An Investigation of Autoantibodies against Tyrosine Hydroxylase in Patients with Vitiligo

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Summary

Vitiligo is an acquired idiopathic hypomelanotic skin disorder characterised by depigmented macules due to loss of cutaneous melanocytes. Evidence suggests that autoimmunity plays a role in the pathogenesis of the disease, since antibodies and T cells against melanocytes can be detected in vitiligo patients. A major goal in vitiligo research is to identify the targets of the immune response in patients, as this will contribute to defining the pathomechanisms of the disease. A better understanding of vitiligo pathogenesis is required in order to allow the development of better diagnostic, prognostic and therapeutic measures.

Previously, the enzyme tyrosine hydroxylase (TH) was identified as a putative B cell autoantigen in vitiligo using phage-display technology. The aims of the present study were to confirm TH as an antibody target in vitiligo, to investigate the prevalence of TH antibodies and to characterise several properties of TH antibodies.

Firstly, a radioimmunoassay (RIA) with [^{35}S]-labelled TH was used to identify TH antibodies in sera from patients with either non-segmental vitiligo (*n*=79), segmental vitiligo (*n*=8) or other autoimmune diseases without concomitant vitiligo (*n*=91). Sera from healthy individuals (*n*=28) were also tested. The results indicated that segmental vitiligo patients, healthy subjects and patients with other autoimmune diseases without concomitant vitiligo were all negative for TH antibody reactivity. Of 79 non-segmental vitiligo patients, 18 (23%) were positive for TH antibodies. A significant increase in the prevalence of TH antibodies was evident in the non-segmental vitiligo patient group when compared with healthy participants (P = 0.003). TH antibody prevalence was also significantly elevated in the group of patients with active vitiligo compared to the group with stable disease (P = 0.009): TH antibodies were detected in 18/64 (28%) of patients with active disease, but not in any of the 20 patients with stable vitiligo.

Secondly, the binding sites of TH antibodies were investigated. Initially, the binding domains for TH antibodies on the protein were identified using [^{35}S]-labelled TH protein fragments in RIAs. Further localisation of TH binding sites (epitopes) was carried out in antibody absorption experiments using synthetic TH peptides and non-radiolabelled *in vitro* expressed TH protein fragments. In addition, antibody binding to the identified TH epitopes was confirmed in TH peptide enzyme-linked immunosorbent assays (ELISA). The results indicated that epitopes for vitiligo patient TH antibodies were located at the N-terminus of

TH between amino acids 1 and 14 (epitope 1-14) and between amino acids 61 and 80 (epitope 61-80). Of 18 vitiligo patients, 17 (94%) had antibodies against epitope 1-14, and 11 (61%) displayed immunoreactivity against epitope 61-80. Antibody binding to both epitopes was demonstrated in 10/18 (56%) of vitiligo patients.

Finally, TH peptide (amino acids 1-14 and 61-80) ELISAs were used to determine the subclass and avidity of TH antibodies. The results showed that antibodies against TH epitope 1-14 were exclusively of the IgG1 subclass. Antibody responses against TH epitope 61-80 were also predominantly of the IgG1 subclass with a minority of subclass IgG3. TH antibody binding was also assessed at increasing NaCl concentrations as a measure of antibody avidity. The results suggested that vitiligo patient TH antibodies were of variable avidity towards their antigenic TH peptide target.

Overall, the work in this thesis confirmed TH as an autoantigen in vitiligo, described the prevalence of TH antibodies in a vitiligo patient cohort, and characterised several properties of TH antibodies including epitopes, subclasses and avidities.

Declaration

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a higher degree. The work reported in this thesis has been carried out by myself except where specifically acknowledged in the text. All sources of information have been specifically acknowledged by means of references.

Dr. Sherif F. E. Rahoma
July 2012

Dedication

I proudly dedicate this thesis to my beloved father Faraj and mother Iftima who are the soul of my life. I also dedicate it to my wife and all my brothers and sisters who have always been there for me and supported me over the years.

Acknowledgments

First and the foremost, praise to ALLAH, the creator of the world, the beneficent and the most merciful. I thank The Almighty for giving me the opportunity and the strength to go through the course. Without his help and guidance, this work would not have been possible to achieve.

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I would like to thank Professor David Gawkrodger for the provision of patient samples and for his advice and supervision, especially during my attendance at dermatology out-patient clinics in the Department of Dermatolgy at the Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield. I am also thankful to Professor Anthony Weetman for his invaluable support, supervision and advice, and for providing patient samples.

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List of Publications

1. **Peer-reviewed original papers:**

Kemp EH, Emhemad S, Akhtar S, Watson PF, Gawkrodger DJ, Weetman AP. Autoantibodies against tyrosine hydroxylase in patients with non-segmental (generalised) vitiligo. **Experimental Dermatology** 2011; 20:35-40.

Rahoma SFE, Sandhu HK, McDonagh AJG, Gawkrodger DJ, Weetman AP, Kemp EH. Epitopes, avidity and IgG subclasses of tyrosine hydroxylase autoantibodies in patients with vitiligo and alopecia areata. **British Journal of Dermatology** 2012; 167:17-28.

2. **Book chapter:**

Kemp EH, Emhemad S, Gawkrodger DJ, Weetman AP. Autoimmunity in vitiligo. In: **Autoimmune Disorders – Pathogenic Aspects**. Mavragani C (ed). InTech, Rijeka, Croatia. 2011; pp271-294.

3. **Abstracts:**

Emhemad S, Akhtar S, Watson PF, Gawkrodger DJ, Weetman AP, Kemp EH. Autoantibodies against tyrosine hydroxylase in patients with non-segmental (generalised) vitiligo. 15th European Society for Pigment Cell Research Meeting, Cambridge, UK. **Pigment Cell and Melanoma Research** 2010; 23:e17.

Emhemad S, Akhtar S, Watson PF, Gawkrodger DJ, Weetman AP, Kemp EH. Autoantibodies against tyrosine hydroxylase in patients with non-segmental (generalised) vitiligo. Meeting of the British Society for Investigative Dermatology, Manchester, UK. **British Journal of Dermatology** 2011; 164:917, P1.

Emhemad S, Sandhu HK, McDonagh AJG, Gawkrodger DJ, Weetman AP, Kemp EH. Identification of tyrosine hydroxylase autoantibody epitopes in vitiligo and alopecia areata patients. Meeting of the British Society for Investigative Dermatology, Exeter, UK. **British Journal of Dermatology** 2012;166:e35, P-20.

List of Abbreviations

AA	Alopecia areata
Ab	Antibody
ACTH	Adrenocorticotrophic hormone
AD	Addison's disease
ADCC	Antibody-dependent cellular cytotoxicity
AIRE	Autoimmune regulator
AIS	Autoimmune susceptibility locus
AH	Autoimmune hypothyroidism
ANCA	Anti-neutrophil cytoplasmic antibodies
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS1	Autoimmune polyendocrine syndrome type 1
ATD	Autoimmune thyroid disease
4a-OH-BH_4	4a-hydroxy-tetrahydrobiopterin
bp	Basepair/s
bFGF	Basic fibroblast growth factor
BH_4	Tetrahydrobipterin
C	Control
cAMP	Cyclic adenosine monophosphate
°C	Degrees centigrade
CCR	Cytokine-chemokine receptor
CD	Cysteinyl-dopaquinone
CLA	Cutaneous lymphocyte-associated antigen
cpm	Counts per minute
CTLA-4	Cytotoxic T lymphocyte antigen-4
DCT	Dopachrome tautomerase
DHI	5,6-dihydroxy-indole
DHICA	5,6-dihydroxy -indole-2-carboxylic acid
DQ	Dopaquinone
ds-DNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
F	Forward (primer)
FcγR	Fc recptors for IgG
FM	Fontana Masson
ET-1	Endothelin-1
g	Gravity
g	Gram/s
GD	Graves' disease
GM-CSF	Granulocyte-macrophage colony stimulating factor
GFRP	Guanosine triphosphate cyclohydrolase I feedback regulatory protein
GTP	Guanosine triphosphate
GTP-CHI	GTP-cyclohydrolase I
h	Hour/s
HEK293	Human embryonic kidney 293
HLA	Human leucocyte antigen
HSP	Heat-shock protein
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
kb	Kilobase/s
kDa	Kilodalton
L	Litre/s
LB	Luria Bertani
LMP	Low molecular weight protein
L-dopa	3,4-dihydroxyphenylalanine
M	Molar
mA	Milli-amperes
mAb	Monoclonal antibody
MAP	Mitogen-activated protein
MART-1	Melanoma antigen recognised by T cells-1
MCH	Melanin-concentrating hormone

MCHR1	Melanin-concentrating hormone receptor 1
MC1R	Melanocortin 1 receptor
mg	Milligram/s
MHC	Major histocompatibility complex
min	Minute/s
MITF	Microphthalmia-associated transcription factor
ml	Millilitre/s
mM	Millimolar
M-MLV	Moloney Murine Leukemia Virus
MOPS	3-[N-morpholino]propanesulphonic acid
N	Amino terminus
α-MSH	α-Melanocyte-stimulating hormone
NB-UVB	Narrow-band-ultraviolet B
NF-κB	Nuclear factor–κB
ng	Nanogram
NGF	Nerve growth factor
nM	Nanomolar
NPY	Neuropeptide Y
NRF2	Nuclear factor E2-related factor
NSV	Non-segmental vitiligo
OCA	Oculocutaneous albinism
PAH	Phenylalanine hydroxylase
PAX3	Paired-box 3
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
POMC	Propiomelanocortin
PUVA	Psoralen with ultraviolet A
qBH_2	Quinonoid dihydropterin
R	Reverse (primer)
RF	Rheumatoid factor
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNP	Ribonucleoprotein

rpm	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
SCD	S-cysteinyldopa
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SL	Smyth line
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism/s
STH	Sheffield Teaching Hospitals
SV	Segmental vitiligo
TBP	Tertiary butylphenol
TCA	Trichloroacetic acid
TEMED	N, N, N, N'-tetramethylethylenediamine
TGF- β	Transforming growth factor-β
TH	Tyrosine hydroxylase
Th	T helper lymphocyte
TNF-α	Tumour necrosis factor-α
TNF-β	Tumor necrosis factor-β
TPH	Tryptophan hydroxylase
TYR	Tyrosinase
TYRP1	Tyrosinase-related protein-1
UV	Ultraviolet
α-MSH	α-melanocyte-stimulating hormone
µg	Microgram/s
µl	Microlitre/s
µM	Micromolar
V	Vitiligo
VETF	Vitiligo European Task Force
VIDA	Vitiligo disease activity
v/v	Volume/volume
w/v	Weight/volume
y	Year

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Chapter 3 - Tables 3.3 and 3.9; Figures 3.2, 3.9, 3.10 and 3.15.
Chapter 4 – Figures 4.4, 4.5, 4.6, 4.7 and 4.8.
Chapter 5 – Table 5.5; Figures 5.2a, 5.2b, 5.3a and 5.3b.

CHAPTER 1

1. General Introduction

1.1 The Skin

The following sections will describe the structure and functions of the skin, with particular emphasis on melanocytes (pigment cells) and melanin (pigment) biosynthesis (melanogenesis). Such information will provide a background to vitiligo, a depigmenting skin disease which is the focus of this project.

1.1.1 Skin structure and functions

The skin is a complex organ which constitutes approximately one-twelfth of the body mass (Millington and Wilkinson 1983) and provides an efficient barrier against physical assault, chemical hazards and pathogens present in the external environment (Proksch *et al.* 2008; Jensen and Proksch 2009). It also functions to prevent fluid loss and to regulate body temperature (Proksch *et al.* 2008). Of course, the many functions of the skin are dependent upon its structure and its various cellular components, and these are described below.

In section, human skin is divided into two main compartments, an outer cellular avascular layer, the epidermis, which is derived from the embryonic ectodermal cells, and the dermis, a heavily vascularised connective tissue layer derived from the embryonic mesoderm (Figure 1.1) (Murphy 2005). The epidermis is mainly composed of keratinocytes which form more than 90% of the cellular component. The primary function of keratinocytes is in the formation of layers of keratin which protect the skin and the underlying tissue from environmental damage such as heat and water loss. These layers are formed through a process called keratinisation in which keratinocytes produce increasing amounts of keratin and eventually undergo programmed cell death. From the inside to the outside of the epidermis, keratinocytes are arranged into the basal cell layer (stratum basalis), the prickle cell layer (stratum spinosum), the granular cell layer (stratum granulosum), the transitional cell layer (stratum lucidum) and the dead horny squamous cell layer (stratum corneum) (Figure 1.1). The innermost basal cell layer consists of a single layer of columnar cells that are attached by hemidesmosomes to the basement membrane which forms a junction between the epidermis and dermis. The basal keratinocytes proliferate and differentiate as they move towards the outer layers. Overlying the basal cell layer is the prickle cell layer which is

composed of 5-10 layers of flattened keratinocytes. The prickle-like appearance of the keratinocytes within this layer is attributed to the protruding desmosomes which are filled with keratin filaments. The granular cell layer is composed of flattened keratinocytes, which are so-called because of the presence of basophilic keratohyaline granules in their cytoplasm. Following full keratinisation and dissolution and loss of their cellular contents, keratinocytes of the granular cell layer form the outermost squamous cell layer and are constantly shed off and replaced by new cells.

Keratinocytes also play a role in modulating skin immunity (Bos and Kapsenberg 1993; Nestle *et al.* 2009). In addition to antimicrobial peptides, they are able to produce an array of cytokines, including interleukin (IL)-1β, IL-1α, IL-4 IL-6, and IL-8, tumor necrosis factor-α (TNF-α), interferon (IFN)-γ and IFN-β (Bos and Kapsenberg 1993; Feldmeyer *et al.* 2007; Nestle *et al.* 2009), and so hold and recruit immune cells into the epidermis. Regulating other skin-resident cells by producing growth factors, including basic fibroblast growth factor (bFGF) (Halaban *et al.* 1988), endothelin-1 (ET-1), stem cell factor (SCF) and transforming growth factor-β (TGF-β) (Imokawa and Moretti 2010), is a further function of epidermal keratinocytes. Finally, keratinocytes contribute to protecting the body from ultraviolet (UV) radiation by taking up melanosomes, vesicles containing melanin, from epidermal melanocytes, and then storing the photoprotective pigment (Scott *et al.* 2002; Watabe *et al.* 2008).

Other cellular components of the epidermal layer include Langerhans cells, Merkel cells and melanocytes. Langerhans cells are specialised dendritic cells which reside in the suprabasal layer and have an antigen presentation capacity through binding, processing and then presenting antigens to effector T cells (Friedmann 1981; Maurer and Stingl 2001; Hunger *et al.* 2004). Merkel cells are scattered throughout the epidermis and are found closely associated with small nerve fibres (Boulais and Misery 2007). Although their function is still unclear, there is evidence that they are associated with touch responses (Maricich *et al.* 2009). Melanocytes are responsible for the synthesis of melanin pigment (Boissy and Nordlund 1995a; Sulaimon and Kitchell 2003), and are detailed in Section 1.1.2.

The dermis is the layer of the skin found between the epidermis and subcutaneous tissues, and is composed of the papillary (stratum papillare) and the reticular (stratum reticulare) dermis (Murphy 2005). Its main roles are to regulate temperature and to supply the epidermis with nutrient-saturated blood. The dermis is mostly made of collagen and elastic fibers which are made by fibroblasts and which lend elasticity and support to the skin. The

dermis is the seat of hair follicles, nerve endings, and pressure receptors. Furthermore, the dermis defends the body against infectious invaders that can pass through the thin epidermis. The papillary dermis is the main agent in dermis function. It is from here that the dermis supplies nutrients to select layers of the epidermis and regulates temperature. Both of these functions are accomplished with a thin but extensive vascular system. The reticular layer is much denser than the papillary dermis. It strengthens the skin, providing structure and elasticity, and supports other skin components such as hair follicles, sweat glands, and sebaceous glands.

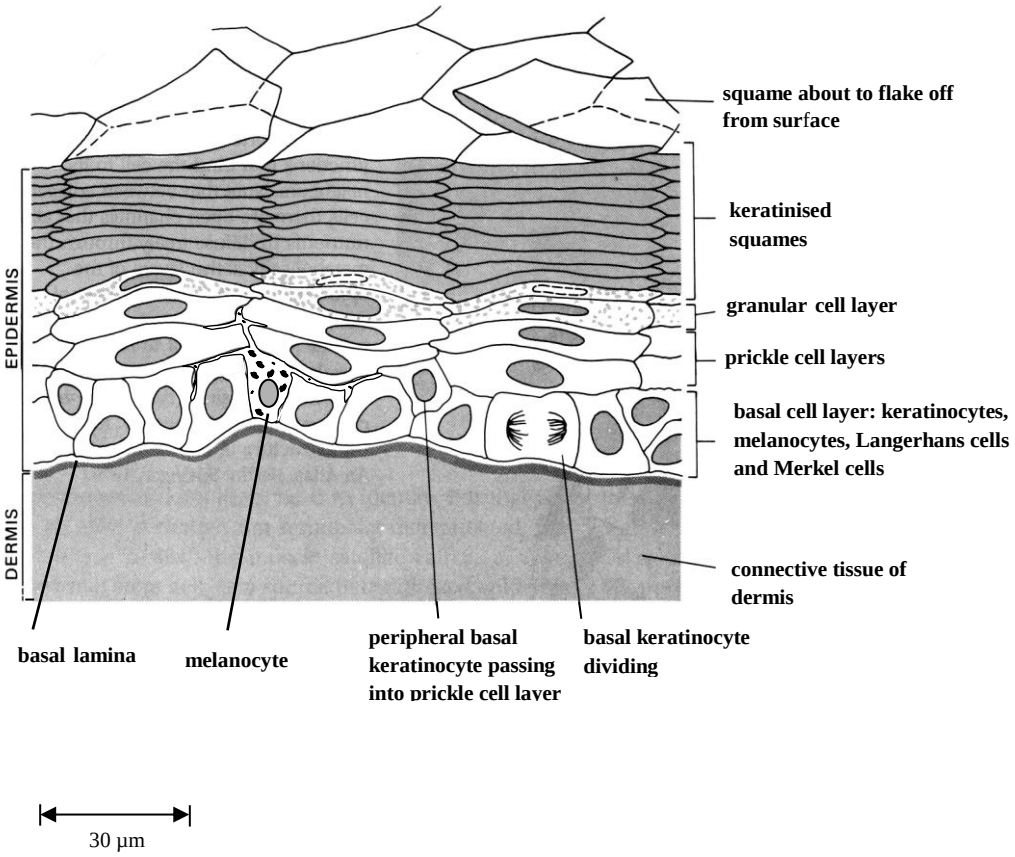

Figure 1.1: A schematic representation of the ultra-structure of the skin.

Drawn with information from (Alberts *et al*. 1989).

1.1.2 Melanocytes

Melanocytes (Figure 1.2) are melanin-producing cells that are found in the skin epidermis, uvea of the eye, inner ear and leptomeninges (Boissy and Nordlund 1995a; Sulaimon and Kitchell 2003), and are responsible for the colour of the hair and skin. They differentiate from melanoblasts (melanocyte precursors) which are derived from the neural crest of the embryonic ectoderm (Boyd 1960). Studies of the early human embryo show that undifferentiated melanoblasts migrate into the developing epidermis at approximately the seventh week of gestation, and subsequently reside in the basal epidermal layer (Holbrook *et al.* 1989; Suder and Bruzewicz 2004). Differentiated melanocytes are found in the epidermis by the eighth week and show early melanin synthesis and contain melanin by the tenth week (Sagebiel and Odland 1970). Several genes influence the differentiation of the melanocyte (Vance and Goding 2004) including paired box-3 (PAX3) (Watanabe *et al.* 1998; Galibert *et al.* 1999; Chen *et al.* 2010; Wang *et al.* 2010), stem cell factor gene (Lahav *et al.* 1994; Luo *et al.* 1995), c-kit proto-oncogene (Lahav *et al.* 1994; Luo *et al.* 1995), microphthalmia-associated transcription factor (MITF) (Watanabe *et al.* 1998; Kawasaki *et al.* 2008; Chen *et al.* 2010; Vachtenheim and Borovansky 2010), and transcription factor SOX10 (Cook *et al.* 2005; Chen *et al.* 2010). Melanocyte differentiation is also influenced by the melanocortin hormones and bFGF (Hirobe 1995).

The population density of melanocytes in the basal cell layer varies in different areas of the skin. The highest melanocyte population density has been noted on the face at a concentration of 2000 cells/mm^2 and the lowest on the trunk at an approximate concentration of 800 cells/mm^2 (Staricco and Pinkus 1957; Fitzpatrick and Szabo 1959). There are no significant differences in skin melanocyte concentration between Caucasian and African American skin (Quevedo *et al.* 1965). Differences in the rate of melanin synthesis (Iwata *et al.* 1990), the type of pigment produced (Quevedo *et al.* 1974), and the way it is distributed within keratinocytes (Szabo *et al.* 1988), account for the different skin colours between ethnic groups.

The main function of melanocytes is in the synthesis of melanin which occurs in membrane-bound organelles called melanosomes (Setaluri 2003; Barral and Seabra 2004; Raposo and Marks 2007; Simon *et al.* 2008). Each melanocyte in the epidermis is surrounded by approximately thirty-six keratinocytes forming the epidermal melanin unit (Fitzpatrick *et al.* 1967), where melanosomes are transported to surrounding keratinocytes via the dendritic tips of melanocytes (Scott *et al.* 2002; Watabe *et al.* 2008). However, the precise mechanism

by which this occurs is not fully understood (Van den Bossche *et al.* 2006). Melanosomes form a cap in the cytoplasm distal to the nuclei where they protect the skin from incident UV radiation by absorbing UV photons and reactive oxygen species thereby minimising UV radiation-induced DNA damage and guarding against skin cancer development (Gasparro 2000; Kadekaro *et al.* 2003; Abdel-Malek *et al.* 2010).

In addition, melanocytes behave as accessory cells in the skin immune response (Das *et al.* 2001). They are capable of producing cytokines, which then mature antigen presenting cells, such as Langerhans cells, and also recruit immune cells into the skin (Zachariae *et al.* 1991; Swope *et al.* 1994). Furthermore, melanocytes can act as antigen presenting cells by expressing on their surface intercellular adhesion molecule-1 (ICAM-1) and major histocompatibility complex (MHC) class I and II molecules (Krasagakis *et al.* 1991; Le Poole *et al.* 1993b; Smit *et al.* 1993; Das *et al.* 2001).

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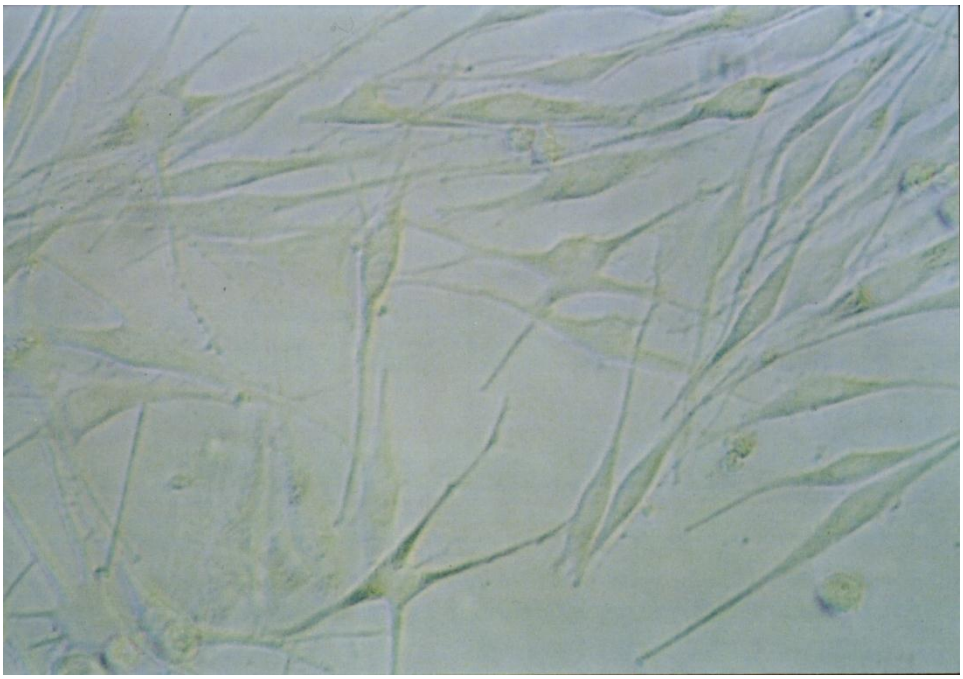


Figure 1.2: Melanocytes growing in culture.

The cells are shown at X200 magnification. The photograph was kindly provided by Miss Charikila Balafa and Prof. Sheila MacNeil, University of Sheffield, Sheffield, UK.

1.1.3 Melanin biosynthesis

The pathways in which melanin is produced are summarised in Figure 1.3, and the most important melanogenic enzymes and proteins with their known functions are given in Table 1.1. The first and rate-limiting step is the conversion of L-tyrosine to L-dopaquinone catalysed by tyrosinase (Kwon *et al.* 1987; Cooksey *et al.* 1997; Riley 1999; Oetting 2000; Simon *et al.* 2008; Ito and Wakamatsu 2011; Kondo and Hearing 2011). For eumelanin synthesis, L-dopaquinone is then cyclised to cyclodopa, which subsequently forms dopachrome with the release of 3,4-dihydroxyphenylalanine (L-dopa) (Tsukamoto *et al.* 1992; Lopez *et al*. 2008). Dopachrome tautomerase (DCT) (formerly tyrosinase-related protein-2/TRP-2) then rapidly catalyses the conversion of dopachrome to 5,6-dihydroxy-indole-2-carboxylic acid (DHICA) (Tsukamoto *et al.* 1992), which is then converted to indole-5,6-quinone-2-carboxylic acid by tyrosinase (Aroca *et al.* 1990). The exact role of tyrosinase-related protein-1 (TYRP1) (formerly TRP-1) in melanogenesis in human melanocytes is not certain (Ito and Wakamatsu 2011; Kondo and Hearing 2011) (. In the absence of DCT, dopachrome can undergo spontaneous decarboxylation to 5,6-dihydroxy-indole (DHI), which is then converted to indole-5,6-quinone by the action of tyrosinase (Korner and Pawelek 1982). In pheomelanin synthesis, L-dopaquinone reacts with thiol compounds such as cysteine or glutathione to form cysteinyldopa and subsequently the red/yellow pigment pheomelanin (Ito and Wakamatsu 2011; Kondo and Hearing 2011). The PMEL protein (formerly Pmel17/gp100) is now regarded as the main component of the melanosomal matrix and previously proposed enzymatic functions (Boissy and Nordlund 1995a) have not been proven.

1.1.4 Regulation of melanogenesis

Melanogenesis is regulated by several factors (Yamaguchi and Hearing 2009; Vachtenheim and Borovansky 2010). Particularly, UV radiation has a direct effect on melanogenesis, stimulating pigment synthesis and melanosome transfer to keratinocytes (Friedmann and Gilchrest 1987; Scott *et al.* 2002). Of particular importance in the control of melanogenesis are the melanotrophic peptides α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotrophic hormone (ACTH) which derive from a common precursor propiomelanocortin (POMC), synthesised in both the pituitary gland and locally in the skin, and which competitively bind to melanocortin 1 receptor (MC1R) on the melanocyte

(Ortonne *et al* 2000). Cell-signalling pathways, including the cyclic adenosine monophosphate (cAMP)-dependent protein kinase pathway and the mitogen-activated protein (MAP) kinase pathway, are activated in response to α-MSH binding to MC1R and, ultimately, these up-regulate melanogenesis (Ortonne *et al*. 2000; Park *et al*. 1999; Park *et al*. 2009).

In addition, several cytokines and growth factors, including TNF-α, ET-1, SCF, bFGF and granulocyte-macrophage colony-stimulating factor (GM-CSF), are known to modulate melanogenesis (Funasaka *et al*. 1998; Yamaguchi *et al*. 2009). For example, ET-1 produced from keratinocytes activates melanocyte proliferation, tyrosinase, stimulates TYRP1 expression and enhances the sensitivity of the MC1R to α-MSH (Imokawa *et al.* 1992; Yohn *et al.* 1993; Hara *et al.* 1995; Funasaka *et al.* 1998; Tada *et al.* 1998). With regard to genetic control, MITF plays an important role in regulating the expression of genes that encode proteins involved in melanogenesis and melanosome biosynthesis (Vachtenheim and Borovansky 2010).

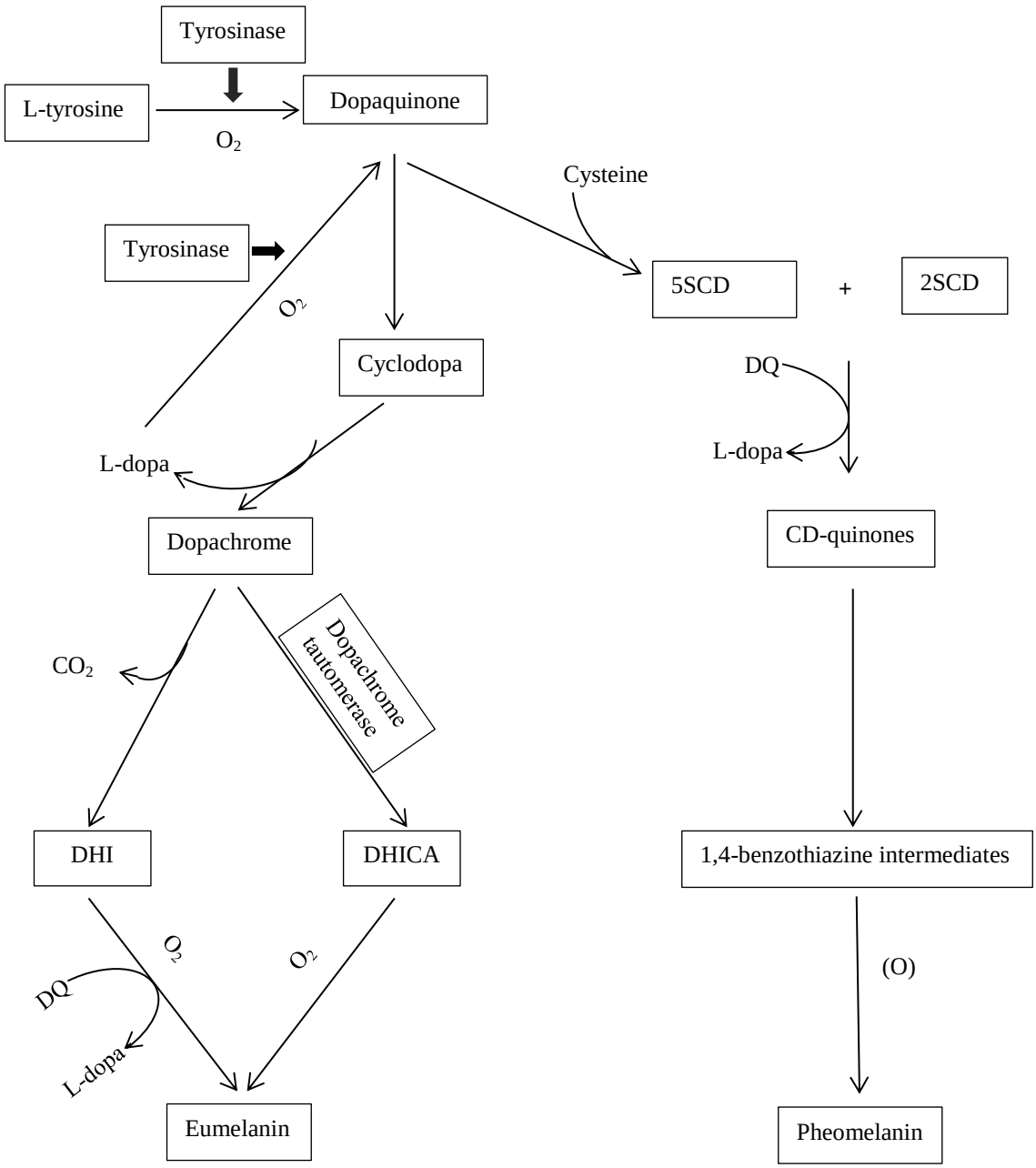


Figure 1.3 A diagrammatic overview of the melanogenesis pathway in human melanocytes.

CD, cysteinyl-dopaquinone; DHICA, 5,6-dihydroxy-indole-2-carboxylic acid; DHI, 5,6-dihydroxy-indole; DQ, dopaquinone; SCD, *S*-cysteinyldopa. Drawn with information from (Boissy and Nordlund 1995a; Marles *et al.* 2003; Sulaimon and Kitchell 2003; Park *et al.* 2009; Ito and Wakamatsu 2011; Kondo and Hearing 2011).

Table 1.1: Important melanogenic proteins

Protein	Size (kDa)	Function	References
Tyrosinase	70	Catalyses the first step in melanin synthesis, the conversion of L-tyrosine to dopaquinone.	(Kwon *et al.* 1987; Oetting 2000; Ito and Wakamatsu 2011; Kondo and Hearing 2011)
Tyrosinase-related protein-1 (TYRP1)	60	Function unclear in human pigmentation.	(Kobayashi *et al.* 1994; Orlow *et al.* 1994; Sturm *et al.* 1995; Setaluri 2003; Ito and Wakamatsu 2011; Kondo and Hearing 2011)
Dopachrome tautomerase (DCT)	59	Catalyses the tautomerisation of dopachrome to 5,6-dihydroxy-indole-2-carboxylic acid (DHICA).	(Aroca *et al.* 1990; Jackson *et al.* 1992; Kroumpouzos *et al.* 1994; Sturm *et al.*1995; Ito and Wakamatsu 2011; Kondo and Hearing 2011)
PMEL	73	Melanosomal protein that forms the fibrillar matrix of the melanosome upon which eumelanin is deposited.	(Johnson and Jackson 1992; Kushimoto *et al.* 2001; Theos *et al.* 2006; Valencia *et al.* 2007; Harper *et al.* 2008; Ito and Wakamatsu 2011; Kondo and Hearing 2011)
Melanoma antigen recognised by T cells 1 (MART-1). Also called MelanA	13	A structural melanosomal protein that complexes with PMEL enhancing its trafficking to the melanosome.	(Hoashi *et al.* 2005)
P-protein	90	Function unclear, but one theory is that it maintains melanosomal pH.	(Rinchik *et al.* 1993; Puri *et al.* 2000; Toyofuku *et al.* 2002)
Lysosome-associated membrane protein-1	90-120	Protection of the melanosomal membrane.	(Das *et al.* 2001)

1.2. Vitiligo: Clinical Features and Treatments

The next sections will describe the skin depigmenting disease vitiligo, including its epidemiology, clinical features and treatments.

1.2.1 Definition

Vitiligo is a common acquired chronic hypomelanotic skin disease characterised by the development of progressive depigmented macules (flat patches or lesions) resulting from the destruction or non-functioning of melanocytes (pigment cells) in the epidermis (Rezaei *et al.* 2007; Taïeb and Picardo 2007).

1.2.2 Epidemiology

Across the world, vitiligo is a common skin disease. Studies have showed a point prevalence of between 1-2% (Mehta *et al.* 1973; Howitz *et al.* 1977; Majumder *et al.* 1993; Boisseau-Garsaud *et al.* 2000a). However, a higher frequency of the disease has been reported in some areas of the world, especially India where the prevalence is up to 8.8% (Sehgal and Srivastava 2007). This elevated frequency may be attributable to a high rate of reporting of the disease, perhaps related to the apparent colour difference between affected and normal skin. The social implications of having vitiligo may also lead to the patient seeking an early consultation (Sehgal and Srivastava 2007). In addition, there may be occupational factors: vitiligo in rural areas could be related to the printing, carpet-making and dyeing industries (Sehgal and Srivastava 2007).

All ages, races and both sexes are equally affected by vitiligo, although females seek early consultation due to the greater psychological impact of the disease on them (Hann and Lee 1996; Le Poole and Boissy 1997; Cho *et al.* 2000; Zaima and Koga 2002; Behl *et al.* 2003; Handa and Dogra 2003). The disease has been reported to start at the age of 20 years in approximately half of all cases, and before the age of 30 years in about 70-80% of patients (Engel 2001; Jaigirdar *et al.* 2002; Behl *et al.* 2003; Herane 2003). Vitiligo is rarely present at birth and, if depigmentation is evident at this stage, consideration must be given to other possible diagnoses such as piebaldism.

1.2.3 Clinical patterns

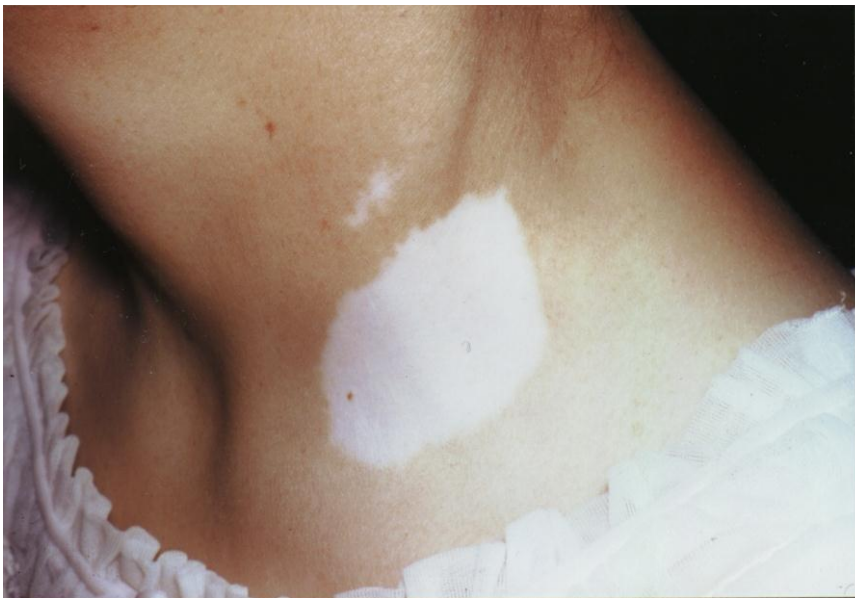
Typical vitiligo lesions consist of milky white macules that vary in diameter from a few millimetres to several centimetres. The border of the lesion is well demarcated and the affected skin is otherwise normal with respect to the texture. Lesions may be single or multiple (Mosher *et al.* 1999). At present, there is no uniform clinical classification of vitiligo but, according to the Vitiligo European Task Force (VETF), the disease is best classified initially into either segmental vitiligo or non-segmental vitiligo, which is also referred to as generalised, types (Taïeb and Picardo 2007). However, both types may co-exist in some patients who are then said to have so-called mixed vitiligo (Gauthier *et al.* 2003b; Mulekar *et al.* 2006; Taïeb and Picardo 2010a).

Segmental vitiligo is found in 5–28% of all patients with vitiligo (Huggins *et al.* 2005). The lesions are unilateral and to a varying extent dermatomal or blaschkoid in distribution (Figure 1.4a). They may appear as limited small patches without an obvious distribution pattern, in which case they are referred to as focal vitiligo (Taïeb and Picardo 2010a). The most common sites for segmental vitiligo are the trigeminal area (50%), and the neck and trunk. Patients do not tend to have a family history and segmental vitiligo is rarely associated with other autoimmune diseases.

Non-segmental vitiligo is the most common clinical type of vitiligo. It is characterised by a wide distribution of the lesions, which are often symmetrical (Figure 1.4b). The extensor surfaces of the elbow, knee, and metatarsal/metacarpal interphalangeal joints are often involved. Other regions affected are the umbilicus, anterior tibia, axilla and the malleoli. Peri-orificial involvement may also be present where the peri-orbital area, the ear, the mucous membranes of the lips and the genitals are affected. Some authors recognise vitiligo that appears in a predominantly peri-orificial distribution in association with involvement of the distal digits, as a subgroup of non-segmental vitiligo described as ‘acro-facial’ (Passeron and Ortonne 2010). Patients with generalised vitiligo often have a family history of the disease and of other autoimmune diseases such as type 1 diabetes mellitus, pernicious anaemia, autoimmune thyroid disease and Addison’s disease and these are detailed in Section 1.2.5.

Non-segmental vitiligo may progress over time and proceed to involve more than 90% of the skin surface area, in which case it is known as ‘vitiligo universalis’, which can be associated with multiple endocrinopathies (Taïeb and Picardo 2010a). Rarely, the lesions of vitiligo have a slightly elevated inflammatory margin and are associated with itching. This is

(a)



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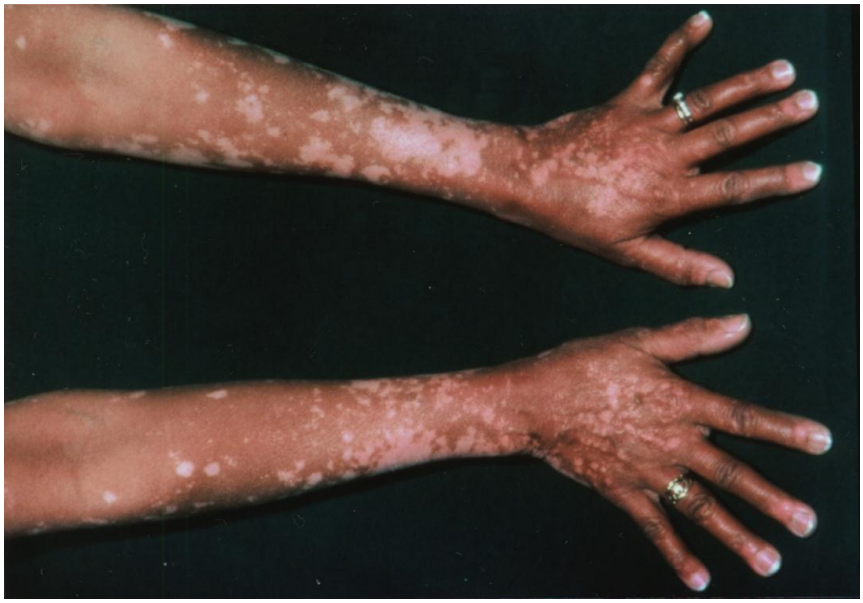


Figure 1.4: Clinical patterns of vitiligo.

(a) Segmental vitiligo affecting one side of the neck. The photograph was kindly provided by Prof. Tony Weetman, Department of Human Metabolism, University of Sheffield, Sheffield, UK. **(b)** Symmetrical vitiligo on the dorsal aspects of the hands. The photograph was kindly provided by Prof. David Gawkrodger, Department of Dermatology, Royal Hallamshire Hospital, Sheffield, UK.

known as inflammatory vitiligo (Buckley and Lobitz 1953; Watzig 1974; Ortonne *et al.* 1979; Ishii and Hamada 1981; Arata and Abe-Matsuura 1994; Lee *et al.* 2000).

In addition to the disease categories described by the distribution of vitiligous skin, a three-grade pathological staging system corresponding to depletion of the melanocytes has been proposed (Gauthier 1994). In this classification scheme, grade I vitiligo exhibits only a partial depletion in epidermal melanocytes and results in the possibility of repigmentation, without a follicular pattern, after phototherapy treatment. Grade II vitiligo has complete depletion of the epidermal melanocytes and corresponds to a follicular pattern of repigmentation, in which melanocytes have migrated from the follicular reservoir to replace the absent epidermal melanocytes, after phototherapy treatment. Grade III vitiligo is characterised by a total depletion of both epidermal and follicular melanocytes and patients at this stage do not respond to medical therapy. More recently, a vitiligo disease activity (VIDA) score has been proposed which measures disease activity in relation to time, as assessed by the patient (Njoo *et al.* 1999).

1.2.4 Psychological effects

In comparison with some diseases, vitiligo might be considered a minor disorder. However, the psychological effects of vitiligo are frequently considerable (Kent & Al'Abadie 1996). This is particularly the case in people with racially darkly pigmented skin where the appearance of the pale patches is very prominent. Individuals with vitiligo may experience feelings of stress, embarrassment or self-consciousness when in social contact with strangers. A perception of discrimination and of low self-esteem are common, especially in patients with white patches in the cosmetically sensitive areas of the hands and face (Ginsburg 1996; Ongenae *et al.* 2005; Ongenae *et al.* 2006; Schmid-Ott *et al.* 2007; Dolatshahi *et al.* 2008; Choi *et al.* 2010).

In areas such as India, patients with vitiligo are subjected to isolation from society, antagonism or insult (Parsad *et al.* 2003). They may also undertake diet restriction and avoidance of some food such as fish and milk because of local beliefs concerning the aetiology of the disease (Parsad *et al.* 2003). Furthermore, the psychosocial burden of vitiligo is greater in females as they may face many social difficulties such as finding a marriage partner (Parsad *et al.* 2003). However, vitiligo can also affect the marital status and sexual

health in men due to embarrassment and low self-esteem (Porter *et al.* 1990; Dolatshahi *et al.* 2008).

Appropriate psychological intervention and assessment of quality of life in vitiligo patients can influence positively the outcome of treatment, as well as improve the patient's self-esteem and confidence (Papadopoulos *et al.* 1999; Parsad *et al.* 2003; Picardi *et al.* 2003).

1.2.5 Associated disorders

Vitiligo is frequently associated with other autoimmune disorders (Table 1.2), particularly autoimmune thyroid disease (Ochi and DeGroot 1969; Boelaert *et al.* 2010), autoimmune polyendocrine syndromes (Neufeld *et al.* 1981; Ahonen *et al.* 1990), pernicious anaemia (Dawber 1969), Addison's disease (Dunlop 1963; Zelissen *et al.* 1995), and alopecia areata (Sharma *et al.* 1996a; Ahmed *et al.* 2007). Furthermore, patients with vitiligo are more likely to suffer from autoimmune conditions than those in the general population (Cunliffe *et al.* 1968; Turnbridge *et al.* 1977; Liu *et al.* 2005; Birlea *et al.* 2008). In a survey of more than 2,600 unselected Caucasian vitiligo patients, elevated frequencies of autoimmune thyroid disease, Addison's disease, systemic lupus erythematosus and pernicious anaemia were found, with approximately 30% of patients being affected with at least one additional autoimmune disorder (Alkhateeb *et al.* 2003). Moreover, these same autoimmune diseases occurred at an increased frequency in the first-degree relatives of the patients studied (Alkhateeb *et al.* 2003). Similarly, in multiplex non-segmental vitiligo families, higher frequencies of psoriasis, rheumatoid arthritis and type 1 diabetes mellitus were noted in addition to autoimmune thyroid disease, Addison's disease, systemic lupus erythematosus and pernicious anaemia (Laberge *et al.* 2005). Such data indicate that individuals can be genetically predisposed to a specific group of autoimmune diseases that includes vitiligo.

Table 1.2: Autoimmune associations of vitiligo

Autoimmune disease	Percentage of patients with vitiligo	Reference
Addison's disease	9.6	(Zelissen *et al.* 1995)
Alopecia areata	3.5 1.8	(Ahmed *et al.* 2007) (Sharma *et al.* 1996b)
Autoimmune polyendocrine syndrome type 1	13 8	(Ahonen *et al.* 1990) (Neufeld *et al.* 1981)
Autoimmune polyendocrine syndrome type 2	4.5 5	(Ahonen *et al.* 1990) (Neufeld *et al.* 1981)
Graves' disease	1.4 7	(Boelaert *et al.* 2010) (Ochi and DeGroot 1969)
Hashimoto's thyroiditis	2.6	(Boelaert *et al.* 2010)
Type 1 diabetes mellitus (juvenile)	1.7	(Macaron *et al.* 1977)
Pernicious anaemia	9	(Dawber 1969)

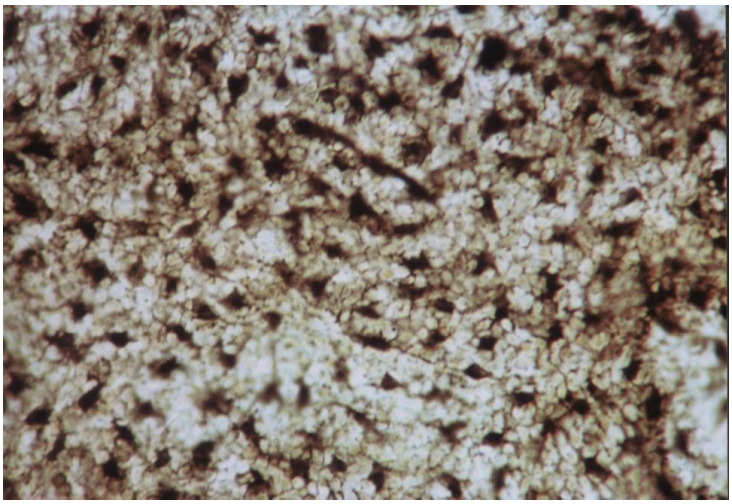
1.2.6 Histopathology

The affected skin in vitiligo shows a loss of melanin and an absence of or decreased numbers of melanocytes in the epidermis (Tobin *et al.* 2000). Figures 1.5a and 1.5b demonstrate the difference in density of epidermal melanocytes between normal and vitiligo skin. The histological findings vary according to the stage of the disease (Aslanian *et al.* 2010). In the early stage, a few mononuclear cells infiltrate the dermo-epidermal junction. Melanocytes, as demonstrated using the Fontana Masson (FM) stain or using an appropriate monoclonal antibody technique, are still present, but they disappear from vitiligo skin as the disease progresses (Ackerman *et al.* 2000).

