004) The authors reported similar results for the transgene controlled by the thyroglobulin promoter, whereas Aire had no effect on thymic deletion of the antigen controlled by a systemic promoter (Liston et al. 2004). Thus, the expression of organ-specific antigens in the thymus is sensitive to small reductions in Aire activity, and the ectopic expression of these transcripts is directly linked to negative selection of T-cells specific for these antigens.

However, recent results from Kuroda and co-workers (2005) on their Aire-deficient mice invoke intriguing additional hypotheses regarding Aire function. The phenotype of their Aire $^{-/-}$ mice included Sjögren's syndrome-like pathology of salivary glands, which was associated with autoimmunity (autoantibodies and proliferation of splenocytes upon antigen stimulation) against α-fodrin, a ubiquitous protein. What makes the results interesting is that the thymic expression of α-fodrin was unaffected in the Aire $^{-/-}$ mice, suggesting that Aire may be involved in the regulation of processing and/or presentation of self proteins in addition to controlling their ectopic expression in the thymus. Similar results were obtained by Anderson and co-workers in a recent study (2005). In their double transgenic system, ovalbumin was expressed under control of the rat insulin promoter. When the effect of Aire was examined it was found that in Aire $^{-/-}$ double transgenic mice, the negative selection of ovalbumin-specific T-cells was substantially impaired. Moreover, the expression levels of ovalbumin were similar in mTECs from Aire $^{-/-}$ and Aire $^{+/+}$ mice. This indicates that in the absence of Aire, negative selection was impaired although the ectopic transcript was expressed at normal levels, implying that Aire might affect the presentation of self-antigens by mTECs in addition to regulating the expression of ectopic genes. This was then shown in an *in vitro* experiment where it was found that mTECs from Aire $^{-/-}$ mice were significantly less effective in presenting antigens. The mechanism for this novel cellular function of Aire remains obscure, as there were normal levels of MHC and common costimulatory molecules expressed in Aire-deficient mTECs. The results are somewhat contradictory to those of Liston et al. (2004); in their system, the expression of HEL under the same rat insulin promoter was absent in the thymus of Aire-deficient mice. Their analysis was not done on isolated mTECs (Liston et al. 2004), otherwise the reason for the difference noted in these two systems is unclear.

In the Aire $^{-/-}$ mouse model no defects in the production of regulatory T-cells have so far been identified. The animals have normal numbers of T_{reg}s, and they show normal suppressive activity, and normal levels of Foxp3 expression (Anderson et al. 2002, Liston et al. 2003, Su and Anderson 2004, Kuroda et al. 2005, Anderson et al. 2005). Furthermore, Anderson et al. (2005) showed that cotransfer of an Aire-positive thymus together with an Aire-negative thymus into a thymusless recipient could not prevent the autoimmunity caused by transfer of the Aire-negative thymus. At the moment it therefore appears that Aire does not have any major effect on differentiation and functionality of the overall population of T_{reg} s.

6.6 Controlling AIRE

The fact that the expression of AIRE is limited to antigen presenting cells, mainly mTECs and dendritic cells, suggests that its expression is strictly controlled. The human AIRE promoter has recently been characterised, and analysis of its structure indicated that its silencing (in tissues other than thymic epithelium and dendritic cells) is most likely mediated by CpG island methylation and histone deacetylation (Murumägi et al. 2003), implicating epigenetic mechanisms at least in the control of AIRE expression at the tissue level.

Data on the effect of the lymphotoxin (LT) pathway on Aire expression has emerged in the form of mice deficient in either LT receptor α or β (Boehm et al. 2003, Chin et al. 2003). The lymphotoxin pathway is involved in the organogenesis of secondary lymphoid tissues, and in the maintenance of their microarchitecture (for a review, see Gommerman and Browning (2003)). In both LT receptor models, both reduced levels of Aire expression in the thymus, and autoimmune disease were seen. The exact mode of regulation of Aire expression in this context is under dispute, as one study shows that the amount of mTECs is significantly reduced, but that Aire is expressed at normal levels in the remaining mTECs (Boehm et al. 2003), whereas the other indicates that the lymphotoxin pathway directly influences Aire expression (Chin et al. 2003).

Another level of Aire regulation was discovered by Akiyama et al. (2005) who found that in tumour necrosis factor receptor-associated factor (TRAF) 6-deficient mice, there was no expression of Aire in mTECs and that the animals had an autoimmune phenotype. Interestingly, the mice had significantly reduced levels of T_{reg}s. It seems that the TRAF6 signalling pathway functions irrespective of the lymphotoxin receptor pathway (Derbinski and Kyewski 2005), and that both are required for the generation of a normal, mature mTEC population, which apparently is a requisite for Aire expression.