In established lesions, the affected skin appears normal apart from absence of melanin pigment from the basal layer as demonstrated by FM stain (Spielvogel and Kantor 2005). Immunohistochemical studies of vitiligo lesion at this stage have shown that melanocytes are usually absent from the basal layer, although they may be present in reduced numbers and can show degenerative changes (van den Wijngaard *et al.* 2000): melanocytes at the edges of vitiligo macules appear to be larger with longer dendritic processes than normal melanocytes (Spielvogel and Kantor 2005). A superficial peri-vascular lymphocytic infiltration may be present at this stage (Wolf *et al.* 2005). The main histological finding in long-standing vitiligo lesions is a prominent absence of pigmentation in the epidermis. No inflammatory infiltrate is normally seen at this stage of the disease (Aslanian *et al.* 2010).

Degenerative changes have been demonstrated in both melanocytes and keratinocytes in apparently normal skin adjacent to vitiligo macules. A few mononuclear cells showing vacuolar degeneration have been detected in the basal cell layer of clinically normal skin with the deposition of extracellular granular substance (Moellmann *et al.* 1982; Bhawan and Bhutani 1983; Anbar *et al.* 2009). Recently, T lymphocyte infiltration at the dermo-epidermal junction with microscopic absence of melanocytes and decreased epidermal pigmentation has been described in clinically uninvolved skin (Wankowicz-Kalinska *et al.* 2003; Pretti Aslanian *et al.* 2007).

(a)



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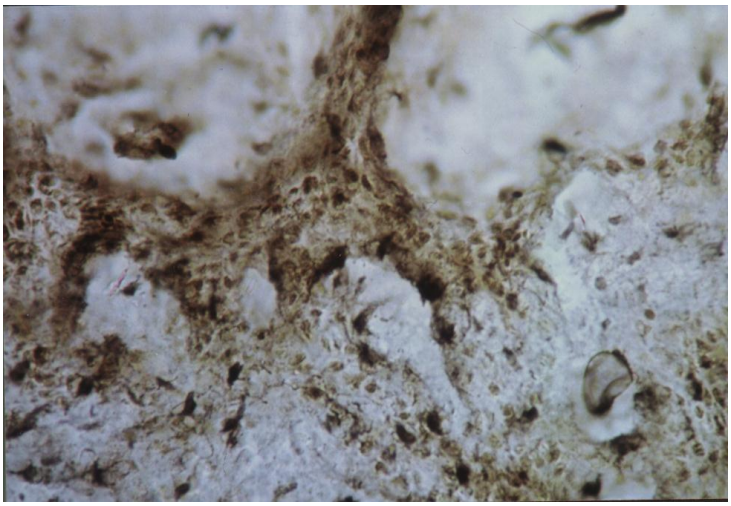


Figure 1.5: Histopathological features of vitiligo.

The skin sections were treated with a L-dopa immunohistochemical stain, which detects tyrosinase activity and reveals the melanocytes in brown. **(a)** Melanocytes in a section of normal cutaneous epidermis. **(b)** Melanocytes in a cutaneous epidermal section from a patient with vitiligo showing a much reduced melanocyte density. Taken with kind permission from Springer Science and Business Media **(**Van Godewijckstraat 30, P.O. Box 17, 3300 AA Dordrecht, The Netherlands) from (Gawkrodger 1998).

1.2.7 Treatment modalities

The aetiology of vitiligo is unclear at present and hence there are no specifically targeted treatments for the disease. In many cases, the use of sunscreens and cosmetic camouflage is the most appropriate intervention rather than the application of a medical or surgical therapy (Gawkrodger 1998; Kovacs 1998; Gawkrodger *et al.* 2010; Taïeb and Picardo 2010b). Nonetheless, there are several treatments which can induce a degree of repigmentation and the best option can be applied following a detailed assessment of the patient's vitiligo.

1.2.7.1 Patient assessment

Before commencing treatment, a complete assessment of the patient is performed to exclude other conditions that may mimic vitiligo such as pityriasis versicolor, piebaldism and idiopathic guttate hypomelanosis (Gawkrodger *et al.* 2010). An accurate and full examination to determine the extent and distribution of depigmented lesions and the clinical type of vitiligo is then made before the available treatment options are discussed with the patient. Quality of life assessments are also helpful and the provision of psychological support is useful for patients who have suffered serious psychological effects or social stigma as a result of the disease (Ongenae *et al.* 2006; Choi *et al.* 2010; Gawkrodger *et al.* 2010; Taïeb and Picardo 2010b). Thyroid function and a test for the presence of anti-thyroid antibodies, and possibly other endocrine organ antibodies, are valuable since vitiligo can be associated with autoimmune thyroiditis in at least 5% of cases (Bjoro *et al.* 2000).

1.2.7.2 Medical treatments

Several medical approaches have been used to treat vitiligo including UV light, topical agents, camouflaging and depigmenting chemicals and, these are discussed in the next sections.

1.2.7.2.1 Topical corticosteroids

At present, the use of topical corticosteroids, which have anti-inflammatory and immunosuppressive effects, is considered to be the first-line treatment in children and adults with segmental or non-segmental vitiligo of recent onset (Gawkrodger 1998; Cockayne *et al.* 2002; Forschner *et al.* 2007; Gawkrodger *et al.* 2008; Abu Tahir *et al.* 2010; Gawkrodger *et*

al. 2010; Hossani-Madani and Halder 2010; Taïeb and Picardo 2010b; Tamesis and Morelli 2010; Van der Veen *et al.* 2010; Whitton *et al.* 2010). Potent (e.g., betamethasone valerate) and highly potent (e.g., clobetasol propionate) topical corticosteroids can induce repigmentation in vitiligo lesions to a variable degree, but this is only seen in a proportion of patients: repigmentation of more than 75% and of 90-100% was achieved in 2/23 and 6/23 vitiligo patients, respectively, using clobetasol propionate and betamethasone valerate (Kandil 1974; Clayton 1977). Furthermore, in combination with UVA, topical fluticasone propionate, a potent steroid, was found to be three times more effective in inducing repigmentation when compared to each treatment alone (Westerhof *et al.* 1999), and topical hydrocortisone 17-butyrate together with 308-nm excimer laser treatment was shown to be synergistically effective in the therapy of recalcitrant vitiligo lesions on the face and neck (Sassi *et al.* 2008). However, despite their efficacy, potent topical corticosteroids can only be used once daily for no more than two months, in order to avoid skin atrophy (Van der Veen *et al.* 2010).

1.2.7.2.2 Topical immunomodulators

Topical immunomodulators such as tacrolimus and pimecrolimus are preferentially used in the treatment of vitiligo lesions on the face and neck in adults and children (Grimes *et al.* 2002; Lepe *et al.* 2003; Mayoral *et al.* 2003; Coskun *et al.* 2005; Sendur *et al.* 2006; Boone *et al.* 2007; Seirafi *et al.* 2007; Hartmann *et al.* 2008; Radakovic *et al.* 2009; Stinco *et al.* 2009; Xu *et al.* 2009; Hossani-Madani and Halder 2010; Lo *et al.* 2010; Whitton *et al.* 2010). Also referred to as calcineurin inhibitors, these reagents down-regulate the activation and maturation of T cells, immune factors which are thought to be involved in vitiligo pathogenesis (Homey *et al.* 1998). In combination with either narrow-band (NB)-UVB (Ostovari *et al.* 2006; Fai *et al.* 2007; Esfandiarpour *et al.* 2009; Majid 2010) or with 308-nm excimer laser (Kawalek *et al.* 2004), tacrolimus also has a synergistic effect in inducing repigmentation. Topical immunomodulators have a better safety profile than topical steroids with respect to skin side-effects, and percutaneous absorption of topical immunomodulators is limited so no significant systemic effects have been reported (De and Kanwar 2008). With regard to efficacy, topical tacrolimus has been shown to be as effective as clobetasol propionate in inducing repigmentation (Lepe *et al.* 2003), and pimecrolimus was found to be more effective than calcipotriol in the treatment of facial lesions (Bilac *et al.* 2009). Overall,

topical immunomodulators are considered a useful alternative to topical steroids in the treatment of vitiligo (Van der Veen *et al.* 2010). However, more studies are required to assess their long-term benefits and safety when used as either a monotherapy or in combination treatment.

1.2.7.2.3 Topical vitamin D analogues

The topical vitamin D analogues, calcipotriol and tacalcitol, have been used in vitiligo therapy as, they can promote tyrosinase activitiy and so initiate pigment synthesis. Repigmentation of vitiligo lesions has been reported in patients treated with topical calcipotriol alone (Gargoom *et al.* 2004), and in combination with either topical corticosteroids, NB-UVB, psoralen with UVA (PUVA) (Section 1.2.7.2.5) or 308-nm excimer laser (Katayama *et al.* 2003; Kullavanijaya and Lim 2004; Travis and Silverberg 2004; Goktas *et al.* 2006; Kumaran *et al.* 2006; Leone *et al.* 2006; Amano *et al.* 2008; Gamil *et al.* 2010). The benefits of vitamin D analogues in vitiligo treatment are, however, controversial, with several studies reporting either topical calcipotriol monotherapy as ineffective or that no additional improvement is noted in repigmentation when they are used in combination with other therapies (Chiaverini *et al.* 2002; Ada *et al.* 2005; Hartmann *et al.* 2005; Goldinger *et al.* 2007; Rodriguez-Martin *et al.* 2009).

1.2.7.2.4 Antioxidants

There is convincing evidence for the occurrence of oxidative stress in the epidermal skin of vitiligo patients (Schallreuter 1999; Schallreuter *et al.* 1999). Antioxidants work by reducing high levels of H_2O_2 in epidermal melanocytes and keratinocytes, thus leading to repigmentation of vitiligo lesions (Schallreuter *et al.* 1995; Schallreuter 1999; Schallreuter *et al.* 1999; Schallreuter *et al.* 2001). Several different reagents have been tested including pseudocatalase (Schallreuter *et al.* 1995) and vitamin E (Akyol *et al.* 2002). Pseudocatalase in a topical cream, and in combination with short-term UVB exposure, resulted in excellent repigmentation in 90% of 33 patients, especially on the dorsum of hands and face (Schallreuter *et al.* 1995). However, further studies are required to evaluate the effectiveness of pseudocatalase in vitiligo treatment. Another antioxidant, vitamin E, in combination with PUVA, induced 75% repigmentation in 60% of vitiligo patients compared with 40% of vitiligo patients for PUVA treatment alone (Akyol *et al.* 2002). In a randomised, double-

blind, placebo-controlled multicentre trial, an oral antioxidant pool containing alpha-lipoic acid and vitamins C and E was found to improve significantly NB-UVB effectiveness with excellent repigmentation in 40% of patients compared with placebo-NB-UVB (Dell'Anna *et al.* 2007). In addition, treatment with the antioxidant pool induced a reduction in oxidative stress in the epidermis of the vitiligo patients (Dell'Anna *et al.* 2007).

1.2.7.2.5 Photochemotherapy

Psoralen is a furocoumarin compound which can stimulate melanocyte proliferation, melanosome formation, melanosome transfer to keratinocytes and tyrosinase activation (Gupta and Anderson 1987; Averbeck 1989; Abdel-Naser *et al.* 1997; Canton *et al.* 2002; Wu *et al.* 2007). Photochemotherapy using orally applied psoralen with UVA (PUVA) is considered as second-line therapy in the adult patients with non-segmental vitiligo affecting more than 10-20% of the skin (Njoo *et al.* 1999; Gawkrodger *et al.* 2008; Falabella and Barona 2009; Alomar 2010; Gawkrodger *et al.* 2010). However, the treatment is not recommended for either children under twelve years of age because of retinal toxicity or for those with a personal history of malignant skin lesions, photoallergy, impaired liver function, pregnancy and cataract (Carrascosa *et al.* 2005; Alomar 2010). In contrast, topical photochemotherapy can be applied to children over two years and to adults with localised vitiligo lesions (Alomar 2010).

1.2.7.2.6 Narrow-band-UVB

As yet, the precise mechanism of action of narrow-band (NB)-UVB in repigmenting vitiligo lesions is unknown. However, NB-UVB can activate the release from keratinocytes of ET-1 and bFGF, which then stimulate melanocyte proliferation (Wu *et al.* 2004), and can stimulate the expression of focal adhesion kinase and matrix metalloproteinases, which may enhance melanocyte migration from the outer root sheath of the hair follicle into the epidermis (Wu *et al.* 2004). When used for vitiligo treatment, more than 75% repigmentation has been achieved in 67% (Westerhof and Nieuweboer-Krobotova 1997) and 53% (Njoo *et al.* 1998) of patients. Furthermore, stabilisation of the disease was evident in 80% of those patients with active disease (Njoo *et al.* 1998). Since these early studies, many others have confirmed the efficacy of NB-UVB for the treatment of vitiligo (Scherschun *et al.* 2001; Samson Yashar *et al.* 2003; Hamzavi *et al.* 2004; Anbar *et al.* 2006; Brazzelli *et al.* 2007; Nicolaidou *et al.* 2007; Kumar

et al. 2009; Leone and Tanew 2010) . This modality now represents the phototherapy of choice for inducing repigmentation in patients with generalised vitiligo where depigmented areas affect multiple sites or large areas of the body. It is also useful for halting the progression of currently active disease. Comparative studies have also indicated the superior efficacy and better safety profile of NB-UVB over PUVA (Westerhof and Nieuweboer-Krobotova 1997; Bhatnagar *et al.* 2007b; Bhatnagar *et al.* 2007a; Yones *et al.* 2007).

1.2.7.2.7 Laser treatments

The 308-nm excimer laser is a new therapeutic option for vitiligo which allows the targeted phototherapy of depigmented lesions (Passeron and Ortonne 2005; Passeron and Ortonne 2006; Leone and Tanew 2010). Although the mechanism of action is not yet understood, excimer laser treatment is thought to stimulate melanocyte migration and proliferation and to act as an immunosuppressant. The efficacy of excimer laser treatment depends on the location of the vitiligo lesions, with the best results being reported for the face and neck, and on treatment frequency, with weekly applications optimal (Hofer *et al.* 2005; Shen *et al.* 2007). Furthermore, a combination of 308-nm excimer laser with topical tacrolimus induces a greater repigmentation response and a shorter duration of treatment compared to excimer laser monotherapy (Kawalek *et al.* 2004; Passeron *et al.* 2004). Overall, excimer laser has a shorter treatment time and fewer adverse side-effects than other phototherapies (Baltas *et al.* 2001; Hofer *et al.* 2005; Le Duff *et al.* 2010; Leone and Tanew 2010), but relapse of repigmentation has been reported to occur in 15% of patients 1-3 years after discontinuation of therapy (Passeron and Ortonne 2005).

1.2.7.2.8 Depigmenting treatments

Depigmentation with topical monobenzyl ether of hydroquinone is considered in patients with extensive depigmentation, usually involving more than 90% of the body surface area, or with depigmentation in cosmetically significant sites such as the face or hands, or for whom other treatments have been ineffective (Mosher *et al.* 1977). The combination of topical 4-methoxyphenol and Q-switched ruby laser therapy has also been shown to elicit effective depigmentation (Njoo *et al.* 2000). Cryotherapy and 4-hydroxyanisole may also produce depigmentation (Radmanesh 2000; Di Nuzzo and Masotti 2010).

1.2.7.2.9 Camouflage

Camouflaging has only recently been recognised as a medical intervention for treating vitiligo (Rajatanavin *et al.* 2008; Gawkrodger *et al.* 2010; Tanioka *et al.* 2010). Self-tanning lotions and pigmented creams can be used to disguise depigmented areas and they can be relatively simple to apply (Jouary and DePase 2010). In addition, tattoing is an option to cover vitiligo lesions, but this technique has not always given satisfactory results (Boissy and Nordlund 1995b; De Cuyper 2008; Holla and Parsad 2010; Jouary and DePase 2010; Singh and Karki 2010).

1.2.7.3 Surgical treatments

Surgical treatment is not a first option for vitiligo and is only used in cases which fail to respond to medical therapies. This is particularly so for vitiligo lesions on the fingers, hands and feet, where there is a low density of hair follicles and, for segmental vitiligo (Holla and Parsad 2010; Olsson 2010). Several surgical techniques have been developed and these are listed in Table 1.3. Combining these techniques with medical treatments can also be beneficial (Sachdev and Krupashankar 2000; Pai *et al.* 2002; Toriyama *et al.* 2004). Selection criteria for suitable patients include the site, number and extent of the lesions, the texture of the recipient skin, the clinical type of vitiligo and disease stability. The outcome of surgical treatments is excellent for patients with stable segmental vitiligo, but for those with non-segmental vitiligo, patients should not have demonstrated the Koebner phenomenon, the extension of old lesions or the development of new lesions over the previous year for a successful result (Gawkrodger *et al.* 2008; Holla and Parsad 2010; Olsson 2010).

1.2.7.4 Summary

In summary, the treatment choice in vitiligo is dependent upon factors which include vitiligo type, patient age and location and stability of lesions (Taieb et al 2010b). However, despite many treatment modalities, repigmentation in the majority of vitiligo patients is rarely complete or long-lasting. A better understanding of the precise pathogenesis of the disease is crucial to improving the efficacy of treatment regimens.

Table 1.3: Surgical treatments for vitiligo

Surgical procedure	Comments	Reference
Autologous grafting e.g., minigrafts, suction blisters, ultra-thin grafting	Cobblestone appearance is common at the recipient site. Relatively easy and inexpensive methods.	(Boersma *et al.* 1995; Boissy and Nordlund 1995b; Kovacs 1998; Fongers *et al.* 2009; Olsson 2010)
Transplantation of cultured epidermis	Results of culturing will depend on skin type and any previous UV therapy. Requires culture facilities and trained personnel.	(Boissy and Nordlund 1995b; Kovacs 1998; Pianigiani *et al.* 2005; Czajkowski *et al.* 2007)
Cultured melanocytes and keratinocytes	Cells are cultured in suspension or on defined membranes. Requires culture facilities and trained personnel.	(Mulekar 2004; Pandya *et al.* 2005; El-Zawahry *et al.* 2010)
Cultured epidermal sheets with CO_2 laser	Large lesions can be treated.	(Toriyama *et al.* 2004)
Autologous grafting with erbium YAG laser	Larger lesions can be treated. No cobblestone appearance.	(Sachdev and Krupashankar 2000; Pai *et al.* 2002)

1.3. Vitiligo: Aetiology and Pathogenesis

The exact aetiology and detailed pathogenesis of vitiligo still remains obscure. There are several proposed causes and these are discussed in the following sections.

1.3.1 Physical trauma

Mechanical trauma or friction injury to the skin, also known as Koebner's phenomenon, may be a triggering and perpetuating factor of melanocyte loss in vitiligo (Gauthier 1996; Taïeb 2000), as it has been observed that the distribution of depigmented lesions can correlates with the site of friction or injury (Gauthier 1996). The reported incidence of Koebner's phenomenon in vitiligo varies from 21-62% of patients (Barona *et al.* 1995; Hann *et al.* 1997; Mazereeuw-Hautier *et al.* 2010; van Geel *et al.* 2011b) and, recently, a new classification has been proposed to allow the evaluation of Koebner's phenomenon in daily practice or in experimental studies (van Geel *et al.* 2011a).

To date, the pathophysiology of Koebner's phenomenon in vitiligo has not been clarified, but may involve factors such as oxidative or immunological stresses which eventually lead to melanocyte death (van Geel *et al.* 2011a). Interestingly, detachment of surviving melanocytes from the basal layer following traumatic friction in non-lesional skin in patients with generalised vitiligo has been demonstrated (Gauthier *et al.* 2003a). This detachment was followed by the trans-epidermal migration of and ultimately the death of pigment cells suggesting that physical stress can be responsible for chronic melanocyte loss in vitiligo (Gauthier *et al.* 2003a).

1.3.2 Psychological stress

There is evidence to indicate that stressful life events can contribute to the onset of vitiligo in some patients (Papadopoulos *et al.* 1998). In contrast, it has been observed that the development of vitiligo is not necessarily related to stressful situations but more to the inability to cope with uncontrollable events (Picardi *et al.* 2003). In addition, a lack of social support can increase susceptibility to vitiligo (Picardi *et al.* 2003). With regard to a pathological mechanism, it has been suggested that psychological stress and emotional trauma may increase the levels of neuropeptide-Y (Al'Abadie *et al.* 1994b; Tu *et al.* 2001) and neuroendocrine hormones (Al'Abadie *et al.* 1994a), which could result in melanocyte damage leading to the acute onset of depigmentation.

1.3.3 Infections

It has been suggested that infection may be a possible factor leading to pigment cell loss in vitiligo (Le Poole *et al.* 1993a). The destruction of melanocytes has been explained by the display of infectious agent particles with major histocompatibility complex (MHC) class I molecules on the infected cell surface thereby triggering a secondary immune response (Le Poole *et al.* 1993a). The cytomegalovirus genome has been identified in lesional and non-lesional skin biopsies of some patients with vitiligo suggesting that viral infection may play a role in disease onset (Grimes *et al.* 1996). In addition, vitiligo has been reported to be associated with other infections including human immunodeficiency virus (Ivker *et al.* 1994; Vin-Christian *et al.* 2000; Antony and Marsden 2003), chronic mucocutaneous candidiasis (Howanitz *et al.* 1981) and leprosy (Shegan 1971).

1.3.4 Genetic factors

The following details and discussion refer to non-segmental since there are few studies that provide information with regard to the genetics of the segmental form of the disease.

1.3.4.1 Genetic epidemiology of vitiligo

The majority of cases of vitiligo are sporadic without a family history of the disease. Nevertheless, 15-20% of patients report at least one affected first-degree relative (Alkhateeb *et al.* 2003), lending evidence for a genetic role in the aetiology of vitiligo. Furthermore, among Caucasians, the risk of vitiligo developing in a patient's sibling is approximately 6.1% (Alkhateeb *et al.* 2003), an increase of sixteen-fold compared to the general Caucasian population where the prevalence of the disease is 0.38% (Howitz *et al.* 1977). Similarly, an increased risk among first-degree relatives is found in Indian-Pakistanis at 6.1% (Alkhateeb *et al.* 2003), in American Hispanic-Latinos at 4.8% (Alkhateeb *et al.* 2003) and in Han Chinese at 2.6% (Sun *et al.* 2006). A simple Mendelian inheritance pattern is not displayed in these familial aggregations of vitiligo cases (Mehta *et al.* 1973; Carnevale *et al.* 1980; Hafez *et al.* 1983; Das *et al.* 1985; Majumder *et al.* 1988; Bhatia *et al.* 1992; Majumder *et al.* 1993; Nath *et al.* 1994; Alkhateeb *et al.* 2003; Laberge *et al.* 2005; Sun *et al.* 2006), suggesting that the disease is probably transmitted as a polygenic trait. Indeed, earlier disease onset in familial cases (Alkhateeb *et al.* 2003; Laberge *et al.* 2005) and reduced risk of vitiligo with increasing genetic distance from the patient (Alkhateeb *et al.* 2003) are indicative of a

polygenic disorder. Formal genetic segregation analyses of vitiligo have also suggested that multiple loci contribute to vitiligo susceptibility (Majumder *et al.* 1993; Nath *et al.* 1994; Sun *et al.* 2006). Seldom have large multi-generation families been reported, where vitiligo segregates in an autosomal dominant pattern (Alkhateeb *et al.* 2005).

Approximately 15-25% of vitiligo patients have an additional autoimmune disorder, for example, autoimmune thyroid disease and type 1 diabetes mellitus (Alkhateeb *et al.* 2003). Moreover, the risk of occurrence of the same autoimmune diseases is elevated in the vitiligo patients' first-degree relatives (Alkhateeb *et al.* 2003; Laberge *et al.* 2005). Such findings indicate that these vitiligo patients and their close relatives have a susceptibility to autoimmune disease, including vitiligo, which is mediated by shared genes, with additional genetic loci determining disease specificity (Spritz 2010b).

Twin studies have also provided evidence of a genetic component to vitiligo aetiology. For vitiligo in monozygotic twins, the concordance is 23% (Alkhateeb *et al.* 2003), a disease risk that is sixty-fold greater than that in the general population (Howitz *et al.* 1977) and four-fold higher than that for a patient's sibling (Alkhateeb *et al.* 2003)

1.3.4.2 Identification of genes involved in vitiligo aetiology

The genetic epidemiological evidence has prompted the search for genes which predispose an individual to the development of vitiligo. Investigations have included families with vitiligo as well as cohorts of patients without a familial history of the disease (Fain *et al.* 2003; Cantón *et al.* 2005). Different approaches have been employed to identify genes which confer susceptibility to vitiligo. Candidate gene association studies rely upon testing known genes and this type of analysis has been extensively used with populations of unrelated vitiligo patients (Blomhoff *et al.* 2005; Cantón *et al.* 2005). In contrast, genome-wide linkage studies scan the whole genome for regions that segregate in a non-random fashion with vitiligo. This approach has been used in analysing genetic factors in vitiligo, but is limited to the analysis of family-related vitiligo (Fain *et al.* 2003; Spritz *et al.* 2004; Chen *et al.* 2005; Liang *et al.* 2007). Genome-wide association studies combine the advantages of candidate gene association and genome-wide linkage analyses and this approach has recently been applied to the study of vitiligo susceptibility genes (Birlea *et al.* 2010; Jin *et al.* 2010a; Quan *et al.* 2010). The genes and genetic loci so far identified have been extensively reviewed (Spritz

2006; Spritz 2007; Spritz 2008; Spritz 2010b; Spritz 2011; Spritz 2012), and are summarised in the following sections.

1.3.4.3 Human leukocyte antigen alleles of the major histocompatibility complex

The studies of human leukocyte antigen (HLA) alleles of the MHC in relation to vitiligo susceptibility are summarised in Table 1.4. Initial case control studies demonstrated an association between predisposition to vitiligo and several different HLA alleles (Metzker *et al.* 1980; Foley *et al.* 1983; Dunston and Halder 1990; Finco *et al.* 1991; Poloy *et al.* 1991; Lorini *et al.* 1992; Orecchia *et al.* 1992; Venneker *et al.* 1992; Ando *et al.* 1993; Schallreuter *et al.* 1993; Venneker *et al.* 1993; Al-Fouzan *et al.* 1995; Venkataram *et al.* 1995; Buc *et al.* 1996). Although these studies showed weak and variable associations, a significant association of HLA-DR4 and vitiligo was demonstrated in several populations (Foley *et al.* 1983; Dunston and Halder 1990; Venneker *et al.* 1992) and a subsequent meta-analysis of a series of case-control studies reported association of vitiligo with HLA-A2 (Liu *et al.* 2007).

More recently, the use of better analytical and statistical methods has revealed associations of vitiligo with HLA-DRB1*04, HLA-DRB1*03 and HLA-DRB1*07 alleles in Turkish patients (Tastan *et al.* 2004), with HLA-DRB4*0101 and HLA-DQB1*0303 in Dutch patients (Zamani *et al.* 2001), HLA-A25-Cw*0602-DQA1*0302, HLA-DQA1*0302, HLA-DQB1*0303 and HLA-DQB1*0503 in Han Chinese patients (Yang *et al.* 2005; Xia *et al.* 2006), and A*33:01, B*44:03, and DRB1*07:01 in Idian and Gujarat patients (Singh *et al.* 2012). Furthermore, a study of 76 Caucasian multiplex vitiligo families found the HLA-DRB1A*04-DQB1*0301 haplotype to be associated with a higher risk of developing vitiligo and with an earlier onset of the disease (Fain *et al.* 2006). Finally, of three genome-wide association studies undertaken on populations of vitiligo patients (Birlea *et al.* 2010; Jin *et al.* 2010a; Quan *et al.* 2010), two reported that predisposition of vitiligo was associated with HLA class I and II antigens (Jin *et al.* 2010a; Quan *et al.* 2010).

1.3.4.4 Other immune-response genes and loci

Studies of immune-response gene variants in relation to predisposition to vitiligo development are summarised in Table 1.5. Several of these genetic associations have been verified while others remain to be confirmed. Of particular note, the allelic variation R620W of the PTPN22 gene, which encodes lymphoid protein tyrosine phosphatase, a molecule

Table 1.4: Association of human leukocyte antigen (HLA) alleles with vitiligo susceptibility

Population	Associated HLA allele	Reference
American (Caucasian)	DR4	(Foley *et al.* 1983)
American (African)	DR4, DQw3	(Dunston and Halder 1990)
American and British (European-derived, Caucasian)	DRB1A*04-DQB1*0301	(Fain *et al.* 2006)
American and British (European-derived, Caucasian)	Class I (specifically A*0201) and II antigens	(Jin *et al.* 2010a)
Chinese (Han)	DQA1*0302, DQB1*0303, DQB1*0503	(Yang *et al.* 2005)
Chinese (Han)	A25-Cw*0602-DQA1*0302	(Xia *et al.* 2006)
Chinese (Han and Uygar)	Class I and II antigens	(Quan *et al.* 2010)
Dutch	DR4, DR6, Cw6	(Venneker *et al.* 1992; Venneker *et al.* 1993)
Dutch	DRB4*0101, DQB1*0303	(Zamani *et al.* 2001)
German (Northern)	A2	(Schallreuter *et al.* 1993)
Hungarian	DR1, DR3	(Poloy *et al.* 1991)
Italian	A30, B27, Cw6, DQw3	(Finco *et al.* 1991)
Italian (Northern)	A3	(Lorini *et al.* 1992)
Italian (Northern)	A30, Cw6, DQw3	(Orecchia *et al.* 1992)
Japanese	A31, Bw46, Cw4	(Ando *et al.* 1993)
Kuwaiti	B21, Cw6	(Al-Fouzan *et al.* 1995)
Moroccan (Jewish)	B13	(Metzker *et al.* 1980)
Omani	Bw6, DR7	(Venkataram *et al.* 1995)
Slovak	A2, Dw7	(Buc *et al.* 1996)
Turkish	DRB1*03, DRB1*04, DRB1*07	(Tastan *et al.* 2004)
Yemeni	Bw35	(Metzker *et al.* 1980)
Indian and Gujarat	A*33:01, B*44:03, DRB1*07:01	(Singh *et al.* 2012)

involved in T cell signalling, has been shown to confer vitiligo susceptibility in several independent reports (Cantón *et al.* 2005; Laberge *et al.* 2008a; Laberge *et al.* 2008b; Jin *et al.* 2010a). In addition, allelic variants in the NLRP1 gene (previously NALP1 or SLEV1), which encodes a key regulator of the innate immune system, have been reproducibly associated with an increased risk of vitiligo in different populations (Nath *et al.* 2001; Spritz *et al.* 2004; Jin *et al.* 2007b; Jin *et al.* 2007c; D'Osualdo and Reed 2012). More recent genome-wide association studies have identified and confirmed the association of vitiligo with variations in several immune-response genes e.g., CCR6, FOXP1, FOXP3, TSLP and XBP1 (Cheong *et al.* 2009; Ren *et al.* 2009; Jin *et al.* 2010a; Jin *et al.* 2010b; Quan *et al.* 2010; Birlea *et al.* 2011).

In contrast, studying variations in the cytotoxic T lymphocyte antigen 4 (CTLA4) gene has yielded conflicting results with respect to vitiligo susceptibility (Kemp *et al.* 1999; Blomhoff *et al.* 2005; Itirli *et al.* 2005; Laberge *et al.* 2008a; Birlea *et al.* 2009; Pehlivan *et al.* 2009; Deeba *et al.* 2010; Birlea *et al.* 2011). Presently, allelic differences in CTLA4 appear to be predominantly associated with vitiligo occurring together with other autoimmune diseases (Blomhoff *et al.* 2005), and it has been suggested, therefore, that the association of CTLA4 with vitiligo is probably secondary to its primary association with disorders such as autoimmune thyroid disease (Spritz 2010b). Similar conflicts of data have arisen with respect to the study of single nucleotide polymorphisms (SNPs) in the autoimmune regulator (AIRE) gene. Mutations in the AIRE gene are the cause of autoimmune polyendocrine syndrome type 1 (APS1) of which vitiligo is a common clinical manifestation (Nagamine *et al.* 1997). Significant associations between SNPs in AIRE and vitiligo susceptibility were found in one (Tazi-Ahnini *et al.* 2008), but not in a second candidate gene association study (Birlea *et al.* 2011), and not in a report analysing multiplex families (Jin *et al.* 2007a).

1.3.4.5 Non-immune-response genes and loci

Many studies have been undertaken to find variations in genes that regulate melanocyte metabolism, development or proliferation that could be associated with susceptibility to vitiligo (Table 1.6). These have included the genes encoding catalase (Casp *et al.* 2002; Gavalas *et al.* 2006; Park *et al.* 2006; Shajil *et al.* 2007; Liu *et al.* 2010; Birlea *et al.* 2011), catechol-*O*-methyltransferase (Türsen *et al.* 2002; Li *et al.* 2009a; Birlea *et al.* 2011),

Table 1.5: Association of immune-response gene variants with vitiligo susceptibility

Gene or locus	Function	Association status	Reference
AIRE	Autoimmune regulator. Transcription factor which determines self-tolerance during T cell maturation in the thymus. Causes autoimmune polyendocrine syndrome type 1.	Data conflicting	(Jin *et al.* 2007a; Tazi-Ahnini *et al.* 2008; Birlea *et al.* 2011)
AIS2	Autoimmune susceptibility locus 2. Function undefined. Associated with autoimmune disease.	Confirmed	(Spritz *et al.* 2004)
AIS3	Autoimmune susceptibility locus 3. Function undefined.	Unconfirmed	(Spritz *et al.* 2004)
CCR6	Cytokine-chemokine receptor for CCL20. Recruits immune cells on binding of ligand. Associated with inflammatory bowel disease.	Confirmed	(Jin *et al.* 2010a; Jin *et al.* 2010b; Quan *et al.* 2010)
CD4	CD antigen. Regulates cell-mediated immune responses. Associated with type 1 diabetes mellitus.	Data conflicting	(Zamani *et al.* 2010; Birlea *et al.* 2011)
C1QTNF6	C1q and tumour necrosis factor-related protein- 6. Associated with rheumatoid arthritis and type 1 diabetes mellitus.	Confirmed	(Jin *et al.* 2010a)
CTLA4	Cytotoxic T lymphocyte antigen 4. Negatively regulates T cell activation. Associated with several autoimmune diseases.	Data conflicting	(Kemp *et al.* 1999; Blomhoff *et al.* 2005; Itirli *et al.* 2005; Laberge *et al.* 2008a; Birlea *et al.* 2009; Pehlivan *et al.* 2009; Deeba *et al.* 2010; Birlea *et al.* 2011)
DDR1	Discoid domain receptor tyrosine kinase 1. Regulates leukocyte function and cell adhesion.	Data conflicting	(Kim *et al.* 2010; Silva de Castro *et al.* 2010; Birlea *et al.* 2011)
FOXP1	Forkhead box P1. Transcription factor which regulates development of immune cells.	Confirmed	(Jin *et al.* 2010a; Jin *et al.* 2010b)
FOXP3	Forkhead box P3. Transcription factor which regulates regulatory T cell development. Causes autoimmune IPEX syndrome.	Confirmed	(Birlea *et al.* 2011)
GZMB	Granzyme B. Regulates cell-mediated immune responses.	Confirmed	(Jin *et al.* 2010a)
IL10	Interleukin 10. Cytokine which inhibits T cell differentiation and proliferation.	Data conflicting	(Abanmi *et al.* 2008; Birlea *et al.* 2011)
IL1RN	Interleukin (IL)-1 receptor antagonist. Modulates action of IL1.	Data conflicting	(Pehlivan *et al.* 2009; Birlea *et al.* 2011)
IL2RA	Interleukin-2 receptor alpha chain. Receptor for cytokine IL2 which induces T and B cell proliferation. Associated with many autoimmune diseases.	Confirmed	(Jin *et al.* 2010a)
LMP2	Low molecular weight polypeptide-2. Functions in processing and presentation of antigens to the immune system.	Unconfirmed	(Casp *et al.* 2003)
LMP7 (PSMB8)	Low molecular weight polypeptide-7. Functions in processing and presentation of antigens to the immune system.	Data conflicting	(Casp *et al.* 2003; Birlea *et al.* 2011)
LPP	LIM domain-containing preferred translocation partner in lipoma. Function unknown. Associated with celiac disease and rheumatoid arthritis.	Confirmed	(Jin *et al.* 2010a)
MBL2	Mannose-binding lectin 2. Functions in the innate immune response.	Data conflicting	(Onay *et al.* 2007; Dwivedi *et al.* 2009; Birlea *et al.* 2011)
NLRP1 (NALP1; SLEV1)	NACHT leucine-rich-repeat protein 1. Functions in the innate immune response. Associated with many	Confirmed	(Nath *et al.* 2001; Spritz *et al.* 2004; Jin *et al.* 2007b;

	autoimmune diseases.		Jin *et al.* 2007c)
PTPN22	Lymphoid protein tyrosine phosphatase. Negatively regulates T cell activation. Associated with many autoimmune diseases.	Confirmed	(Cantón *et al.* 2005; Laberge *et al.* 2008a; Laberge *et al.* 2008b; Jin *et al.* 2010a)
TAP1	Transporter associated with antigen processing protein-1. Functions in processing and presentation of antigens to the immune system.	Data conflicting	(Casp *et al.* 2003; Birlea *et al.* 2011)
TGFBR2	Transforming growth factor beta receptor 2. Receptor for cytokine TGF which regulates many immune responses and can inhibit melanocyte activity.	Data conflicting	(Yun *et al.* 2010; Birlea *et al.* 2011)
TNF	Tumor necrosis factor-alpha. Proinflammatory cytokine which can inhibit melanocyte proliferation and melanogenesis.	Data conflicting	(Yazici *et al.* 2006; Namian *et al.* 2009; Birlea *et al.* 2011)
TSLP	Thymic stromal lymphopoietin. Cytokine which induces naïve CD4+ T cells to produce Th2 cytokines.	Confirmed	(Cheong *et al.* 2009; Birlea *et al.* 2011)
UBASH3A	Ubiquitin-associated and SH3 domain-containing A gene. Regulates T cell receptor signalling. Associated with type 1 diabetes mellitus.	Confirmed	(Jin *et al.* 2010a)
XBP1	X-box binding protein 1. Transcription factor which regulates MHC class II gene expression. Associated with inflammatory bowel disease.	Confirmed	(Ren *et al.* 2009; Birlea *et al.* 2011)

and ET-1 (Kim *et al.* 2007a; Lan *et al.* 2009; Birlea *et al.* 2011). However, in the majority of cases, the data are conflicting and will require further analyses to confirm or deny an association with vitiligo.

Of particular interest is the association determined between vitiligo and a major allelic variant of TYR (402Q), the gene which encodes the melanogenic enzyme tyrosinase (Jin *et al.* 2010a). The protein encoded by the minor 402Q variant of TYR is produced in small amounts, is thermosensitive, not correctly glycosylated and is retained in the endoplasmic reticulum (Tripathi *et al.* 1991; Toyofuku *et al.* 2001). Interestingly, the minor TYR allele (402Q) is associated with susceptibility to malignant melanoma (Gudbjartsson *et al.* 2008; Bishop *et al.* 2009). These observations have led to the postulation that the TYR 402Q variant is less available to the immune system such that neoplastic melanocytes may escape immune surveillance in melanoma patients with this minor allele (Jin *et al.* 2010a; Spritz 2010a). Conversely, 402R tyrosinase is perhaps more efficiently presented to the immune system resulting in a more effective immune response against pigment cells in melanoma and but predisposing to vitiligo (Jin *et al.* 2010a; Spritz 2010a).

1.3.4.6 Summary

Overall, there is considerable evidence for the importance of genes in the development of vitiligo (Spritz 2006; Spritz 2007; Spritz 2008; Spritz 2010b; Spritz 2011; Spritz 2012). Interestingly, the majority of the confirmed vitiligo susceptibility loci encode proteins which play a role in the immune response (Tables 1.6 and 1.7), thus strongly supporting an autoimmune basis for the disease. However, exactly how the different gene variants function to contribute to the vitiligo phenotype has not yet been determined. Of interest also, is that the genetic underpinnings of the disease may vary, for example, between patients without a family history of vitiligo and those with family-related vitiligo (Ando *et al.* 1993; Silva de Castro *et al.* 2010). Patients with vitiligo and associated autoimmune disease may also have different genetic factors underlying their disease compared with individuals presenting with isolated depigmentation (Kemp *et al.* 1999; Blomhoff *et al.* 2005). In addition, differences have been reported between gene variants in non-segmental and those in segmental vitiligo, although few studies have addressed this issue (Yang *et al.* 2005; Xia *et al.* 2006; Li *et al.* 2008; Liu *et al.* 2009). Furthermore, different genes may be involved in the pathogenesis of vitiligo in different populations (Fain *et al.* 2003; Spritz *et al.* 2004; Chen *et al.* 2005; Liang

et al. 2007; Laddha *et al.* 2008). Finally, the limited concordance in identical twins (Alkhateeb *et al.* 2003) indicates that other factors, probably environmental, are also involved in causing the disorder making vitiligo a complex, polygenic, multi-factorial disease.

Table 1.6: Association of variants of non-immune-response genes with vitiligo susceptibility

Gene or locus	Function	Association status	Reference
ACE	Angiotensin-converting enzyme inhibitor. Regulates blood pressure and inflammation.	Data conflicting	(Jin *et al.* 2004a; Akhtar *et al.* 2005; Dwivedi *et al.* 2008; Birlea *et al.* 2011)
CAT	Catalase. Regulates breakdown of H_2O_2 and so protects against oxidative stress.	Data conflicting	(Casp *et al.* 2002; Gavalas *et al.* 2006; Park *et al.* 2006; Shajil *et al.* 2007; Liu *et al.* 2010; Birlea *et al.* 2011)
CLEC11A (SCGF)	C-type lectin (Stem cell growth factor). Keratinocyte-derived growth factor which egulates growth and function of melanocytes.	Data conflicting	(Lan *et al.* 2009; Birlea *et al.* 2011)
COMT	Catechol-*O*-methyl transferase. Degrades toxic o-quinones.	Data conflicting	(Türsen *et al.* 2002; Li *et al.* 2009a; Birlea *et al.* 2011)
COX2 (PTGS2)	Cyclooxygenase. Functions in melanocyte proliferation and melanogenesis.	Data conflicting	(Li *et al.* 2009b; Birlea *et al.* 2011)
EDN1	Endothelin-1. Keratinocyte-derived growth factor which regulates growth and function of melanocytes.	Data conflicting	(Kim *et al.* 2007a; Lan *et al.* 2009; Birlea *et al.* 2011)
ESR1	Estrogen receptor 1. Transcription factor but exact function in melanocytes is unknown.	Data conflicting	(Jin *et al.* 2004b; Birlea *et al.* 2011)
FBXO11 (VIT1)	F-box protein 11. Regulates melanocyte proliferation and apoptosis.	Data conflicting	(Le Poole *et al.* 2001; Birlea *et al.* 2011)
FGFR1OP	Fibroblast growth factor receptor 1 oncogene partner. Functions in proliferation and differentiation of melanocytes.	Unconfirmed	(Quan *et al.* 2010)
FOXD3 (AIS1)	Forkhead box D3. Transcription factor. Regulates melanoblast differentiation and development. Found in a single large family with autoimmune-associated vitiligo. Autosomal dominant.	Data conflicting	(Alkhateeb *et al.* 2002; Fain *et al.* 2003; Alkhateeb *et al.* 2005; Birlea *et al.* 2011)
GSTT1	Glutathione S-transferase theta 1. Protects against oxidative stress.	Data conflicting	(Uhm *et al.* 2007; Liu *et al.* 2009; Birlea *et al.* 2011)
GSTM1	Glutathione S-transferase mu 1. Protects against oxidative stress.	Data conflicting	(Uhm *et al.* 2007; Liu *et al.* 2009; Birlea *et al.* 2011)
KITLG (SCF)	KIT ligand (Stem cell factor). Keratinocyte-derived growth factor which regulates growth and function of melanocytes.	Data conflicting	(Lan *et al.* 2009; Birlea *et al.* 2011)
MYG1 (C12orf10)	Melanocyte proliferating gene 1. Functions in skin development.	Data conflicting	(Kingo *et al.* 2006; Philips *et al.* 2010; Birlea *et al.* 2011)
NFE2L2	Nuclear factor-(erythroid-derived 2)-like 2. Transcription factor which egulates	Data conflicting	(Guan *et al.* 2008b; Birlea *et al.* 2011)

	expression of detoxifying and antioxidant genes.		
PDGFRA	Platelet-derived growth factor receptor alpha. Receptor for PDGF□□α which functions in the differentiation of melanocytes.	Data conflicting	(Xu *et al.* 2010; Birlea *et al.* 2011)
RERE	Atrophin-like protein 1. Transcriptional co-repressor thought to regulate apoptosis.	Confirmed	(Jin *et al.* 2010a)
RNASET2	RNase T2. Regulates oxidative stress.	Unconfirmed	(Quan *et al.* 2010)
SMOC2	SPARC-related modular calcium binding 2. Function unknown.	Confirmed	(Birlea *et al.* 2010)
TNFRSF6 (FAS; APO1)	Tumor necrosis factor receptor superfamily, member 6. Modulates apoptosis.	Data conflicting	(Li *et al.* 2008; Birlea *et al.* 2011)
TYR	Tyrosinase. Functions in melanin biosynthesis.	Confirmed	(Jin *et al.* 2010a)
TXNDC5	Thioredoxin domain containing 5. Functions in protein folding and chaperoning.	Data conflicting	(Jeong *et al.* 2010; Birlea *et al.* 2011)
UVRAG	UV radiation resistance-associated gene. Functions in autophagosome formation.	Data conflicting	(Jeong *et al.* 2010; Birlea *et al.* 2011)
VDR	Vitamin D receptor.	Data conflicting	(Birlea *et al.* 2006; Birlea *et al.* 2011)

1.3.5 Neural factors

The neural hypothesis of vitiligo pathogenesis is supported by several clinical, histological and pathophysiological findings in patients with the disease.

1.3.5.1 Clinical evidence

Adverse neurochemical-mediated effects on melanocytes could explain the often symmetrical distribution of vitiligo lesions or the dermatomal pattern of segmental vitiligo (Lerner 1959). It has also been noted that vitiligo spares denervated skin, for example, below the level of neurological damage in certain patients with severe spinal cord injury (Lerner 1959). Spontaneous repigmentation of lesions occurs occasionally in vitiligo patients whose nervous system has been compromised by diabetic neuropathy (Bose 1994). In contrast, vitiligo-like hypopigmented macules are sometimes produced in inflammatory diseases affecting the peripheral nervous system such as leprosy, and in neurodysplasias such as neurofibromatosis and tuberous sclerosis (Boisseau-Garsaud *et al.* 2000b; Oiso *et al.* 2007; Nanda 2008). Additional indirect evidence is provided by the requirement for innervation for repigmentation to occur following skin transplantation (Lerner 1959). While vitiligo patients do not complain of sensory abnormalities in lesional skin, autonomic dysfunction has been reported, including: increased skin surface temperature and an increased sweating response in vitiliginous areas, when compared to normal skin, and an abnormal sympathetic skin response quantified by electrical measurement of sympathetic nerve activity (Chanco-Turner and Lerner 1965; Dutta and Mandal 1982; Merello *et al.* 1993).

1.3.5.2 Histological and ultrastructural abnormalities

Immunohistochemical studies of lesional and peri-lesional vitiligo skin have demonstrated abnormalities in the levels of skin neuropeptides. In 5 of 10 patients with symmetrical vitiligo, levels of neuropeptide Y (NPY) were found to be elevated in the margins of vitiligo lesions and, of these patients, those with active disease also had increased NPY within lesional and perilesional skin (Al'Abadie *et al.* 1994b; Hristakieva *et al.* 2000; Lazarova *et al.* 2000). Elevated NPY plasma levels have also been demonstrated in patients with vitiligo (Tu *et al.* 2001). In addition, the number of nerve fibres immunoreactive to nerve growth factor (NGF) and calcitonin gene-related peptide has been shown to be increased in vitiligo lesions

compared to the uninvolved and control skin (Liu *et al.* 1996). Studies of the ultra-structure of dermal nerves using electron microscopy have also demonstrated regenerative and degenerative changes (Al'Abadie *et al.* 1994b), as well as direct cell-cell contact with melanocytes (Morohashi *et al.* 1977), in vitiliginous skin. Furthermore, melanocytes originate from the neural crest and, in many cases of vitiligo, perilesional melanocytes appear progressively more neural in their behaviour, both in an increasingly dendritic morphology and in an ability to synthesise adrenalin (Iyengar and Misra 1987; Iyengar and Misra 1988).

1.3.5.3 Neurotransmitters

Several studies have reported increased plasma levels of the catecholamine neurotransmitters (e.g., adrenalin, noradrenalin) in early and progressive vitiligo (Morrone *et al.* 1992; Cucchi *et al.* 2000; Cucchi *et al.* 2003). Furthermore, elevated synthesis of catecholamines by epidermal keratinocytes has been reported in vitiligo skin (Schallreuter *et al.* 1992). High levels of catecholamines are directly toxic. Indirectly, increased catecholamines can cause a build-up of melanocyte-damaging oxidative stress (Section 1.3.6) by stimulating severe vasoconstriction and the generation of oxyradicals from hypoxia (Morrone *et al.* 1992; Gillbro *et al.* 2004; Namazi 2007). In turn, hypoxia can lead to the development of extracellular acidosis, which may contribute to the activation of the adaptive immune response against melanocytes (Section 1.3.9.6) (Martinez *et al.* 2007).

1.3.6 Biochemical factors

Biochemical and cellular defects have been proposed to contribute to the loss of melanocytes in vitiligo. The theory is supported by clinical and experimental observations, as well as the applicability of certain treatment regimens and possible genetic susceptibilities. These are discussed below.

1.3.6.1 Accumulation of toxic metabolites

The accumulation of toxic metabolites has been proposed to play a role in vitiligo aetiology. Melanocytes and keratinocytes are capable of the *de novo* synthesis, recycling and regulation of 6-tetrahydrobiopterin (6-BH_4), and this is illustrated in Figure 1.6 (Schallreuter *et al.* 1994a; Schallreuter *et al.* 1994b). 6-BH_4 is required as a cofactor by phenylalanine

hydroxylase (PAH) in the synthesis of tyrosine which is needed for melanin and catecholamine production (Figure 1.6). Phenylalanine hydroxylase activity has been demonstrated to be low in the epidermis of vitiligo patients with the consequent accumulation of high levels of toxic 6-BH_4 and 7-BH_4, which have been suggested to impair melanocyte function (Schallreuter *et al.* 1994a; Schallreuter *et al.* 1994b; Schallreuter *et al.* 1998; Schallreuter *et al.*2008b). Indeed, 6-biopterin, a metabolite of 6-BH_4, is highly toxic to cultured melanocytes (Schallreuter *et al.* 1994b). However, this pathway needs to verified by examining the expression of PAH in melanocytes.

Increased levels of toxic catecholamines in vitiligo patients (Morrone *et al.* 1992; Cucchi *et al.* 2000; Cucchi *et al.* 2003) have already been discussed (Section 1.3.5.2). In addition, the activity of catechol-*O*-methyl transferase (Table 1.7), the enzyme which inactivates catecholamines, is increased in vitiligo skin indicating the presence of higher levels of these compounds (Le Poole *et al.* 1994). Furthermore, elevated activity of monoamine oxidase A (Table 1.7) in the epidermis of patients with vitiligo has been related to defective catecholamine synthesis (Schallreuter *et al.* 1996).

With respect to melanin biosythesis, a reduced stability of TYRP1 and the subsequent production of toxic melanin intermediates, which could contribute to melanocyte damage, has also been reported in vitiligo melanocytes (Jimbow *et al.* 2001).

1.3.6.2 Oxidative stress

Inappropriate production of reactive oxygen species and a failure to clear these effectively from the cell can cause damage to DNA, proteins and cell membranes. Indeed, oxidative stress has long been proposed as a mechanism for melanocyte destruction in vitiligo: melanocytes from normally pigmented skin of vitiligo patients exhibit a high *in vitro* susceptibilty to chemically applied oxidants (Maresca *et al.* 1997; Jimbow *et al.* 2001; Boissy and Manga 2004). In addition, the H_2O_2-scavanging enzyme catalase (Table 1.7) is present at decreased levels in vitiligo patient blood and in cultured vitiligo melanocytes (Schallreuter *et al.* 1991; Schallreuter *et al.* 1999; Schallreuter *et al.* 2001). This has been attributed to variations in the gene encoding catalase which alter the expression of the enzyme (Table 1.7) (Shajil *et al.* 2007; Wood *et al.* 2008). Other antioxidant enzymes including thioredoxin reductase (Table 1.7) (Schallreuter *et al.* 1987) and xanthine oxidase (Table 1.7) (Shalbaf *et al.* 2008) also have impaired function in vitiligo skin and could contribute to an imbalance of

intracellular antioxidants. The effective use of antioxidants in the treatment of vitiligo (Schallreuter *et al.* 1995; Schallreuter 1999; Schallreuter *et al.* 2001) is detailed in Section 1.2.7.2.4. Such

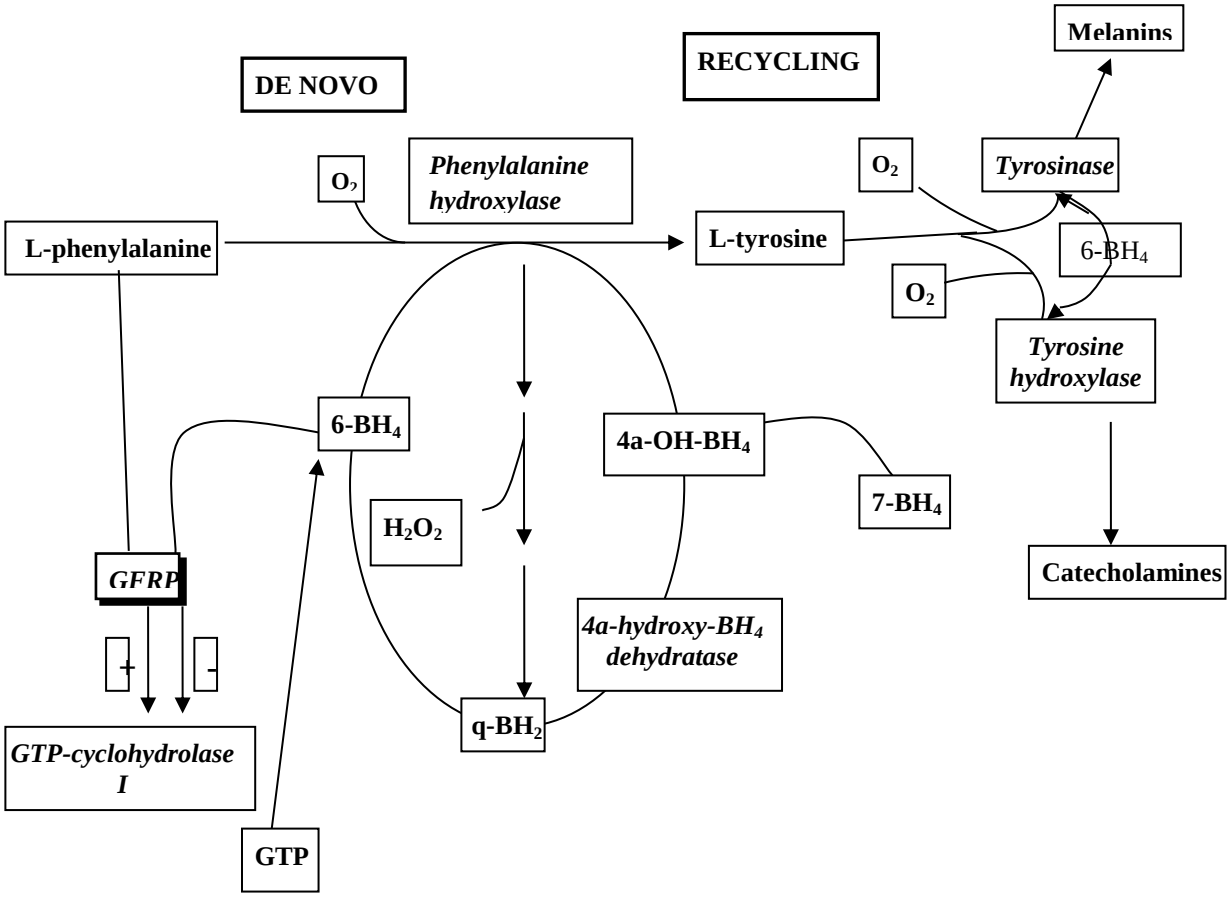


Figure 1.6: Synthesis, recycling and regulation of 6-tetrahydrobiopterin.

Melanocytes and keratinocytes are capable of the *de novo* synthesis, recycling and regulation of 6-tetrahydrobiopterin (6-BH_4). 6-BH_4 acts as a cofactor for the hydroxylation of phenylalanine to tyrosine by phenylalanine hydroxylase. In addition, 6-BH_4 is an uncompetitive inhibitor of tyrosinase activity and serves as a cofactor for the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-dopa) by tyrosine hydroxylase. 6-BH_4 is produced from guanosine triphosphate (GTP) by the action of GTP-cyclohydrolase I (GTP-CHI). GTP-CHI is regulated by GTP-CHI feedback regulatory protein (GFRP), which responds to 6-BH_4 levels. 4a-hydroxy-tetrahydrobiopterin (4a-OH-BH_4) is formed as an intermediate in the 6-BH_4 synthesis pathway and is converted to quinonoid dihydropterin (qBH_2) by 4a-OH-BH_4 dehydratase. By a non-enzymatic process, 7-tetrahydrobiopterin (7-BH_4) is produced from 4a-OH-BH_4. There is a short circuit which produces qBH_2 from 6-BH_4 and which releases hydrogen peroxide (H_2O_2). Drawn with information from (Schallreuter *et al.* 2001).

Table 1.7: Biochemical pathways implicated in vitiligo aetiology

Enzyme or cofactor	Normal function	Experimental observations in relation to vitiligo	Proposed contribution to disease aetiology	Reference
Tetrahydrobiopterin	Regulatory role in melanogenesis since it is a rate-limiting cofactor in L-phenylalanine → L-tyrosine, and L-tyrosine→L-dopa conversions.[1]	Phenylalanine accumulation seen in epidermis of vitiligo patients.[2,3] 6-biopterin, metabolite of tetrahydrobiopterin, is highly toxic to cultured melanocytes.[1]	Defective melanogenesis leads to toxic metabolites which cause the melanocytes to self-destruct.[1]	[1] (Schallreuter *et al.* 1994b) [2] (Schallreuter *et al.* 1998) [3](Schallreuter *et al.* 2005)
Thioredoxin reductase	A free-radical scavenging enzyme located on melanocyte membrane. Also involved in conversion L-tyrosine→L-phenylalanine. Action inhibited by calcium.	Vitiligo melanocytes[4] and keratinocytes[5] have decreased capacity for calcium uptake. Extracellular Ca^{2+} is, therefore, higher in vitiligo skin.	Inhibition of thioredoxin reductase, by ↑ extracellular Ca^{2+}, leads to a build-up in free-radicals which may lead to melanocyte damage.[5,6] Inhibition may also affect melanogenesis leading to the build-up of toxic metabolites.[7]	[4] (Schallreuter *et al.* 1996) [5] (Schallreuter and Pittelkow 1988) [6] (Ortonne and Bose 1993) [7] (Schallreuter *et al.* 1994b)
Catalase	H_2O_2 scavenging enzyme (H_2O_2 → $H_2O + O_2$). ↓oxidative stress in epidermis after UVB irradiation.	Decreased levels of catalase recorded in vitiligo patient blood and in cultured vitiligo melanocytes.[8]	Toxic levels of H_2O_2 may accumulate destroying the melanocyte.[8]	[8] (Schallreuter *et al.* 1991)
Catechol-*O*-methyl transferase (COMT)	Enzyme which inactivates catecholamines by methylation.	Increased COMT in lesional vitiligo skin,[9] indicative of high levels of catecholamines. Increased catecholamines in vitiligo patient skin.[10] Elevated synthesis of catecholamines by epidermal keratinocytes in vitiligo patients.[10]	Catecholamines are directly cytotoxic in high levels. Indirectly, catecholamines can cause a build-up of oxidative stress as follows; abnormal release of catecholamines may cause increased vasoconstriction→ hypoxia-ischaemia→ reoxygenation→ increased production of oxidative species.[11]	[9] (Le Poole *et al.* 1994) [10] (Schallreuter *et al.* 1992) [11] (Morrone *et al.* 1992)
Xanthine oxidase	Hydroxylation of hypoxanthine to xanthine → uric acid	Allantoin present in lesional and non-lesional epidermis of patients with	Presence of allantoin is a biomarker for oxidative stress. [13]	[12] (Nishino *et al.* 2005) [13] (Shalbaf *et al.*

	in purine degradation pathway, the process that yields allantoin and H_2O_2.[12]	active vitiligo and absent in healthy subjects.[13]		2008)
Monoamine oxidase	Catalyses deamination of norepinephrine and epinephrine → ammonia, 3,4-dihydroxymandelic aldehyde and H_2O_2.[14]	Monoamine oxidase activity significantly increased in lesional and nonlesional vitiligo skin.[15]	Accumulation of high amount of H_2O_2 and 6-BH_4 in the epidermis → toxic damage of epidermal cells.[14]	[14] (Yasuhara *et al.* 1993) [15] (Schallreuter *et al.* 1996)

therapeutic approaches, which have also been used in combination with immunosuppressive reagents (Akyol *et al.* 2002), also suggest a role for oxidative stress in vitiligo aetiology.

More recent studies have shown that the enzyme heme oxygenase-1 protects human melanocytes from H_2O_2-induced oxidative stress (Jian *et al.* 2011) and UV radiation (Elassiuty *et al.* 2011) via the nuclear factor E2-related factor (NRF2)-antioxidant response element pathway. Polymorphisms in the NRF2 gene promoter have been associated with an increased risk of developing vitiligo (Guan *et al.* 2008b; Zhou *et al.* 2008), and altered cellular localisation of NRF2 protein in the lesional skin of vitiligo patients has also been reported (Guan *et al.* 2008a). Furthermore, expression of the antioxidant response genes which are controlled by NRF2 is not elevated in lesional vitiligo skin following oxidative stress (Natarajan *et al.* 2010), indicating an impairment of the NRF2- antioxidant response element pathway in vitiligo.

1.3.6.3 Chemically-induced vitiligo

Chemical compounds, which are aliphatic or aromatic derivatives of phenols or catechols, are cytotoxic to melanocytes and can cause depigmentation or leukoderma in exposed individuals (Bleehen *et al.* 1968; Lerner 1971; Cummings and Nordlund 1995). Again, this clinical observation indicates that toxic biochemicals can induce vitiligo. Experimental studies using phenolic derivatives including 4-tertiary butylphenol (4-TBP) have shown that TYRP1 mediates the cytotoxic effects of such reagents (Manga *et al.* 2002). 4- tertiary butylphenol undergoes enzymatic conversion into semiquinon free radicals and reactive oxygen species which damage melanocytes (Shvedova *et al.* 2000; Boissy and Manga 2004). In addition, 4-TBP can increase the melanocyte membrane expression of stress protein heat-shock protein 70 (HSP70) resulting in sensitisation of melanocytes to dendritic cell-mediated cytotoxicity (Denman *et al.* 2008).

1.3.7 Melanocyte growth factors

Melanogenesis is regulated by several melanogenic cytokines and growth factors such as ET-1, SCF, bFGF and GM-CSF. These factors are produced by keratinocytes and fibroblasts and co-ordinate with their respective receptors on melanocytes to exhibit their effects on melanin synthesis (Imokawa and Moretti 2010).

An alteration of the expression of keratinocyte melanogenic cytokines has been suggested to occur in vitiligo. For example, a decreased level of SCF mRNA and protein has been detected in the depigmented skin of vitiligo patients (Moretti *et al.* 2002a; Moretti *et al.* 2002b; Lee *et al.* 2005; Moretti *et al.* 2009). In addition, the protein levels of melanogenic cytokines GM-CSF and bFGF (Moretti *et al.* 2002a; Moretti *et al.* 2002b; Lee *et al.* 2005), as well as ET-1 transcript levels (Moretti *et al.* 2009), have been determined to be reduced in the depigmented vitiligo epidermis. The exact involvement that these melanogenic cytokine alterations play in the development of vitiligo has yet to be ascertained.

1.3.8 Melanocortin hormones

The melanocortin peptide α-MSH is an important regulatory agent in skin pigmentation, inflammatory modulation and response to stress (Slominski *et al.* 1991; Abdel-Malek *et al.* 1995; Luger *et al.* 2003; Brzoska *et al.* 2008; Meyer *et al.* 2009). The hormone binds to MC1R on the melanocyte (Ortonne and Ballotti 2000) (Section 1.1.4).

Few studies have addressed the alterations of α-MSH in vitiligo. Vitiligo patients exhibit significantly lower α-MSH levels in their plasma compared with normal individuals (Pichler *et al.* 2006). In addition, the level and expression of α-MSH in the melanocytes of lesional and perilesional vitiligo skin are lower than in the melanocytes from normal skin (Wakamatsu *et al.* 1997; Graham *et al.* 1999). Furthermore, the proportion of α-MSH immuno-positive melanocytes is significantly reduced in the lesional and perilesional skin of vitiligo patients compared to controls (Graham *et al.* 1999). Although not yet verified, low α-MSH levels could contribute to melanocyte damage in vitiligo through increased oxidative stress (Graham *et al.* 1999).

1.3.9 Autoimmunity

Several lines of evidence suggest that autoimmunity plays a role in the pathogenesis of vitiligo and these are discussed below.

1.3.9.1 Association of vitiligo with autoimmune diseases

The association of vitiligo with other autoimmune diseases has been discussed earlier in Section 1.2.5.

1.3.9.2 Association of vitiligo with immune-response gene polymorphisms

The association of vitiligo with immune-response gene polymorphisms has been discussed earlier in Section 1.3.4.

1.3.9.3 Animal models of vitiligo

The study of animal models has added credence to the theory that immune mechanisms play a part in the development of vitiligo. Several spontaneous animal models of vitiligo exist, although the exact relevance of such models to the equivalent human disorder remains to be established (Boissy and Lamoreux 1988). The well-documented Smyth line (SL) chickens express a genetically inherited form of vitiligo-like depigmentaton resulting from the loss of melanocytes in feather and ocular tissues (Smyth 1989). In this avian model, vitiligo begins with an inherent melanocyte defect that is followed by an autoimmune response involving both humoral and cellular reactions that eliminate abnormal pigment cells (Lamont and Smyth 1981; Boissy *et al.* 1984; Boyle *et al.* 1987; Pardue *et al.* 1987). An increase in T and B cells in the feather pulp and circulating inflammatory leukocytes has been shown in Smyth chickens prior to the onset, and during the development of, vitiligo (Erf *et al.* 1995; Erf and Smyth 1996; Shi and Erf 2012). Antibodies to chicken melanocytes have also been detected in the sera of 100% of SL chicks but not in the sera of normally pigmented birds (Austin *et al.* 1992). These antibodies were found to be present both before and during the presentation of vitiligo (Searle *et al.* 1993), and the primary target antigen was identified as TYRP1 (Austin and Boissy 1995).

More recently, the expression of immune function-related cytokines in growing feathers has been investigated throughout SL vitiligo development and progression (Shi and Erf 2012). Expression levels of cytokines IFN-γ, IL-10, and IL-21 began to increase near visible SL vitiligo onset, reached peak levels during active disease, and decreased to near pre-vitiligo levels after the complete loss of melanocytes. The results suggest that SL vitiligo appears to be a Th1-polarised autoimmune disease, whereby IFN-γ expression is strongly associated with parallel increases in IL-10 and IL-21, particularly during early and active stages of the disease.

In other animals with vitiligo including horses, cats and dogs, antibody reactivity occurs against a similar pattern of melanocyte antigens to that found in patients with the

disease (Naughton *et al.* 1983a; Naughton *et al.* 1986a), suggesting that similar immunological responses may occur in both animals and humans.

1.3.9.4 Melanocyte abnormalities in vitiligo

Several studies have shown abnormal expression of MHC class II antigen HLA-DR and increased expression of ICAM-1 by perilesional melanocytes in vitiligo compared with melanocytes from normal skin (Al Badri *et al.* 1993a; Hedley *et al.* 1998; van den Wijngaard *et al.* 2000). Since these molecules have important roles in antigen presentation and in the activation of helper T cells, their expression by melanocytes could contribute to the anti-melanocyte cellular immune responses that are seen in vitiligo (Ogg *et al.* 1998; van den Boorn *et al.* 2009). Both vitiligo and normal melanocytes are also capable of expressing MHC class I molecules (Hedley *et al.* 1998), which could allow interaction with destructive cytotoxic T cells. Furthermore, melanocytes have an antigen processing and presenting capability which can make them target cells for T cell-mediated cytotoxicity (Le Poole *et al.* 1993b).

In perilesional vitiligo biopsies, melanocytes express macrophage markers CD68 and CD36 (van den Wijngaard *et al.* 2000) and reduced levels of membrane regulators of complement activation, including decay acceleration factor and membrane cofactor protein (van den Wijngaard *et al.* 2002), which suggests a vulnerability of these cells to attack by macrophages and the complement system, respectively.

1.3.9.5 Immunosuppressive treatments of vitiligo

Repigmentation in vitiligo patients receiving treatment with immunosuppressive agents (Section 1.2.7) indirectly supports the theory that immune-mediated processes are involved in vitiligo pathogenesis. For example, topically applied tacrolimus (FK506), a therapeutic agent which exerts a potent immunosuppressive effect on T cells by blocking the action of the cytokine gene-activating cofactor calcineurin (Homey *et al.* 1998), has resulted in successful repigmentation responses in vitiligo patients (Boone *et al.* 2007; Hartmann *et al.* 2008).

In addition, topical corticosteroids, which have anti-inflammatory and immunosuppressive actions, are considered to be an effective first-line treatment in children and adults with segmental or non-segmental vitiligo of recent onset (Abu Tahir *et al.* 2010; Gawkrodger *et al.* 2010), and, indeed, following treatment of vitiligo patients with systemic

steroids, a reduction in anti-melanocyte antibody levels and in antibody-mediated anti-melanocyte cytotoxicity has been demonstrated (Takei *et al.* 1984; Hann *et al.* 1993a).

Finally, PUVA is used as a second-line therapy for vitiligo (Alomar 2010; Gawkrodger *et al.* 2010). Following PUVA treatment, a reduction in the number of Langerhans cells and a decrease in the expression of vitiligo-associated melanocyte antigens, which could lead to a blocking of antibody-dependent cell-mediated cytotoxicity against melanocytes, have been noted in vitiligo patients (Kao and Yu 1992; Viac *et al.* 1997). In addition, ultraviolet radiation can induce the expression of anti-inflammatory cytokines, modulate the expression of ICAM-1, and induce apoptosis of skin-infiltrating T lymphocytes (Duthie *et al.* 1999; Krutmann and Morita 1999).

1.3.9.6 Humoral immune responses in vitiligo

Several studies have reported circulating autoantibodies directed against melanocytes in vitiligo patients in addition to other organ-specific autoantibodies.

1.3.9.6.1 Anti-melanocyte antibodies

Antibodies to melanocytes occur at a significantly increased frequency in the sera of vitiligo patients compared with healthy individuals (Naughton *et al.* 1983a; Naughton *et al.* 1983b; Cui *et al.* 1992; Cui *et al.* 1995; Hann *et al.* 1996a; Hann and Lee 1996; Rocha *et al.* 2000; Farrokhi *et al.* 2005). As well as circulating antibodies, antibody deposits have been noted in the basement membrane zones of depigmented areas in patients with vitiligo (Uda *et al.* 1984). However, no B cells or specific antibodies have yet been isolated from vitiligo lesions. Interestingly, correlations can also exist between the incidence and level of melanocyte antibodies and both the activity and extent of vitiligo (Naughton *et al.* 1986b; Aronson and Hashimoto 1987; Harning *et al.* 1991; Yu *et al.* 1993; Kemp *et al.* 2011b), indicating that melanocyte antibodies are possible markers of disease progression.

Predominantly, melanocyte antibodies have been characterised as immunoglobulin (Ig) G (Naughton *et al.* 1983b; Naughton *et al.* 1983a; Uda *et al.* 1984; Cui *et al.* 1992; Cui *et al.* 1995; Hann *et al.* 1996a; Hann *et al.* 1996b; Rocha *et al.* 2000; Farrokhi *et al.* 2005) and as belonging to subclasses IgG1, IgG2 and IgG3 (Xie *et al.* 1991), although anti-melanocyte IgA antibodies have also been reported (Aronson and Hashimoto 1987). Initial immunoprecipitation studies using melanoma cell extracts revealed that antibodies in vitiligo

patients were most commonly directed against antigens with molecular weights of 35, 40-45, 75, 90 and 150 kDa (Cui *et al.* 1992). Several of the proteins (40-45, 75 and 150 kDa) appeared to be common tissue antigens, while others (35 and 90 kDa) were preferentially expressed on melanocytes (Cui *et al.* 1992). In immunoblotting studies with melanocyte extracts, antigens of 45, 65, and 110 kDa have been identified (Hann *et al.* 1996b; Park *et al.* 1996), while vitiligo-associated antibodies have been demonstrated to recognise melanoma cell proteins of 68, 70, 88, 90, 110 and 165 kDa (Hann *et al.* 1996a; Rocha *et al.* 2000).

The identity of several melanocyte-associated antibody targets has been reported and these are summarised in Table 1.8. Included are the melanogenic enzymes tyrosinase (Song *et al.* 1994; Baharav *et al.* 1996; Kemp *et al.* 1997a) and DCT (Kemp *et al.* 1997b; Okamoto *et al.* 1998), and the melanosomal matrix protein PMEL (Kemp *et al.* 1998b).

1.3.9.6.2 Other antibodies

Circulating organ-specific autoantibodies (Table 1.9), particularly to the thyroid, adrenal glands, gastric parietal cells, and pancreatic islet cells are commonly detected in the sera of vitiligo patients (Brostoff 1969; Betterle *et al.* 1976; Zauli *et al.* 1986; Mandry *et al.* 1996). Moreover, antinuclear antibody and IgM-rheumatoid factor have been detected at a significant frequency in vitiligo patients (Farrokhi *et al.* 2005). Anti-keratinocyte intracellular antibodies that correlate with disease extent and activity have also been detected in vitiligo patients (Yu *et al.* 1993). The technique of peptide phage-display has identified the melanin-concentrating hormone receptor 1 (MCHR1) as a target of vitiligo patient antibodies (Kemp *et al.* 2002). Recent proteomic analysis has also revealed lamin A is a vitiligo-associated antigen (Li *et al.* 2011).

1.3.9.6.3 Pathogenic mechanisms

With respect to pathogenic effects, vitiligo-associated antibodies are able to destroy melanocytes and melanoma cells *in vitro* and *in vivo* by complement-mediated damage and antibody-dependent cellular cytotoxicity (ADCC) (Norris *et al.* 1988; Fishman *et al.* 1993; Gottumukkala *et al.* 2006). Complement-mediated cytolysis of melanocytes by vitiligo patient antibodies appears to be cell selective and more common in individuals with active

Table 1.8: Anti-melanocyte antibodies in vitiligo patients

Antigen	Number of patients with antibodies (%)	Number of controls with antibodies (%)	Reference
PMEL	3/53 (5.9)	0/20 (0)	(Kemp *et al.* 1998b)
SOX10	3/93 (3.2)	0/65 (0)	(Hedstrand *et al.* 2001)
SOX9	1/93 (1.1)	0/65 (0)	(Hedstrand *et al.* 2001)
Tyrosinase	16/26 (61)	0/31 (0)	(Song *et al.* 1994)
Tyrosinase	7/18 (39)	0/12 (0)	(Baharav *et al.* 1996)
Tyrosinase	5/46 (10.9)	0/20 (0)	(Kemp *et al.* 1997a)
TYRP1	3/53 (5.9)	0/20 (0)	(Kemp *et al.* 1998c)
TYRP1	8/84 (9.5)	Not reported	(Li *et al.* 2011)
DCT	3/53 (5.9)	0/20 (0)	(Kemp *et al.* 1997b)
DCT	10/15 (67)	0/21 (0)	(Okamoto *et al.* 1998)
DCT	20/30 (67)	1/35 (2)	(Okamoto *et al.* 1998)
GTP-binding protein Rab38	8/53 (15)	0/53 (0)	(Waterman *et al.* 2002)

Table 1.9: Other antibodies detected in vitiligo patients

Antibody reactivity	Number of patients with antibodies (%)	Reference
Gastric parietal cells	11/65 (17)	(Zauli *et al.* 1986)
Gastric parietal cells	6/20 (30)	(Mandry *et al.* 1996)
Gastric parietal cells	17/80 (21)	(Brostoff 1969)
Gastric parietal cells	13/96 (13.7)	(Betterle *et al.* 1976)
Thyroid cytoplasm	22/80 (28)	(Brostoff 1969)
Thyroid peroxidase	10/20 (50)	(Mandry *et al.* 1996)
Thyroid peroxidase	19/96 (20)	(Betterle *et al.* 1976)
Thyroglobulin	8/20 (40)	(Mandry *et al.* 1996)
Thyroglobulin	7/80 (9)	(Brostoff 1969)
Adrenal gland	3/80 (4)	(Brostoff 1969)
Pancreatic islet cells	7/96 (7.2)	(Betterle *et al.* 1976)
Anti-nuclear antibody	4/55 (7.3)	(Farrokhi *et al.* 2005)
IgM-rheumatoid factor	6/55(10.8)	(Farrokhi *et al.* 2005)
Lamin A	24/84 (28.6)	(Li *et al.* 2011)
MCHR1[1]	9/55 (16.4)	(Kemp *et al.* 2002)
MCHR1[1]	12/84 (14.3)	(Li *et al.* 2011)
MCHR1[1]	24/145 (16.55)	(Zhou *et al.* 2011)
Gamma-enolase	4/53 (8)	(Waterman *et al.* 2002)
Alpha-enolase	5/53 (9)	(Waterman *et al.* 2002)
Heat-shock protein 90	7/53 (13)	(Waterman *et al.* 2002)
Heat-shock protein 70	16/60 (26)	(Kim *et al.* 2011)
Fibrin beta	16/60 (26)	(Kim *et al.* 2011)
Osteopontin	5/53 (9)	(Waterman *et al.* 2002)
Ubiquitin-conjugating enzyme	8/53 (15)	(Waterman *et al.* 2002)
Translation-initiation factor 2	3/53 (6)	(Waterman *et al.* 2002)

[1]MCHR1, melanin-concentrating hormone receptor 1.

disease (Cui *et al.* 1993). Passive immunisation of nude mice grafted with human skin has also indicated that IgG from vitiligo patients can induce melanocyte destruction (Gilhar *et al.* 1995). Furthermore, IgG melanocyte antibodies from individuals with vitiligo can induce HLA-DR and intercellular adhesion molecule-1 expression on and release of interleukin-8 from melanocytes (Yohn *et al.* 1993). Such changes that may enhance the antigen-presenting activity of melanocytes allowing antigen-specific immune effector cell attack resulting in melanocyte destruction.

Antibodies against MCHR1 have been shown to block the function of the receptor in a heterologous cell line (Gottumukkala *et al.* 2006). Stimulation of MCHR1 in cultured melanocytes with melanin-concentrating hormone (MCH) can down regulate the actions of α-MSH, including the production of melanin, suggesting that the MCH/MCHR1 signalling pathway has a role with the melanocortins in regulating melanocyte function (Hoogduijn *et al.* 2002). Any adverse effects of MCHR1 antibodies upon the functioning of the receptor in melanocytes could potentially disrupt normal melanocyte behaviour, a feature that could precede the clinical manifestation of vitiligo. However, this has not yet been reported and is still the object of study.

More recent work has found that 69% (9/13) of vitiligo patient sera tested induced melanocyte detachment in a reconstructed epidermis model, although this was unrelated to either the extent or the activity of the disease (Cario-Andre *et al.* 2007). Further studies are needed to confirm that this serum effect is antibody mediated and, if so, that the antibody activity is specific to vitiligo patient sera.

1.3.9.6.4 The origin of anti-melanocyte antibodies in vitiligo

How anti-melanocyte antibodies arise in vitiligo has not yet been elucidated and several mechanisms could account for their presence. Antibodies might result from a genetic dysregulation of the immune system at the B or T cell level resulting in a lack of tolerance to melanocyte antigens and the subsequent appearance of anti-pigment cell antibodies (Cooke and Fehervari 2007; Sercarz and Raja-Gabaglia 2007). Particularly, this may be the case with respect to individuals with AIRE gene mutations who develop vitiligo as part of their APS1 (Nagamine *et al.* 1997).

Alternatively, antigens released from melanocytes disrupted by cellular immune reactions (Ogg *et al.* 1998; Lang *et al.* 2001; Palermo *et al.* 2001; Kroll *et al.* 2005) or non-immune processes (Kroll *et al.* 2005; Schallreuter *et al.* 2005; Dell'Anna and Picardo 2006)

could initiate humoral immune responses that target melanocytes. Indeed, antibodies against DCT have been identified in melanoma patients during immunotherapy with this pigment cell-specific antigen (Okamoto *et al.* 1998), indicating that exposure to melanogenic proteins can stimulate B cell reactivity. Interestingly, TYRP1 and PMEL have been found to be expressed transiently on the surface of melanocytes and, therefore, are accessible as an antibody targets (Takechi *et al.* 1996; Leonhardt *et al.* 2011). Other autoantigens, including the MCHR1, are cell-surface receptors so are exposed to possible antibody interaction without the need for cellular damage (Cui *et al.* 1992; Park *et al.* 1996; Kemp *et al.* 2002; Farrokhi *et al.* 2005; Gottumukkala *et al.* 2006). Conceivably, a humoral immune response to pigment cells could also occur in vitiligo patients if melanocytes expose antigens that are similar to either an infecting agent or to other cells that are themselves the primary target of antibodies. Indeed, several vitiligo autoantigens appear to be expressed on cells other than melanocytes (Cui *et al.* 1992; Kemp *et al.* 2002).

In summary, it has not been determined how melanocyte antibodies arise and this process may be patient-specific and/or antigen-dependent. Whether or not melanocytes are the targets of a primary or secondary humoral immune response, a major question remains concerning the contribution pigment cell antibodies make to the development of depigmentation in vitiligo.

1.3.9.7 Cellular immune responses in vitiligo

Many studies have reported cellular immune responses directed against melanocytes in vitiligo patients and these are described in the next sections.

1.3.9.7.1 Macrophages

Macrophage infiltration has been demonstrated in vitiligo lesions, with increased numbers present in perilesional skin (Le Poole *et al.* 1996; van den Wijngaard *et al.* 2000). It is possible that macrophages are involved in clearing melanocytes that have been induced to apoptose by cytotoxic T lymphocytes. Additional evidence for the active involvement of macrophages in vitiligo pathogenesis is demonstrated by their expression of immunoglobulin receptors: in a mouse model, it has been shown that macrophages, expressing the common gamma (γ) chain of the activating Fc receptors, can mediate vitiligo in the presence and absence of complement C3 fraction (Trcka *et al.* 2002).

1.3.9.7.2 Dendritic cells

The density of Langerhans cells in vitiliginous skin has been variously reported as normal, increased and decreased compared with pigmented skin from the same patients and from control subjects (Riley 1967; Claudy and Rouchouse 1984; Hatchome *et al.* 1987; Searle *et al.* 1993). The differences in the documented densities of Langerhans cells may be due to the type of vitiligo, the sampling techniques used or the site of skin biopsies. An increase in the number of Langerhans cells could contribute to the immunological processes that damage melanocytes. However, although degenerative changes in Langerhans cells have been observed in vitiligo skin lesions, their role in vitiligo still remains unclear.

More recently, dendritic cell-mediated destruction of melanocytes has been demonstrated *in vivo* and *in vitro* (Kroll *et al.* 2005). This process is related to the release of HSP70 by stressed melanocytes, which induces an immune response against the cells from which it is produced. At the same time there is an increased expression of TNF-related apoptosis which induces ligand receptors on stressed melanocytes making them more prone to killing by dendritic cells (Kroll *et al.* 2005; Denman *et al.* 2008).

1.3.9.7.3 Natural killer cells

Studies of natural killer (NK) cells in vitiligo are controversial. The mean percentage of peripheral NK cells was reported to be significantly higher in vitiligo patients than in healthy individuals (Hann *et al.* 1993b). However, no significant difference (Abdel-Naser *et al.* 1992), and a lower NK cell number (Mahmoud *et al.* 2002) have also been described in patients with vitiligo compared to healthy subjects. Abnormalities in NK cells have been observed in vitiligo including increased cell activity, elevated activatory receptor and decreased inhibitory receptor, all indicating a possible role of NK cells in vitiligo pathogenesis (Basak *et al.* 2008).

1.3.9.7.4 CD4+ helper and CD8+ cytotoxic T lymphocytes

Autoimmune disorders are often associated with an expansion of peripheral CD4+ helper T cells. However, with respect to vitiligo, inconsistent data regarding abnormalities in circulating helper T cells have been reported. An increase in the number of activated CD4+ helper T cells was detected in patients with stable vitiligo as well as in their first-degree

relatives when compared with healthy individuals (Soubiran *et al.* 1985; D'Amelio *et al.* 1990; Abdel-Naser *et al.* 1992). In contrast, a decrease in the CD4+ helper T cell population has also been observed in individuals with vitiligo (Grimes *et al.* 1986; Halder *et al.* 1989). No simple explanation exists for these differences but they could be attributable to the factors such as the population of patients under study, disease characteristics and received treatments.

Circulating melanocyte-specific CD8+ cytotoxic T lymphocytes that target melanocyte-specific antigens, including MelanA (MART-1), PMEL and tyrosinase, have been detected in vitiligo patients (Ogg *et al.* 1998; Lang *et al.* 2001; Le Gal *et al.* 2001; Palermo *et al.* 2001; Mandelcorn-Monson *et al.* 2003; Le Poole *et al.* 2004; Garbelli *et al.* 2005; Le Poole and Luiten 2008; van den Boorn *et al.* 2009). They were found to express high levels of the skin-homing receptor cutaneous lymphocyte-associated antigen and their frequency correlated with both the extent and activity of the disease (Lang *et al.* 2001). In addition, melanocyte-specific T cells showed cytotoxic reactivity towards melanocytes (Ogg *et al.* 1998).

Histological studies of skin biopsies from vitiligo patients have demonstrated that infiltrating cytotoxic and helper T cells are most prominent at the periphery of vitiligo lesions (Al Badri *et al.* 1993b; van den Wijngaard *et al.* 2000). Many of the inflammatory cells are activated, as indicated by the expression of the MHC class II antigen HLA-DR, and a significant number also exhibit high levels of the receptor cutaneous lymphocyte-associated antigen, typical of skin-homing T cells (Al Badri *et al.* 1993b; van den Wijngaard *et al.* 2000). Local activation of cytotoxic T cells at the perilesional epidermal/dermal junction of vitiliginous skin is also suggested by the presence of granzyme B+ and perforin+ cells (van den Wijngaard *et al.* 2000). There is evidence for IL-2 receptor and IFN-γ receptor expression by the lymphocytic infiltrate (Abdel-Naser *et al.* 1994), and also for down-regulation of the helper T cell 2-dependent CDw60 molecule in the vitiliginous epidermis suggesting that infiltrating T cells may exhibit a helper T cell 1-type cytokine production pattern which is consistent with cell-mediated organ-specific autoimmunity (Le Poole *et al.* 2003). In addition, perilesional T cell clones exhibit a predominant type-1-like cytokine secretion profile (Wankowicz-Kalinska *et al.* 2003). More recently it has been demonstrated that T lymphocytes obtained from perilesional skin biopsies are enriched for cytotoxic T cells that recognise melanocyte antigens tyrosinase, PMEL and MelanA (van den Boorn *et al.* 2009). Moreover, upon infiltration of autologous pigmented skin, isolated perilesional T

lymphocytes efficiently kill melanocytes, providing direct evidence that cytotoxic T cells can cause the depigmentation seen in vitiligo (van den Boorn *et al.* 2009).

1.3.9.7.5 Regulatory T lymphocytes

Regulatory $CD25^{+}CD4^{+}$ T cells (Tregs) have been shown to inhibit $CD25^{+}CD4^{+}$ T effector cells and regulate autoreactive T and B cells and so may have an important role in controlling autoimmunity (Jonuleit *et al.* 2001; Danese and Rutella 2007). Tregs play a critical role in clearing self-reactive T cells that have escaped clonal deletion (Sakaguchi *et al.* 2001). Reduced numbers of Tregs have been observed in lesional, perilesional and non-lesional vitiligo skin in comparison to normal skin (Klarquist *et al.* 2010). Serum level of TGF-β, which is synthesised by Tregs, is also lower in vitiligo patients than in controls, and the level of reduction is correlated with the skin surface area involved (Basak *et al.* 2009; Tu *et al.* 2011). The reduction in Tregs numbers and function may allow the unchecked destruction of melanocytes by cytotoxic T cells in vitiligo lesions (Klarquist *et al.* 2010).

1.3.9.7.6 T helper 17 lymphocytes

T helper 17 lymphocytes (Th17) cells have been implicated in the pathogenesis of many inflammatory disorders such as Crohn's disease, multiple sclerosis, psoriasis and rheumatoid arthritis (Matusevicius *et al.* 1999; Duerr *et al.* 2006; Kirkham *et al.* 2006; Krueger *et al.* 2007; Miossec *et al.* 2009). Recently, the involvement of Th17 cells in vitiligo pathogenesis has been investigated. Reports of elevated levels of serum IL-17 (Basak *et al.* 2009) in vitiligo patients, and the presence of $IL\text{-}17A^{+}$ T cells in the active border of vitiligo skin biopsies have been made (Wang *et al.* 2011). However, further studies are required to determine the exact role of Th17 cells in vitiligo pathogenesis.

1.3.9.7.7 Cytokines

An imbalance of several melanogenic cytokines that can affect melanocyte activity and survival has been shown in vitiligo lesional skin (Moretti *et al.* 2002a), and these are described in Section 1.3.7. Inflammatory cytokines have also been analysed in relation to vitiligo pathogenesis. For example, serum levels of soluble IL-2 receptor, which can be used to monitor *in vivo* immune activation, is significantly increased in vitiligo patients when compared with controls, indicating that the activation of T cells is a component in the

pathogenesis of vitiligo (Caixia *et al.* 1999; Galadari 2005). The production of IL-6 by mononuclear cells is also elevated in vitiligo patients (Yu *et al.* 1997). This cytokine can induce the expression of ICAM-1 on melanocytes thereby facilitating leukocyte-melanocyte interactions and consequently immunological damage (Kirnbauer *et al.* 1992; Moretti *et al.* 2002b; Zailaie 2005). Increased production of IL-8, which can attract neutrophils to vitiligo lesions amplifying destructive inflammatory reactions, has also been reported in vitiligo patients (Yu *et al.* 1997; Zailaie 2005). Significantly higher expression of IFN-γ, IL-10, and IL-1β also occurs in the lesional and peri-lesional skin of vitiligo patients compared with skin of healthy control subjects (Grimes *et al.* 2004).

The expression of TNF-α, an inflammatory mediator involved in the pathogenesis of autoimmune disease, is significantly elevated in vitiligo skin (Moretti *et al.* 2002b; Grimes *et al.* 2004; Zailaie 2005). The cytokine TNF-α can increase the production of H_2O_2 leading to oxidative stress and death of cells (Haycock *et al.* 2000; Dell'Anna and Picardo 2006). Moreover, TNF-α regulates the down-stream signaling molecule nuclear factor-κB (NF-κB) (Hsu *et al.* 1996), which promotes the expression of genes that prevent cell apoptosis (May and Ghosh 1998). Inhibition of NF-κB activation by TNF-α was found to induce apoptosis of cultured keratinocytes (Kim *et al.* 2007b). In vitiligo, it has been suggested that impaired NF-κB activity can lead to TNF-α-dependent keratinocyte death and, consequently, melanocyte loss (Imokawa and Moretti 2010). The significant reduction in TNF-α in vitiligo skin after successful repigmentation induced by tacrolimus may also indicate a possible role of this cytokine in vitiligo pathogenesis (Grimes *et al.* 2004).

Overall, the precise effects of abnormal alterations in inflammatory cytokine levels in the pathogenesis of vitiligo is not fully understood. Interestingly, certain inflammatory cytokines such as TNF-α, IL-1α and IL-6 have been found to act as paracrine inhibitors of melanocyte proliferation and of melanogenesis in a dose-dependent manner both *in vivo* and *in vitro* (Swope *et al.* 1991; Martinez-Esparza *et al.* 1998).

1.3.10 The convergence theory

The conversion theory suggests that several of the previously described factors could act synergistically or independently to induce the disappearance of melanocytes from the skin resulting in vitiligo, and that this could vary from patient to patient (Le Poole *et al.* 1993a) Furthermore, different clinical sub-types of vitiligo could result from different

pathomechanisms. For example, the neural theory (Lerner 1959; Cucchi *et al.* 2000) is often related to the development of segmental vitiligo while autoimmunity (Ongenae *et al.* 2003; Garbelli *et al.* 2005) is normally associated with non-segmental (generalised) vitiligo (Taïeb 2000).

More recently, Le Poole and Luiten (Le Poole and Luiten 2008) have described the elicitation phase of vitiligo as incorporating physical trauma to the skin (Gauthier *et al.* 2003a), emotional stresses (Papadopoulos *et al.* 1998), and imbalances of endogenous neural factors (Lerner 1959; Cucchi *et al.* 2000), metabolites (Schallreuter *et al.* 1994a; Schallreuter *et al.* 1994b) or hormones (Pichler *et al.* 2006) (Figure 1.7). Such factors can lead to oxidative stress within melanocytes which respond by actively secreting HSP70 and chaperoned melanocyte antigens (Le Poole and Luiten 2008). Stimulated dendritic cells can then initiate an immune response against pigment cells in the activation phase (Le Poole and Luiten 2008) (Figure 1.7). To support this theory, a link and a temporal sequence between oxidative stress, autoimmune responses and depigmentation have been shown in vitiligo (Kroll *et al.* 2005). Once activated, dendritic cells migrate to the regional lymph nodes where presentation of the processed melanocyte antigen to T lymphocytes occurs. Autoreactive, anti-melanocyte cytotoxic T cells (van den Boorn *et al.* 2009) can subsequently destroy epidermal pigment cells. An absence of functional skin-infiltrating regulatory T cells may also contribute to the on-going immune response (Ben Ahmed *et al.* 2012). Autoantibodies against melanocyte proteins such as tyrosinase (Song *et al.* 1994; Baharav *et al.* 1996; Kemp *et al.* 1997a) are likely generated as a response to melanocyte damage, but it is possible they can damage pigment cells by complement activation and/or ADCC (Norris *et al.* 1988; Fishman *et al.* 1993; Gottumukkala *et al.* 2006). Overall, genetic susceptibility factors can influence the development of vitiligo, particularly in regard to the immune activation phase (Spritz 2007).