6.7 Future prospects

Several questions remain unanswered with regard to the function of AIRE at the molecular level. If AIRE indeed also represses transcription, as has been shown in the AIRE-deficient mice (Johnnidis et al. 2005), identifying the components of the AIRE protein complexes (Halonen et al. 2004) would clarify whether there is direct involvement in repression of transcription (exemplified by the presence of histone deacetylases), or just activation, in which case the repression could be a downstream effect of AIRE-activated genes. In the same line, identifying the other components of AIRE nuclear bodies would elucidate their function, and possibly how AIRE is regulated through mechanisms affecting its subcellular localisation.

As AIRE seems to direct the expression of only a subset of ectopic transcripts in mTECs, further study is required to identify how the target genes are selected. A more detailed resolution of the regulation of AIRE expression is also warranted.

As the expression levels of (Aire-specific) ectopic transcripts are very sensitive to small changes in Aire activity (Liston et al. 2004), it would be interesting to assess the effect of heterozygous AIRE mutations on the development of the component autoimmune diseases of APECED, especially type I diabetes. Although it appears that the control of expression of ectopic genes by AIRE is directly linked to negative selection (Liston et al. 2004), more information on the role of AIRE in this pathway is needed. The possibility that AIRE also participates in the production of APECED autoantigen-specific regulatory T-cells should still be addressed.

7. SUMMARY AND CONCLUSIONS

In this thesis the biochemical functions, interactions, and subcellular localisation of the AIRE protein are characterised. When AIRE is inactivated by mutations, a breakdown of tolerance to a multitude of self-antigens, manifesting as autoimmune destruction of several, mainly endocrine organs, ensues.

It is shown that the AIRE protein is a potent transcriptional activator when fused with a heterologous DNA binding domain, and that it can also activate the interferon beta minimal promoter. The activation domain is mapped to the PHD fingers in the C terminus. The CREB-binding protein is identified as an AIRE interaction partner, and it is shown that it can coactivate AIRE in model systems of transactivation. AIRE and CBP can also co-operatively activate transcription of endogenous interferon beta mRNA.

At the subcellular level, it is shown that the HSR domain of AIRE facilitates binding to cytoplasmic filaments. By using an inhibitor of nuclear export it is demonstrated that AIRE is subject to nuclear export, also probably mediated by the HSR domain. Furthermore, the conserved nuclear localisation signal in the N terminus is functional. In addition, there seems to be another motif in the C terminus of AIRE that can also mediate nuclear import in the absence of the conserved NLS. By using time-lapse microscopy of living cells it is shown that the subcellular localisation of AIRE is under the control of the proteasome-ubiquitin pathway, and that AIRE is ubiquitinated by an unknown Ubiquitin E3 ligase, but that AIRE does not show self-ubiquitination activity. Most of nuclear AIRE is found in AIRE nuclear bodies which are shown to be components of the protein-containing nuclear matrix. No colocalisation of either histone H2B or acetylated histone H3 in AIRE nuclear bodies is seen, suggesting that they are not actual sites of transcription.

In more detailed studies of the AIRE nuclear bodies, it is shown that AIRE competes for CBP colocalisation with PML and that under certain conditions (when CBP is overexpressed or PML is not present), CBP is a component of AIRE nuclear bodies. CBP is also seen in AIRE nuclear bodies in a small subset of AIRE-positive cells in human thymus sections. Using three-dimensional reconstruction of confocal microscopy images it is shown that in AIRE nuclear bodies containing CBP, the outer surface of the body seems to contain only AIRE, whereas CBP is only seen inside the bodies.

In conclusion, the first biochemical function of the AIRE protein, transcriptional activation, is identified and characterised in this thesis. The AIRE protein domains responsible for activation, nuclear shuttling, and localisation in cytoplasmic filaments are defined. It is further

shown that the common coactivator CBP binds AIRE and functions as an AIRE coactivator in model systems of transcription. AIRE and CBP also co-operatively activate expression of endogenous interferon beta mRNA.

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Live today like it's your first / don't ever let your problems bring out your worst / don't ever take your mind off whatever is your goal / be true to yourself and follow your soul

from Follow your soul by Albert Cummings
Blind Pig Records, 2004

Tampere, November 2005

Jukka Pitkänen

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