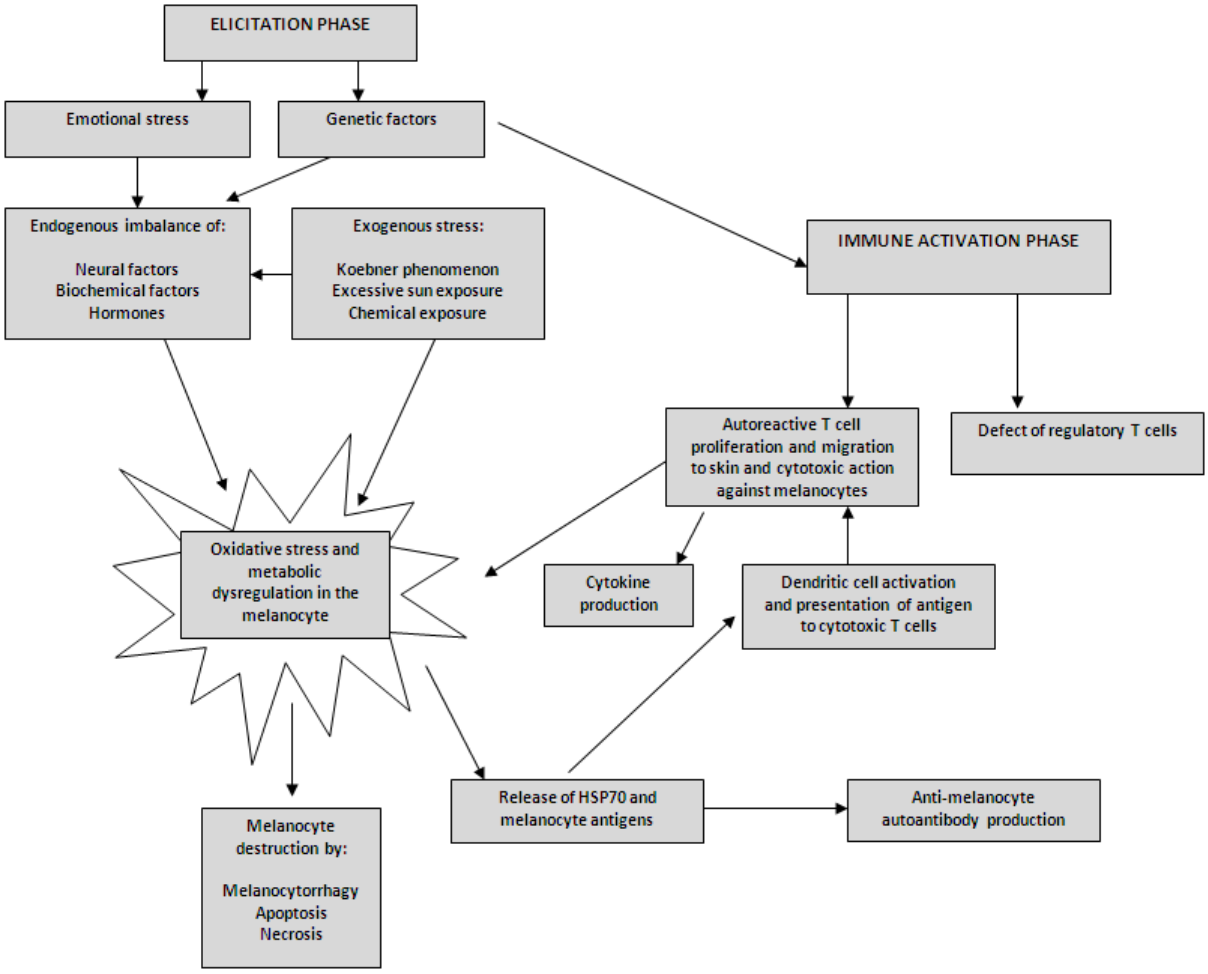


Figure 1.7: A schematic representation of the convergence theory for vitiligo aetiology.

A theoretical explanation of the interaction between immunological and non-immune factors which can lead to vitiligo. The convergence theory suggests that several factors can act synergistically or independently to induce the disappearance of melanocytes. Genetic predisposition, metabolic deregulation together with impaired redox status, environmental aggressions and neural dysfunction probably act in an interrelated way to trigger local inflammation and damage of cells. These 'danger' signals can then promote the activation of antigen-presenting cells with the subsequent activation of anti-melanocyte autoreactive cytotoxic T lymphocytes. The decreased number and function of regulatory T cells may promote the loss of control of cytotoxic T lymphocytes. Drawn with information from (Ben Ahmed *et al.* 2012).

1.4 Aims of Current Project

Much evidence from recent studies supports a role for autoimmune factors in vitiligo pathogenesis (Rezaei *et al.* 2007) and, as discussed earlier (Section 1.3.9), several targets (autoantigens) of antibodies and autoreactive T cells have been documented in vitiligo (Baharav *et al.* 1996; Kemp *et al.* 1998a; Okamoto *et al.* 1998; Xie *et al.* 1999; Kemp *et al.* 2002; Waterman *et al.* 2002). Identifying and characterising the targets of vitiligo-associated antibodies would be of benefit in:

(i) Providing diagnostic and prognostic tests, as well as a means for vitiligo classification and treatment monitoring.
(ii) Defining the factors involved in vitiligo pathogenesis. For example, vitiligo-associated autoantibodies, even if they are not inherently pathogenic, could serve as markers of T cell responses in patients with the disease. Such knowledge would help in the understanding of the aetiology and pathogenic mechanisms in vitiligo, which is essential for the development of better therapeutic measures.

In the current project, antibodies to tyrosine hydroxylase (TH) will be investigated in vitiligo patients. This enzyme was previously reported as a putative autoantigen in vitiligo in experiments using phage-display technology (Waterman *et al.* 2010).

The aims of this project were:

(i) To investigate the frequency of TH antibodies in vitiligo patients using a radioimmunoassay specific for TH antibodies.
(ii) To determine if there were any associations between the presence of TH antibodies and the clinical features of vitiligo.
(iii) To analyse the vitiligo patient TH antibody binding sites (epitopes) on TH. This will be done using deletion derivatives of TH in radioimmunoassays.
(iv) To evaluate the characteristics of TH antibodies in terms of titres, subclasses and avidities.

CHAPTER 2

2. General Materials and Methods

2.1 Participants

Sera were obtained from 87 vitiligo patients who had attended either Dermatology (Professor David Gawkrodger) or Endocrinology (Professor Anthony Weetman) clinics in the Sheffield Teaching Hospitals NHS Trust, Sheffield, UK. Details of these patients are summarised in Table 2.1. In addition, 28 healthy controls (10 male, 18 female; mean age: 34 years with range 21-59 years), who had no present or past history of vitiligo or autoimmune disorders, were included in the study. Finally, 91 patients with autoimmmune diseases who had no concomitant vitiligo were also included as a disease control group. The patients were as follows: 27 with Graves' disease (6 male, 21 female; mean age: 43 years with range 16-84 years); 19 with Addison's disease (7 male, 12 female; mean age: 49 years with range 26-77 years); 25 with autoimmune hypoparathyroidism (25 female; mean age: 51 years with range 25-74 years); and 20 with systemic lupus erythematosus (1 male, 19 female; mean age: 46 years with range 21-66 years).

Serum was separated from whole blood samples (10-20 ml) by centrifugation in a Sorvall® RT6000-D centrifuge at 3,000 revolutions per minute (rpm) for 5-10 min. Sera were kept frozen at –80^{o}C until required.

The study was approved by the Sheffield Research Ethics Committee, Sheffield, UK (REC Reference Number 09/H1308/91) and informed consent was obtained from participants. Research Governance for the project was administered by the Research Department, Sheffield Teaching Hospitals (STH) NHS Foundation Trust, Sheffield, UK (STH Number STH15257).

2.2 Chemicals and plasticware

The majority of chemicals and media components were purchased from either Sigma-Aldrich (Poole, UK) or Melford Laboratories (Ipswich, UK). Solvents and acids were obtained from Fisher Scientific UK Ltd. (Loughborough, UK). Where appropriate, the source of some chemicals and buffers is indicated in the text. Sterile plasticware was from Sarstedt Ltd.

Table 2.1: Details of vitiligo patients

Patient detail	Non-segmental vitiligo patients	Segmental vitiligo patients
Demographic details		
Number of patients	79	8
Male/Female	30/49	3/5
Mean age (range)	46 years (7-77 years)	33 years (16-58 years)
Mean onset age (range)	33 years (1-73 years)	33 years (14-54 years)
Mean disease duration (range)	15 years (< 1-50 years)	2 years (< 1-4 years)
Vitiligo clinical sub-type		
Symmetrical	63	
Symmetrical/periorificial	9	
Symmetrical with segmental patch	3	
Periorificial	2	
Universal	1	
Occupational	1	
Vitiligo activity[1]		
Active vitiligo	64	3
Stable vitiligo	15	5
Vitiligo and autoimmune disease		
No associated autoimmune disease	55	6
Autoimmune thyroid disease	14	
Autoimmune thyroid disease + Addison's disease	1	
Autoimmune thyroid disease + hyperparathyroidism	1	
Autoimmune thyroid disease + pernicious anaemia	1	
Alopecia areata	2	2
Type 1 diabetes mellitus	1	
Psoriasis	1	
Systemic lupus erythematosus	2	
Scleroderma	1	

[1]Active vitiligo was defined by currently progressing depigmentation. Stable vitiligo was defined by depigmentation that had shown no progression for the previous six months.

(Numbrecht, Germany), Starlab (UK) Ltd. (Milton Keynes, UK), Corning Incorporated (Corning, NY, USA) or Sterilin Ltd. (Caerphilly, UK).

2.3 Bacterial strains

The bacterial strains used in this study are derivatives of *Escherichia coli* K-12 and are given in Table 2.2. Derivatives of bacterial strains carrying plasmid vectors or recombinant plasmids were constructed by transformation (Section 2.15).

2.4 Growth and storage of bacterial strains

All *E. coli* strains were routinely grown from frozen stocks by initially streaking on to Luria Bertani (LB) agar plates (Section 2.5), containing selective antibiotic(s) where required (Section 2.6). Plates were then incubated at 37˚C overnight. A single colony of the desired bacterial strain was then inoculated into LB broth with the appropriate antibiotic supplementation, and incubated at 37˚C overnight in a rotary incubator shaking at 250 rpm. For long-term storage at -80˚C, 0.7-ml aliquots of an overnight-grown bacterial culture were mixed with 0.3-ml aliquots of sterile 50% (v/v) glycerol in deionised water.

2.5 Luria Bertani medium

Luria Bertani (LB) broth was prepared in deionised water and contained: 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. Luria Bertani agar was prepared by adding 2% (w/v) agar to LB medium. After autoclaving, the agar was left to cool to about 40-45˚C before adding the appropriate antibiotic. The agar was poured into 90-mm petri-dishes and allowed to set. Plates were then dried and stored at 4˚C until required.

2.6 Antibiotics

Antibiotics were prepared as 1000x concentrated stocks in deionised water and sterilised by filtration through 0.22 micron Millex® Filter Units (Millipore Corp., Bedford, MA, USA). Antibiotic solutions were stored at –20˚C and used at the following concentrations in culture medium and agar plates: ampicillin (sodium salt), 100 µg/ml; kanamycin sulphate, 50 µg/ml.

Table 2.2: Bacterial strains

Strain[1]	Details	Source
JM109	A bacterial strain used in the propagation of plasmid vectors and recombinant plasmids.	Promega (Southampton, UK)
JM109 pcDNA3	Carries plasmid vector pcDNA3 (Table 2.3).	Dr. Helen Kemp (University of Sheffield, Sheffield, UK)
JM109 pcDNA3-TH	Carries recombinant plasmid pcDNA3-TH (Table 2.3).	Dr. Helen Kemp (University of Sheffield, Sheffield, UK)
JM109 pcMCHR1	Carries recombinant plasmid pcMCHR1 (Table 2.3).	Dr. Helen Kemp (University of Sheffield, Sheffield, UK)
JM109 pSP64-Poly(A)-PAH	Carries recombinant plasmid pSP64-Poly(A)-PAH (Table 2.3).	Dr. Helen Kemp (University of Sheffield, Sheffield, UK)
JM109 pSP64-Poly(A)-TPH	Carries recombinant plasmid pSP64-Poly(A)-TPH (Table 2.3).	Dr. Helen Kemp (University of Sheffield, Sheffield, UK)
JM109 pcDNA3-TYR	Carries recombinant plasmid pcDNA3-TYR (Table 2.3).	Dr. Helen Kemp (University of Sheffield, Sheffield, UK)
JM109 pTH140	Carries recombinant plasmid pTH140 (Table 2.3).	This study (Chapter 4)
JM109 pTH170	Carries recombinant plasmid pTH170 (Table 2.3).	This study (Chapter 4)
JM109 pTH200	Carries recombinant plasmid pTH200 (Table 2.3).	This study (Chapter 4)
JM109 pTH240	Carries recombinant plasmid pTH240 (Table 2.3).	This study (Chapter 4)
JM109 pTH280	Carries recombinant plasmid pTH280 (Table 2.3).	This study (Chapter 4)
JM109 pTH320	Carries recombinant plasmid pTH320 (Table 2.3).	This study (Chapter 4)
JM109 pTH360	Carries recombinant plasmid	This study (Chapter 4)

	pTH360 (Table 2.3).	
JM109 pTH400	Carries recombinant plasmid pTH400 (Table 2.3).	This study (Chapter 4)
JM109 pTH440	Carries recombinant plasmid pTH440 (Table 2.3).	This study (Chapter 4)
JM109 pTH480	Carries recombinant plasmid pTH480 (Table 2.3).	This study (Chapter 4)
JM109 pTH1-80	Carries recombinant plasmid pTH1-80 (Table 2.3).	This study (Chapter 4)
JM109 pTH81-140	Carries recombinant plasmid pTH81-140 (Table 2.3).	This study (Chapter 4)
JM109 pTH141-497	Carries recombinant plasmid pTH141-497 (Table 2.3).	This study (Chapter 4)

[1]All strains are derivatives of *Escherichia coli* K-12.

2.7 Plasmids

The plasmids used in this study are listed in Table 2.3. They were stored at –20˚C in sterile TE buffer (10 mM Tris-HCl; 1 mM ethylenediaminetetraacetic acid (EDTA); pH 8.0) (Promega, Southampton, UK). Diagrams of plasmids pcDNA3 (Invitrogen, Paisley, UK) and pcDNA3-TH (Dr. Helen Kemp, University of Sheffield, Sheffield, UK) are shown in Figures 2.1 and 2.2, respectively.

2.8 Small-scale plasmid preparations

The Wizard® *Plus* SV Minipreps DNA Purification System (Promega) was used to purify plasmid DNA from a 5-10 ml overnight culture of the required bacterial strain, according to the manufacturer's protocol. Briefly, a single colony of the desired bacterial strain was isolated by streaking out 20 µl of frozen bacterial stock on to LB agar containing the appropriate antibiotic(s). A single colony from the selective plate was then used to inoculate 10 ml of LB containing the relevant antibiotic(s) which was subsequently shaken in a rotary incubator at 250 rpm at 37˚C overnight. The bacterial cell pellet was obtained by centrifugation at 10,000 *g* for 10 min. The pellet was resuspended in 250 µl of Cell Resuspension Solution (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A), and the cells were lysed by the addition of 250 µl of Cell Lysis Solution (0.2 M NaOH; 1% (w/v) sodium dodecyl sulphate). The cell extract was then neutralised by the addition of 350 µl of Neutralisation Solution (0.759 M potassium acetate; 4.09 M guanidine hydrochloride; 2.12 M glacial acetic acid; pH 4.2), mixed gently and centrifuged at 10,000 *g* for 10 min at room temperature. The cleared lysate was then loaded on to a Wizard® SV Minicolumn which was centrifuged at 10,000 *g* for 1 min at room temperature and the flow-through discarded. The Minicolumn was washed with 750 µl of Column Wash Solution (60% (v/v) ethanol; 60 mM potassium acetate; 8.3 mM Tris-HCl; 0.04 mM EDTA) and then with 250 µl of Column Wash Solution with centrifugation at for 2 min at room temperature each time. To recover the DNA bound to the column, 100 µl of nuclease-free water was added to the column and this was centrifuged at 10,000 *g* for 1 min at room temperature.

Table 2.3: Plasmids

Plasmid	Details	Source
pcDNA3	A 5.4-kb[1] expression vector with a selectable resistance marker to ampicillin. Contains promoters for T7 and SP6 polymerases flanking a diverse multiple cloning site (Figure 2.1).	Invitrogen (Paisley, UK)
pcDNA3-TH	pcDNA3 vector containing TH (isoform 2) cDNA (1.5-kb[1]) cloned into the *Hin*dIII-*Bam*HI restriction site (Figure 2.3).	Dr. Helen Kemp (University of Sheffield, Sheffield, UK)
pcMCHR1	pcDNA3 vector containing melanin-concentrating hormone receptor 1 (MCHR1) cDNA (1.3 kb[1]) cloned into the *Eco*RI-*Xba*I restriction site.	Dr. Helen Kemp (University of Sheffield, Sheffield, UK)
pSP64-Poly(A)-PAH	pSP64-Poly(A) vector (Promega, Southampton, UK) containing phenylalanine hydroxylase (PAH) cDNA(1.4 kb[1]).	Prof. Olle Kampe (University Hospital, Uppsala University, Uppsala, Sweden)
pSP64-Poly(A)-TPH	pSP64-Poly(A) vector containing tryptophan hydroxylase (TPH) cDNA (1.3 kb[1]) cloned into the *Hin*dIII-*Bam*HI restriction site.	Prof. Olle Kampe (University Hospital, Uppsala University, Uppsala, Sweden)
pcDNA3-TYR	pcDNA3 vector containing tyrosinase cDNA (2.0 kb[1]) cloned into the *Kpn*I-*Xba*I restriction site.	Dr. Helen Kemp (University of Sheffield, Sheffield, UK)
pTH140	pcDNA3 vector containing TH cDNA base pairs 1-420 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH170	pcDNA3 vector containing TH cDNA base pairs 1-510 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)

pTH200	pcDNA3 vector containing TH cDNA base pairs 1-600 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH240	pcDNA3 vector containing TH cDNA base pairs 1-720 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH280	pcDNA3 vector containing TH cDNA base pairs 1-840 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH320	pcDNA3 vector containing TH cDNA base pairs 1-960 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH360	pcDNA3 vector containing TH cDNA base pairs 1-1080 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH400	pcDNA3 vector containing TH cDNA base pairs 1-1200 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH440	pcDNA3 vector containing TH cDNA base pairs 1-1320 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH480	pcDNA3 vector containing TH cDNA base pairs 1-1440 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH1-80	pcDNA3 vector containing TH cDNA base pairs 1-240 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH81-140	pcDNA3 vector containing TH cDNA base pairs 241-420 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)
pTH141-497	pcDNA3 vector containing TH cDNA base pairs 421-1491 cloned into the *Hin*dIII-*Bam*HI restriction site.	This study (Chapter 4)

[1]kb, kilobase.

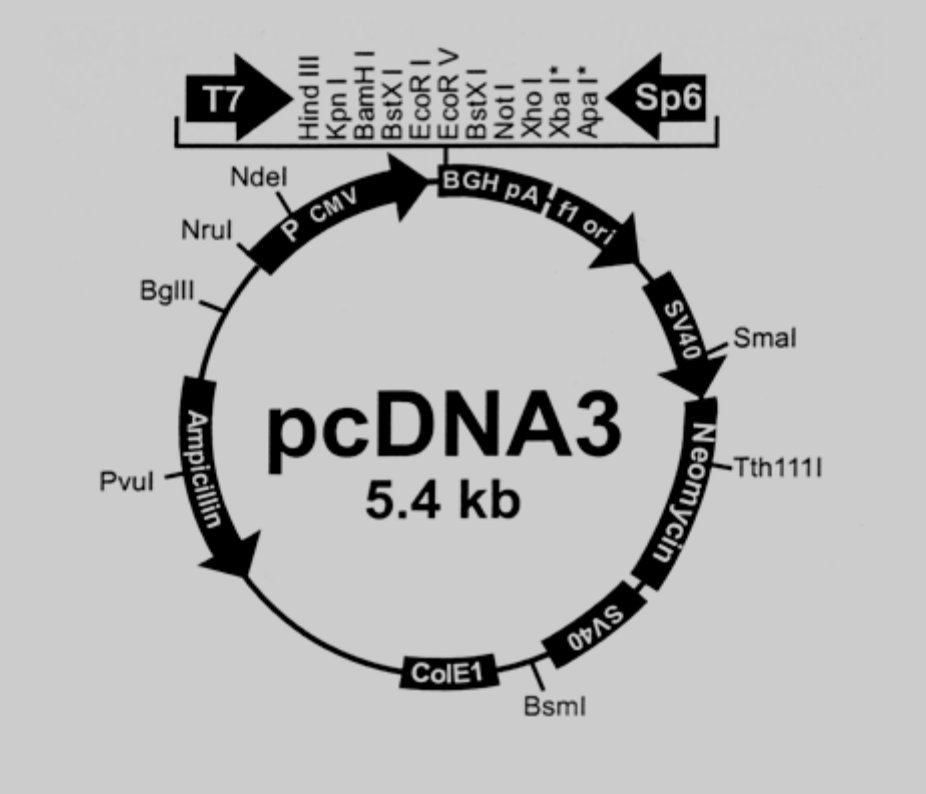


Figure 2.1: A map of the pcDNA3 vector. Restriction enzyme sites and sequencing primer binding sites are shown.

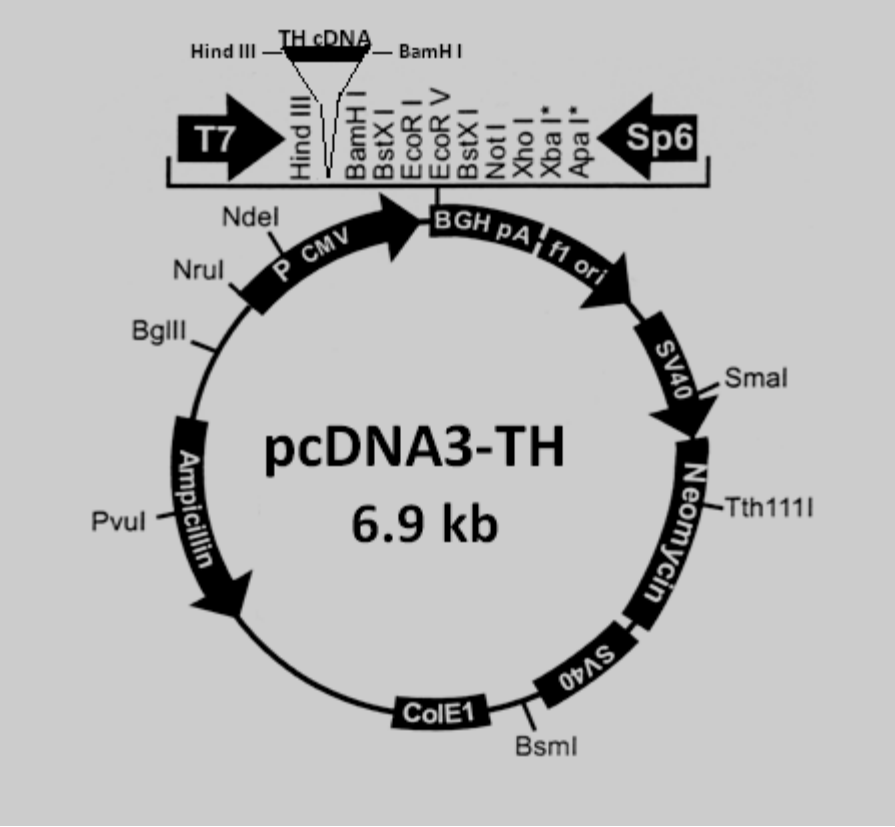


Figure 2.2: A map of the pcDNA3-TH plasmid. Restriction enzyme sites and sequencing primer binding sites are shown. TH cDNA is a 1.5-kb fragment cloned into the *Hin*dIII-*Bam*HI cloning site.

2.9 Large-scale plasmid preparations

A large-scale culture of the bacterial strain carrying the desired plasmid was prepared by inoculation of 0.5-1 litres of LB containing the appropriate antibiotic(s), with a 10-ml starter culture. This was followed by incubation overnight with shaking at 37˚C. The culture was then centrifuged at 4,000 *g* for 30 min and plasmid extracted from the cell pellet using a Qiagen Plasmid DNA Maxiprep Kit (Qiagen Ltd., Crawley, UK) as per the kit instructions. The bacterial cell pellet was first resuspended in 10 ml of P1 Buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 μg/ml RNase A). An equal volume of P2 Buffer (0.2 M NaOH; 1% (w/v) sodium dodecyl sulphate) was then added to the resuspended cells and mixed by gentle inversion, followed by incubation at room temperature for 5 min. The tube was then mixed gently again after the addition of 10 ml of P3 Buffer (1.32 M potassium acetate, pH 5.5) and incubated on ice for 20 min. The mixture was subsequently centrifugated at 20,000 *g* for 30 min at 4^{o}C resulting in a clear supernatant. A Qiagen column was equilibrated by adding QBT Buffer (750 mM NaCl; 50 mM 3-[N-morpholino]propanesulphonic acid (MOPS), pH 7.0; 15% (v/v) isopropanol and 0.15% (v/v) Triton X-100) and was allowed to empty by gravity flow. The clear supernatant was then loaded on to the column and left to flow through, followed by washing of the column twice with 30 ml of QC Buffer (1 M NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) isopropanol). Plasmid DNA was subsequently eluted with 15 ml of QF Buffer (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% (v/v) isopropanol) and precipitated by the addition of 10.5 ml of 100% isopropanol and centrifugation at 15,000 *g* for 30 min at 4^{o}C. The resulting DNA pellet was washed with 70% (v/v) ethanol, centrifuged at 15,000 *g* for 10 min at 4^{o}C and finally resuspended in 300 μl of TE buffer. The concentration of the DNA was ascertained by spectrophotometry at 260 nm using a NanoDrop ND-1000 Spectrometer (Labtech, Wilmington, DE, USA) and NanoDrop Software (Labtech).

2.10 Agarose gel electrophoresis

For analysis of DNA, agarose gels, 0.8-1% (w/v), were prepared by boiling molecular biology grade agarose (Sigma) in TAE electrophoresis buffer (40 mM Tris-acetate; 1 mM EDTA; pH 8.3) (Promega) for 1-2 min in a microwave oven. One microlitre of ethidium bromide solution

(10 mg/ml) (Promega) was added for every 50 ml of the gel solution. The molten agarose was cooled and poured into the casting deck of a Sub-Cell® Horizontal Electrophoresis System (Bio-Rad Ltd., Hemel Hempstead, UK). After it had set, the combs were removed, and the electrophoresis tank was filled with TAE as a running buffer. Blue/Orange Loading Dye 6x (0.4% orange G; 0.03% (w/v) bromphenol blue; 0.03% xylene cyanol FF; 15% Ficoll® 400; 10 mM Tris-HCl, pH 7.5; 50 mM EDTA, pH 8.0) (Promega) was added to the DNA sample at 1/6th of the volume, and the sample was then loaded into a gel slot. To size DNA products after they had migrated through the gel, a 'marker' lane was included. This contained a 0.5-1.0-µg sample of either *Hin*dIII-restricted bacteriophage λ DNA (125-23,130-bp DNA fragments) (Promega) or 1-kb DNA Ladder (500-10,000-bp DNA fragments) (New England Biolabs®, Hitchin, UK). The gels were run in Sub-Cell® Horizontal Electrophoresis System (Bio-Rad Ltd.) at 50-70 volts using a PowerPac Basic Power Supply (Bio-Rad Ltd.), and subsequently viewed and recorded using a GBOX Gel Documentation System (Syngene, Cambridge, UK) and GeneSnap Image Acquisition Software (Syngene).

2.11 Restriction enzyme digests

All restriction enzymes (Table 2.4) and restriction enzyme buffers (Table 2.4) were supplied by Promega. Restriction enzyme digests of plasmids and polymerase chain reaction (PCR) amplification products were carried out in 0.5 ml tubes in a volume not normally exceeding 25 µl and contained up to 1 µg of DNA, 10 units of enzyme(s) and 0.1 volumes of the appropriate 10x restriction enzyme buffer. Each reaction proceeded for 90 min at 37^{o}C, unless otherwise stated. Restriction digests were analysed by agarose gel electrophoresis (Section 2.10).

Table 2.4: Restriction enzymes and buffers

Enzyme[1]	Buffer (10x concentration)[1]	Restriction site
*Bam*HI	Buffer E: 60 mM Tris-HCl, pH 7.5; 1 M NaCl; 60 mM $MgCl_2$; 10 mM dithiothreitol.	5'-GGATCC-3'
*Hin*dIII	Buffer E: 60 mM Tris-HCl, pH 7.5; 1 M NaCl; 60 mM $MgCl_2$; 10 mM dithiothreitol.	5'-GAATTC-3'

[1]All enzymes and buffers were supplied by Promega (Southampton, UK).

2.12 Polymerase chain reaction amplification

Preceding the PCR amplification reaction, forward and reverse oligonucleotide primers were appropriately designed to amplify the required regions of a DNA fragment. Primers were synthesised to order by Eurofins Genetic Services Ltd. (London, UK) and were stored at -40^{o}C in sterile, nuclease-free at a concentration of 100 pmol/µl. A list of the PCR primers used in this study is given in Table 2.5, and the exact primers used for PCR amplification of a particular DNA are given at the relevant points in the text.

Reactions were carried out in 0.5 ml tubes in 50-µl volumes comprising, unless indicated, 50 ng of template DNA, 0.01-1 µM of each required (forward and reverse) primer, 1.25 units of GoTaq® Flexi DNA polymerase (Promega), 0.2 mM deoxynucleotides (dATP, dGTP, dCTP and dTTP) (Promega), 1.5 mM $MgCl_2$ (Promega) and 0.2 volumes of 5x GoTaq® Flexi Buffer (50 mM Tris-HCl, pH 8.5; 0.05% (w/v) gelatine; 250 mM KCl; 0.5% (v/v) Tween 20; 0.5% (v/v) Nonidet P-40) (Promega). Reactions without template DNA and without primers were included as controls. Each reaction was overlaid with mineral oil to prevent evaporation during heating, and was then subjected to PCR amplification in a DNA Thermal Cycler (Perkin-Elmer/Cetus Norwalk, CT, USA).

The cycling conditions were denaturation at 95^{o}C for 1 min, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 2 min for 30 cycles, unless stated otherwise. A final extension at 72^{o}C for 10 min, followed by a soak at 4^{o}C, completed the reactions. Amplification products were routinely analysed by agarose gel electrophoresis (Section 2.10).

2.13 Extraction and purification of DNA fragments from agarose gels

When electrophoresis was performed to purify a required DNA fragment, generated either from a PCR amplification reaction or a restriction enzyme digest, the DNA fragment was recovered from an agarose gel using a Wizard® PCR Preps DNA Purification Kit (Promega). Briefly, the area of the gel containing the relevant band of DNA was visualised using an ultra-violet transilluminator, excised using a clean scalpel and placed in a 1.5 ml Eppendorf tube. One millilitre of Wizard® PCR Preps DNA Purification Resin was used to dissolve the gel slice and

Table 2.5: Oligonucleotide PCR amplification primers

Primer[1]	Sequence[2]
THF1	5'-ttaagcttgccgcc**atg**cccacccccgacgcc-3'
THR480	5'-aaggatcccgcctatcactccagggagcgccg-3'
THR440	5'-aaggatcccgc**ctatca**ggcgtcactgaagct-3'
THR400	5'-aaggatcccgc**ctatca**caggagctccccgta-3'
THR360	5'-aaggatcccgc**ctatca**atccgaggcccccag-3'
THR320	5'-aaggatcccgc**ctatca**gggcgaggacgcgtg-3'
THR280	5'-aaggatcccgc**ctatca**cttcaggaagcggga-3'
THR240	5'-aaggatcccgc**ctatca**cttcagcgtggtgta-3'
THR200	5'-aaggatcccgc**ctatca**gcggtacacctggtc-3'
THR170	5'-aaggatcccgc**ctatca**cacttttcttgggaa-3'
THR140	5'-aaggatcccgc**ctatca**gtcccctcggcgcac-3'
THF141	5'-ttaagcttgccgcc**atg**atgctggccgccctgctc-3'
THR497	5'-aaggatcccgc**ctatca**gccaatggcactcag-3'
THF81	5'-ttaagcttgccgcc**atg**gtgctaaacctgctc-3'
THR80	5'-aaggatcccgc**ctatca**ggccttcccctcctt-3'

[1]All oligonucleotide PCR primers were supplied by Eurofins Genetic Services Ltd. (London, UK).

[2]The *Hin*dIII restriction site is underlined in the forward (F) primer. The *Bam*HI restriction site is underlined in each of the reverse (R) primers. The ATG translation start codon in the forward primer is in bold type. The TGA and TAG translation stop codons in the reverse primers are in bold type.

the resulting mixture was applied via a 2 ml syringe to a Wizard® Minicolumn followed by 2 ml of 80% (v/v) isopropanol. The Wizard® Minicolumn was centrifuged at 10,000 *g* for 2 min, to remove excess isopropanol, prior to DNA elution with 30 µl of sterile TE buffer. The DNA then was stored at -20˚C until required. Purified DNA fragments were analysed by agarose gel electrophoresis (Section 2.10).

2.14 DNA ligations

Ligation of vector and DNA fragments was performed using T4 DNA ligase (Promega) in a reaction volume of 10-20 µl. An estimation of the concentration of each vector and insert was performed by agarose gel electrophoresis next to molecular size markers of a known concentration. Approximately 200 ng of vector DNA were mixed together with insert (the amount used was calculated using a vector:insert molar ratio of between 1 and 3) in a clean 0.5 ml tube, and the required volume of nuclease-free water added. The mixture was heated to 65^{o}C and gradually cooled to 16^{o}C in a DNA Thermal Cycler (Perkin-Elmer/Cetus) to allow the DNA to anneal slowly before the addition of 1-2 units of T4 DNA ligase (Promega) and 0.1 volumes of 10x DNA ligase buffer (300 mM Tris-HCl, pH 7.8; 100 mM $MgCl_2$; 100 mM dithiothreitol; 10 mM ATP) (Promega). The reaction was subsequently incubated at 15^{o}C overnight before using to transform bacterial cells (Section 2.15).

2.15 Bacterial transformation

When required, a 50-µl aliquot of chemically competent *E. coli* JM109 (Promega) cells was thawed from storage at -80^{o}C. The appropriate DNA sample (plasmid DNA or DNA in a ligation reaction) was gently mixed with the cells and this was incubated on ice for 5-10 min. The cells were then heat shocked at exactly 42^{o}C for 45 sec and returned to ice. After 2 min, the cells were transferred to a culture tube containing 900-950 µl of chilled SOC medium (2% (w/v) tryptone; 0.5% (w/v) yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) (Invitrogen). The culture tube was placed in the rotary incubator shaking at 150 rpm and grown for 1 h, to allow expression of the antibiotic resistance genes carried by the transforming plasmid DNA. A 100-µl aliquot of undiluted transformed cells, and of 1:10 and 1:100 dilutions,

were then spread on agar plates containing the appropriate antibiotic(s), and the plates incubated at 37^{o}C overnight. As a control in each transformation experiment, an aliquot of untransformed cells was also plated on to selective medium. Individual colonies were subsequently purified by streaking on to fresh LB agar plates with selective antibiotic(s) for growth overnight at 37˚C.

2.16 DNA sequencing

Automated DNA sequencing was carried out by the Genetics Core Facility at the Medical School, University of Sheffield, Sheffield, UK. DNA templates (plasmids or PCR products) and oligonucleotide sequencing primers were provided to the service at 50-100 ng/µl and 1 pmol/µl, respectively. Sequencing primers M13 Forward, M13 Reverse, T7 and SP6 (Table 2.6) were purchased from Promega. Other primers (Table 2.6) were synthesised to order by Eurofins Genetic Services Ltd. (London, UK). Sequencing reactions were performed using a BigDye® Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 Capillary Sequencer (Applied Biosystems).

2.17 DNA and protein analyses

Protein and DNA homology searches against the GenBank database were performed using the BLAST service of the National Center for Biotehnology Information (NCIB) (http://www.ncbi.nlm.nih.gov/) (Bethesda, MD, USA). Other analyses of DNA and protein sequences were carried out using the network facilities of the European Bioinformatics Institute-European Molecular Biology Laboratory (EBI-EMBL) (http://www.ebi.ac.uk/) (Cambridge, UK) and of the Bayler College of Medicine HGSC (http://searchlauncher.bcm.tmc.edu/) (Houston, TX, USA).

2.18 In vitro coupled transcription-translation of cDNA

A TnT® T7- or SP6-Coupled Reticulocyte Lysate System (Promega) was used to produce the protein of interest labelled with [^{35}S]-methionine. Reactions were set up in 0.5 ml tubes in 50-µl volumes containing: 0.5 µg of plasmid DNA carrying the appropriate cDNA, 0.5 volumes of TnT® Rabbit Reticulocyte Lysate, 10 units of TnT® T7 RNA Polymerase, 0.02 mM amino acid

Table 2.6: Oligonucleotide sequencing primers

Primer[1]	Sequence
T7	5'-taatacgactcactataggg-3'
SP6	5'-attaaccctcactaaaggga-3'
M13 Reverse	5'-caggaaacagctatgac-3'
M13 Forward	5'-tgtaaaacgacggccagt-3'
THF400	5'-tcgtgcgcctcgaggt-3'
THF839	5'-aggagcgcacgggctt-3'
THF1239	5'-ccctgaggctgcgg-3'
THR1	5'-tgcctgcgcccaa-3'

[1]Oligonucleotide sequencing primers were supplied by Promega (Southampton, UK) or Eurofins Genetic Services Ltd. (London, UK).

mixture minus methionine, 0.04 volumes of 25x TnT® Reaction Buffer, 40 units of RNasin® Ribonuclease Inhibitor (Promega) and 0.04 volumes of 10 mCi/ml translation-grade [^{35}S]-methionine (1,000 Ci/mmol) (Perkin-Elmer LAS UK Ltd., Beaconsfield, UK). Reactions were incubated at 30^{o}C for 120 min and then stored at -40^{o}C until needed.

The percentage incorporation of [^{35}S]-methionine was determined by trichloroacetic acid (TCA)-precipitation as detailed by the manufacturer (Promega). In brief, 2 µl of the reaction mixture were added to 98 µl of 1 M NaOH/2% H_2O_2 and incubated at 37^{o}C for 10 min. Susequently, 900 µl of ice-cold 25% (w/v) TCA/2% (w/v) casamino acids (Difco, Detroit, MI, USA) were added and the reaction incubated on ice for 30 min. To collect the precipitated translation products, 250 µl of the TCA reaction mix were vacuum filtered on to a Whatman GF/A glass fibre filter (Whatman International Ltd., Maidstone, UK) pre-wetted with cold 5% (w/v) TCA. The filter was rinsed three times with 1 ml of ice-cold 5% (w/v) TCA, once with 1 ml of acetone and then allowed to dry at room temperature before immersing in 3 ml of Ultima-Gold® XR scintillation fluid (Packard Bioscience, Groningen, The Netherlands) and counting in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc., Fullerton, CA, USA). To determine total counts per min (cpm) present in the reaction, a 5-µl aliquot of the TCA reaction mix was spotted directly on to a filter. This was dried for 10 min before counting as above. The percent incorporation of [^{35}S]-methionine was determined as: 100 x (cpm of washed filter/cpm of unwashed filter x 50), and ranged from 7-15% depending on the protein being labeled.

Radiolabelled protein products were also analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Section 2.19).

2.19 SDS-PAGE and autoradiography

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples was performed in 8%, 10% or 12% (w/v) SDS-polyacrylamide resolving gels. The exact constitution of gels and associated buffers is shown in Table 2.7. The percentage acrylamide of the gel varied according to the size of the protein products to be electrophoresed.

The gels were created using a Bio-Rad Mini-Protean Tetra Cell apparatus (Bio-Rad Ltd.). Briefly, glass plates and spacers were assembled using the dedicated equipment according to the manufacturer's instructions. The resolving gel solution was poured into the space between the plates and overlaid with 1 ml of butan-1-ol. After the gel had polymerized, the butanol was poured off and a gel comb was inserted before the solution for the stacking gel was poured in on top. Subsequently, the gel comb was removed and the full apparatus assembled into the gel running tank. Laemmli buffer (250 mM Tris-base; 0.1% (w/v) SDS; 0.2 M glycine) was poured into the tank until the bottom of the plates was covered and the top buffer reservoir was full.

Prior to loading, radiolabelled protein products made by *in vitro* coupled transcription-translation (Section 2.18) were mixed with SDS-sample buffer (2% (w/v) SDS; 25% (v/v) glycerol; 0.01% (w/v) bromophenol blue; 2% (v/v) 2-mercaptoethanol; 62.5 mM Tris-HCl, pH 6.8) (Bio-Rad Ltd.) and heated at 85^{o}C for 5 min. Samples were then loaded into the wells along with either Prestained SDS-PAGE Standards, Low Range (21-103 kDa) (Bio-Rad Ltd.) or Precision Plus Protein All Blue Standards (10-250 kDa) (Bio-Rad Ltd.). Gels were run at 35 milli-amperes for 3-5 h or until the visible dye front had reached the bottom of the plates.

The apparatus was subsequently dismantled and the gel transferred to a plastic tray and covered with Coomassie® Blue stain (0.05% (w/v) Coomassie® Brilliant Blue (Bio-Rad Ltd.); 10% (v/v) glacial acetic acid; 25% (v/v) isopropanol). This was placed on a rocking platform. After a minimum of 30 min staining, the gel was destained by repeated fresh additions of a solution containing 10% (v/v) glacial acetic acid and 25% (v/v) isopropanol, until the protein markers were clearly visible. The destain was discarded and the gel was soaked for 30 min in Amersham Amplify™ Fluorographic Reagent (GE Healthcare Life Sciences, Little Chalfont, UK), before being dried for 2 h at 60^{o}C on to 3MM Whatman paper (Whatman International Ltd.) in a Bio-Rad Gel Dryer 583 (Bio-Rad Ltd.). Dried gels were subjected to autoradiography by exposure to Fuji RX x-ray film (Genetic Research Instrumentation Ltd., Dunmow, UK) in a Hypercassette™ (GE Healthcare BioSciences) at room temperature for 24 h. The film was subsequently developed using Photosol CD18 x-ray developer (Photosol Ltd., Basildon, UK) for 3 min, rinsed in water and then fixed for 3 min in Photosol CF40 fixer (Photosol Ltd.).

Table 2.7: Constituents of SDS-PAGE[1] gels

(a) Resolving gel

Constituent	Final concentration in gel
Buffer A: Tris-base SDS[2]	 0.4 M 0.1% (w/v)
Acrylamide:bisacrylamide (37.5:1) (Bio-Rad Laboratories Ltd.)	10-12% (w/v)
Ammonium persulphate	0.04% (w/v)
TEMED[3]	0.0004% (v/v)

(b) Stacking gel

Constituent	Final concentration in gel
Buffer B: Tris-Base SDS[2]	 0.125 M 0.1% (w/v)
Acrylamide: bisacrylamide (37.5: 1)	4% (w/v)
Ammonium persulphate	0.05% (w/v)
TEMED[3]	0.075% (v/v)

[1]SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

[2]SDS, sodium dodecyl sulphate.

[3]TEMED, N, N, N, N'-tetramethylethylenediamine.

2.20 Radioimmunoassays

For each radioimmunoassay (RIA) (Figure 2.3) reaction, a 1-2-µl aliquot of the required *in vitro* coupled transcription-translation reaction (equivalent to 10,000-20,000 cpm of TCA-precipitable material) was suspended in 50 µl immunoprecipitation buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% (v/v) Triton X-100; 10 mg/ml aprotinin) in a 1.5 ml tube. Serum or animal antiserum was then added to the required final dilution and the tube incubated overnight with shaking at 4^{o}C. 50 µl of protein G Sepharose^{TM} 4 Fast Flow (GE Healthcare Life Sciences), prepared according to the manufacturer, were added to the tube which was then incubated for a further 90 min at 4^{o}C. Subsequently, the protein G Sepharose-antibody-antigen complexes were collected by centrifugation at 5,000 *g* and washed six times for 15 min in immunoprecipitation buffer at 4^{o}C. The complexes were then transferred to 1 ml of Ultima-Gold® XR scintillation fluid contained in a 10 ml scintillation vial (Molecular Devices, Sunnyvale, CA, USA). Immunoprecipitated radioactivity was evaluated in a Beckman LS 6500 Multi-Purpose Scintillation Counter. Serum or antibody samples were always tested in duplicate in each experiment and the mean cpm was calculated from the two samples.

Antibody levels were expressed as an antibody (Ab) index. This was calculated for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by the population of healthy control sera. Each serum was tested in at least two experiments and the mean Ab index was calculated from these values. The upper limit of normal for RIAs was calculated using the mean Ab index + 3SD of the population of healthy controls. Any serum sample with an Ab index above the upper limit of normal was designated as positive for antibody reactivity.

For analysis by SDS-PAGE and autoradiography, the protein G Sepharose-antibody complexes were resuspended in 100 µl of SDS-sample buffer, boiled, centrifuged and the supernatants recovered for electrophoresis in SDS-polyacrylamide gels (Section 2.19).

To determine antibody titres, sera were analysed in RIAs at final dilutions of 1:100, 1:200, 1:500, 1:1000 and 1:2000. An Ab index for each sample at each dilution was calculated as described above. Sera were tested in two experiments and the mean Ab index calculated.

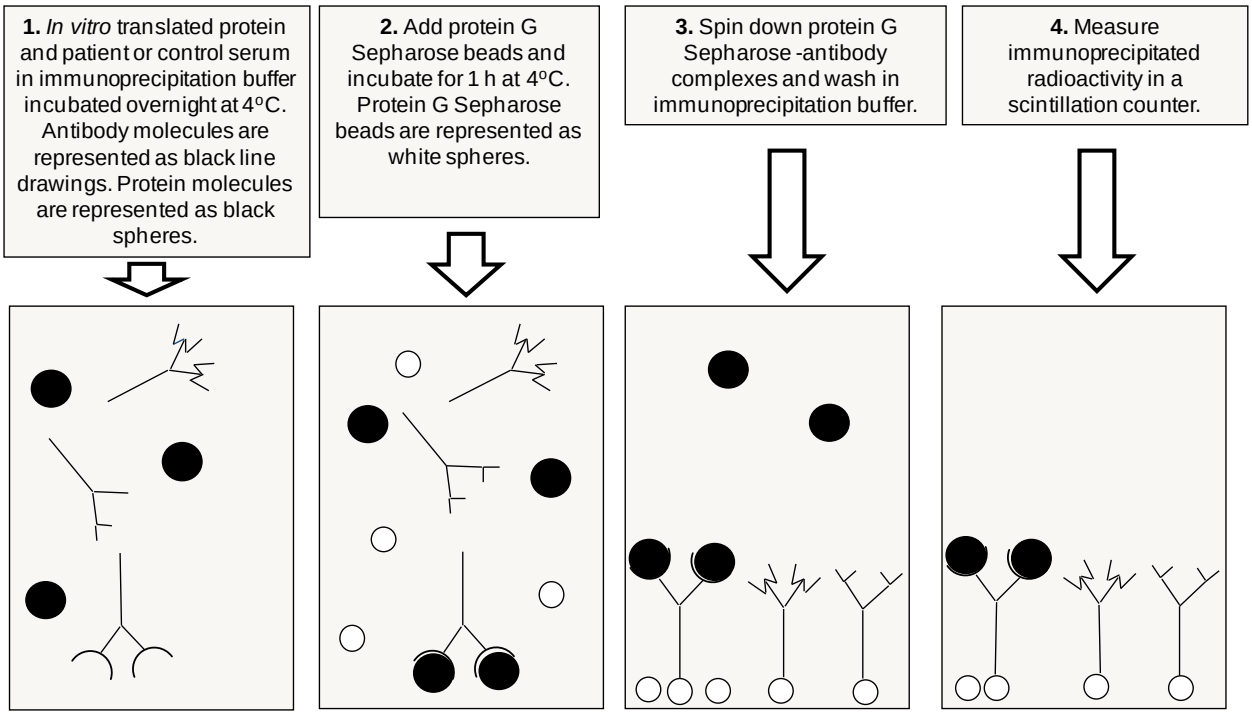


Figure 2.3: Schematic representation of the radioimmunoassay protocol.

2.21 Animal antisera

The animal antisera used in this study are listed in Table 2.8. All antisera were stored in accordance with the manufacturers' instructions at –20˚C.

2.22 Antibody absorption experiments using cell extracts

All cell extracts used in antibody absorption experiments were a gift from Dr. Helen Kemp (Department of Human Metabolism, University of Sheffield, Sheffield, UK). Extracts had been prepared from untransfected human embryonic kidney 293 (HEK293) cells and from HEK293 cells expressing TH, tyrosinase or MCHR1, resulting from prior transfection with pcDNA3-TH, pcDNA3-TYR or pcMCHR1 (Table 2.3), respectively. The expression of the heterologous proteins had been confirmed using immunoblotting with antigen-specific animal antisera (Table 2.8). The cell extracts were in extract buffer (150 mM NaCl; 10 mM Tris-HCl, pH 7.4; 1% (v/v) Triton X-100; Protease Cocktail Inhibitor) (Sigma-Aldrich), contained equivalent amounts of total protein (1 mg/ml) and were stored at -80°C until required.

For antibody absorption experiments, 100-µl samples of sera were incubated at 4°C for 16 h with 400 µl of cell extract made from either untransfected HEK293 cells or HEK293 cells containing expressed TH, tyrosinase or MCHR1. Sera were also incubated in cell extract buffer under the same conditions, but without any cell extract added. Anti-TH antiserum was also incubated in the same way to act as a positive control. Following incubation, samples were centrifuged at 4°C and 45,000 *g* for 1 h and the absorbed serum (supernatant) recovered. The TH antibody RIA was then set up as detailed previously (Section 2.20), to measure immunoreactivity against TH in the unabsorbed and pre-absorbed sera.

2.23 Synthetic peptides

The synthetic peptides used in this study were obtained from Severn Biotech Ltd. (Kidderminster, UK) and details of them are given in Table 2.9. They were stored at –80˚C until required.

Table 2.8: Animal antisera

Antiserum	Details	Source
ab59276: Polyclonal rabbit anti-TH[1] antiserum	Raised against a synthetic peptide close to the N-terminus of human TH[1].	Abcam, Inc. (Cambridge, MA, USA)
α-PEP7:Polyclonal rabbit anti-tyrosinase antiserum	Raised against a synthetic peptide corresponding to the C-terminus of mouse tyrosinase. Cross reacts with human tyrosinase.	Prof. Vincent Hearing (National Institutes of Health, Bethesda, MD, USA)
MCHR11-S: Polyclonal rabbit anti-MCHR1[2] antiserum	Raised against a 16-amino acid peptide close to the C-terminus of human MCHR1[2].	Alpha Diagnostics International, Inc. (San Antonio, TX, USA)
sc-15109: Polyclonal goat anti-PAH[3] antiserum	Raised against a synthetic peptide close to the N-terminus of human PAH[3].	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
sc-15114: Polyclonal goat anti-TPH[4] antiserum	Raised against a synthetic peptide in the internal region of human TPH[4].	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)

[1]TH, tyrosine hydroxylase.

[2]MCHR1, melanin-concentrating hormone receptor 1.

[3]PAH, phenylanine hydroxylase.

[4]TPH, tryptophan hydroxylase.

Table 2.9: Synthetic peptides

Peptide[1]	Sequence
TH-1-14[2]	MPTPDATTPQAKGF
TH-15-29[2]	RRAVSELDAKQAEAI
TH-30-44[2]	MSPRFIGRRQSLIED
TH-45-60[2]	ARKEREAAVAAAAAAV
TH-61-80[2]	PSEPGDPLEAVAFEEKEGKA
MCHR1-105-118[3]	ISYINIIMPSVFGT

[1]Synthetic peptides were supplied by Severn Biotech Ltd. (Kidderminster, U.K.).

[2]TH amino acid residue numbers for National Center for Biotechnology Information (NCBI) (Bethesda, MD, USA) sequence reference number NM000360.

[3]MCHR1 amino acid residue numbers for NCBI sequence reference number NM005297.

2.24 Antibody absorption experiments using TH peptide fragments

For use in antibody absorption experiments, unlabelled TH peptide fragments and MCHR1 were produced in an *in vitro* TnT® T7-Coupled Reticulocyte Lysate System as detailed above (Section 2.18), but using non-radiolabelled methionine in the reaction. In absorption experiments, sera at a 1:100 dilution in 50 µl of immunoprecipitation buffer were pre-incubated at 4°C for 2 h with 10 µl of *in vitro* translation reaction which contained the appropriate non-radiolabelled TH fragment or non-radiolabelled MCHR1. Following the addition of *in vitro* translation reaction containing radiolabelled TH, the TH antibody RIA was completed as above (Section 2.20).

2.25 Antibody absorption experiments using synthetic TH peptides

In peptide absorption experiments, sera were pre-incubated at a dilution of 1:100 with synthetic peptides (Table 2.9) at 100 µg/ml in 50 µl of immunoprecipitation buffer. After incubation at 4°C for 2 h, *in vitro* translation reaction containing radiolabelled TH was added to the samples which were then analysed in TH antibody RIAs as detailed above (Section 2.20).

2.26 Peptide enzyme-linked immunosorbent assays

For testing antibody reactivity against specific peptides in an enzyme-linked immunosorbent assay (ELISA) format, Corning polystyrene 96-well microtitre plates (Bibby Sterilin Ltd., Mid-Glamorgan, UK) were coated with 3 µg of the required synthetic peptide in 50 µl of coating buffer (1.5 mM Na_2CO_3; 3.5 mM $NaHCO_3$; pH 9.2). The plates were then incubated overnight at 4°C. Wells were washed with phosphate-buffered saline (0.137 M NaCl; 0.0027M KCl; 0.0081 Na_2HPO_4; 0.0015 M KH_2PO_4; pH7.4; PBS) (Oxoid, Basingstoke, UK)/0.1% (w/v) Tween-20, blocked with 3% (w/v) bovine serum albumin in PBS at room temperature for 1 h and then washed PBS/0.1% (w/v) Tween-20. Aliquots (100-µl) of serum at the required dilution were added to wells and PBS was applied as a control. The plates were incubated at room temperature for 2 h and then washed six times with PBS/0.1% (w/v) Tween-20. Aliquots (100-µl) of anti-human IgG alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1:1000 in PBS/0.1% (w/v) Tween-20 were added to the wells for 1 h at room temperature. After washing six times with PBS/0.1% (w/v) Tween-20, 100 µl of alkaline phosphatase substrate (Sigma Fast *p*-Nitrophenyl

Phosphate Tablet Set) (Sigma-Aldrich) were applied to each well and plates incubated at room temperature for 30 min. A LabSystems Integrated EIA Management System (Life Sciences International, Hampshire, UK) was used to read absorption of the wells at 405 nm.

All sera were tested in at least duplicate and the average OD_{405} value taken. The binding reactivity of each patient and control sera to each TH peptide was expressed as an Ab index calculated as: mean OD_{405} of tested serum/mean OD_{405} of the population of healthy control sera. Each serum was tested in two experiments and the mean Ab index was calculated from the resulting Ab index values. The upper limit of normal for each TH peptide ELISA was calculated using the mean Ab index + 3SD of the population of healthy control sera. Patient sera with an Ab index greater than the upper limit of normal were regarded as positive for binding to the TH peptide used in the ELISA.

To determine antibody titres, sera were analysed in ELISAs at final dilutions of 1:100, 1:200, 1:500, 1:1000 and 1:2000. An Ab index for each sample at each dilution was calculated as described above. Sera were tested in two experiments and the mean Ab index calculated.

For TH antibody IgG subclass determination, anti-human IgG1, IgG2, IgG3 and IgG4 AP-conjugates (SouthernBiotech, Birmingham, AL, USA) were applied as the secondary antibody in the ELISA experiments at a 1:1000 dilution.

For avidity evaluations, sera were diluted at 1:100 in PBS/0.1% (w/v) Tween-20 containing NaCl at concentrations of 0.15, 0.5, 1.5 and 3.0 M, prior to testing antibody binding in ELISA experiments.

2.27 Statistical analyses

Statistical analyses included: Fisher's exact tests for 2 x 2 contingency tables for categorical data; non-parametric Mann-Whitney U tests for comparing two unpaired groups for continuous data; and paired *t* tests for comparing two paired groups for continuous data. The tests were carried out as appropriate using GraphPad InStat 3 software (GraphPad Software, CA, USA). In all tests, *P* values < 0.05 (two-tailed) were regarded as significant.

CHAPTER 3

3. Detection of tyrosine hydroxylase antibodies in vitiligo patients using a radioimmunoassay

3.1 Introduction

3.1.1 Tyrosine hydroxylase

Tyrosine hydroxylase (TH) catalyses the conversion of tyrosine to L-dopa, the precursor molecule of catecholamine (e.g., dopamine) biosynthesis (Lewis *et al.* 1993; Nagatsu 1995). Four isoforms of TH (TH1-TH4) exist, these differing in the N-terminal regulatory domain (Lewis *et al.* 1993; Nakashima *et al.* 2009). The most widely expressed forms are TH1 and TH2 which are found mostly in the central nervous system (Lewis *et al.* 1993). Tyrosine hydroxylase expression and activity has also been shown in human keratinocytes (Ramchand *et al.* 1995; Marles *et al.* 2003). Controversy surrounds the presence of TH in melanocytes. Both TH1 mRNA and active TH were reported in human epidermal melanocytes, the enzyme being located on the melanosomal membrane together with tyrosinase (Marles *et al.* 2003). This finding led to the suggestion that TH directly supplies the preferred substrate and activator L-dopa for tyrosinase, which then initiates melanin synthesis (Figure 3.1) (Marles *et al.* 2003). In contrast, undetectable or insignificant levels of TH mRNA were found in several melanocyte and melanoma cell lines (Kågedal *et al.* 2004). To date, the function of TH in melanogenesis is not clearly defined and the *in vivo* expression and activity of TH, which may be higher than that found *in vitro* (Kågedal *et al.* 2004) also remains to be thoroughly investigated.

Tyrosine hydroxylase shows a high degree of amino acid homology with enzymes tryptophan hydroxylase (TPH) and PAH with an overall linear homology of about 35%, and in the catalytic domain, a homology of 70% (Figure 3.2) (Hufton *et al.* 1995). Both PAH and TPH are also involved in the synthesis of neurotransmitters: PAH catalyses the conversion of L-phenylalanine to L-tyrosine, and TPH hydroxylates L-tryptophan as part of the serotonin biosynthetic pathway (Hufton *et al.* 1995).

The first 165 amino acid residues at the N-terminal of TH constitute a regulatory domain that can be removed without significantly altering the enzyme activity (Daubner *et al*. 1993). A recent study has shown that phosphorylation of serine residue 40 imparts greater flexibility on the regulatory domain of the enzyme, and it has been suggested that TH exists in open and closed conformations depending on phosphorylation at serine 40 (Wang *et al.* 2011). However, the lack of a structural model of the regulatory domain of TH has limited the understanding of the structural changes associated with regulatory mechanisms that modulate the activity of the enzyme.

The active site of TH resides in the catalytic C-terminal and is dependent upon the presence of ferrous iron (Fitzpatrick 1989). The crystal structure of the catalytic and tetramerisation domains of TH have revealed a novel alpha-helical basket holding the catalytic iron and a 40-Ångstrom long anti-parallel coiled coil which forms the core of the tetramer (Goodwill *et al.* 1997). The catalytic iron is located 10 Ångstroms below the enzyme surface in a 17-Ångstrom deep active site pocket and is coordinated by the conserved residues histidine 331, histidine 336 and glutamine 376 (Goodwill *et al.* 1997).

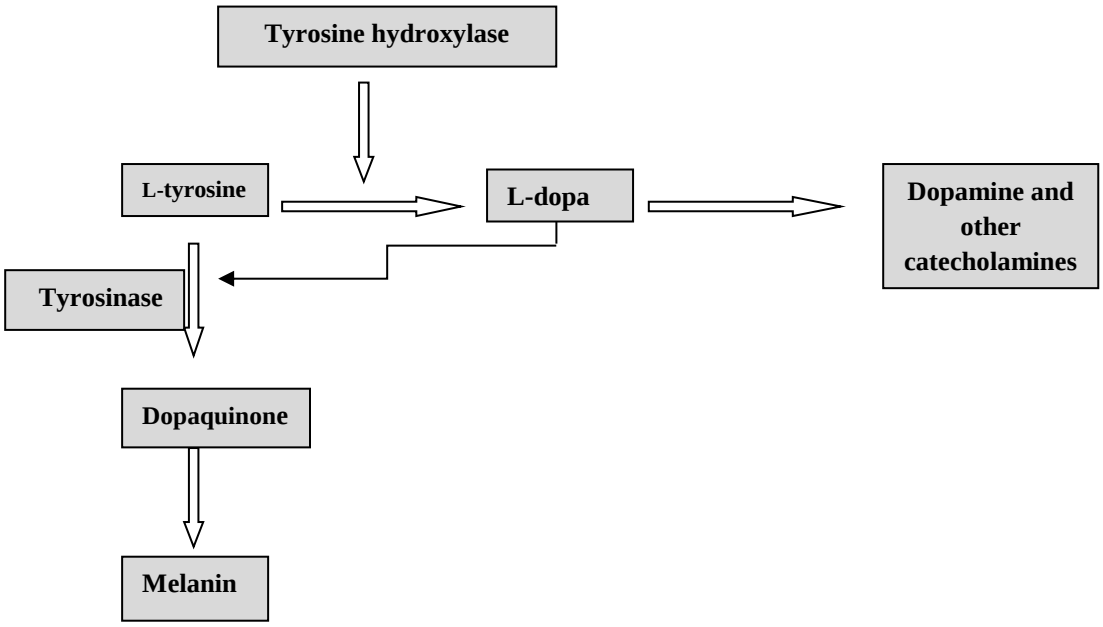


Figure 3.1: A possible role for tyrosine hydroxylase in melanogenesis.

Tyrosine hydroxylase (TH) possibly directly supplies L-dopa to facilitate the activation of tyrosinase enzyme which subsequently initiates melanogenesis by converting L-tyrosine to dopaquinone. Drawn with information from (Marles *et al.* 2003).

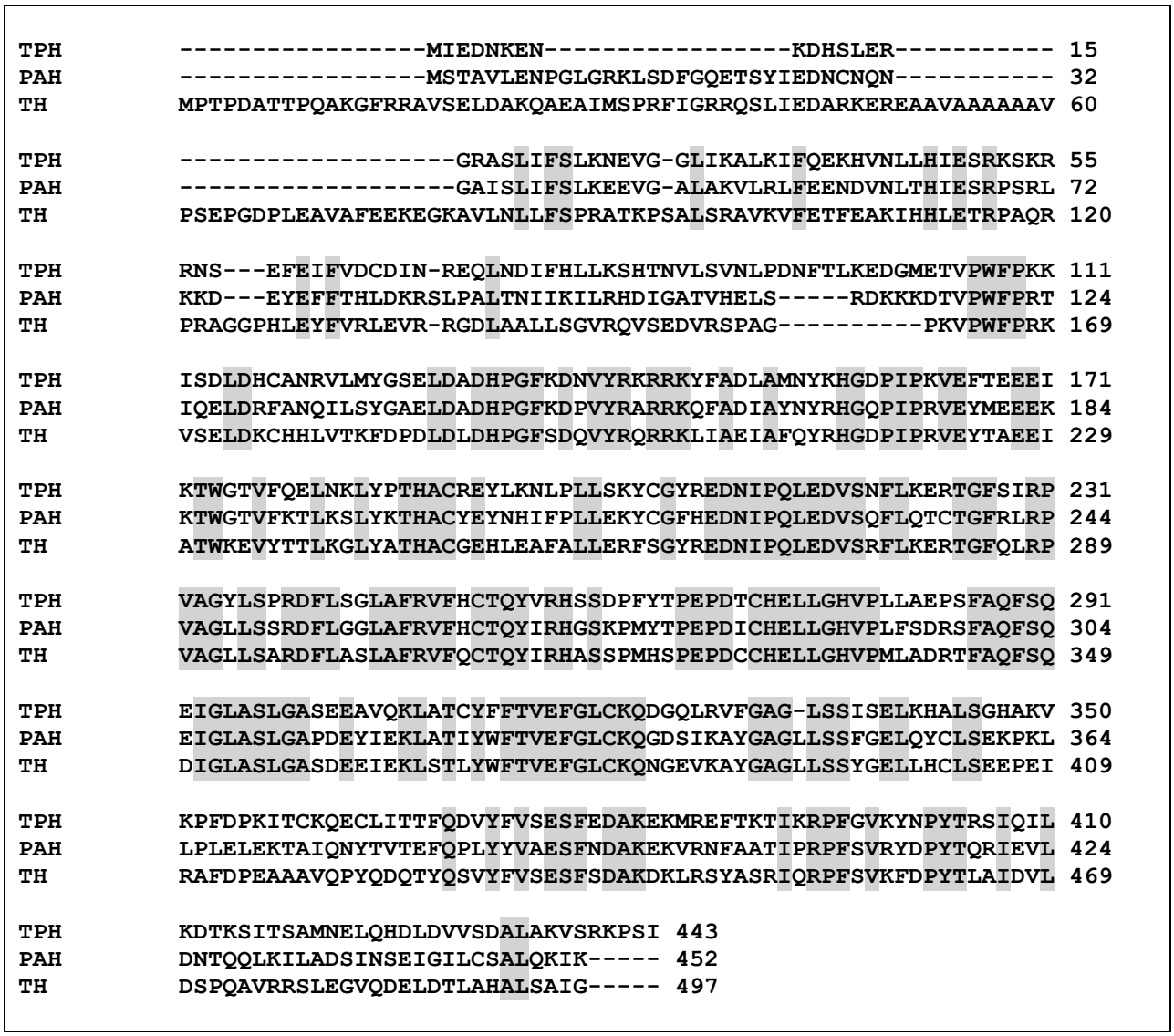


Figure 3.2: Amino acid sequence homologies of hydroxylase enzymes.

The aligned amino acid sequences of TH (NCBI Reference Sequence: NM000360.3), phenylalanine hydroxylase (PAH) (NCBI Reference Sequence: NM000277.1), and tryptophan hydroxylase (TPH) (NCBI Reference Sequence: NM004179.2) are also shown. Identical amino acid residues in the three sequences are highlighted. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

3.1.2 Tyrosine hydroxylase as an autoantigen

Tyrosine hydroxylase antibodies were reported first in APS1, also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), patients, including 57% of those individuals displaying vitiligo as part of their clinical manifestations (Hedstrand *et al.* 2000). However, a significant association was not noted between the presence of TH antibodies and vitiligo in APS1 patients. In contrast, immunoreactivity against TH was strongly associated with the presence of another ectodermal condition, alopecia areata (Hedstrand *et al.* 2000). Furthermore, TH was previously identified as a putative antibody target in vitiligo using phage-display enrichment of a melanocyte cDNA phage-display library with vitiligo patient IgG (Section 1.4) (Kemp *et al.* 2002; Waterman *et al.* 2010). This finding was the foundation for the current study to investigate TH as a possible antibody target in vitiligo. Interestingly, the related enzymes PAH and TPH are also autoantigens in APS1 (Ekwall *et al.* 1999; Ekwall *et al.* 2000).

3.1.3 Cloning of TH cDNA

In order to investigate possible immunoreactivity against TH in vitiligo patient sera, it was necessary to clone TH cDNA so that it could be used to express TH protein which in turn could be employed as a ligand in RIAs (Kemp *et al.* 2002; Kemp *et al.* 2011b). In brief, TH cDNA was isolated as a 1.5-kilobase (kb) fragment by Dr. Helen Kemp (Department of Human Metabolism, University of Sheffield, Sheffield, UK) using reverse transcriptase (RT)-PCR. Melanocyte RNA was reversed transcribed with Moloney Murine Leukemia Virus (M-MLV) RT to isolate melanocyte cDNA. The cDNA was then subjected to PCR amplification with TH-specific forward and reverse primers, THF1 and THR497 (Table 2.5), respectively. Subsequently, the TH cDNA was cloned into pcDNA3 at the *Hin*dIII-*Bam*HI restriction sites and the recombinant plasmid designated pcDNA3-TH (Figure 2.2). The TH cDNA was cloned in the correct orientation for expression of the protein from the T7 promoter in the pcDNA3 vector (Figure 2.1). Sequencing of the cloned TH cDNA using primers T7, SP6, THR1, THF400, THF839 and THR1239 (Table 2.6) indicated that the cDNA corresponded to TH isoform 2 (Lewis *et al.* 1993; Kemp *et al.* 2011b) with NCBI sequence reference number NM000360. The DNA sequence and amino acid sequence of TH are given in Figures 3.3 and 3.4, respectively.

Figure 3.3: TH cDNA sequence.

The cDNA sequence matches that of TH cDNA isoform 2 with National Center for Biotechnology Information (NCBI) (Bethesda, MD, USA) sequence reference number NM000360. The ATG translation start codon and the TAG translation termination codon are shown in bold-type face. The cDNA has 1491 base pairs.

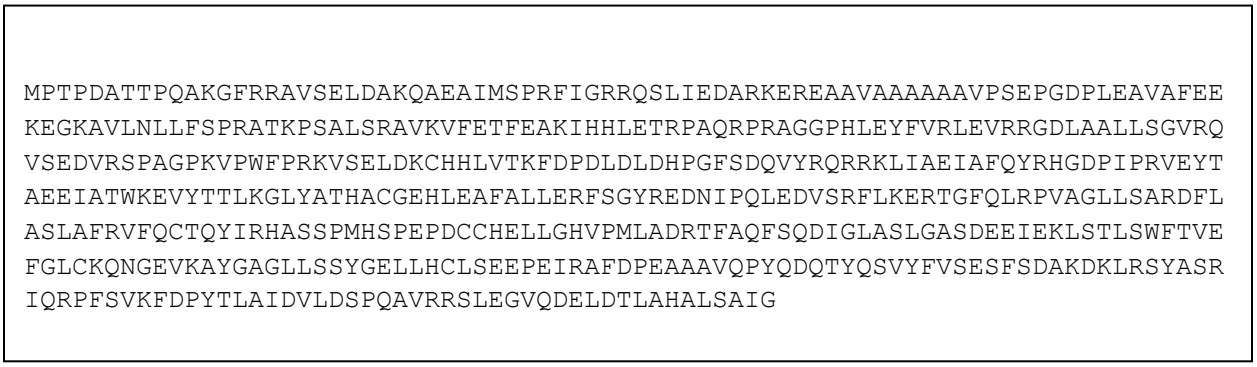


Figure 3.4: TH amino acid sequence.

Amino acids are designated by their one letter symbol. The protein has 497 amino acids residues.

3.1.4 Anti-TH antibodies in vitiligo

As stated above, TH was previously identified as a putative antibody target in vitiligo using phage-display enrichment of a melanocyte cDNA phage-display library with vitiligo patient IgG (Section 1.4) (Kemp *et al.* 2002; Waterman *et al.* 2010). The current study aimed to further investigate TH as a possible antibody target in vitiligo by measuring antibody reactivity against the enzyme in a panel of sera from patients with this cutaneous depigmenting disease.

3.2 Aims

The aims of this part of the study were:

- To develop a radioimmunoassay (RIA) to detect and measure antibodies against TH.

- To investigate the frequency of TH antibodies in a panel of vitiligo patients using the RIA.

- To determine any associations between the presence of TH antibodies and the clinical or demographic features of the vitiligo patients.

3.3 Experiments and Results

3.3.1 Preparation of plasmid pcDNA3-TH

Initially, *E.coli* JM109 pcDNA3-TH (Table 2.2) was streaked out from a frozen stock on to a LB agar plate containing 100 μg/ml ampicillin. The plate was incubated overnight at 37^{o}C. A single colony was inoculated into 10 ml of LB with ampicillin at 100 μg/ml and grown for 6 h at 37^{o}C. This starter culture was then inoculated into 500 ml of LB with ampicillin at 100 μg/ml and grown overnight at 37^{o}C. Plasmid pcDNA3-TH was prepared from the culture using a Qiagen Plasmid DNA Maxiprep Kit (Section 2.9). The plasmid was analysed by agarose gel electrophoresis in a 0.8% (w/v) agarose gel (Section 2.10) and quantified using a NanoDrop ND-1000 Spectrometer (Section 2.9). The results indicated successful purification of the pcDNA3-TH plasmid, which is shown in Figure 3.5. The plasmid preparation had a concentration of 1.2 mg/ml.

The plasmid was also checked for the presence of the correctly sized insert by restriction enzyme digestion with *Hin*dIII and *Bam*HI. The 25-μl reactions contained 1 μg of pcDNA3-TH, 10 units of *Hin*dIII and/or *Bam*HI, and 2.5 μl of restriction buffer E (Table 2.4). The reactions were incubated for 90 min at 37^{o}C before analysis by agarose gel electrophoresis in a 0.8% (w/v) agarose gel. The results showed a DNA insert at approximately 1.5 kb was released from pcDNA3-TH after restriction with both *Hin*dIII and *Bam*HI (Figure 3.5). This estimated size was close to that of the expected size of 1491 base pairs as determined from previous sequencing of the TH cDNA fragment (Figure 3.3).

As a final check that the plasmid was correct, the prepared pcDNA3-TH was subjected to DNA sequencing (Section 2.16) using primers T7, SP6, THR1, THF400, THF839 and THR1239 (Table 2.6). The results verified that the plasmid contained TH cDNA with the sequence matching that in Figure 3.3.

3.3.2 *In vitro* transcription-translation of TH cDNA

In order to produce [^{35}S]-TH for use in a RIA, plasmid pcDNA3-TH was transcribed and translated in a TnT® T7-Coupled Reticulocyte Lysate System as detailed in Section 2.18. To

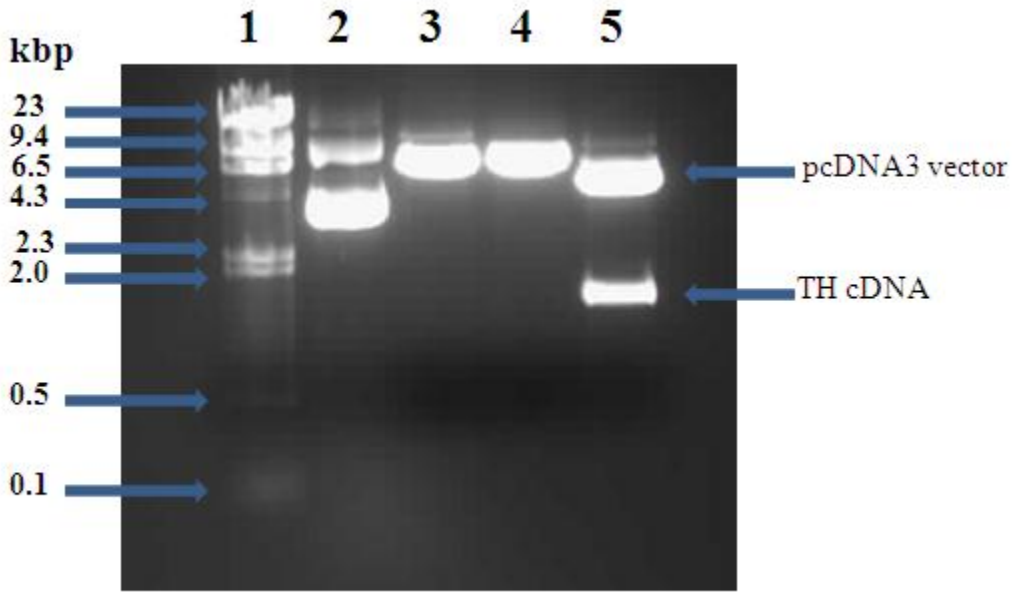


Figure 3.5: Agarose gel electrophoresis of plasmid pcDNA3-TH.

The unrestricted plasmid and plasmid restricted with *Hin*dIII and/or *Bam*HI were subjected to electrophoresis in a 0.8% (w/v) agarose gel. The gel shows: *Hin*dIII-restricted bacteriophage λ DNA with DNA fragments from 0.1-23 kb (lane 1); unrestricted pcDNA3-TH (lane 2); *Hin*dIII-restricted pcDNA3-TH (lane 3); *Bam*HI-restricted pcDNA3-TH (lane 4); *Hin*dIII and *Bam*HI-restricted pcDNA3-TH (lane 5).

analyse the radiolabelled product qualitatively, a 5-µl aliquot of the *in vitro* transcription-translation reaction containing [^{35}S]-TH was added to 20 µl of SDS-sample buffer (Section 2.19) and heated to 85^{o}C for 5 min before analysing a 10-µl sample by SDS-PAGE in a 10% (w/v) SDS-polyacrylamide gel and subsequent autoradiography (Section 2.19).

The results revealed a protein product with an estimated molecular weight of approximately 59 kDa (Figure 3.6), which agreed well with the molecular weight of 55 kDa predicted from the amino acid sequence of the protein with 497 residues (Figure 3.4).

3.3.3 Immunoreactivity of [^{35}S]-TH in a TH antibody RIA

In order to analyse the immunoreactivity of the *in vitro* transcribed and translated [^{35}S]-TH, a TH antibody RIA was carried out (Section 2.20) using a panel of animal antisera raised against different antigens (Table 2.8). Antiserum dilutions used in duplicate in the RIA are given in parentheses: ab59276 (anti-TH; 1:500), α-PEP7 (anti-tyrosinase; 1:100), MCHR11-S (anti-MCHR1; 1:100), sc-15109 (anti-PAH; 1:100) and sc-15114 (anti-TPH; 1:100). An immunoprecipitation reaction without antiserum was also included as a background control. In each TH antibody RIA experiment, a TH Ab index was calculated for each antiserum as: cpm immunoprecipitated by tested antiserum/cpm immunoprecipitated by the background control. Each antiserum was tested in four experiments and the mean TH Ab index was calculated from these values. The TH Ab indices of the different antisera were compared using Mann-Whitney tests (Section 2.27). In all tests, *P* values <0.05 (two-tailed) were regarded as significant.

The mean TH Ab index ± SD for each antiserum was: ab59276 (4.12 ± 0.53), α-PEP7 (1.06 ± 0.03), MCHR11-S (1.06 ± 0.11), sc-15109 (0.91 ± 0.10) and sc-15114 (0.85 ± 0.15) (Figure 3.7). In Mann-Whitney tests, the TH Ab index of ab59276 was significantly higher when compared with that of either α-PEP7, MCHR11-S, sc-15109 or sc-15114: *P* values were all <0.05, indicating a specific immunoprecipitation reaction between [^{35}S]-TH and the anti-TH antiserum. No significant differences were detected when comparing the TH Ab indices of the other animal antisera to each other: *P* values were all > 0.05.

To analyse qualitatively the TH protein immunoprecipitated in the TH antibody RIA by antiserum ab59276, protein G Sepharose-antibody-antigen complexes were suspended in 50 µl of SDS-sample buffer, heated at 85^{o}C for 5 min, centrifuged and the supernatant recovered for

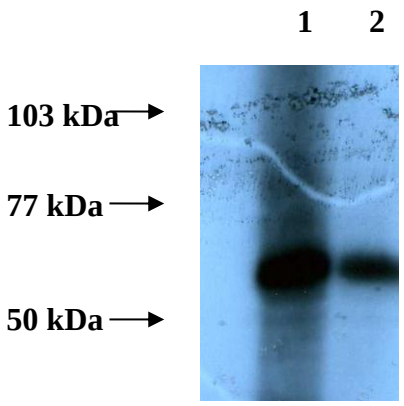


Figure 3.6: SDS-PAGE and autoradiography of [^{35}S]-TH and immunoprecipitated [^{35}S]-TH.

Radiolabelled TH was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System using plasmid pcDNA3-TH. [^{35}S]-TH was then immunoprecipitated in a TH antibody RIA with anti-TH antiserum ab59276. Immunoprecipitated proteins were analysed by SDS-PAGE in a 10% (w/v) SDS-polyacrylamide gel followed by autoradiography. The results are shown for: *In vitro* translated, non-immunoprecitated [^{35}S]-TH (lane 1); [^{35}S]-TH immunoprecipitated with anti-TH antiserum ab59276 (lane 2). Prestained SDS-PAGE Standards, Low Range (Bio-Rad Ltd.) are indicated. This gel was run and processed by Dr. Helen Kemp (Department of Human Metabolism, University of Sheffield, Sheffield, UK).

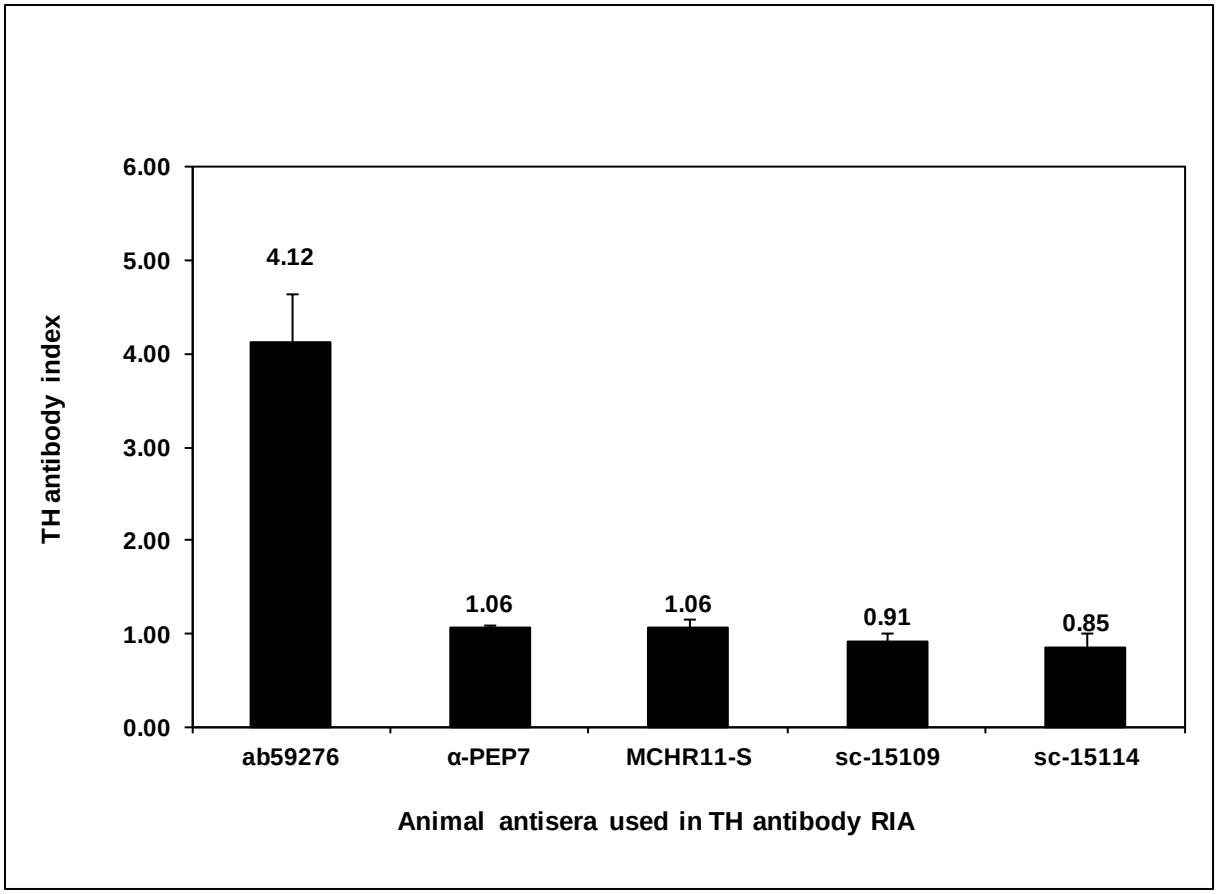


Figure 3.7: TH antibody RIA using different animal antisera.

The immunoreactivity of the *in vitro* transcribed and translated [^{35}S]-TH was tested in the TH antibody RIA with animal antisera ab59276 (anti-TH), α-PEP7 (anti-tyrosinase), MCHR11-S (anti-MCHR1), sc-15109 (anti-PAH) and sc-15114 (anti-TPH). An immunoprecipitation reaction without any serum was included as a background control. The mean TH Ab index (± SD) of four experiments is shown for each antiserum: ab59276 (4.12 ± 0.53), α-PEP7 (1.06 ± 0.03), MCHR11-S (1.06 ± 0.11), sc-15109 (0.91 ± 0.10) and sc-15114 (0.85 ± 0.15).

SDS-PAGE in a 10% (w/v) SDS-polyacrylamide gel (Section 2.19). The gel was then subjected to autoradiography (Section 2.19).

The results showed that the antiserum ab59276 immunoprecipitated a protein band equivalent in size (59 kD) to that of *in vitro* translated [^{35}S]-TH (Figure 3.6).

Overall, the results of these experiments indicated that, potentially, radiolabelled TH could be used as an antigen in a RIA to detect TH antibodies.

3.3.4 Analysis of the specificity of anti-TH antiserum ab59276

In order to analyse the specificity of the antiserum ab59276, it was used in RIAs with a panel of different radiolabelled antigens including: tyrosinase, MCHR1, PAH and TPH.

Radiolabelled [^{35}S]-tyrosinase and [^{35}S]-MCHR1 were produced in a TnT® T7-Coupled Reticulocyte Lysate System (Section 2.18) from plasmids pcDNA3TYR (Table 2.3) and pcMCHR1 (Table 2.3), respectively. Labelled proteins [^{35}S]-PAH and [^{35}S]-TPH were transcribed and translated from plasmids pSP64-Poly(A)-PAH (Table 2.3) and pSP64-Poly(A)-TPH (Table 2.3), respectively, in a TnT® SP6-Coupled Reticulocyte Lysate System (Section 2.20). RIAs using these labeled antigens with duplicate samples of antiserum ab59276 at a 1:100 (1:500 for TH antigen) dilution were carried out as detailed in (Section 2.20). Appropriate positive control antisera were included in each RIA at the dilution given in parentheses: α-PEP7 (1:100), MCHR11-S (1:100), sc-15109 (1:500) and sc-15114 (1:500). An immunoprecipitation reaction without antiserum was also included in each RIA as a background control. In each RIA experiment, an Ab index was calculated for each antiserum as: cpm immunoprecipitated by tested antiserum/cpm immunoprecipitated by the background control. Each antiserum was tested in four experiments and the mean Ab index was calculated from these values. Ab indices were compared using Mann-Whitney tests (Section 2.27). In all tests, *P* values <0.05 (two-tailed) were regarded as significant.

The mean Ab index ± SD for the antiserum ab59276 in RIAs against the different antigens was: TH (4.12 ± 0.53), tyrosinase (0.77 ± 0.10), MCHR1 (1.03 ± 0.05), PAH (0.88 ± 0.07) and TPH (0.77 ± 0.02) (Figure 3.8). The mean Ab index (± SD) for the antisera α-PEP7, MCHR11-S, sc-15109 and sc-15114 in RIAs against their respective antigens was: tyrosinase (14.34 ± 1.49), MCHR1 (10.06 ± 0.39), PAH (5.34 ± 0.23) and TPH (7.93 ± 0.50) (Figure 3.8).

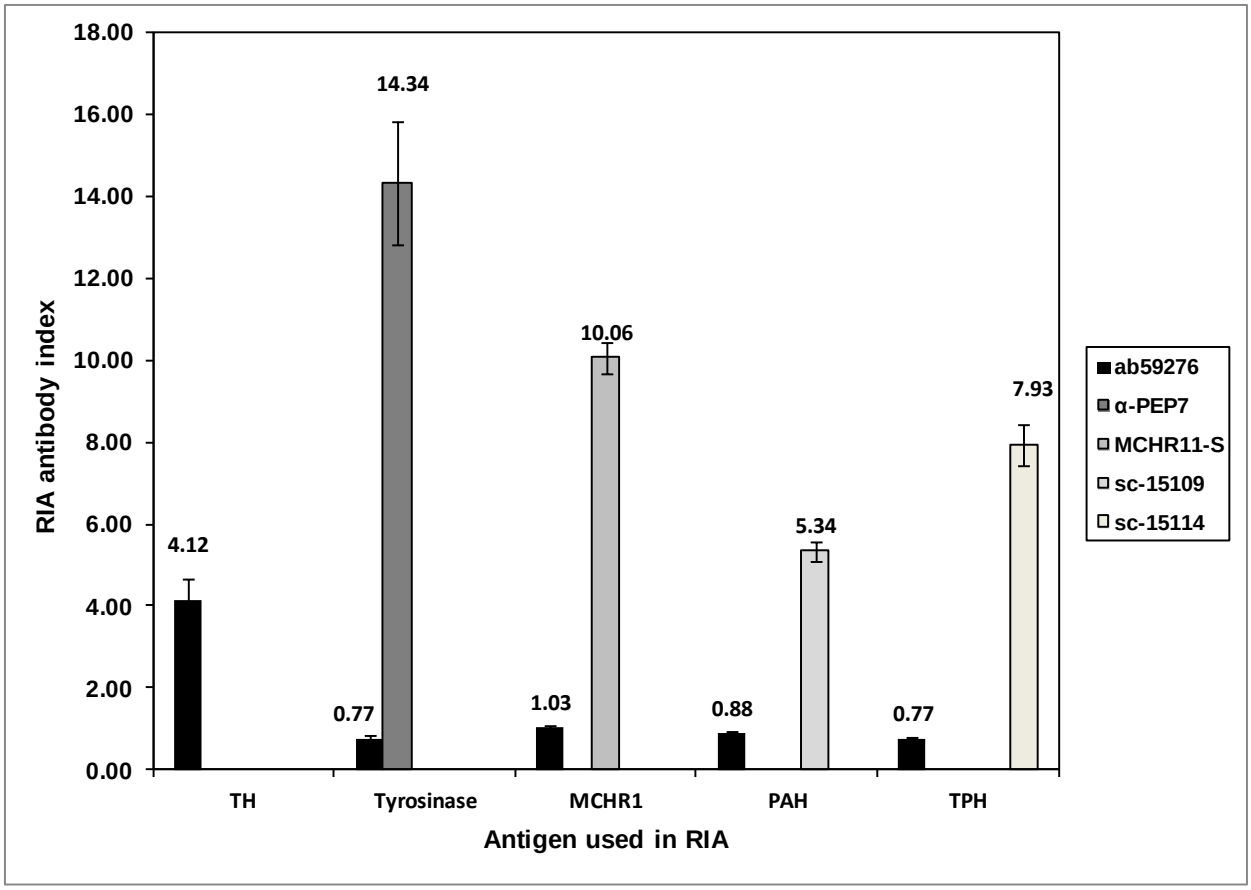


Figure 3.8: RIAs with different antigens and with anti-TH antiserum ab59276.

The immunoreactivity of the anti-TH antiserum ab59276 was tested against *in vitro* transcribed and translated [^{35}S]-TH, [^{35}S]-tyrosinase, [^{35}S]-MCHR1 [^{35}S]-PAH [^{35}S]-TPH in RIAs. Animal antisera α-PEP7 (anti-tyrosinase), MCHR11-S (anti-MCHR1), sc-15109 (anti-PAH) and sc-15114 (anti-TPH) were included in RIAs as positive controls for immunoprecipitation of their respective antigens. An immunoprecipitation reaction without any serum was included as a background control in each RIA. The mean Ab index (± SD) of four experiments is shown for the antiserum ab59276 in RIAs against the different antigens: TH (4.12 ± 0.53), tyrosinase (0.77 ± 0.10), MCHR1 (1.03 ± 0.05), PAH (0.88 ± 0.07) and TPH (0.77 ± 0.02). The mean Ab index (± SD) of four experiments is also shown for the antisera α-PEP7, MCHR11-S, sc-15109 and sc-15114 in RIAs against their respective antigens: tyrosinase (14.34 ± 1.49), MCHR1 (10.06 ± 0.39), PAH (5.34 ± 0.23) and TPH (7.93 ± 0.50).

In Mann-Whitney U tests, the Ab index of the antiserum ab59276 in the different RIAs was compared to that of the positive control antiserum for the particular RIA in question. In all cases, the Ab index of ab59276 was significantly lower: *P* values were all < 0.05, indicating there was no significant immunoprecipitation of other antigens by the anti-TH antiserum.

Overall, the results of these experiments indicated that antiserum ab59276 did not immunopreciptate either homologous proteins PAH and TPH or vitiligo-associated antigens tyrosinase and MCHR1. It could be used therefore as a positive control for TH antibodies in TH antibody RIAs.

3.3.5 TH antibody RIA using patient and control sera

To analyse patient and control sera for the presence of TH antibodies, TH antibody RIAs were carried as detailed in (Section 2.22) using [^{35}S]-TH as the radiolabelled antigen. The sera analysed were from patients with: non-segmental vitiligo (*n*=79), segmental vitiligo (*n*=8), Graves' disease (*n*=27), autoimmune hypothyroidism (*n*=25), Addison's disease (*n*=19) and systemic lupus erythematosus (*n*=20), and from healthy controls (*n*=28) (Section 2.1; Table 2.1). All sera were assayed in duplicate at a final dilution of 1:100. Antiserum ab59276 was included in each assay set as a positive control at a dilution of 1:500.

In each assay set, antibody levels were expressed as a TH Ab index. This was calculated for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 28 healthy control sera. Each serum was tested in at least two experiments and the mean TH Ab index was calculated from these values. The upper limit of normal for the assay was calculated using the mean TH Ab index + 3SD of the population of 28 healthy controls. Any serum sample with a TH Ab index above the upper limit of normal was designated as positive for TH antibody reactivity.

The results of the TH antibody RIAs are shown in Table 3.1 and Figure 3.9, and indicated that the upper limit of normal for the RIA was a TH Ab index of 1.09, calculated as above from the TH Ab indices of the control group. Healthy controls, patients with segmental vitiligo, and patients with other autoimmune diseases were all TH antibody-negative. Of the 79 non-segmental vitiligo patient sera, 18 (23%) were considered TH antibody-positive, as their TH Ab indices (Table 3.2) were above the upper limit of normal value of 1.09.

Table 3.1: Results of TH antibody RIAs

Patient or control group	TH antibody indices (mean ± SD)	TH antibody indices (range)	Number positive for TH antibodies[1]
Non-segmental vitiligo	1.25 ± 0.63	0.83-3.39	18/79
Segmental vitiligo	1.03 ± 0.04	0.94-1.06	0/8
Graves' disease	1.00 ± 0.04	0.85-1.08	0/27
Addison's disease	1.00 ± 0.04	0.92-1.07	0/19
Autoimmune hypothyroidism	1.01 ± 0.04	0.93-1.07	0/25
Systemic lupus erythematosus	1.00 ± 0.02	0.96-1.04	0/20
Healthy controls	1.00 ± 0.03	0.95-1.08	0/28

[1]Patient sera with a TH Ab index above the upper limit of normal value of 1.09, calculated from the mean TH Ab index + 3SD of 28 healthy controls, were considered positive for TH antibodies.

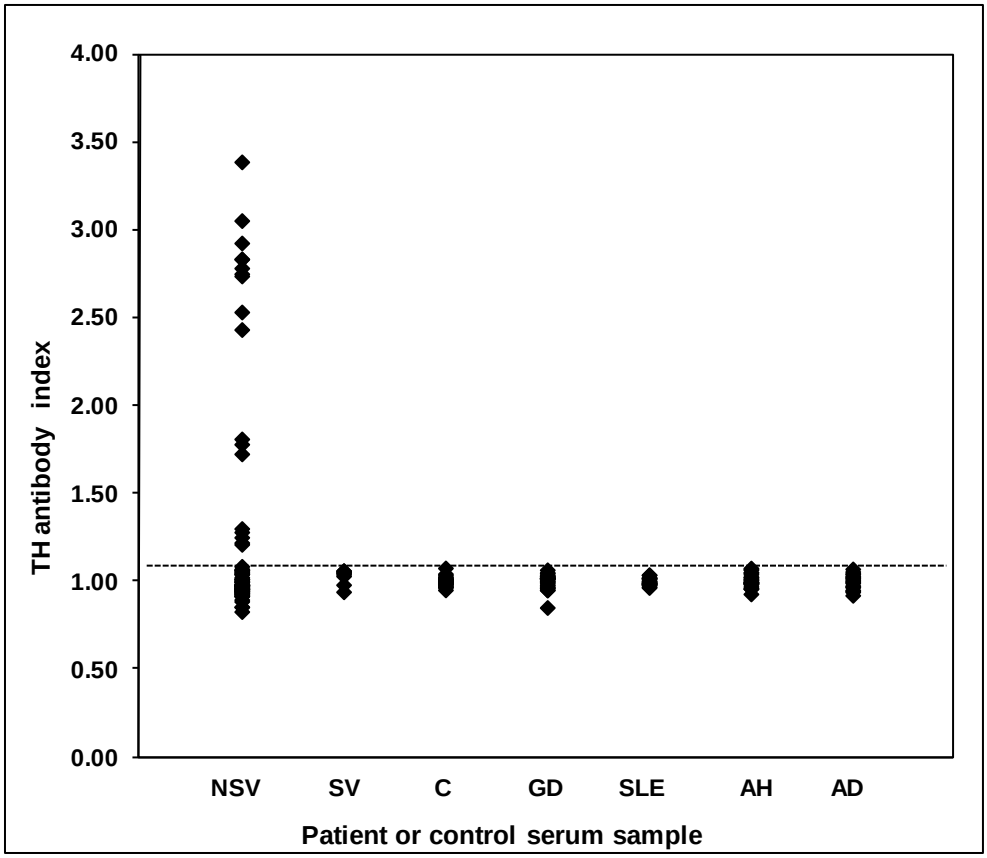


Figure 3.9: RIA for TH antibodies in patient and control sera.

Patient and control sera were evaluated for TH antibodies in the TH antibody RIA. The TH Ab indices are shown for sera from non-segmental vitiligo (NSV) (*n*=79), segmental vitiligo (SV) (*n*=8), systemic lupus erythematosus (SLE) (*n*=20), Graves' disease (GD) (*n*=27), autoimmune hypothyroidism (AH) (*n*=25), and Addison's disease (AD) (*n*=19) patients, and healthy controls (C) (*n*=28). The TH Ab index shown for each serum is the mean of at least two experiments. The upper limit of normal (mean TH Ab index + 3SD of 28 healthy controls) for the TH antibody RIA was a TH Ab index of 1.09, and this is shown by the dotted line. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Kemp *et al.* 2011a).

Table 3.2: TH antibody indices of TH antibody-positive vitiligo patients

Patient	TH antibody index[1]
V4	2.93 (± 0.06)
V8	2.53 (± 0.22)
V9	2.83 (± 0.05)
V10	2.43 (± 0.14)
V20	3.39 (± 0.04)
V25	2.74 (± 0.13)
V27	1.73 (± 0.05)
V30	1.81 (± 0.08)
V33	2.78 (± 0.07)
V39	2.75 (± 0.33)
V62	3.05 (± 0.31)
V63	1.30 (± 0.03)
V64	1.28 (± 0.07)
V69	1.22 (± 0.03)
V70	1.25 (± 0.04)
V82	1.21 (± 0.03)
V86	1.78 (± 0.06)
V87	2.84 (± 0.13)

[1]The TH Ab index shown for each TH antibody-positive vitiligo patient serum is the mean (±SD) of at least two experiments.

To analyse qualitatively proteins immunoprecipitated in the TH antibody RIA by vitiligo patients and control sera, protein G Sepharose-antibody-antigen complexes were suspended in 50 µl of SDS-sample buffer, heated at 85^{o}C for 5 min, centrifuged and the supernatant recovered for SDS-PAGE in 10% (w/v) SDS-polyacrylamide gels and subsequent autoradiography (Section 2.19).

The results indicated that all of the 18 TH antibody-positive vitiligo sera tested immunoprecipitated a protein band of 59 kDa, which is of the expected size for TH in SDS-polyacrylamide gels. Figure 3.10 illustrates the results for three TH antibody-positive vitiligo patient sera and three healthy control sera.

3.3.6 Analysis of TH antibody frequency in vitiligo patients and healthy controls

The frequency of TH antibodies was compared between vitiligo patient groups and healthy controls in Fisher's exact tests for 2 x 2 contingency tables (Section 2.27). *P* values <0.05 (two-tailed) were regarded as significant.

Table 3.3 shows the comparison of TH immunoreactivity in vitiligo patients and controls. Compared to controls, a significant increase in TH antibody frequency was noted in non-segmental vitiligo patients: *P* value was < 0.05. The frequency of TH antibodies was also significantly increased in the patient groups with active vitiligo compared to the patient cohorts with stable disease: *P* value was < 0.05. However, there was no difference in TH antibody frequency when comparing vitiligo patients with autoimmune diseases to those without autoimmune disorders: *P* value was > 0.05.

3.3.7 Determination of TH antibody titres in vitiligo patients

In order to measure TH antibody titres, all of the 18 TH antibody-positive vitiligo patient and 6 healthy control sera were analysed in the TH antibody RIA at final dilutions of 1:100, 1:200, 1:500, 1:1000 and 1:2000. TH Ab indices were calculated for each serum tested as: cpm immunoprecipitated by tested serum at each dilution/the cpm immunoprecipitated by six healthy control sera at each dilution. Samples were tested in two experiments and the mean TH Ab index was calculated from these values for each serum.

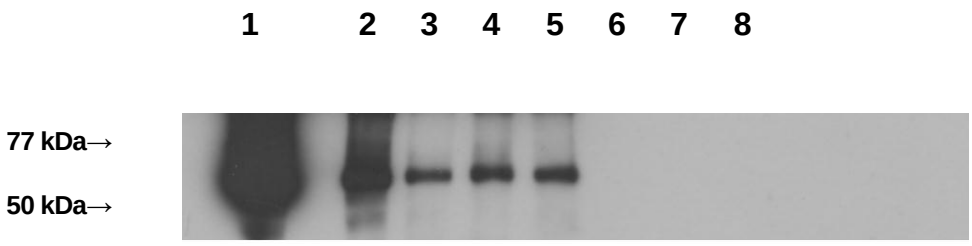


Figure 3.10: SDS-PAGE and autoradiography of [^{35}S]-TH and immunoprecipitated [^{35}S]-TH.

Radiolabelled TH was produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System and then immunoprecipitated in TH antibody RIAs with either vitiligo or healthy control sera. Immunoprecipitated proteins were analysed by SDS-PAGE in a 10% (w/v) SDS-polyacrylamide gel followed by autoradiography. The results are shown for 3 vitiligo patient and 3 healthy control sera: *In vitro* translated, non-immunoprecitated [^{35}S]-TH (lane 1); [^{35}S]-TH immunoprecipitated with: anti-TH antiserum ab59276 (lane 2); serum from TH antibody-positive vitiligo patient V4 (lane 3); serum from TH antibody-positive vitiligo patient V20 (lane 4); serum from TH antibody-positive vitiligo patient V62 (lane 5); serum from healthy controls - C1, C2 and C3 (lanes 6-8). Prestained SDS-PAGE Standards, Low Range (Bio-Rad Ltd.) are indicated. This gel was run and processed by Dr. Helen Kemp (Department of Human Metabolism, University of Sheffield, Sheffield, UK). Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Kemp *et al.* 2011a).

Table 3.3: Analysis of immunoreactivity against TH in vitiligo patient and control sera

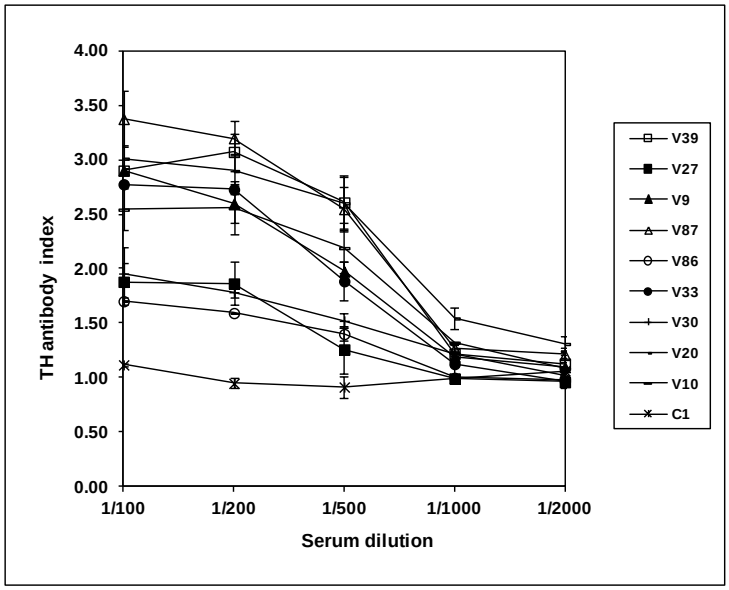
Detail of group analysed in TH antibody RIA	TH antibody-positive samples	*P* value[1]	*P* value[2]
Patient or control group			
Non-segmental vitiligo	18/79 (23%)	0.003	-
Segmental vitiligo	0/8 (0%)	-	-
Autoimmune disease (without vitiligo)	0/91 (0%)	-	-
Healthy controls	0/28 (0%)	-	-
Non-segmental vitiligo sub-type			
Symmetrical	13/63 (21%)	0.008	-
Symmetrical/periorificial	4/9 (44%)	0.002	-
Symmetrical with segmental patch	1/3 (33%)	0.100	-
Periorificial	0/2 (0%)	-	-
Universal	0/1 (0%)	-	-
Occupational	0/1 (0%)	-	-
Vitiligo activity			
Active vitiligo (all patients)	18/67 (27%)	0.001	0.009
Stable vitiligo (all patients)	0/20 (0%)	-	
Active non-segmental vitiligo	18/64 (28%)	0.001	0.017
Stable non-segmental vitiligo	0/15 (0%)	-	
Vitiligo and autoimmune disease			
Vitiligo with autoimmune disease (all patients)	4/26 (15%)	0.047	0.567
Vitiligo with no autoimmune disease (all patients)	14/61 (23%)	0.040	
Non-segmental vitiligo with autoimmune disease	4/24 (17%)	0.039	0.561
Non-segmental vitiligo with no autoimmune disease	14/55 (25%)	0.002	

[1]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.27) for comparing the prevalence of TH antibody reactivity in vitiligo patient and control groups. $P < 0.05$ was considered significant.

[2]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.27) for comparing the prevalence of TH antibody reactivity in vitiligo patient groups (e.g., active *versus* stable vitiligo; vitiligo with *versus* with no autoimmune disease). $P < 0.05$ was considered significant.

-, *P* values were not determined due to lack of TH antibody-positive samples.

The results of the dilution experiments showed that TH antibodies could still be detected at levels above the upper limit of normal in serum dilutions of 1:500, 1:1000, 1:2000, in 8, 6 and 4 patient samples, respectively (Figure 3.11). The TH antibody titres in the vitiligo patient sera are summarised in Table 3.4.



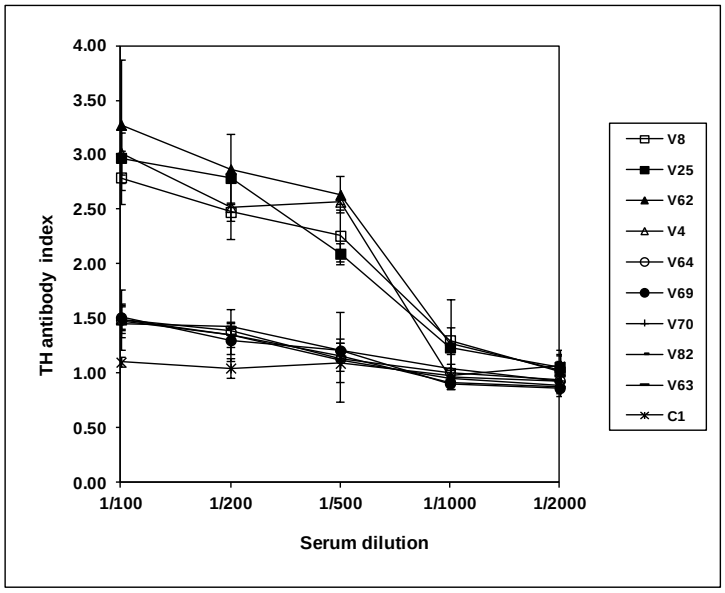


Figure 3.11: TH antibody titres in vitiligo patients.

The 18 TH antibody-positive vitiligo (V) sera and 6 healthy control (C) sera were analysed in the TH antibody RIA at dilutions of 1:100, 1:200, 1:500, 1:1000, and 1:2000. The TH Ab index (± SD) of each serum at each dilution is shown and is the mean of two experiments. The results are shown for the 18 vitiligo patients and for 1 control.

Table 3.4: TH antibody titres in TH antibody-positive vitiligo patients

Patient	TH antibody titre[1]
V4	1:500
V8	1:1000
V9	1:2000
V10	1:1000
V20	1:2000
V25	1:1000
V27	1:500
V30	1:1000
V33	1:1000
V39	1:2000
V62	1:1000
V63	1:500
V64	1:500
V69	1:500
V70	1:500
V82	1:500
V86	1:500
V87	1:2000

[1]The TH antibody titre is given as the dilution at which immunoreactivity of the serum sample could still be detected at levels above the upper limit of normal in the TH antibody RIA.

3.3.8 Evaluation of the specificity of TH antibodies in vitiligo patients

Absorption experiments were carried out to determine whether or not vitiligo patient TH antibodies reacted specifically against TH. In these experiments, sera from the 18 TH-antibody positive vitiligo patients and six healthy controls were mixed with extract made from either untransfected HEK293 cells or HEK293 cells containing either expressed TH, tyrosinase or MCHR1 (Section 2.22). Following absorption with cell extracts, duplicate serum samples (equivalent to a 1:100 dilution of the original serum) were tested in the TH antibody RIA for binding to [^{35}S]-TH. A TH Ab index was calculated for each serum tested as: cpm immunoprecipitated by tested serum/the cpm immunoprecipitated by six healthy control sera. Samples were tested in two experiments and the mean TH Ab index was calculated from these values. The TH Ab indices for the absorbed and unabsorbed vitiligo patient sera were compared using paired *t* tests (Section 2.27). In all tests, *P* values <0.05 (two-tailed) were regarded as significant.

The results of the absorption experiments are shown in Figures 3.12, where the TH Ab index for each sample is shown for unabsorbed sera and sera pre-absorbed with either untransfected HEK293 cell extract or extract made from HEK293 cells expressing TH, tyrosinase or MCHR1. TH Ab indices were compared in paired *t* tests. The tests indicated that compared to unabsorbed sera, the TH Ab indices of the TH-antibody positive vitiligo patient sera were significantly reduced when pre-absorbed with extract made from HEK293 cells expressing TH: *P* values were < 0.05 (Table 3.5). In contrast, significant reductions in TH Ab indices were not observed when TH-antibody positive vitiligo patient sera were pre-absorbed with extract prepared from untransfected HEK293 cells, HEK293 cells expressing tyrosinase or HEK293 cells expressing MCHR1: *P* values were > 0.05.

The absorption experiments indicated that sera from all the 18 TH antibody-positive vitiligo patients contained TH antibodies which specifically recognised the antigen and did not cross-react with either tyrosinase or MCHR1.

3.3.9 Analysis of vitiligo patient sera for tyrosinase and MCHR1 antibodies

Radioimmunoassays for tyrosinase and MCHR1 antibodies (Kemp *et al.* 1997a; Kemp *et al.* 2002) were carried out on all the vitiligo patient sera, in order to determine if there was any

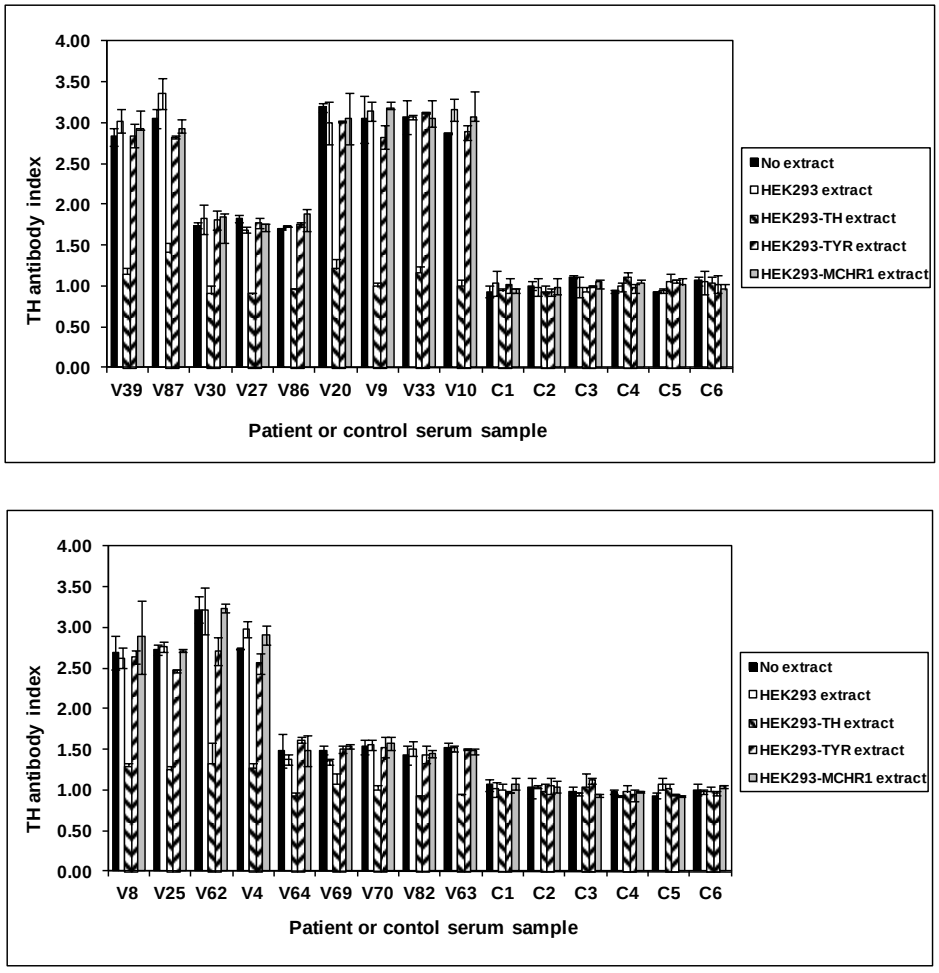


Figure 3.12: Antibody absorption experiments.

The 18 TH antibody-positive vitiligo (V) patient sera and 6 healthy control (C) sera were pre-absorbed with extract prepared from either untransfected HEK293 cells or from HEK293 cells expressing tyrosine hydroxylase (TH), tyrosinase (TYR) or melanin-concentrating hormone receptor 1 (MCHR1). Unabsorbed and pre-absorbed serum samples were then analysed in the TH antibody RIA. The results show the mean TH Ab index (± SD) of two experiments for unabsorbed and pre-absorbed sera. The results are shown for the 18 vitiligo patients and for 1 control.

Table 3.5: Comparison of TH Ab indices in antibody absorption experiments

Patient	*P* value[1]
V4	0.02
V8	0.04
V9	0.04
V10	0.02
V20	0.04
V25	0.002
V27	0.03
V30	0.04
V33	0.04
V39	0.04
V62	0.04
V63	0.03
V64	0.03
V69	0.04
V70	0.02
V82	0.04
V86	0.004
V87	0.002

[1]*P* values were calculated in paired *t* tests (Section 2.27) for comparing the TH Ab indices of TH antibody-positive patient sera without pre-absorption to those with pre-absorption using extract from HEK293 cells expressing TH. *P* values < 0.05 (two-tailed) were regarded as significant.

association between the presence of TH antibodies and the occurrence of antibodies against either tyrosinase or MCHR1.

Firstly, in order to produce [^{35}S]-tyrosinase and [^{35}S]-MCHR1 for use in RIAs, plasmids pcDNA3TYR (Table 2.3) and pcMCHR1 (Table 2.3), respectively, were transcribed and translated in a TnT® T7-Coupled Reticulocyte Lysate System (Section 2.18). The radiolabelled antigens (Figure 3.13) were then used to analyse patient and control sera for the presence of either tyrosinase or MCHR1 antibodies in RIAs, which were carried out as detailed in (Section 2.20). The sera analysed were from patients with non-segmental vitiligo (*n*=79) and segmental vitiligo (*n*=8), and from healthy controls (*n*=28) (Table 2.1; Section 2.1). All sera were assayed in duplicate at a final dilution of 1:100. As appropriate, either antiserum α-PEP7 (Table 2.8) or antiserum MCHR11-S (Table 2.8) was included in each assay set as a positive control at a dilution of 1:100 (Figure 3.13).

In each assay set, antibody levels were expressed as an Ab index. This was calculated for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 28 healthy control sera. Each serum was tested in at least two experiments and the mean Ab index was calculated from these values. The upper limit of normal for the assay was calculated using the mean Ab index + 3SD of the population of 28 healthy controls. Any serum sample with an Ab index above the upper limit of normal was designated as positive for either tyrosinase or MCHR1 antibody reactivity.

The results of the tyrosinase and MCHR1 antibody RIAs are shown in Table 3.6. All healthy controls and patients with segmental vitiligo were negative for tyrosinase and MCHR1 antibodies. Of the 79 non-segmental vitiligo patient sera, 11 (14%) and 14 (18%) were considered positive for antibodies against tyrosinase and MCHR1, respectively, with Ab indices above the upper limits of normal at values of 1.29 for the tyrosinase antibody RIA (Table 3.7), and 1.45 for the MCHR1 antibody RIA (Table 3.8). The tyrosinase and MCHR1 antibody frequencies were found to be similar to those reported in previous studies (Kemp *et al.* 1997a; Kemp *et al.* 2002).

The frequencies of tyrosinase and MCHR1 antibodies were compared between the non-segmental vitiligo patients and healthy controls in Fisher's exact tests for 2 x 2 contingency tables (Section 2.27). *P* values < 0.05 (two-tailed) were regarded as significant. The results

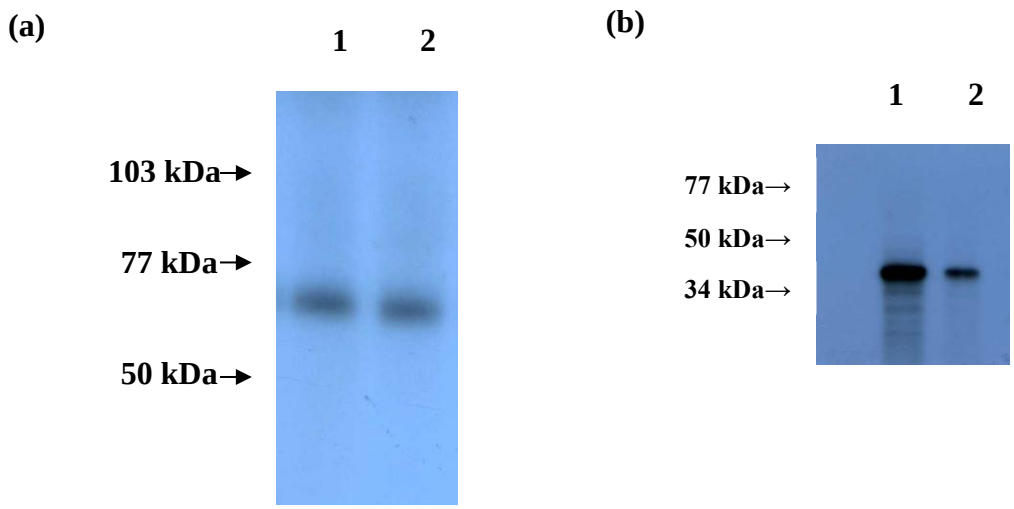


Figure 3.13: SDS-PAGE and autoradiography of [^{35}S]-tyrosinase and [^{35}S]-MCHR1 and immunoprecipitated [^{35}S]-tyrosinase and [^{35}S]-MCHR1.

Radiolabelled tyrosinase and MCHR1 were produced *in vitro* in a TnT® T7-Coupled Reticulocyte Lysate System using plasmids pcDNA3TYR and pcMCHR1, respectively. [^{35}S]-tyrosinase and [^{35}S]-MCHR1 were then immunoprecipitated in RIAs with antiserum α-PEP7 or antiserum MCHR11-S, respectively. Immunoprecipitated proteins were analysed by SDS-PAGE in a 10% (w/v) SDS-polyacrylamide gel followed by autoradiography. The results are shown for: **(a)** *In vitro* translated, non-immunoprecitated [^{35}S]-tyrosinase (57 kDa) (lane 1); [^{35}S]-tyrosinase immunoprecipitated with antiserum α-PEP7 (lane 2). **(b)** *In vitro* translated, non-immunoprecitated [^{35}S]-MCHR1 (45 kDa) (lane 1); [^{35}S]-MCHR1 immunoprecipitated with antiserum MCHR11-S (lane 2). Prestained SDS-PAGE Standards, Low Range (Bio-Rad Ltd.) are indicated. These gels were run and processed by Dr. Helen Kemp (Department of Human Metabolism, University of Sheffield, Sheffield, UK).

Table 3.6: Results of RIAs for antibodies against tyrosinase, MCHR1, PAH and TPH

Patient or control group → / **Antibody RIA ↓**	**Non-segmental vitiligo patients (*n*=79)**	**Segmental vitiligo patients (*n*=8)**	**Healthy controls (*n*=28)**
Tyrosinase antibody RIA[1]			
Tyrosinase Ab indices (mean ± SD)	1.20 ± 0.50	1.02 ± 0.07	1.02 ± 0.09
Tyrosinase Ab indices (range)	0.82-3.37	0.87-1.12	0.82-1.14
Number positive for tyrosinase antibodies	11 (14%)	0 (0%)	0 (0%)
MCHR1 antibody RIA[1]			
MCHR1Ab indices (mean ± SD)	1.14 ± 0.68	0.90 ± 0.25	0.96 ± 0.16
MCHR1Ab indices (range)	0.44-3.81	0.76-1.42	0.72-1.25
Number positive for MCHR1 antibodies	14 (18%)	0 (0%)	0 (0%)
PAH antibody RIA[1]			
PAH Ab indices (mean ± SD)	1.00 ± 0.04	1.01 ± 0.04	1.05 ± 0.05
PAH Ab indices (range)	0.93-1.08	0.96-1.07	0.85-1.06
Number positive for PAH antibodies	0 (0%)	0 (0%)	0 (0%)
TPH antibody RIA[1]			
TPH Ab indices (mean ± SD)	0.98 ± 0.06	0.96 ± 0.07	1.00 ± 0.07
TPH Ab indices (range)	0.85-1.12	0.89-1.05	0.79-1.18
Number positive for TPH antibodies	0 (0%)	0 (0%)	0 (0%)

[1]Patient sera with an Ab index above the upper limit of normal, calculated from the mean Ab index + 3SD of 28 healthy controls, were considered positive for antibodies. The upper limits of normal were: 1.29, 1.45, 1.15 and 1.21, for the tyrosinase, MCHR1, PAH and TPH antibody RIAs, respectively.

Table 3.7: Tyrosinase antibody indices of tyrosinase antibody-positive vitiligo patients

Patient	Tyrosinase antibody index[1]
V10	1.68 (± 0.09)
V18	1.43 (± 0.11)
V21	1.52 (± 0.12)
V27	1.93 (± 0.05)
V38	1.86 (± 0.14)
V53	2.16 (± 0.08)
V54	3.36 (± 0.32)
V55	2.37 (± 0.07)
V63	1.59 (± 0.03)
V78	2.11 (± 0.13)
V82	2.43 (± 0.21)

[1]The tyrosinase Ab index shown for each tyrosinase antibody-positive vitiligo patient serum is the mean (±SD) of two experiments.

Table 3.8: MCHR1 antibody indices of MCHR1 antibody-positive vitiligo patients

Patient	MCHR1 antibody index[1]
V6	2.18 (± 0.10)
V9	3.11 (± 0.29)
V12	2.01 (± 0.05)
V13	1.71 (± 0.08)
V18	3.22 (± 0.77)
V29	2.82 (± 0.36)
V44	1.64 (± 0.23)
V46	1.77 (± 0.16)
V50	1.53 (± 0.01)
V52	1.86 (± 0.12)
V54	1.58 (± 0.09)
V55	3.79 (± 0.72)
V62	3.81 (± 0.28)
V82	1.72 (± 0.14)

[1]The MCHR1 Ab index shown for each MCHR1 antibody-positive vitiligo patient serum is the mean (±SD) of two experiments.

indicated that antibodies against MCHR1, but not against tyrosinase, were significantly more frequent in non-segmental vitiligo patients than in controls: *P* values were 0.019 and 0.063, respectively.

3.3.10 Analysis of vitiligo patient sera for PAH and TPH antibodies

Radioimmunoassays for detecting PAH and TPH antibodies (Ekwall *et al.* 1999; Ekwall *et al.* 2000) were carried out on all the vitiligo patient sera, in order to determine if antibodies to these closely related proteins, in terms of amino acid sequence, were present in vitiligo patients.

Firstly, in order to produce [^{35}S]-PAH and [^{35}S]-TPH for use in RIAs, plasmids pSP64-Poly(A)-PAH (Table 2.3) and pSP64-Poly(A)-TPH (Table 2.3), respectively, were transcribed and translated in a TnT® SP6-Coupled Reticulocyte Lysate System (Section 2.18). The radiolabelled antigens (Figure 3.14) were then used to analyse patient and control sera for the presence of either PAH or TPH antibodies in RIAs which were carried out as detailed in (Section 2.20). The sera analysed were from patients with non-segmental vitiligo (*n*=79) and segmental vitiligo (*n*=8), and from healthy controls (*n*=28) (Table 2.1; Section 2.1). All sera were assayed in duplicate at a final dilution of 1:100. As appropriate, either antiserum sc-15109 (Table 2.8) or antiserum sc-15114 (Table 2.8) was included in each assay set as a positive control at a dilution of 1:500 (Figure 3.14).

In each assay set, antibody levels were expressed as an Ab index. This was calculated for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 28 healthy control sera. Each serum was tested in at least two experiments and the mean Ab index was calculated from these values. The upper limit of normal for the assay was calculated using the mean Ab index + 3SD of the population of 28 healthy controls. Any serum sample with an Ab index above the upper limit of normal was designated as positive for either PAH or TPH antibody reactivity.

The results of the PAH and TPH antibody RIAs are shown in Table 3.6. Immunoreactivity to either PAH or TPH was not apparent in any vitiligo patient sera or control sera with all PAH and TPH Ab indices below the upper limits of normal at values of 1.15 for the PAH antibody RIA, and 1.21 for the TPH antibody RIA. These results indicated that patient TH antibodies were

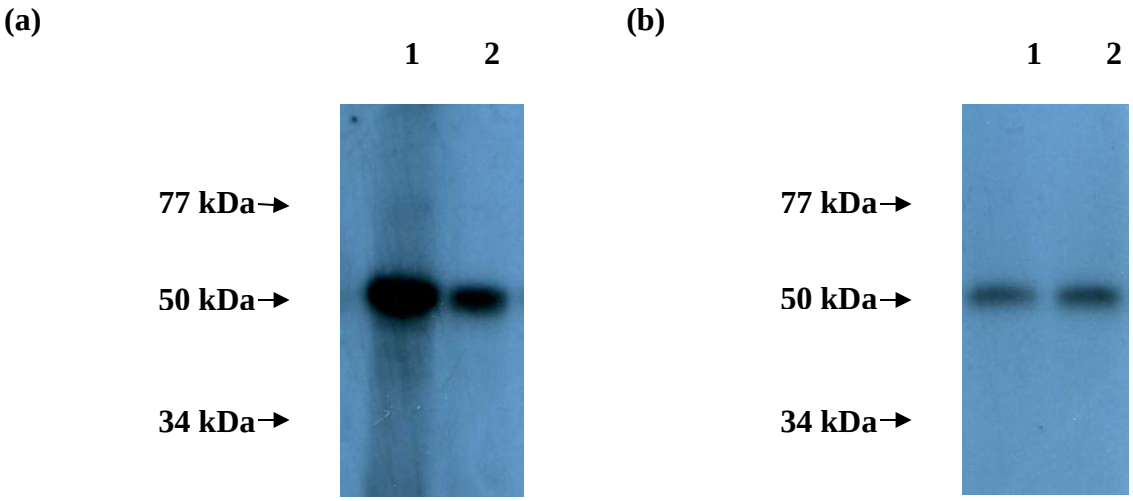


Figure 3.14: SDS-PAGE and autoradiography of [^{35}S]-PAH and [^{35}S]-TPH and immunoprecipitated [^{35}S]-PAH and [^{35}S]-TPH.

Radiolabelled PAH and TPH were produced *in vitro* in a TnT® SP6-Coupled Reticulocyte Lysate System using plasmids pSP64-Poly(A)-PAH and pSP64-Poly(A)-TPH, respectively. [^{35}S]-PAH and [^{35}S]-TPH were then immunoprecipitated in RIAs with antiserum sc-15109 and antiserum sc-15114, respectively. Immunoprecipitated proteins were analysed by SDS-PAGE in a 10% (w/v) SDS-polyacrylamide gel followed by autoradiography. The results are shown for: **(a)** *In vitro* translated, non-immunoprecitated [^{35}S]-PAH (50 kDa) (lane 1); [^{35}S]-PAH immunoprecipitated with antiserum antiserum sc-15109 (lane 2). **(b)** *In vitro* translated, non-immunoprecitated [^{35}S]-TPH (49 kDa) (lane 1); [^{35}S]-TPH immunoprecipitated with antiserum sc-15114 (lane 2). Prestained SDS-PAGE Standards, Low Range (Bio-Rad Ltd.) are indicated. These gels were run and processed by Dr. Helen Kemp (Department of Human Metabolism, University of Sheffield, Sheffield, UK).

not cross-reactive with either PAH or TPH, probably recognising unique epitopes, despite a high degree of homology between the amino acid sequences of the three enzymes.

3.3.11 Co-incidence of TH, tyrosinase and MCHR1 antibody reactivity in the non-segmental vitiligo patients

The co-incidence of TH, tyrosinase and MCHR1 antibody reactivity in the non-segmental vitiligo patient group is illustrated in Figure 3.15. Only 1/79 (1.3%) of the patients was positive for antibody reactivity to all three antigens. An antibody response to one and to two of the antigenic targets, respectively, was detected in 24/79 (30%) and in 8/79 (10%) of the patients. In addition, the prevalence of tyrosinase and MCHR1 antibodies was not significantly increased in non-segmental vitiligo patients with TH antibodies when compared to those without TH antibodies (Table 3.9). Overall, there was no indication that the presence of antibodies to the different target antigens was associated.

3.3.12 Comparison of the demographic, clinical and serological details of the TH antibody-positive and TH antibody-negative non-segmental vitiligo patient groups

The demographic, clinical and serological details of the TH antibody-positive and TH antibody-negative non-segmental vitiligo patient groups were compared in Fisher's exact tests for 2 x 2 contingency tables (Section 2.27) or in Mann-Whitney tests, as appropriate (Section 2.27). *P* values <0.05 (two-tailed) were regarded as significant.

The results are summarised in Table 3.9. No significant difference was observed between the two groups either in terms of gender, age of patients, disease duration, age at onset of vitiligo, presence of additional autoimmune diseases, the clinical subtype of non-segmental vitiligo or the presence of specific antibodies against tyrosinase, MCHR1, PAH and TPH: *P* values were >0.05. However, the frequency of patients with active vitiligo was significantly higher in the TH antibody-positive patient group: *P* value was <0.05.

The demographic, clinical and serological details of the 18 TH antibody-positive non-segmental vitiligo patients are summarised in Table 3.10.

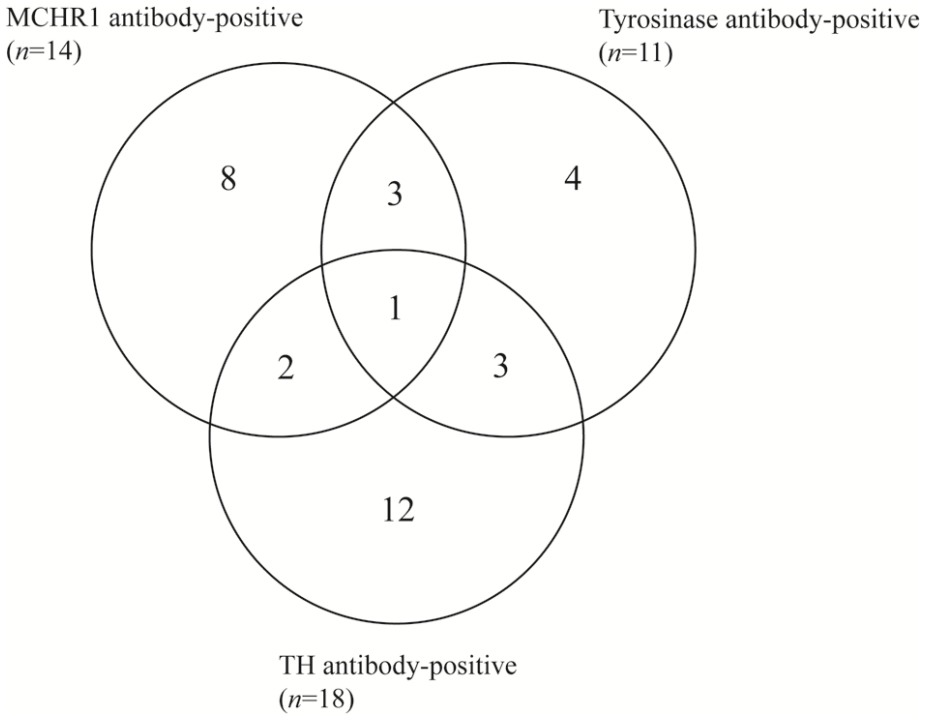


Figure 3.15: A schematic representation of the antibody response to TH, tyrosinase and MCHR1.

Of 79 non-segmental vitiligo patients, only one was positive for antibody reactivity to all three antigens. An antibody response to one and to two of the antigenic targets, respectively, was detected in 24/79 and in 8/79 of the patients. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Kemp *et al.* 2011a).

Table 3.9: Comparison of the demographic, clinical and serological details in non-segmental vitiligo patients with and without TH antibodies

Patient detail	TH antibody-positive non-segmental vitiligo patients (*n*=18)	TH antibody-negative non-segmental vitiligo patients (*n*=61)	*P* value
Demographic details			
Sex: Male/Female	8/10 (44%/56%)	22/39 (35%/64%)	0.586[1]
Mean age (range)	47 y (7-77 y)	47 y (14-74 y)	0.836[2]
Mean onset age (range)	28y (5-70 y)	35 y (<1-73 y)	0.157[2]
Mean disease duration (range)	18 y (1-50 y)	13 y (<1-49 y)	0.550[2]
Non-segmental vitiligo clinical sub-type			
Symmetrical	13 (72%)	50 (82%)	0.504[1]
Symmetrical/periorificial	4 (22%)	5 (8%)	0.198[1]
Symmetrical with segmental patch	1 (6%)	2 (3%)	0.527[1]
Periorificial	0 (0%)	2 (3%)	1.000[1]
Universal	0 (0%)	1 (2%)	1.000[1]
Occupational	0 (0%)	1 (2%)	1.000[1]
Vitiligo activity			
Active vitiligo	18 (100%)	46 (75%)	0.017[1]
Stable vitiligo	0 (0%)	15 (25%)	0.017[1]
Vitiligo and autoimmune disease			
With autoimmune disease	4 (22%)	20 (33%)	0.561[1]
With no autoimmune disease	14 (78%)	41 (67%)	0.561[1]
Antibody reactivity			
Tyrosinase antibody-positive	4 (22%)	7 (11%)	0.261[1]
MCHR1 antibody-positive	3 (17%)	11 (18%)	1.000[1]
PAH antibody-positive	0 (0)%	0 (0)%	-
TPH antibody-positive	0 (0)%	0 (0)%	-

[1]*P* value calculated using Fisher's exact test for 2 x 2 contingency tables (Section 2.27) for comparing the prevalence of demographic, clinical and serological features in the TH antibody-positive and TH antibody-negative non-segmental vitiligo patient groups. *P* values <0.05 were considered significant.

[2]*P* value calculated using Mann-Whitney tests (Section 2.27) for comparing the age, onset age and disease duration in the TH antibody-positive and TH antibody-negative non-segmental vitiligo patient groups. *P* values <0.05 were considered significant.

-, *P* values were not determined due to lack of antibody-positive samples.

Table 3.10: Clinical demographic and serological details of non-segmental vitiligo patients with TH antibodies

Patient	Sex	Autoimmune disease	Vitiligo subtype	Disease activity	Age at sample (y)	Onset age (y)	Disease duration (y)	Tyrosinase antibodies[1]		MCHR1 antibodies[1]	
V4	M	-	Symmetrical and peri-orificial	Active	77	70	7	-	0.97 (± 0.07)	-	0.93 (± 0.05)
V8	F	-	Symmetrical with segmental patch	Active	37	10	27	-	1.03 (± 0.09)	-	1.04 (± 0.07)
V9	F	-	Symmetrical	Active	76	40	36	-	0.99 (± 0.10)	+	**3.11** (± 0.29)
V10	M	-	Symmetrical	Active	60	56	4	+	**1.68** (± 0.09)	-	0.47 (± 0.06)
V20	M	Alopecia areata	Symmetrical	Active	70	20	50	-	0.94 (± 0.04)	-	0.59 (± 0.04)
V25	F	-	Symmetrical	Active	19	6	13	-	0.92 (± 0.03)	-	0.43 (± 0.03)
V27	F	-	Symmetrical	Active	23	19	4	+	**1.93** (± 0.05)	-	0.84 (± 0.07)
V30	F	-	Symmetrical	Active	53	52	1	-	1.03 (± 0.08)	-	1.15 (± 0.07)
V33	F	-	Symmetrical	Active	54	51	3	-	0.96 (± 0.06)	-	0.68 (± 0.10)
V39	M	-	Symmetrical	Active	32	13	19	-	0.95 (± 0.01)	-	1.06 (± 0.29)
V62	F	Autoimmune thyroid disease	Symmetrical	Active	27	7	20	-	0.99 (± 0.08)	+	**3.81** (± 0.28)
V63	M	-	Symmetrical	Active	45	44	1	+	**1.59** (± 0.03)	-	1.09 (± 0.10)
V64	M	Psoriasis	Symmetrical	Active	47	5	42	-	1.08 (± 0.07)	-	0.97 (± 0.12)
V69	F	-	Symmetrical and peri-orificial	Active	70	39	31	-	0.98 (± 0.11)	-	0.93 (± 0.11)
V70	F	Autoimmune thyroid disease	Symmetrical and peri-orificial	Active	48	20	28	-	0.94 (± 0.05)	-	0.90 (± 0.04)
V82	F	-	Symmetrical	Active	7	6	1	+	**2.43** (± 0.21)	+	**1.72** (± 0.14)
V86	M	-	Symmetrical	Active	76	35	41	-	1.01 (± 0.02)	-	0.86 (± 0.07)
V87	M	-	Symmetrical and peri-orificial	Active	23	19	4	-	0.96 (± 0.04)	-	0.81 (± 0.07)

[1]+, positive for antibody reactivity; -, negative for antibody reactivity. The mean Ab indices (±SD) are shown.

3.4 Summary of Results

The results indicated that:

- A TH antibody RIA could be used to detect and measure TH antibodies.
- TH antibodies were present in 18/79 (23%) of patients with non-segmental vitiligo.
- Immunoreactivity against TH was not evident in any of the 8 patients with segmental vitiligo.
- Antibodies against TH were not found in patients with other autoimmune diseases or in healthy controls.
- The frequency of TH antibodies was significantly increased in the patient group with active vitiligo compared to the patient cohort with stable disease.
- The frequency of patients with active non-segmental vitiligo was significantly higher in the TH antibody-positive patient group than the TH antibody-negative patient group.

- TH antibodies did not cross-react with tyrosinase or MCHR1, targets of antibodies which can be associated with vitiligo.

- Antibodies against PAH and TPH were not detected in vitiligo patients.

- Antibodies against tyrosinase and MCHR1 were detected at frequencies comparable to those in earlier studies.

- The presence of TH antibodies was not associated with the occurrence of antibodies against either tyrosinase or MCHR1.

3.5 Discussion

The enzyme TH catalyses the conversion of tyrosine to L-dopa, the precursor molecule in catecholamine neurotransmitter biosythesis (Lewis *et al.* 1993; Nagatsu 1995). Antibodies against TH were reported first in APS1 patients, including 57% of those individuals displaying vitiligo as part of their clinical manifestations (Hedstrand *et al.* 2000), although the association was not significant. Initial evidence for its potential as an autoantigen in vitiligo arose from the enrichment of the TH peptide from a melanocyte cDNA expression phage-display library in biopanning experiments with vitiligo patient IgG (Kemp *et al.* 2002; Waterman *et al.* 2010). In the current study, we investigated the prevalence of immunoreactivity to TH in vitiligo patients.

TH antibodies were demonstrated in 23% (18/79) of those individuals with a non-segmental pattern of disease. The TH antibody response was detected using TH2 as the assay ligand, although four isoforms of TH (TH1-TH4) do exist which differ in the N-terminal regulatory domain (Lewis *et al.* 1993; Nakashima *et al.* 2009). It is likely that vitiligo patient TH antibodies also recognise other forms of the enzyme which are highly similar in amino acid sequence (Nagatsu 1995) and, indeed, this has been shown for TH antibodies in APS1 patients (Hedstrand *et al.* 2000).

Although the frequency of TH antibodies in vitiligo patients at 23% appears to be relatively low, earlier studies have detected antibodies to several autoantigens in patients with the disease at a similarly low prevalence: tyrosinase antibodies at 11% (Kemp *et al.* 1997a), TYRP1 antibodies at 5% (Kemp *et al.* 1998c), DCT antibodies at 5% (Kemp *et al.* 1997b), PMEL antibodies at 5% (Kemp *et al.* 1998b), and MCHR1 antibodies at 16% (Kemp *et al.* 2002).

Autoimmunity has been suggested to play a role in the development of non-segmental vitiligo: it is frequently associated with autoimmune disorders (Taïeb 2000; Alkhateeb *et al.* 2003), responds less well to autologous melanocyte grafting (Gauthier and Surleve-Bazeille 1992; Taïeb 2000) and is responsive to treatment with immunosuppressive agents (Lepe *et al.* 2003). In contrast, it has been proposed that segmental vitiligo has an aetiology which does not involve autoimmune pathomechanisms (Taïeb 2000). In keeping with this, TH antibody responses were only detected in non-segmental vitiligo patients. However, samples from only eight segmental vitiligo patients were available for testing, so firm conclusions cannot be drawn as to prevalence of antibody reactivity in this disease sub-type.

Notably, TH was an antibody target in patients with or without concomitant autoimmune disease. This finding contrasts to previous studies in which antibodies directed against melanocyte-specific proteins PMEL, TYRP1 and DCT were only detected in vitiligo patients who had other autoimmune diseases (Kemp *et al.* 1997b; Kemp *et al.* 1998b; Kemp *et al.* 1998c). Tyrosinase and transcription factor SOX10 antibodies were also predominantly detected in vitiligo patients with additional autoimmune disorders (Kemp *et al.* 1997a; Hedstrand *et al.* 2001). In addition, the results demonstrated that the frequency of TH antibodies was significantly increased in the group of non-segmental vitiligo patients with active disease. However, only 15 patients with stable disease were available for study, so further verification of this result is necessary.

A comparison of TH antibody-positive and TH antibody-negative vitiligo patients demonstrated no significant association between the presence of TH antibodies and either disease duration, patient age, patient gender or age at disease onset, although an association with vitiligo activity was noted.

Interestingly, antibodies against TPH and PAH, enzymes involved in neurotransmitter biosynthesis which are closely related to TH, have also been detected in patients with APS1 (Ekwall *et al.* 1999; Ekwall *et al.* 2000). Again, PAH and TPH antibody responses were apparent in APS1 patients with vitiligo, but no association between the incidence of either PAH or TPH antibodies and depigmentation was evident. In this study and in contrast to TH, immunoreactivity to either PAH or TPH was not demonstrated in our vitiligo patient cohort, indicating that these enzymes are not autoantigens in isolated vitiligo and that TH antibodies in vitiligo patients do not cross-react with TPH or PAH. A further enzyme which also functions in the production of neurotransmitters, namely aromatic amino acid decarboxylase, is an additional target of the antibody response in APS1 patients where it does significantly associate with the presence of vitiligo (Husebye *et al.* 1997). It would therefore be of interest to determine if immune responses to aromatic amino acid decarboxylase occur in patients with isolated vitiligo and, if so, whether or not this correlates with the presence of TH antibodies.

Due to the intracellular location of the enzyme, TH antibodies most probably arise by exposure of the antigen following damage to melanocytes by oxidative stress (Kroll *et al.* 2005; Schallreuter *et al.* 2005; Dell'Anna and Picardo 2006; Shalbaf *et al.* 2008) or cytotoxic T cells (Ogg *et al.* 1998; Lang *et al.* 2001; Palermo *et al.* 2001; van den Boorn *et al.* 2009). In this

respect, it is interesting that a significantly increased frequency of TH antibodies occurred in patients with actively progressing vitiligo lesions. Several possibilities including the formation of neo-antigens, the exposure of cryptic epitopes and the modification of proteins during apoptosis have been suggested to account for immune responses to intracellular melanocyte antigens (Namazi 2007; Westerhof and d'Ischia 2007). Following processing by mature Langerhans cells, antigenic peptides could be presented to autoreactive T cells which have escaped clonal deletion or to naïve T lymphocytes which have not been tolerised against cryptic epitopes (Namazi 2007; Westerhof and d'Ischia 2007). Antibodies could then be produced following the stimulation of autoreactive B lymphocytes by activated autoreactive CD4+ T cells (Namazi 2007) and activated autoreactive cytotoxic T cells could directly attack melanocytes expressing antigenic peptides on their surface in the context MHC class I molecules (Hedley *et al.* 1998; Namazi 2007; Westerhof and d'Ischia 2007). Indeed, many studies have underlined the role of cytotoxic T cells in the initiation and progression of vitiligo: melanocyte-specific T lymphocytes against tyrosinase, MelanA (MART-1) and PMEL are present in perilesional vitiligo skin (Ogg *et al.* 1998; Lang *et al.* 2001; Palermo *et al.* 2001; van den Boorn *et al.* 2009), melanocyte-specific T lymphocytes can have cytotoxic activity against pigment cells (Ogg *et al.* 1998; Lang *et al.* 2001; Palermo *et al.* 2001; van den Boorn *et al.* 2009), and melanocyte-specific T lymphocytes can have the capacity to destroy melanocytes within skin tissue (van den Boorn *et al.* 2009). With regard to this, studies to identify T cell reactivity to TH would be of interest.

With limited access to the target antigen within viable cells, TH antibodies are unlikely to have any adverse effects upon the function of TH and, although the destruction of melanocytes by vitiligo-associated antibodies has been reported involving either complement-mediated mechanisms (Norris *et al.* 1988) or ADCC (Norris *et al.* 1988; Gilhar *et al.* 1995), TH antibodies may only serve as markers for melanocyte disruption and/or a cellular immune response against TH.

In summary, TH has been identified as a novel B cell autoantigen in vitiligo which needs to be further characterised.

CHAPTER 4

4. Mapping of the B cell epitopes of vitiligo patient TH antibodies

4.1 Introduction

4.1.1 Antibody-antigen interactions

In order for binding to take place between an antibody and its antigen, there must be complementarity between surface structures on the antigen and the combining site of the antibody. The precise region of the antibody's combining site which contacts with the antigen is termed the paratope, and the part of the antigen recognised by the paratope is termed the epitope. Antigens are highly diverse in size and primary sequence, as well as in their secondary structure which can be adapted by post-translational modifications such as glycosylation (Westwood and Hay 2001). Consequently, antigenic epitopes are extremely varied but can be loosely classified in two categories as: (i) linear or continuous epitopes which constitute part of a linear amino acid sequence on a polypeptide chain, and (ii) conformational or discontinuous epitopes which are formed by two or more stretches of the primary sequence that are distant from each other, but are brought together in the folded secondary or tertiary structure of the antigen. The size of an epitope is subject to controversy, but it is thought that linear epitopes can range in length from 5-6 amino acids (Roitt and Delves 1997) to up to 15-22 amino acids (von Mikecz *et al.* 1995) and that conformational epitopes can consist of approximately discontinuous 16 amino acid side-chains (Roitt and Delves 1997). The specificity of a particular antibody is not absolute, given that both paratope and epitope are deformable, and there is the possibility of an antibody binding to multiple cross-reactive epitopes.

In contrast to the processed linear epitopes presented to T cells, B lymphocytes usually recognise antigen in the context of the native molecule. The most likely areas of a protein to contain B cell epitope regions are, therefore, parts of peptide chains that protrude from the globular surface. These external structures are likely to be hydrophilic. Using this information, and the fact that some amino acids are thought to be more antigenic than others (Westwood and

Hay 2001), it is possible to predict areas of probable antigenicity on a particular protein. However, in order to determine the exact location of epitopes on an antigen, experimental epitope mapping techniques need to be applied.

4.1.2 Methods used for mapping B cell epitopes

The techniques currently available for mapping linear and conformational B cell epitopes are summarised in Table 4.1. The most precise available method of defining the area of an antigen making contact with an antibody is x-ray crystallography of the antigen-antibody complex. This technique has the advantage of allowing the study of the three-dimensional structures and, therefore, can yield information about conformational epitopes. However, it is costly and requires sufficiently large quantities of a pure monoclonal antibody (or fragment of) and its antigen to produce crystal.

When the primary amino acid sequence of the antigen is known, there are two main approaches to B cell epitope mapping. The first method relies on molecular manipulation at the cDNA level using techniques such as PCR, site-directed mutagenesis, and restriction with DNA exonuclease and endonucleases. Substitutions and/or deletions are created in the amino acid sequence followed by comparative analysis of the immunoreactivity of these recombinant proteins by either immunoblotting, immunoprecipitation or ELISA (Pettersson 1992). Examples of studies in which B cell epitopes have been identified in autoimmune disease using recombinant proteins are given in Table 4.2. The second technique begins at the peptide level and involves the analysis of antibody reactivity to either synthetic peptides or enzymatically digested protein fragments (Pettersson 1992). However, both these methods can only accurately detect linear epitopes and short sequences that may constitute part of a conformational epitope.

More recently, the use of phage-display technology has allowed the identification of conformational peptide epitopes (Williams *et al.* 2001), which can also be defined using chimeric molecules (Hu *et al.* 2007).

Table 4.1: Techniques for mapping linear and conformational B cell epitopes

Technique	Suitable for type/s of epitope	Reference
X-RAY CRYSTALLOGRAPHY[1]	Conformational and linear	(Amit *et al.* 1986)
PHAGE-DISPLAY[2] i) Random-peptide library ii) Single chain Fv[3] fragment (scFv) library	Conformational and linear	(Williams *et al.* 2001)
SYNTHETIC PEPTIDES[4] i) Peptide synthesiser (Generates peptides >10 amino acid residues) ii) 'PEPSCAN'[5] (Peptides ≤ 10 amino acid residues)	Mostly linear	(Pettersson 1992) (Sumar 2001)
PEPTIDE FRAGMENTS[6] Derived enzymatically from the whole protein	Mostly linear	(Pettersson 1992)
RECOMBINANT PROTEIN[7] Subcloning and expression[8] of cDNA encoding peptide fragments generated by: i) Naturally occurring restriction sites ii) Exonuclease digestion iii) Polymerase chain reaction (PCR) iv) Site-directed mutagenesis	Mostly linear	(Pettersson 1992) (Perdue 2001)

[1]Allows direct analysis of the 3-dimensional structure of an antigen-antibody complex.

[2]Epitope regions are determined by affinity selection screening with either (i) antibody, or (ii) antigen.

[3]Fv, variable region of the fragment with antigen binding (Fab) of an immunoglobulin.

[4]Can be used in a number of different assay systems including enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation using monoclonal antibody (mAb) or polyclonal sera.

[5]'PEPSCAN' involves the synthesis of short overlapping peptide sequences on to solid supports (polystyrene pins) which are then used in a modified ELISA.

[6]Can be used in a number of different assay systems including ELISA and immunoprecipitation using mAb or polyclonal sera.

[7]Can be used in a number of different assay systems including immunoblotting, ELISA, immunoprecipitation and radioimmunoassays (RIA) using mAb or polyclonal sera.

[8]A variety of expression vectors/systems may be used. Vectors may fuse a tag to the protein for purification, or allow the incorporation of a radiolabelled amino acid into the recombinant protein for RIA.

Table 4.2: Examples of previous studies employing recombinant proteins to map B cell epitopes in autoimmune disease

Antigen	Disease	Techniques	Reference
Thyroid-stimulating hormone receptor	Graves' disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria. Immunoprecipitation experiments with patient sera.	(Burch *et al.* 1993)
Steroid 17α-hydroxylase	Autoimmune Addison's disease	Subcloning of endonuclease restricted cDNA fragments and exonuclease digestion. Expressed in bacteria and immunoblotted with patient sera.	(Peterson and Krohn 1994)
Steroid 21-hydroxylase	Autoimmune Addison's disease	Subcloning of endonuclease restricted cDNA fragments. Site-directed mutagenesis. Expressed in bacteria. Immunoprecipitation experiments with patient sera.	(Volpato *et al.* 1998)
Ribosomal protein L7	Systemic lupus erythematosus and mixed connective tissue disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria and immunoblotted with patient sera. Also immunoprecipitation experiments with patient sera.	(von Mikecz *et al.* 1995)
Glutamate decarboxylase	Type 1 diabetes mellitus	Subcloning of endonuclease restricted cDNA fragments and cDNA amplified by PCR. Expressed in bacteria. Immunoprecipitation experiments with patient sera.	(Daw *et al.* 1996)
'BP 180' (collagen XVII)	Bullous pemphigoid	Subcloning of cDNA amplified by PCR. Expressed in bacteria and immunoblotted with patient sera.	(Lin *et al.* 1999)
Sodium iodide symporter	Autoimmune thyroid disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria. Immunoprecipitation experiments with patient sera.	(Kemp *et al.* 2001)

4.1.3 The role of studying autoantigen B cell epitopes in autoimmune disease

Mapping of B cell epitopes on autoantigens can provide an understanding of the association of an autoantigen with autoimmune pathogenesis. For example, epitopes may reside in functional domains of autoantigenic proteins. Indeed, autoantibodies in myasthenia gravis, directed against the acetylcholine receptor, are known to bind to an epitope in the acetylcholine-binding site which can directly inhibit the receptor function (Holbrook *et al.* 1989). Similarly, the autoantigen epitopes in systemic lupus erythematosus have been found to reside in highly conserved regions of proteins and can thus inhibit functions of both structural proteins and enzymes (Casiano and Tan 1996). For example, anti-nuclear antibodies targeting tRNA synthetase and DNA polymerase delta auxillary protein have been shown to functionally inhibit the aminoacylation of tRNA and DNA replication and repair, respectively, in *in vitro* studies (Tan *et al.* 1994).

Epitope mapping may provide insights into the initiation of the autoimmune process. Autoimmunity may be triggered by an initial infection with a foreign pathological organism (Wucherpfennig 2001). One of the mechanisms by which this might induce immune disease is the phenomenon of molecular mimicry, in which a microbial peptide has sufficient structural similarity with a self-peptide to evoke a cross-reactive autoimmune response. For example, similarities between epitopes on the parasite *Trypanosoma cruzi* and the cardiac muscle protein myosin cause the aberrant immune response in Chagas' disease (Roitt and Delves 1997). In addition, bacterial and viral proteins have homology with endothelial cell components which can be damaged by cross-reacting antibodies resulting from infections (Luo *et al.* 2010; Liu *et al.* 2011).

As well as providing insights into the mechanisms of autoimmune pathogenesis, the molecular characterisation of B cell epitopes can also allow for new and more specific assays to be established (Lernmark 2001). For example, if the epitope of a pathogenic antibody can be precisely defined, recombinant proteins containing only the significant epitope region could be used to measure titres of pathogenic antibodies within the heterogeneous antibody population of a patient's serum (Ishii *et al.* 1997).

Finally, B cell epitope mapping studies have given interesting information with regard to the overlapping binding sites of several autoimmune disease-specific antibodies which otherwise have different functionalities (Kemp *et al.* 2010; Majumdar *et al.* 2012). Examples include

antibodies against the thyroid-stimulating hormone and calcium-sensing receptors (Kemp *et al.* 2010; Majumdar *et al.* 2012). In the case of thyroid peroxidase, a major autoantigen in autoimmune thyroid disease, epitope identification has also subsequently enabled the characterisation of thyroid peroxidase antibodies with respect to their restricted light chain gene usage (McIntosh *et al.* 1997).

4.1.4 TH antibodies in vitiligo

The previous chapter of this study described the identification of antibodies against TH in vitiligo patients. In this part of the study, the binding sites (epitopes) of TH antibodies were to be determined as this might give a better understanding of the association of the humoral immune response against TH with vitiligo.

4.2 Aims

The aims of this part of the study were:

- To construct a series of deletion derivatives of TH cDNA using PCR amplification and subcloning.

- To express the TH cDNA deletion derivatives in an *in vitro* transcription-translation system to produce radiolabelled TH fragments.

- To investigate the immunoreactivity of vitiligo patient sera against the TH fragments using RIAs and so determine the antibody binding sites (epitopes) on TH.

- To confirm immunoreactivity of vitiligo patient sera against any identified TH epitopes by using ELISAs with synthetic TH peptides containing the putative TH antibody binding site(s).

4.3 Experiments and Results

4.3.1 Patients and controls

Participants were 18 vitiligo patients (8 male, 10 female; mean age, 47 years with range 7-77 years; mean disease duration, 18 years with range 1-50 years; mean age at disease onset 28 years with range 5-70 years) with active, non-segmental disease and TH antibodies (Chapter 3). Fourteen patients had no other autoimmune disorder and no family history of autoimmune disease. Four patients had one other autoimmune disorder: autoimmune thyroid disease 2; alopecia areata, 1; psoriasis, 1. Twenty-eight healthy individuals (10 male, 18 female; mean age, 34 years; age range 21-59 years) were used as controls.

4.3.2 Construction of a series of deletion derivatives of TH cDNA

Thirteen TH cDNA fragments of varying lengths were generated from TH cDNA present in pcDNA3-TH (Figure 2.2) by PCR amplification. The required TH cDNA fragments and the oligonucleotide primers used to generate them are shown in Table 4.3. Restriction sites for *Hin*dIII and *Bam*HI were incorporated into the 5' and 3' oligonucleotide primers, respectively, in order to allow cloning of the PCR amplification products between the *Hin*dIII and *Bam*HI restriction enzyme sites in pcDNA3 (Figure 2.1).

4.3.2.1 PCR amplification of fragments of TH cDNA

The composition of each PCR amplification reaction was as previously described (Section 2.12), and contained 50 ng of pcDNA3-TH DNA which were subjected to 30 cycles of amplification in a DNA Thermal Cycler using the following conditions: 95^{o}C, 1 min; 55^{o}C, 1 min; 72^{o}C, 2 min; and 72^{o}C for 10 min to terminate the reaction (Section 2.12). To check for the correctly sized PCR products, a 5-µl aliquot of each PCR amplification reaction was electrophoresed in a 0.8% (w/v) agarose gel (Section 2.10). The results are shown in Figures 4.1a-d. In all cases, products of the estimated size in base pairs were visible.

Subsequently, each of the 13 PCR amplification products was purified from a 0.8% (w/v) agarose gel using a Wizard PCR Preps DNA Purification System (Section 2.13). The total of

Table 4.3: Oligonucleotide primers used to generate TH cDNA fragments by PCR amplification

TH base pairs[1] (size in base pairs)	Primer name	Primer sequence[2]
1-420 (420)	THF1 THR140	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**gtcccctcggcgcac-3'
1-510 (510)	THF1 THR170	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**cacttttcttgggaa-3'
1-600 (600)	THF1 THR200	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**gcggtacacctggtc-3'
1-720 (720)	THF1 THR240	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**cttcagcgtggtgta-3'
1-840 (840)	THF1 THR280	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**cttcaggaagcggga-3'
1-960 (960)	THF1 THR320	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**gggcgaggacgcgtg-3'
1-1080 (1080)	THF1 THR360	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**atccgaggcccccag-3'
1-1200 (1200)	THF1 THR400	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**caggagctccccgta-3'
1-1320 (1320)	THF1 THR440	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**ggcgtcactgaagct-3'
1-1440 (1440)	THF1 THR480	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**ctccagggagcgccg-3'
1-240 (240)	THF1 THR80	5'-tt<u>aagctt</u>gccgcc**atg**cccacccccgacgcc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**ggccttcccctcctt-3'
241-420 (180)	THF81 THR140	5'-tt<u>aagctt</u>gccgcc**atg**gtgctaaacctgctc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**gtcccctcggcgcac-3'
421-1491 (1071)	THF141 THR497	5'-tt<u>aagctt</u>gccgcc**atg**atgctggccgccctgctc-3' 5'-aa<u>ggatcc</u>cgc**ctatca**gccaatggcactcag-3'

[1]Numbers correspond to the base pair of TH cDNA with the A residue of the initiating ATG codon assigned as base pair number one.

[2]The *Hin*dIII and *Bam*HI restriction sites are underlined. The translation initiation codon (ATG) and the translation termination codons (TGA and TAG) in the forward (F) and reverse (R) primers, respectively, are shown in bold-type face.

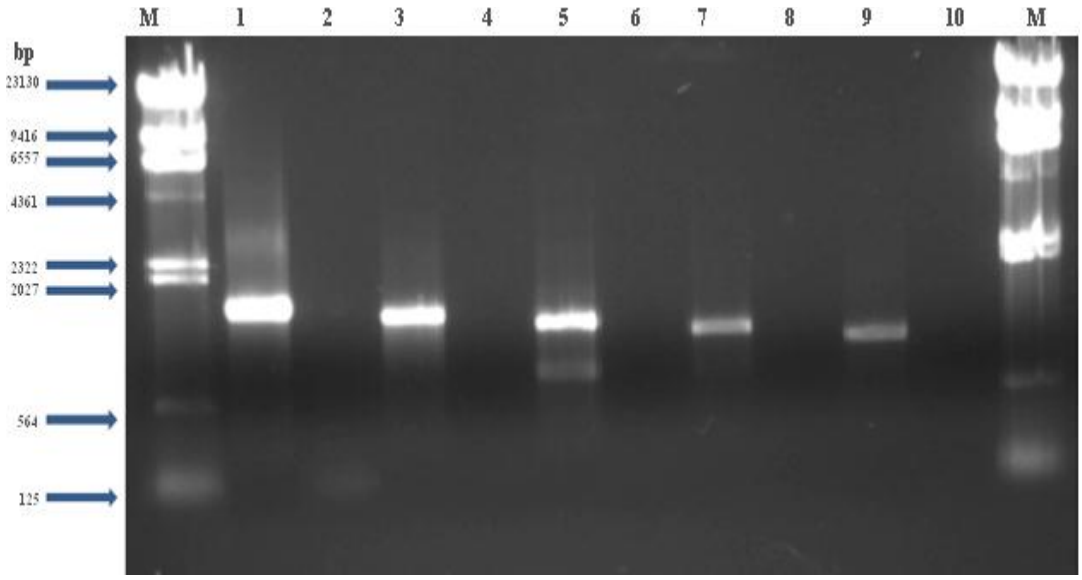


Figure 4.1a: Agarose gel of PCR amplification products amplified from pcDNA3-TH.

Plasmid pcDNA3-TH was subjected to PCR amplification using appropriately designed oligonucleotide primers. The PCR amplification products were analysed by electrophoresis in a 0.8% (w/v) agarose gel. The gel shows: Lambda DNA/*Hin*dIII Markers with DNA fragments from 125 to 23,130 bp (lane M); Plasmid pcDNA3-TH amplified with primers THF1 and THR480 (lane 1); Control - no DNA with primers THF1 and THR480 (lane 2); Plasmid pcDNA3-TH amplified with primers THF1 and THR440 (lane 3); Control - no DNA with primers THF1 and THR440 (lane 4); Plasmid pcDNA3-TH amplified with primers THF1 and THR400 (lane 5); Control - no DNA with primers THF1 and THR400 (lane 6); Plasmid pcDNA3-TH amplified with primers THF1 and THR360 (lane 7); Control - no DNA with primers THF1 and THR360 (lane 8); Plasmid pcDNA3-TH amplified with primers THF1 and THR320 (lane 9); Control - no DNA with primers THF1 and THR320 (lane 10).

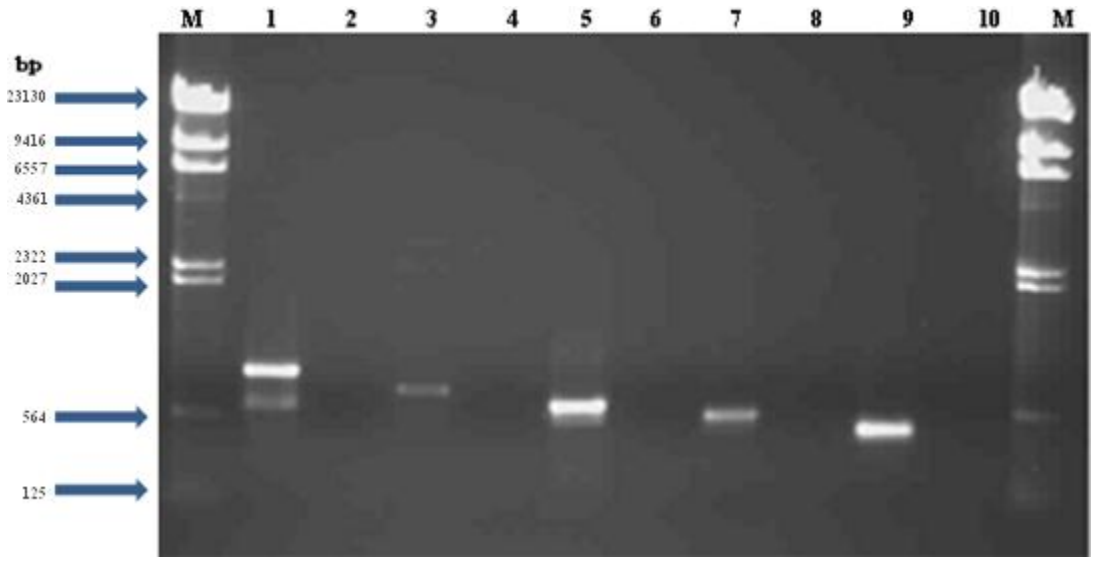


Figure 4.1b: Agarose gel of PCR amplification products amplified from pcDNA3-TH.

Plasmid pcDNA3-TH was subjected to PCR amplification using appropriately designed oligonucleotide primers. The PCR amplification products were analysed by electrophoresis in a 0.8% (w/v) agarose gel. The gel shows: Lambda DNA/*Hin*dIII Markers with DNA fragments from 125 to 23,130 bp (lane M); Plasmid pcDNA3-TH amplified with primers THF1 and THR280 (lane 1); Control - no DNA with primers THF1 and THR280 (lane 2); Plasmid pcDNA3-TH amplified with primers THF1 and THR240 (lane 3); Control - no DNA with primers THF1 and THR240 (lane 4); Plasmid pcDNA3-TH amplified with primers TH-F1 and THR200 (lane 5); Control - no DNA with primers THF1 and THR200 (lane 6); Plasmid pcDNA3-TH amplified with primers THF1 and THR170 (lane 7); Control - no DNA with primers THF1 and THR170 (lane 8); Plasmid pcDNA3-TH amplified with primers TH-F1 and TH-R140 (lane 9); Control - no DNA with primers THF1 and THR140 (lane 10).

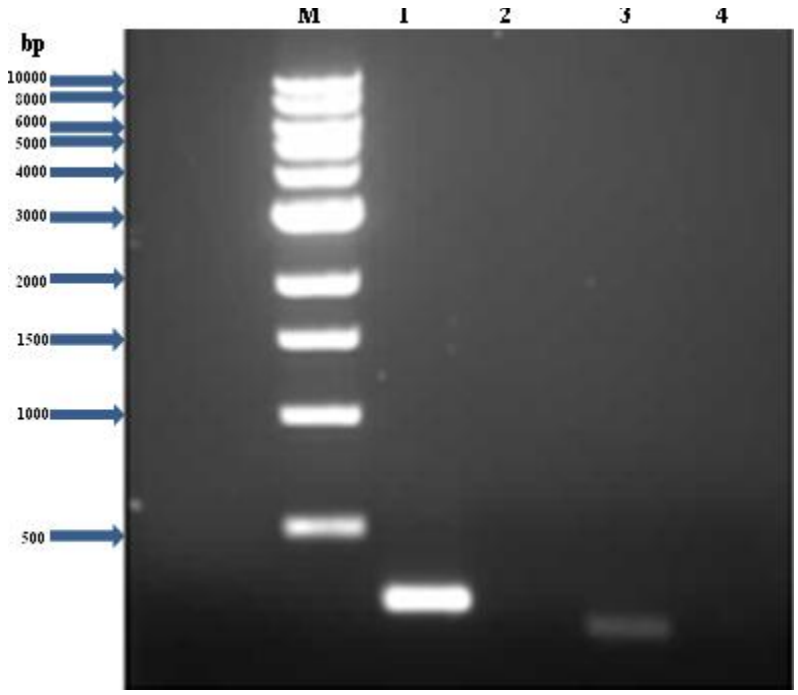


Figure 4.1c: Agarose gel of PCR amplification products amplified from pcDNA3-TH.

Plasmid pcDNA3-TH was subjected to PCR amplification using appropriately designed oligonucleotide primers. The PCR amplification products were analysed by electrophoresis in a 0.8% (w/v) agarose gel. The gel shows: 1-kb DNA Markers with DNA fragments from 500 to 10,000 bp (M); Plasmid pcDNA3-TH amplified with primers THF1 and THR80 (lane 1); Control - no DNA with primers THF1 and THR80 (lane 2); Plasmid pcDNA3-TH amplified with primers THF81 and THR140 (lane 3); Control - no DNA with primers THF81 and THR140 (lane 4).

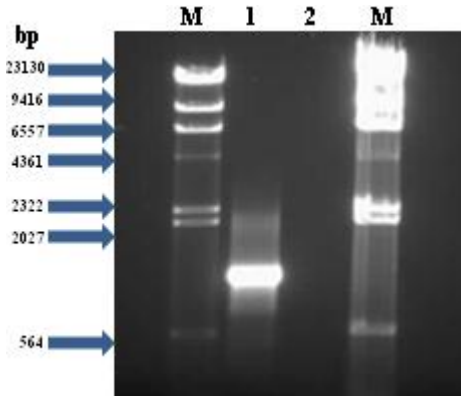


Figure 4.1d: Agarose gel of PCR amplification product amplified from pcDNA3-TH.

Plasmid pcDNA3-TH was subjected to PCR amplification using appropriately designed oligonucleotide primers. The PCR amplification products were analysed by electrophoresis in a 0.8% (w/v) agarose gel. The gel shows: Lambda DNA/*Hin*dIII Markers with DNA fragments from 500 to 23,130 bp (lane M); Plasmid pcDNA3-TH amplified with primers THF141 and THR497 (lane 1); Control - no DNA with primers THF141 and THR497 (lane 2).

each of the recovered products was restricted in a 40-µl reaction with *Hin*dIII and *Bam*HI (Section 2.11) and then re-purified from a 0.8% (w/v) agarose gel using a Wizard PCR Preps DNA Purification System. Finally, a 3-µl aliquot of each restricted and purified product was analysed by agarose gel electrophoresis in a 0.8% (w/v) gel, and then stored at -20^{o}C ready for cloning into pcDNA3.

4.3.2.2 Preparation of vector pcDNA3

For cloning of the PCR products, 1 µg of vector pcDNA3 was digested with *Hin*dIII and *Bam*HI in a 25-µl reaction. The linearised vector was purified from a 0.8% (w/v) agarose gel using a Wizard PCR Preps DNA Purification System. A 3-µl aliquot of the restricted and purified vector was checked on a 0.8% (w/v) agarose gel, before storage at -20^{o}C.

4.3.2.3 Cloning of TH cDNA fragments into pcDNA3

The *Hin*dIII and *Bam*HI-restricted TH cDNA fragments were cloned into *Hin*dIII and *Bam*HI-digested pcDNA3 by setting up 20-µl ligation reactions as described in Section 2.14. A 5-µl aliquot of each ligation reaction was then used to transform 50 µl of chemically competent *E. coli* JM109 cells (Section 2.15), with transformants being selected on LB agar (Section 2.5) containing ampicillin at 100 µg/ml (Section 2.6). Following overnight incubation at 37^{o}C, 10 individual bacterial colonies from each transformation were purified by streaking on to fresh LB agar containing ampicillin at 100 µg/ml and subsequently incubating at 37^{o}C overnight.

4.3.2.4 Screening of E. coli JM109 transformants for recombinant plasmids

For screening of *E. coli* JM109 transformants for recombinant plasmids that contained the correct TH cDNA fragment, 10 individual bacterial clones from each transformation were inoculated separately into 10 ml of LB with ampicillin at 100 µg/ml, and then incubated at 37^{o}C overnight. Plasmid DNA was purified from individual transformants using a Wizard Miniprep DNA Purification System (Section 2.8). Plasmids were then checked for the presence of the correctly sized DNA insert by restriction enzyme digestion of a 0.5-µg sample with *Hin*dIII and *Bam*HI in a 25-µl reaction, followed by analysis by electrophoresis in 0.8% (w/v) agarose gels.

The frequency of recombinant plasmids for each cloning experiment was 100%, with 10/10 plasmids having a DNA insert. One recombinant plasmid from each of the cloning experiments is shown in an agarose gel in Figures 4.2a-e, which illustrate that a DNA insert at approximately the correct size in base pairs was released from the plasmids after restriction with both *Hin*dIII and *Bam*HI. The recombinant plasmids are listed in Table 4.4.

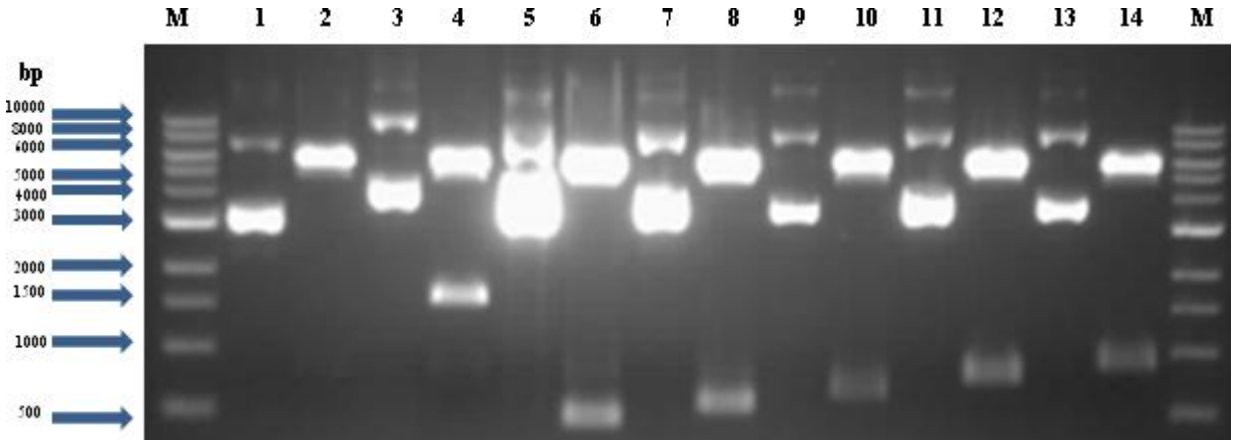


Figure 4.2a: Agarose gel of pcDNA3-TH deletion plasmids.

The pcDNA3-TH deletion plasmids were restricted with *Hin*dIII and *Bam*HI to show the presence of a TH cDNA insert. The gel shows: 1-kb DNA Markers with DNA fragments from 500-10,000 bp (lane M); unrestricted pcDNA3 (lane 1); *Hin*dIII and *Bam*HI-restricted pcDNA3 (lane 2); unrestricted pcDNA3-TH (lane 3); *Hin*dIII and *Bam*HI-restricted pcDNA3-TH (lane 4); unrestricted pTH140 (lane 5); *Hin*dIII and *Bam*HI-restricted pTH140 (lane 6); unrestricted pTH170 (lane 7); *Hin*dIII and *Bam*HI-restricted pTH170 (lane 8); unrestricted pTH200 (lane 9); *Hin*dIII and *Bam*HI-restricted pTH200 (lane 10); unrestricted pTH240 (lane 11); *Hin*dIII and *Bam*HI-restricted pTH240 (lane 12) ; unrestricted pTH280 (lane 13); *Hin*dIII and *Bam*HI-restricted pTH280 (lane 14).

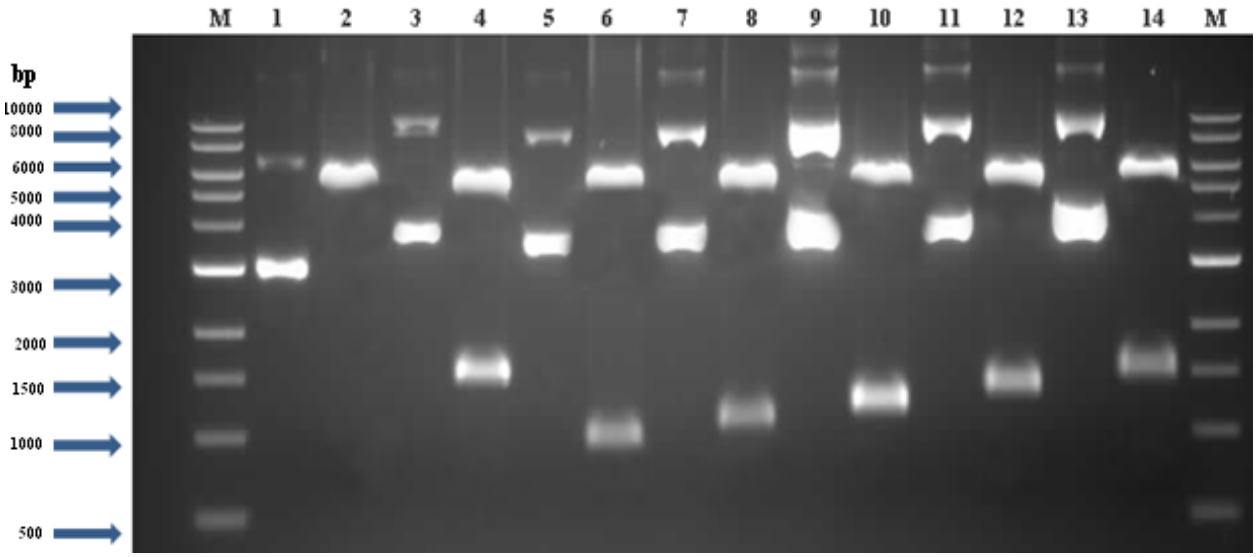


Figure 4.2b: Agarose gel of pcDNA3-TH deletion plasmids.

The pcDNA3-TH deletion plasmids were restricted with *Hin*dIII and *Bam*HI to show the presence of a TH cDNA insert. The gel shows: 1-kb DNA Markers with DNA fragments from 500 to 10,000 bp (lane M); unrestricted pcDNA3 (lane 1); *Hin*dIII and *Bam*HI-restricted pcDNA3 (lane 2); unrestricted pcDNA3-TH (lane 3); *Hin*dIII and *Bam*HI-restricted pcDNA3-TH (lane 4); unrestricted pTH320 (lane 5); *Hin*dIII and *Bam*HI-restricted pTH320 (lane 6); unrestricted pTH360 (lane 7); *Hin*dIII and *Bam*HI-restricted pTH360 (lane 8); unrestricted pTH400 (lane 9); *Hin*dIII and *Bam*HI-restricted pTH400 (lane 10); unrestricted pTH440 (lane 11); *Hin*dIII and *Bam*HI-restricted pTH440 (lane 12); unrestricted pTH480 (lane 13); *Hin*dIII and *Bam*HI-restricted pTH480 (lane 14).

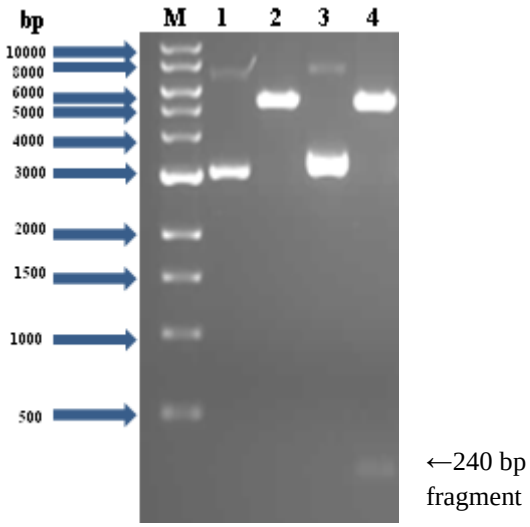


Figure 4.2c: Agarose gel of pcDNA3-TH deletion plasmid.

The pcDNA3-TH deletion plasmid was restricted with *Hin*dIII and *Bam*HI to show the presence of a TH cDNA insert. The gel shows: 1-kb DNA Markers with DNA fragments from 500 to 10,000 bp (lane M); unrestricted pcDNA3 (lane 1); *Hin*dIII and *Bam*HI-restricted pcDNA3 (lane 2); unrestricted pTH1-80 (lane 3); *Hin*dIII and *Bam*HI-restricted pTH1-80 (lane 4).

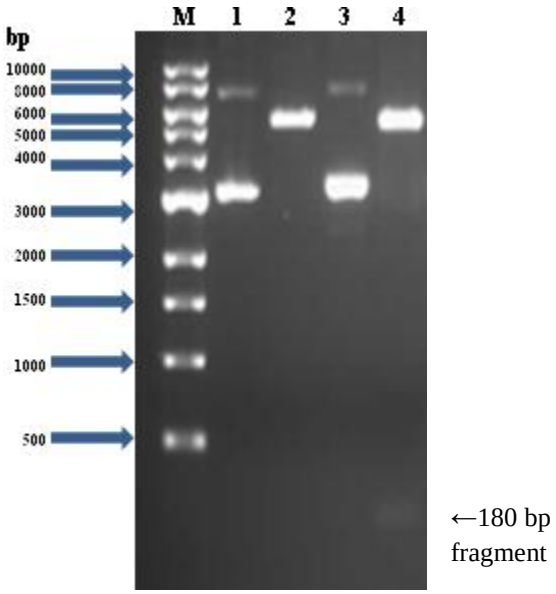


Figure 4.2d: Agarose gel of pcDNA3-TH deletion plasmid.

The pcDNA3-TH deletion plasmid was restricted with *Hin*dIII and *Bam*HI to show the presence of a TH cDNA insert. The gel shows: 1-kb DNA Markers with DNA fragments from 500 to 10,000 bp (lane M); unrestricted pcDNA3 (lane 1); *Hin*dIII and *Bam*HI-restricted pcDNA3 (lane 2); unrestricted pTH81-140 (lane 3); *Hin*dIII and *Bam*HI-restricted pTH81-140 (lane 4).

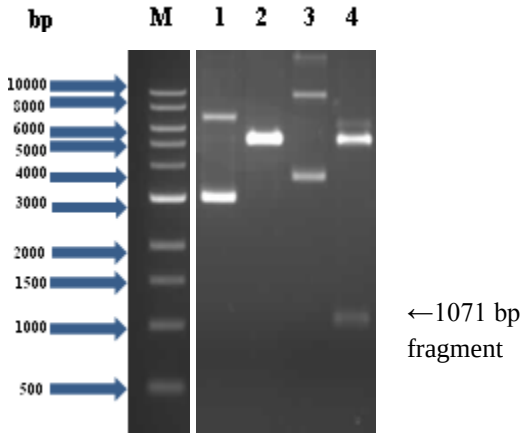


Figure 4.2e: Agarose gel of pcDNA3-TH deletion plasmid.

The pcDNA3-TH deletion plasmid was restricted with *Hin*dIII and *Bam*HI to show the presence of a TH cDNA insert. The gel shows: 1-kb DNA Markers with DNA fragments from 500 to 10,000 bp (lane M); unrestricted pcDNA3 (lane 1); *Hin*dIII and *Bam*HI-restricted pcDNA3 (lane 2); unrestricted pTH141-497 (lane 3); *Hin*dIII and *Bam*HI-restricted pTH141-497 (lane 4).

Table 4.4: Details of recombinant plasmids from cloning of TH cDNA fragments into vector pcDNA3

Plasmid	TH amino acids encoded[1] (base pairs included[2])
pTH140	1-140 (1-420)
pTH170	1-170 (1-510)
pTH200	1-200 (1-600)
pTH240	1-240 (1-720)
pTH280	1-280 (1-840)
pTH320	1-320 (1-960)
pTH360	1- 360 (1-1080)
pTH400	1- 400 (1-1200)
pTH440	1-440 (1-1320)
pTH480	1- 480 (1-1440)
pTH1-80	1-80 (1-240)
pTH81-140	81-140 (241-420)
pTH141-497	141-497 (421-1491)

[1]Numbers correspond to the amino acid residues of TH with the initiating methionine as residue number one.

[2]Numbers correspond to the base pair of TH cDNA with the A residue of the initiating ATG codon assigned as base pair number one.

4.3.2.5 Sequencing of cloned TH cDNA fragments

As a final check that the recombinant plasmids were correct and to verify that no sequence errors had been introduced to the TH cDNA fragment during PCR amplification, all plasmids were subjected to DNA sequencing (Section 2.16) using primers T7, SP6, THR1, THF400, THF839 and THR1239, as required (Table 2.6). The results verified that each recombinant plasmid contained the correct TH cDNA fragment when compared with the sequence of full-length TH cDNA (Appendices I and II) using the ClustalW2 Multiple Sequence Alignment tool at the network facilities of the EBI-EMBL (Section 2.17).

4.3.3 *In vitro* transcription-translation of TH cDNA deletion derivatives

Each TH cDNA fragment was inserted in pcDNA3 in the correct orientation to allow expression of the encoded TH peptide from the T7 promoter (Figure 2.1). Each DNA template also contained appropriate start and stop codons to ensure accurate translation. To produce and label TH deletion derivatives with [^{35}S]-methionine for use in RIAs, the plasmid constructs listed in Table 4.4 were used in a TnT® T7-Coupled Reticulocyte Lysate System (Section 2.18). Full-length TH was also produced and radiolabelled by using pcDNA3-TH in TnT® T7-Coupled Reticulocyte Lysate System. Transcription-translation reactions were stored at –20^{o}C until needed.

To analyse the radiolabelled products qualitatively, a 5-µl aliquot of each the *in vitro* transcription-translation reactions was added to 20 µl of SDS-sample buffer (Section 2.19) and heated to 85^{o}C for 5 min before analysing a 10-µl sample by SDS-PAGE in a SDS-polyacrylamide gel with subsequent autoradiography (Section 2.19).

The results revealed a major band representing the [^{35}S]-labelled TH protein fragment (Figure 4.3 and Table 4.5). The estimated molecular weight of each TH protein fragment agreed well with the molecular weight predicted from the amino acid sequence of the deleted protein (Table 4.5).

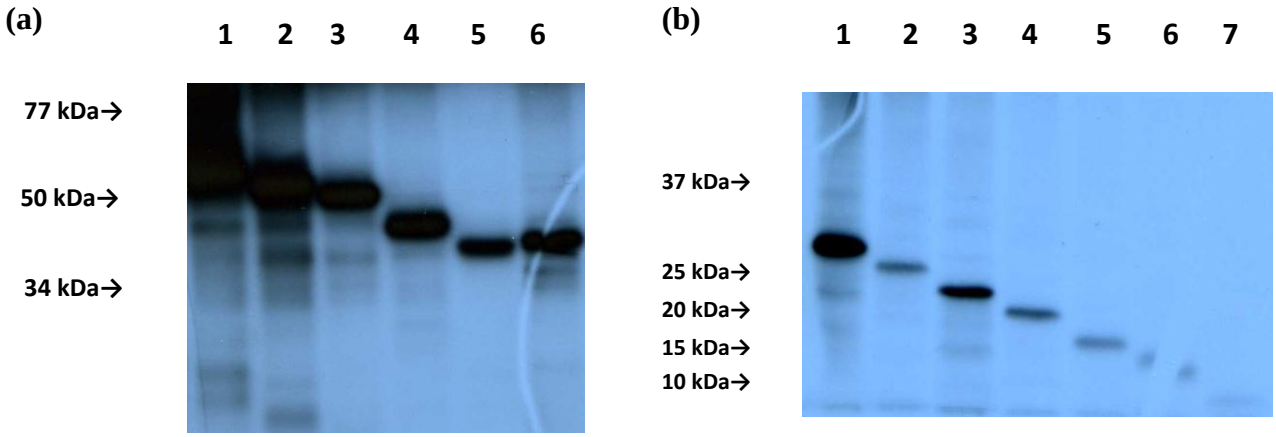


Figure 4.3: SDS-polyacrylamide gel electrophoresis and autoradiography of products arising from *in vitro* translation of TH cDNA deletion derivatives.

TH cDNA deletion derivatives were translated *in vitro* in a TnT® T7 Coupled Reticulocyte Lysate System from the appropriate plasmid. Subsequently, 5 µl of the reaction were added to 20 µl of SDS sample buffer, boiled for 5 min, and 10 µl of this mixture were then analysed by SDS-PAGE in a 12.5 or 15% (w/v) SDS-polyacrylamide gel. The results are shown for: **(a)** Deletion derivatives of TH *in vitro* translated from: pTH480 (lane 1); pTH440 (lane 2); pTH400 (lane 3); pTH360 (lane 4); pTH320 (lane 5); pTH141-497 (lane 6). Prestained SDS-PAGE Standards, Low Range (Bio-Rad Ltd.) are indicated. **(b)** pTH280 (lane 1); pTH240 (lane 2); pTH200 (lane 3); pTH170 (lane 4); pTH140 (lane 5); pTH1-80 (lane 6); pTH81-140 (lane 7). Precision Plus Protein All Blue Standards (Bio-Rad Ltd.) are indicated. These gels were run and processed by Dr. Helen Kemp (Department of Human Metabolism, University of Sheffield, Sheffield, UK).

Table 4.5: TH protein fragments encoded by recombinant plasmids

Plasmid	Protein	Predicted molecular weight of protein[1] (kD[2])	Estimated molecular weight of protein[3] (kD[2])
pTH140	TH140	15.4	16
pTH170	TH170	18.7	20
pTH200	TH200	22.0	23
pTH240	TH240	26.4	27
pTH280	TH280	30.8	32
pTH320	TH320	35.2	38
pTH360	TH360	39.6	41
pTH400	TH400	44.0	45
pTH440	TH440	48.4	51
pTH480	TH480	52.8	53
pTH1-80	TH1-80	8.8	10
pTH81-140	TH81-140	6.6	8
pTH141-497	TH141-497	39.3	40

[1]Predicted from the amino acid sequence of the protein.

[2]kD, kilodalton.

[3]Estimated from the mobility of the protein in SDS-polyacrylamide gels.

4.3.4 Radioimmunoassays with TH fragments and vitiligo patient and control sera

To localise the antibody epitope regions on TH, radiolabelled TH fragments containing amino acids 1-140, 1-170, 1-200, 1-240, 1-280, 1-320, 1-360, 1-400, 1-440, 1-480 and 141-497 (Section 4.3.3) were used in RIAs (Section 2.20) to test the immunoreactivity of sera from 18 TH antibody-positive vitiligo patients and 28 healthy controls. All sera were assayed in duplicate at a final dilution of 1:100. Antiserum ab59276 was included in each assay set as a positive control at a dilution of 1:500.

In each assay set, antibody binding was expressed as an Ab index, which was calculated for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 28 healthy control sera. Each serum was tested in at least two experiments and the mean Ab index was calculated from these values. The upper limit of normal for each RIA was calculated using the mean Ab index + 3SD of the population of 28 healthy controls. Any serum sample with an Ab index above the upper limit of normal was designated as positive for antibody reactivity against the TH ligand used in the RIA.

The results of the RIAs are shown in Figure 4.4 and summarised in Tables 4.6 and 4.7. In summary, none of the healthy individuals was positive for antibodies against the TH fragments used as radiolabelled ligands in the RIAs (Table 4.6). In addition, none of the 18 TH antibody-positive vitiligo patient sera or antiserum ab59276 was positive for antibodies against the TH fragment containing amino acids 141-497 (Table 4.6). In contrast, all of the 18 (100%) TH antibody-positive vitiligo patient sera and antiserum ab59276 were positive for immunoreactivity against TH fragments containing amino acids 1-140, 1-170, 1-200, 1-240, 1-280, 1-320, 1-360, 1-400, 1-440 and 1-480 (Table 4.6 and 4.7). The results suggested that the binding sites for vitiligo patient TH antibodies were present at the N-terminus of the protein between amino acids 1-140, with no epitopes being detected in the remainder of the protein.

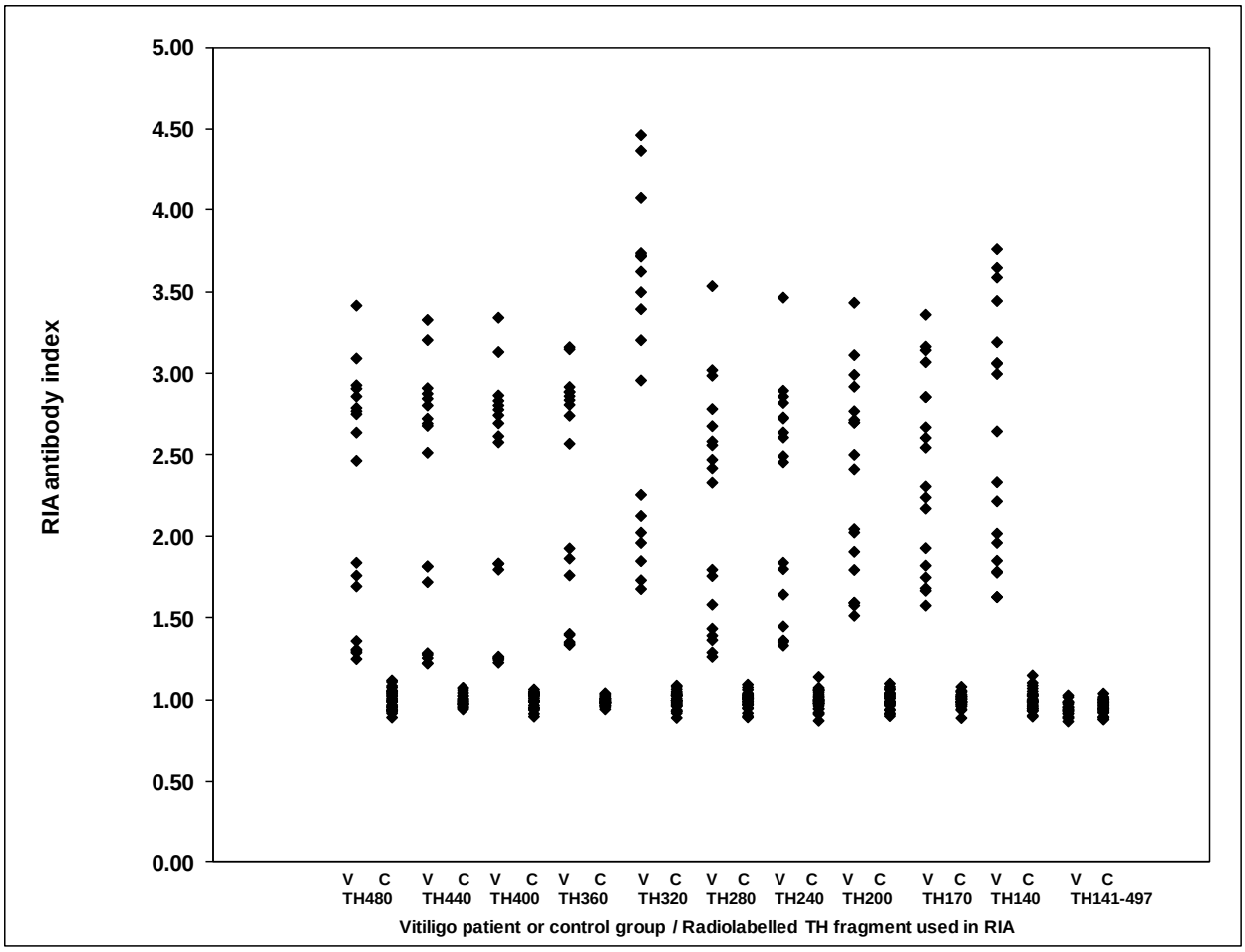


Figure 4.4: Immunoreactivity of vitiligo patient and control sera against TH deletion derivatives.

Serum samples from 18 vitiligo (V) patient and 28 healthy controls (C) were evaluated for antibodies against TH fragments in RIAs. The Ab index is shown for each sample in each RIA and is the mean Ab index of at least two experiments. The upper limits of normal (mean Ab index + 3SD of 28 healthy controls) were Ab indices of 1.18, 1.12, 1.12, 1.06, 1.15, 1.15, 1.18, 1.15, 1.12, 1.18 and 1.09 for TH1-480, TH1-440, TH1-400, TH1-360, TH1-320, TH1-280, TH1-240, TH1-170, TH1-140 and TH141-497 antibody RIAs, respectively. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

Table 4.6: Results of RIAs using TH fragments as the radiolabelled ligands

TH fragment used in RIA	Mean Ab index ± SD of vitiligo patient group	Mean Ab indices ± SD of control group	Upper limit of normal for the RIA	Number of vitiligo patients positive for TH antibodies[1]	Number of healthy controls positive for TH antibodies[1]	Mean Ab index ± SD of positive control antiserum ab59276
TH140	2.59 ± 0.76 (range: 1.63-3.65)	1.00 ± 0.06 (range: 0.90-1.15)	1.18	18/18	0/28	3.24 ± 0.13
TH170	2.41 ± 0.59 (range: 1.58-3.36)	1.00 ± 0.04 (range: 0.94-1.06)	1.12	18/18	0/28	3.60 ± 0.26
TH200	2.35 ± 0.61 (range: 1.52-3.44)	1.00 ± 0.04 (range: 0.90-1.10)	1.15	18/18	0/28	3.88 ± 0.14
TH240	2.22 ± 0.69 (range: 1.33-3.47)	1.00 ± 0.06 (range: 0.88-1.14)	1.18	18/18	0/28	3.61 ± 0.07
TH280	2.18 ± 0.70 (range: 1.27-3.54)	1.00 ± 0.05 (range: 0.92-1.10)	1.15	18/18	0/28	3.64 ± 0.14
TH320	3.02 ± 0.96 (range: 1.68-4.37)	1.00 ± 0.05 (range: 0.89-1.08)	1.15	18/18	0/28	3.84 ± 0.07
TH360	2.29 ± 0.71 (range: 1.35-3.17)	0.99 ± 0.02 (range: 0.95-1.04)	1.06	18/18	0/28	3.56 ± 0.90
TH400	2.23 ± 0.75 (range: 1.23-3.35)	1.00 ± 0.04 (range: 0.90-1.07)	1.12	18/18	0/28	2.84 ± 0.10
TH440	2.24 ± 0.76 (range: 1.23-3.33)	1.00 ± 0.04 (range: 0.95-1.07)	1.12	18/18	0/28	3.02 ± 0.22
TH480	2.25 ± 0.75 (range: 1.25-3.42)	1.00 ± 0.06 (range: 0.90-1.11)	1.18	18/18	0/28	2.91 ± 0.21
TH141-497	0.95 ± 0.04 (range: 0.87-1.03)	0.96 ± 0.04 (range: 0.88-1.04)	1.09	0/18	0/28	0.91 ± 0.05

[1]Sera with an Ab index above the upper limit of normal value, calculated from the mean Ab index + 3SD of 28 healthy controls, were considered positive for antibodies against the TH ligand used in the RIA.

Table 4.7: Antibody indices of vitiligo patient sera in RIAs using TH fragments as the radiolabelled ligands

Patient	Ab index with TH140[1]		Ab index with TH170[1]		Ab index with TH200[1]		Ab index with TH240[1]		Ab index with TH280[1]		Ab index with TH320[1]		Ab index with TH360[1]		Ab index with TH400[1]		Ab index with TH440[1]		Ab index with TH480[1]	
V4	3.76 ± 0.07	+	3.15 ± 0.17	+	3.00 ± 0.22	+	2.73 ± 0.30	+	2.48 ± 0.08	+	3.74 ± 0.02	+	2.89 ± 0.08	+	2.81 ± 0.05	+	2.91 ± 0.08	+	2.91 ± 0.04	+
V8	2.33 ± 0.12	+	2.31 ± 0.02	+	2.42 ± 0.01	+	2.50 ± 0.13	+	2.33 ± 0.00	+	3.73 ± 0.04	+	2.89 ± 0.10	+	2.75 ± 0.05	+	2.68 ± 0.00	+	2.64 ± 0.02	+
V9	3.06 ± 0.13	+	2.86 ± 0.55	+	2.70 ± 0.51	+	2.73 ± 0.17	+	2.68 ± 0.26	+	4.08 ± 0.16	+	2.92 ± 0.13	+	2.87 ± 0.03	+	2.88 ± 0.01	+	2.93 ± 0.05	+
V10	2.65 ± 0.00	+	2.55 ± 0.12	+	2.51 ± 0.01	+	2.86 ± 0.23	+	2.99 ± 0.38	+	3.63 ± 0.18	+	2.81 ± 0.25	+	2.58 ± 0.07	+	2.52 ± 0.09	+	2.47 ± 0.08	+
V20	3.65 ± 0.13	+	3.36 ± 0.07	+	3.44 ± 0.03	+	3.47 ± 0.07	+	3.54 ± 0.15	+	4.47 ± 0.12	+	3.15 ± 0.01	+	3.35 ± 0.17	+	3.33 ± 0.04	+	3.42 ± 0.04	+
V25	3.07 ± 0.16	+	3.07 ± 0.31	+	2.92 ± 0.12	+	2.90 ± 0.42	+	3.02 ± 0.58	+	3.72 ± 0.08	+	2.57 ± 0.05	+	2.62 ± 0.06	+	2.70 ± 0.03	+	2.76 ± 0.06	+
V27	2.02 ± 0.09	+	1.93 ± 0.04	+	1.91 ± 0.11	+	1.84 ± 0.04	+	1.80 ± 0.01	+	2.26 ± 0.02	+	1.93 ± 0.24	+	1.84 ± 0.16	+	1.72 ± 0.06	+	1.76 ± 0.04	+
V30	1.79 ± 0.14	+	1.82 ± 0.09	+	1.80 ± 0.07	+	1.80 ± 0.07	+	1.76 ± 0.07	+	2.13 ± 0.02	+	1.76 ± 0.09	+	1.84 ± 0.07	+	1.82 ± 0.07	+	1.84 ± 0.02	+
V33	3.00 ± 0.03	+	2.67 ± 0.41	+	2.70 ± 0.29	+	2.61 ± 0.44	+	2.57 ± 0.56	+	3.40 ± 0.01	+	2.84 ± 0.13	+	2.70 ± 0.02	+	2.73 ± 0.05	+	2.77 ± 0.03	+
V39	3.20 ± 0.07	+	2.61 ± 0.22	+	2.72 ± 0.21	+	2.46 ± 0.28	+	2.42 ± 0.41	+	3.50 ± 0.00	+	2.75 ± 0.13	+	2.78 ± 0.01	+	2.85 ± 0.19	+	2.79 ± 0.06	+
V62	3.59 ± 0.11	+	3.17 ± 0.01	+	3.12 ± 0.01	+	2.82 ± 0.32	+	2.79 ± 0.41	+	4.37 ± 0.02	+	3.17 ± 0.10	+	3.14 ± 0.01	+	3.21 ± 0.04	+	3.10 ± 0.04	+
V63	1.78 ± 0.01	+	1.75 ± 0.01	+	1.60 ± 0.04	+	1.45 ± 0.04	+	1.44 ± 0.05	+	1.68 ± 0.02	+	1.41 ± 0.21	+	1.23 ± 0.02	+	1.28 ± 0.07	+	1.36 ± 0.06	+
V64	1.63 ± 0.05	+	1.58 ± 0.06	+	1.52 ± 0.01	+	1.36 ± 0.03	+	1.40 ± 0.01	+	1.73 ± 0.00	+	1.35 ± 0.04	+	1.27 ± 0.07	+	1.23 ± 0.05	+	1.31 ± 0.00	+
V69	1.85 ± 0.07	+	1.67 ± 0.16	+	1.60 ± 0.04	+	1.36 ± 0.07	+	1.29 ± 0.04	+	1.85 ± 0.01	+	1.36 ± 0.08	+	1.26 ± 0.04	+	1.23 ± 0.02	+	1.29 ± 0.00	+
V70	1.63 ± 0.02	+	1.68 ± 0.09	+	1.58± 0.00	+	1.33 ± 0.06	+	1.27 ± 0.08	+	2.03 ± 0.07	+	1.40 ± 0.15	+	1.25 ± 0.03	+	1.29 ± 0.02	+	1.25 ± 0.00	+
V82	1.96 ± 0.13	+	2.17 ± 0.45	+	2.03 ± 0.47	+	1.37 ± 0.02	+	1.37 ± 0.09	+	1.96 ± 0.09	+	1.34 ± 0.09	+	1.26 ± 0.05	+	1.26 ± 0.04	+	1.30 ± 0.01	+
V86	2.22 ± 0.08	+	2.24 ± 0.08	+	2.05 ± 0.07	+	1.65 ± 0.27	+	1.59 ± 0.17	+	2.96 ± 0.02	+	1.87 ± 0.15	+	1.80 ± 0.09	+	1.82 ± 0.06	+	1.70 ± 0.06	+
V87	3.45 ± 0.12	+	2.86 ± 0.58	+	2.77 ± 0.52	+	2.64 ± 0.27	+	2.59 ± 0.23	+	3.21 ± 0.07	+	2.87 ± 0.07	+	2.84 ± 0.08	+	2.81 ± 0.01	+	2.86 ± 0.00	+

[1]The Ab index shown for each vitiligo patient serum is the mean ± SD of at least two experiments; +, denotes positive for antibody reactivity.

4.3.5 Absorption of TH antibodies by TH peptide fragments

The use of several TH fragments enabled the identification of a major epitope region corresponding to amino acids 1-140 (Section 4.3.4). To further identify epitopes within this TH domain, non-radiolabelled TH fragments containing amino acids 1-80 and 81-140 were used in absorption experiments to investigate their effects on the binding of TH antibodies to *in vitro* translated TH.

Non-radioactive TH fragments containing amino acids 1-80 and 81-140 (Table 4.5) were produced in an *in vitro* TnT® T7-Coupled Reticulocyte Lysate System (Section 2.18), using non-radiolabelled methionine in the reaction. Unlabelled MCHR1 was also synthesised *in vitro* from plasmid pcMCHR1 (Table 2.3) and was then used as an experimental control.

For absorption experiments, duplicate serum samples from 18 TH antibody-positive vitiligo patients and 6 healthy controls were pre-incubated at 4^{o}C for 2 h with 10 µl of *in vitro* translation reaction which contained the appropriate non-radiolabelled TH fragment or non-radiolabelled MCHR1. Sera were at a 1:100 dilution in 50 µl of immunoprecipitation buffer. Following the addition of *in vitro* translation reaction containing radiolabelled TH, the TH antibody RIA was completed as previously detailed (Section 2.20). Samples of unabsorbed vitiligo patient and control sera were included in each assay set.

For each serum sample, TH antibody binding was expressed as a TH Ab index which was calculated as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 6 healthy control sera. Each serum was tested in at least two experiments and the mean TH Ab index was calculated from these values. The TH Ab indices for the pre-absorbed and unabsorbed vitiligo patient sera were compared using paired *t* tests (Section 2.27). In all tests, *P* values < 0.05 (two-tailed) were regarded as significant.

The results are shown in Figure 4.5. The immunoreactivity of all 18 vitiligo patient sera against TH was significantly reduced when pre-absorbed with TH fragment 1-80, in comparison to unabsorbed serum samples (*P* values were < 0.05) (Table 4.8). This contrasted to the results obtained when prior absorption was with TH fragment 81-140 or with MCHR1, where no significant effects on TH antibody binding were observed (*P* values were > 0.05). The data indicated that at least one TH epitope(s), for patient TH antibodies, was located between amino acids 1-80.

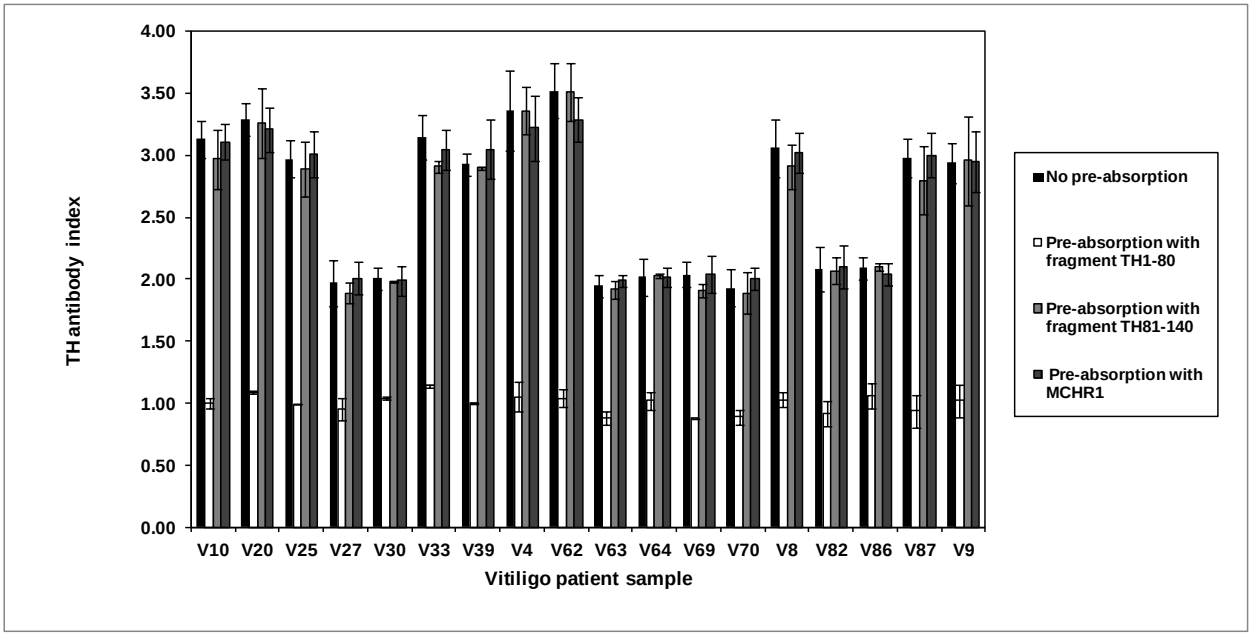


Figure 4.5: Absorption of TH antibodies by TH peptide fragments.

Non-radiolabelled TH fragments containing amino acids 1-80 and 81-140 were used to pre-absorb 18 TH antibody-positive vitiligo (V) patient sera prior to the analysis of immunoreactivity against TH in TH antibody RIAs. *In vitro* translated, non-radiolabelled MCHR1 was used as a pre-absorption control peptide. The TH Ab index (± SD) is shown for each patient serum sample with and without pre-absorption and is the mean of two experiments. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

Table 4.8: Comparison of the TH Ab indices of vitiligo patient sera with and without pre-absorption with TH fragment 1-80

Patient	*P* value[1]
V4	0.04
V8	0.02
V9	0.04
V10	0.004
V20	0.03
V25	0.02
V27	0.01
V30	0.02
V33	0.04
V39	0.003
V62	0.04
V63	0.03
V64	0.04
V69	0.03
V70	0.04
V82	0.04
V86	0.02
V87	0.03

[1]*P* values were calculated using paired *t* tests (Section 2.27) for comparing the TH Ab indices of TH antibody-positive patient sera without pre-absorption to those with pre-absorption using TH fragment 1-80. *P* values < 0.05 (two-tailed) were regarded as significant.

4.3.6 Absorption of TH antibodies by synthetic TH peptides

To identify epitope(s) located between amino acids 1-80 of TH, a second series of absorption experiments were carried out using synthetic peptides corresponding to TH amino acids 1-14, 15-29, 30-44, 45-60 and 61-80 (Table 2.9). MCHR1 peptide (amino acids 105-118 of MCHR1) was used as a control (Table 2.9). The effects of the peptides on TH antibody binding to *in vitro* translated TH were then investigated using TH antibody RIAs.

For absorption experiments, 18 TH antibody-positive vitiligo patient and 6 healthy control sera were pre-incubated at a dilution of 1:100 with synthetic peptides at 100 µg/ml in 50 µl of immunoprecipitation buffer. After incubation at 4^{o}C for 2 h, *in vitro* translation reaction containing radiolabelled TH was added to the samples which were then analysed in TH antibody RIAs as previously detailed (Section 2.20). Samples of unabsorbed vitiligo patient and control sera were included in each assay set.

For each serum sample, TH antibody binding was expressed as a TH Ab index which was calculated as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 6 healthy control sera. Each serum was tested in at least two experiments and the mean TH Ab index was calculated from these values. The TH Ab indices for the pre-absorbed and unabsorbed vitiligo patient sera were compared using paired *t* tests (Section 2.27). In all tests, *P* values <0.05 (two-tailed) were regarded as significant.

The results are given in Figure 4.6. TH antibody binding to TH in 17/18 (94%) patient sera was significantly reduced in the presence of TH peptide 1-14 (*P* values were <0.05) (Table 4.9), when compared with unabsorbed samples. Similarly, 11/18 (61%) patient sera were significantly pre-absorbed of TH antibodies with TH peptide 61-80 (*P* values were <0.05) (Table 4.9). In contrast, a significant decrease in TH immunoreactivity did not occur following pre-incubation with TH peptides 15-29, 30-44 and 45-60, and control MCHR1 peptide (all *P* values were > 0.05). The results suggested the presence of TH antibody binding sites between amino acids 1-14 and 61-80, with the majority (10/18 = 56%) of patient sera displaying immunoreactivity against both these epitopes.

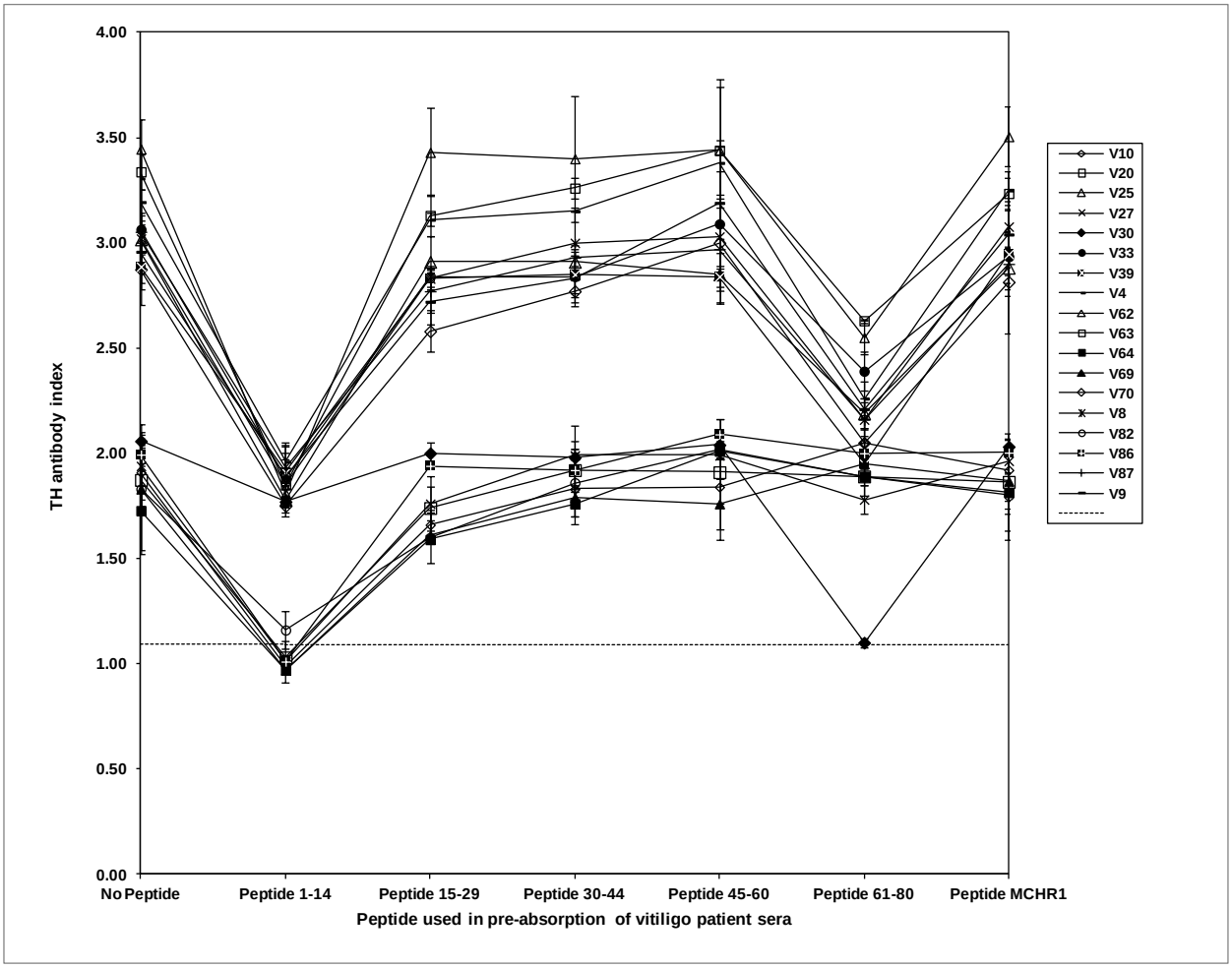


Figure 4.6: Absorption of TH antibodies by synthetic TH peptides.

Synthetic peptides corresponding to TH amino acids 1-14, 15-29, 30-44, 45-60 and 61-80 were used to pre-absorb 18 TH antibody-positive vitiligo (V) patient sera prior to the analysis of immunoreactivity against TH in TH antibody RIAs. Synthetic MCHR1 peptide was used as a pre-absorption control peptide. The TH Ab index (± SD) is shown for each patient serum sample with and without pre-absorption and is the mean of two experiments. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

Table 4.9: Comparison of the TH Ab indices of vitiligo patient sera with and without pre-absorption with synthetic TH peptides 1-14 and 61-80

Patient	*P* value[1,2]	*P* value[1,3]
V4	0.005	0.004
V8	0.04	0.01
V9	0.02	0.003
V10	0.03	0.03
V20	0.04	0.02
V25	0.04	0.04
V27	0.02	0.37
V30	0.13	0.007
V33	0.03	0.04
V39	0.01	0.03
V62	0.03	0.03
V63	0.02	0.12
V64	0.02	0.30
V69	0.04	0.66
V70	0.03	0.22
V82	0.03	0.11
V86	0.02	0.13
V87	0.006	0.02

[1]*P* values were calculated using paired *t* tests (Section 2.27) for comparing the TH Ab indices of TH antibody-positive patient sera without pre-absorption to those with pre-absorption using synthetic TH peptides 1-14[2] and 61-80[3]. *P* values < 0.05 (two-tailed) were regarded as significant.

4.3.7 TH peptide ELISAs with vitiligo patient and control sera

To confirm immunoreactivity of vitiligo patient sera against TH epitopes 1-14 and 61-80, samples from 18 vitiligo and 10 healthy controls were analysed in an ELISA format with peptides TH-1-14 and TH-61-80 as antigen (Table 2.9). TH peptide TH-30-44 was used as a control (Table 2.9) and ELISAs were carried out as detailed in Section 2.26. All sera were assayed in duplicate at a final dilution of 1:100. In each assay set, the binding reactivity of each patient and control sera to each TH peptide was expressed as an Ab index calculated as: mean OD_{405} of tested serum/mean OD_{405} of a population of 10 healthy control sera. Each serum was tested in two experiments and the mean Ab index was calculated from the resulting Ab index values. The upper limit of normal for each TH peptide ELISA was calculated using the mean Ab index + 3SD of 10 control sera. Patient sera with an Ab index greater than the upper limit of normal were regarded as positive for binding to the TH peptide used in the ELISA.

The results of the TH peptide ELISAs are shown in Figure 4.7 and summarised in Tables 4.10 and 4.11. All control sera were negative for antibody reactivity against all the TH peptides tested, and all samples were negative for anti-TH peptide TH-30-44 antibodies (Table 4.10). Of the vitiligo patient sera analysed, 17/18 (94%) and 11/18 (61%), respectively, were positive for antibody reactivity against TH peptides TH-1-14 and TH-61-80, and immunoreactivity against both epitopes was evident in 10/18 (56%) of the patients (Tables 4.10 and 4.11). The results correlated with the findings of the peptide displacement experiments for each of the vitiligo patient samples.

4.3.8 Comparison of patient details with TH antibody responses

The demographic, clinical and MCHR1 and tyrosinase antibody details of the vitiligo patients are given in Table 4.12 along with TH antibody epitopes recognised by the patient sera. There was no apparent association of patient gender, disease duration, age of disease onset, the presence of tyrosinase or MCHR1 antibodies, or the presence of autoimmune disease with recognition of the different TH antibody-binding sites.

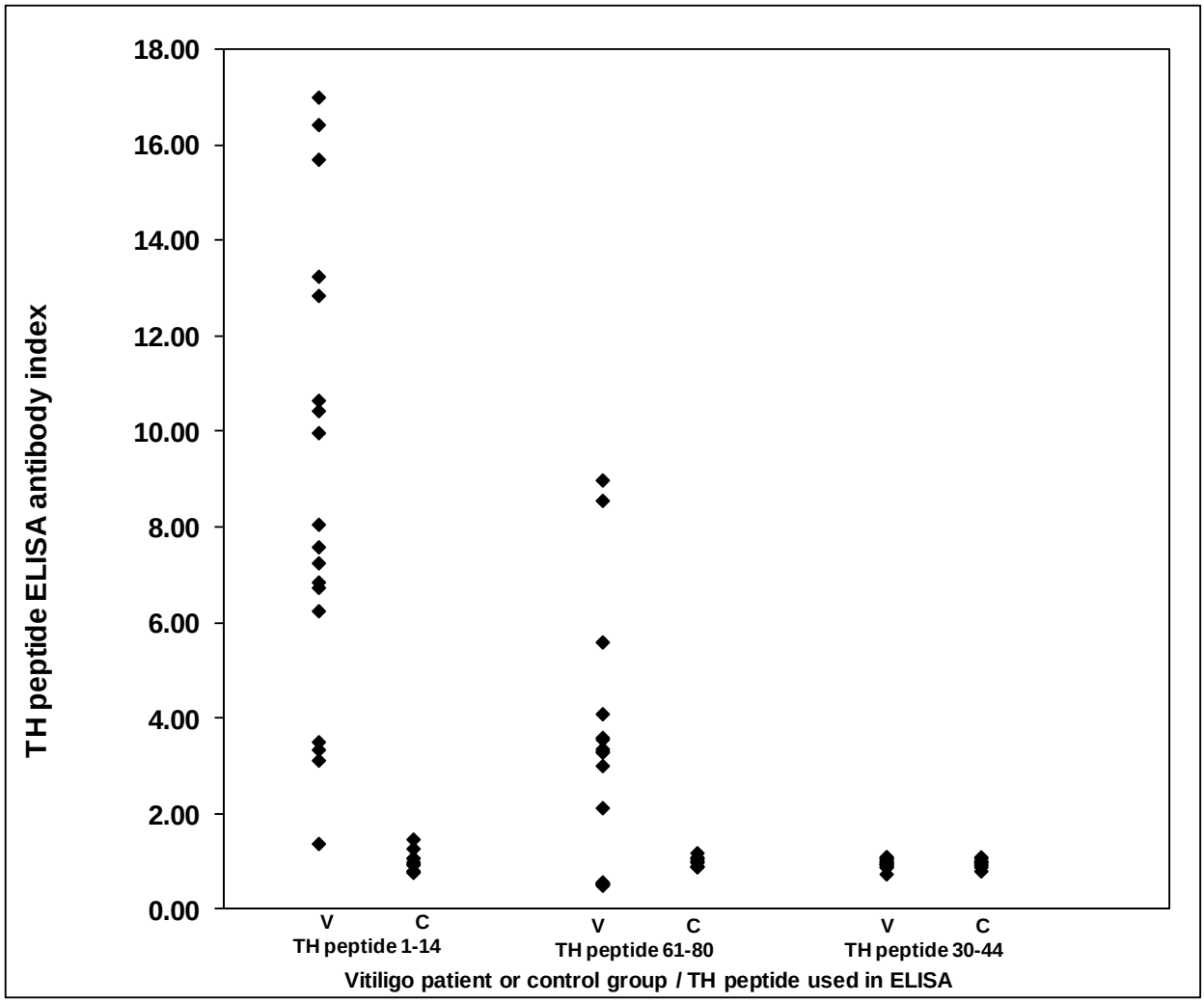


Figure 4.7: Immunoreactivity of vitiligo patient and control sera in TH peptide ELISAs.

Serum samples from 18 vitiligo (V) patient and 10 control (C) sera were analysed for antibody binding to TH peptides in an ELISA format. The Ab index is shown for each sample in each ELISA and is the mean Ab index of at least two experiments. The upper limits of normal (mean Ab index + 3SD of 10 healthy controls) were Ab indices of 1.69, 1.31 and 1.24 for TH peptide 1-14, 61-80 and 30-44 ELISAs, respectively. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

Table 4.10: Results of ELISAs using TH peptides

TH peptide used in ELISA	Mean Ab index ± SD of vitiligo patient group	Mean Ab indices ± SD of control group	Upper limit of normal for the ELISA	Number of vitiligo patients positive for antibodies[1]	Number of healthy controls positive for antibodies[1]
TH-1-14	8.92 ± 4.73 (range: 1.39-17.00)	1.02 ± 0.22 (range: 0.79-1.48)	1.69	17/18	0/10
TH-61-80	2.97 ± 2.64 (range: 0.51-8.99)	1.02 ± 0.10 (range: 0.90-1.19)	1.31	11/18	0/10
TH-30-44	0.98 ± 0.09 (range: 0.75-1.11)	0.98 ± 0.09 (range: 0.81-1.11)	1.24	0/18	0/10

[1]Sera with an Ab index above the upper limit of normal value, calculated from the mean Ab index + 3SD of 10 healthy controls, were considered positive for antibodies against the TH peptide used in the ELISA.

Table 4.11: Antibody indices of vitiligo patient sera in ELISAs using TH peptides

Patient	Ab index with TH peptide TH-1-14[1]		Ab index with TH peptide TH-61-80[1]	
V4	9.98 ± 1.91	+	3.30 ± 0.17	+
V8	15.70 ± 1.78	+	8.57 ± 0.53	+
V9	17.00 ± 2.20	+	2.14 ± 0.10	+
V10	10.66 ± 1.13	+	5.60 ± 0.33	+
V20	7.26 ± 1.02	+	3.61 ± 0.20	+
V25	6.86 ± 0.89	+	3.37 ± 0.18	+
V27	6.26 ± 0.69	+	0.56 ± 0.01	-
V30	1.39 ± 0.10	-	8.99 ± 0.56	+
V33	10.44 ± 2.06	+	3.57 ± 0.19	+
V39	12.85 ± 2.85	+	4.10 ± 0.13	+
V62	16.43 ± 2.01	+	3.32 ± 0.18	+
V63	7.59 ± 1.13	+	0.54 ± 0.04	-
V64	3.52 ± 0.80	+	0.51 ± 0.06	-
V69	3.35 ± 0.75	+	0.54 ± 0.08	-
V70	3.13 ± 0.69	+	0.70 ± 0.09	-
V82	8.07 ± 1.28	+	0.54 ± 0.03	-
V86	6.74 ± 1.85	+	0.54 ± 0.03	-
V87	13.25 ± 1.98	+	3.02 ± 0.16	+

[1]The Ab index shown for each vitiligo patient serum is the mean ± SD of at least two experiments; +, denotes positive for antibody reactivity; -, denotes negative for antibody reactivity.

Table 4.12: TH antibody epitope reactivity shown with the demographic and clinical details as well as antibody responses of TH antibody-positive vitiligo patients

Patient	Sex	Autoimmune disease[1]	Onset age (years)	Disease duration (years)	Antibodies[2]			
					MCHR1[3]	TYR[3]	TH epitope 1-14 antibodies	TH epitope 61-80 antibodies
V4	M	None	70	7	-	-	+	+
V8	F	None	10	27	-	-	+	+
V9	F	None	40	36	+	-	+	+
V10	M	None	56	4	-	+	+	+
V20	M	AA	20	50	-	-	+	+
V25	F	None	6	13	-	-	+	+
V27	F	None	19	4	-	+	+	-
V30	F	None	52	1	-	-	-	+
V33	F	None	51	3	-	-	+	+
V39	M	None	13	19	-	-	+	+
V62	F	ATD	7	20	+	-	+	+
V63	M	None	44	1	-	+	+	-
V64	M	Psoriasis	5	42	-	-	+	-
V69	F	None	39	31	-	-	+	-
V70	F	ATD	20	28	-	-	+	-
V82	F	None	6	1	-	-	+	-
V86	M	None	35	41	+	+	+	-
V87	M	None	19	4	-	-	+	+

[1]ATD, autoimmune thyroid disease; AA, alopecia areata.

[2]+, denotes positive for antibody reactivity; -, denotes negative for antibody reactivity.

[3]Tyrosinase (TYR) and MCHR1 antibody data are taken from Chapter 3.

4.3.9 Sequence analysis of the antibody binding sites on TH

The amino acid sequence of TH (NCBI Reference Sequence: NM000360.3) was compared with related proteins PAH (NCBI Reference Sequence: NM000277.1) and TPH (NCBI Reference Sequence: NM004179.2) using the ClustalW2 Multiple Sequence Alignment tool at the network facilities of the EBI-EMBL (Section 2.17). The results indicated that the TH epitopes mapped to regions of the protein that were not homologous (Figure 4.8). In addition, the antibody binding sites identified were present in the more specific regulatory N terminal domain of the TH molecule and not in the highly homologous catalytic domain at the C-terminus of the protein (Figure 4.8) (Ekwall *et al.* 2000).

Amino acid sequence homologies between the characterised TH epitopes and microbial and viral polypeptides were not demonstrated in searches against international databases performed using the BLAST service of the NCIB (Section 2.17).

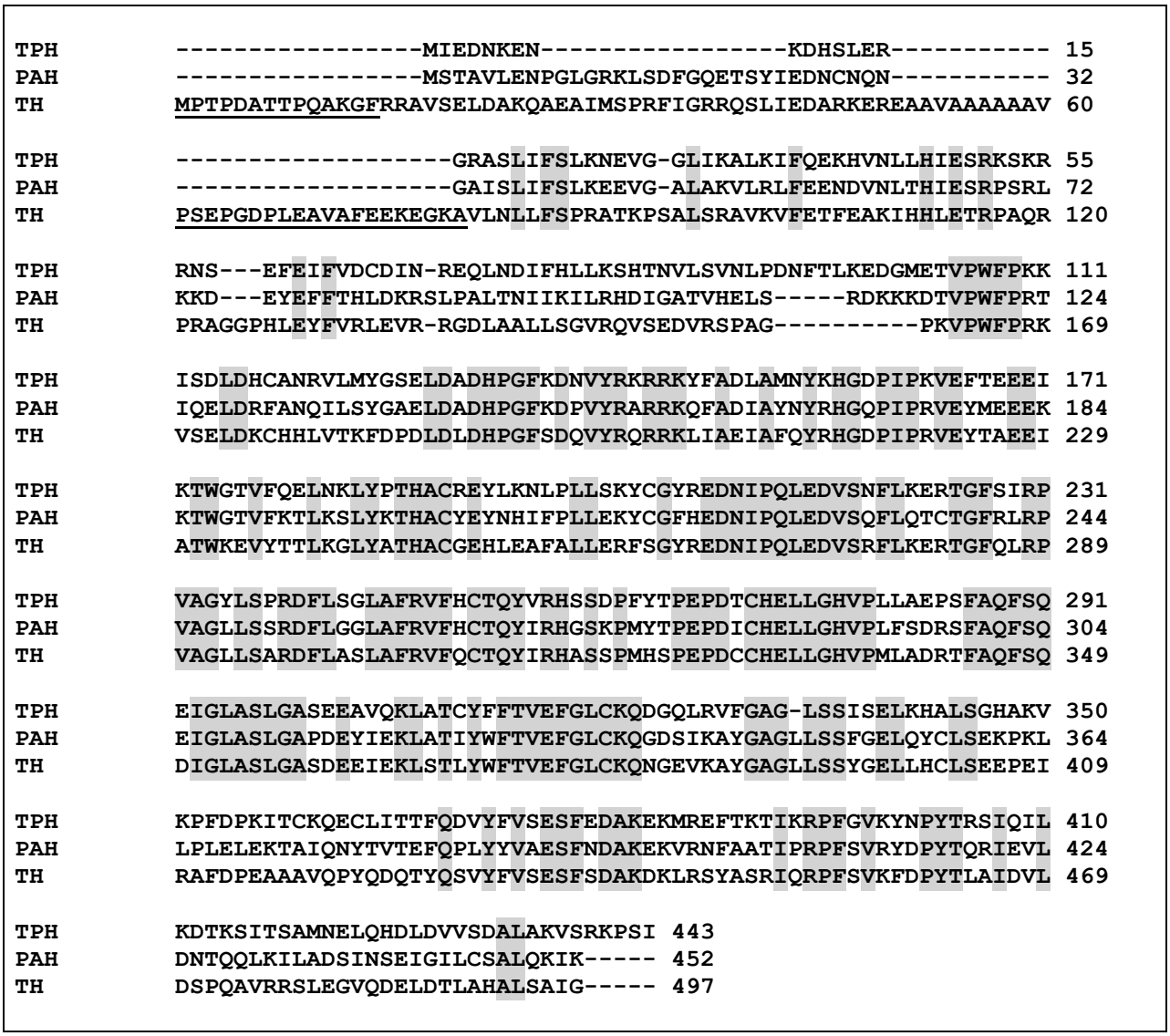


Figure 4.8: Amino acid sequence homologies between tyrosine hydroxylase, phenylalanine hydroxylase and tryptophan hydroxylase.

The amino acid sequences of TH epitopes recognised by vitiligo patient TH antibodies are underlined at amino acids 1-14 and 61-80 at the N terminus of TH (NCBI Reference Sequence: NM000360.3). Aligned amino acid sequences of phenylalanine hydroxylase (PAH) (NCBI Reference Sequence: NM000277.1) and tryptophan hydroxylase (TPH) (NCBI Reference Sequence: NM004179.2) are also shown. Identical amino acid residues in the three proteins are highlighted. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

4.4 Results Summary

The results indicated that:

- Two major binding sites for TH antibodies in vitiligo patients were located at the N-terminus of the protein between amino acids 1-14 and 61-80.
- Of 18 vitiligo patients, 17 (94%) had antibodies against epitope 1-14, and 11 (61%) displayed immunoreactivity against epitope 61-80.
- Immunoreactivity against both epitopes was evident in 10/18 (56%) of the vitiligo patients, suggesting that the humoral immune response to TH in vitiligo is heterogeneous in nature.
- There was no apparent association of patient gender, disease duration, age of disease onset, the presence of tyrosinase or MCHR1 antibodies, or the presence of autoimmune disease with recognition of the different TH antibody-binding sites.
- The TH epitopes mapped to regions of TH that were not homologous with related proteins TPH and PAH.
- Amino acid sequence homologies between the characterised TH epitopes and microbial and viral polypeptides were not demonstrated.

4.5 Discussion

In this part of the study, radiolabelled TH protein fragments coupled with absorption experiments enabled the identification of two major autoantibody specificities at the N-terminus of TH between amino acid residues 1 and 14 (epitope 1-14) and 61 and 80 (epitope 61-80). Subsequently, antibody binding to the identified epitopes in vitiligo patient sera was confirmed in ELISAs. Of 18 vitiligo patients, 17 (94%) had antibodies against epitope 1-14, and 11 (61%) displayed immunoreactivity against epitope 61-80. It was not apparent that epitope recognition was associated with patient gender, disease duration, age of disease onset, the presence of tyrosinase or MCHR1 antibodies or the presence of autoimmune disease.

Immunoreactivity to both epitopes was detected in the majority (10/18 = 56%) of vitiligo patients, indicating a heterogeneous humoral immune response to TH. Similarly, antibodies in autoimmune thyroid disease and type 1 diabetes mellitus have been reported to react against multiple epitopes on thyroid peroxidase and tyrosine phosphatase-like IA-2 autoantigens, respectively (Zanelli *et al.* 1992; Lampasona *et al.* 1996). This has been explained by intramolecular spreading of an autoimmune response from a single or few epitope(s) to multiple epitopes during disease progression. For example, in type 1 diabetes mellitus, a temporal spreading of the antibody responses has been described from immunodominant epitopes in an early pre-clinical phase to lesser immunogenic domains at the manifestation of disease (Naserke *et al.* 1998). In the absence of longitudinal serum samples, however, no evidence of TH epitope spreading in the patients could be ascertained.

Although TH shares a high degree (approximately 35%) of amino acid sequence homology with both the related enzymes TPH and PAH (Ekwall *et al.* 2000), neither antigen-specific nor cross-reacting antibodies to either protein were detected in vitiligo patients displaying a humoral immune response to TH (Chapter 3) (Kemp *et al.* 2011a). In keeping with this previous finding (Kemp *et al.* 2011a), major TH epitopes were demonstrated in areas of the protein which had no homology with TPH or PAH. Likewise, although their exact positions have not been mapped, epitopes unique for TH have also been proposed for TH antibodies found in patients with APS1 (Ekwall *et al.* 2000).

The potential applications of the molecular characterisation of B cell epitopes in vitiligo include: (i) a greater understanding of the association of an autoantigen with disease

pathogenesis, (ii) possible insights into the initiation of the disease process, and (iiii) the establishment of novel and more specific assays for measuring antibodies in patient sera. The ways in which these criteria were met by the study undertaken are discussed below.

Firstly, an association of TH as an autoantigen with vitiligo pathogenesis may be revealed by examining the location of the identified epitopes with respect to the functional domains of the protein. For example, if the epitopes reside in, or contribute to, the structure of the active site of TH, antibodies targeting these epitopes might inhibit enzyme activity. However, the antibody binding sites identified did not map to the C-terminal catalytic domain of the enzyme (Ekwall *et al.* 2000), which indicates that the epitope-specific TH antibodies characterised here may not have functional effects. Even so, it will be important to undertake further investigations in relation to the possible functional effects of TH antibodies in vitiligo patients, since functional TH antibodies may well recognise conformational epitopes not identified by the method used here. Interestingly, a previous study concerning APS1 patients has demonstrated inhibitory effects of TH antibodies on TH function (Hedstrand *et al.* 2000).

Secondly, no apparent homology was identified between the specified epitope regions and any microbial proteins, suggesting that molecular mimicry does not initiate the production of TH antibodies in vitiligo patients. However, as yet uncharacterised B cell epitopes on TH, which may be revealed by a study employing conformational epitope mapping techniques, may demonstrate cross-reactivity. Moreover, the current computer databases are unlikely to represent all microbial proteins.

Finally, since TH had multiple epitope regions and the antibody response provoked in vitiligo patients was heterogeneous, it is unlikely that a specific assay for measuring immunoreactivity to TH, limited to either of the epitopes identified here, would be of prognostic value for vitiligo.

The *in vitro* translation system is a useful procedure for producing both intact and modified [^{35}S]-methionine-labelled proteins, which can then be used for testing antibody reactivity and for epitope mapping (Wedlock *et al.* 1993; Daw *et al.* 1996; Volpato *et al.* 1998; Kemp *et al.* 2001). In this chapter, the technique was used to radiolabel deletion derivatives of TH, which were then used in RIAs to identify antigenic regions on the protein. A disadvantage of the use of deletion derivatives is in the inevitable loss of native conformation and, therefore, of

epitope regions which are brought together by the secondary structure of the protein (Pettersson 1992). In Graves' disease, for example, it has been reported that the autoantibodies to the thyrotropin receptor, which are responsible for disease activity, recognise a number of different conformational epitopes (Morgenthaler *et al.* 1999). In so far as short linear sequences may contribute to conformational epitopes, it may be possible to identify at least part of some conformational epitopes using the methodology applied in this study. In addition, a previous study employing site-directed mutagenesis to dissect reactivity to a conformational autoepitope of steroid 21-hydroxylase in autoimmune endocrinological diseases, suggested that testing of radiolabelled antigens in liquid-phase immunoprecipitation assay can be conformation-sensitive (Nikoshkov *et al.* 1999). Nevertheless, it would be of interest to apply phage-display technology (Scott 1992; Williams *et al.* 2001) to future epitope mapping studies of TH. Phage-display is more suited to the study of conformational epitopes since expressed proteins are able to fold into their correct three-dimensional structures in the periplasmic space of the bacterial host, and can maintain a native arrangement once displayed on the surface of a phage particle (Wilson and Finlay 1998). Although potentially important TH epitopes which may be dependent upon protein conformation and so were not characterised in this part of the study, the significant absorption of TH antibodies by TH peptides 1-14 and 61-80, confirms that at least two widely recognised TH antibody binding sites are linear in nature.

The use of human sera in the mapping of B cell epitopes can be problematic because sera contain multiple antibody species against a particular autoantigen and the immune response tends to diversify with duration. It is difficult therefore to examine the reactivity of a specific autoantibody in isolation and it is not possible to discriminate between a single autoantibody targeted at an epitope and a set of closely-related autoantibodies directed at the same epitope. In order to characterise the array of autoantibodies present in a particular serum and the epitope specificity of a particular autoantibody, the production of human monoclonal antibodies from the patient is usually required. Indeed, monoclonal antibodies isolated from individuals with type 1 diabetes mellitus have been successfully employed to identify the antibody binding sites on glutamic acid decarboxylase, an autoantigen in this disease (Syren *et al.* 1996). Although the monoclonal antibody approach can allow the precise characterisation of epitopes recognised by a specific antibody, it is difficult to know the relative frequencies of the different monoclonal

antibodies in the patient under investigation. Despite this, the isolation of monoclonal antibodies from vitiligo patients will allow a more complete and detailed analysis of the epitopes recognised by TH antibodies.

In summary, mapping of conformational epitopes and identifying TH antibodies with functional effects will give more information on the association of TH antibodies with vitiligo.

CHAPTER 5

5. Characterisation of IgG subclasses, avidities and titres of vitiligo patient TH antibodies

5.1 Introduction

5.1.1 Immunoglobulins

The immunoglobulin G is a major effector molecule of the humoral immune response in man and accounts for about 75% of the total immunoglobulins in plasma of healthy individuals. The immunoglobulins of the other four classes, IgM, IgA, IgD and IgE, each of which has characteristic properties and functions, constitute the other 25% of the immunoglobulins (Spiegelberg 1974). Antibodies of the IgG class express their predominant activity during a secondary antibody response. Thus, the appearance of specific IgG antibodies generally corresponds with the 'maturation' of the antibody response, which is switched on upon repeated contact with an antigen. In comparison to antibodies of the IgM class, IgG antibodies have a relatively high affinity and persist in the circulation for a long time.

5.1.2 IgG subclasses

Four distinct subgroups of human IgG have been identified designated IgG1, IgG2, IgG3 and IgG4, respectively (Gergely 1967). Quantitatively, the relative serum concentrations of the human IgG subclasses are: IgG1>IgG2>IgG3=IgG4 (Table 5.1) (Shakib and Stanworth 1980; French 1986). The four subclasses have structural differences and, as a consequence, show differences in some of their biological properties which are summarised in Table 5.1. For example, the capacity of the four human IgGs to activate complement decreases in the order: IgG3>IgG1>IgG2, with IgG4 unable to activate complement (Flanagan and Rabbitts 1982; van Loghem 1986). The four human IgG subclasses also show differences in their interaction with Fc receptors for IgG (FcγR). The latter are expressed primarily on effector cells of the immune system, in particular macrophages, monocytes, myeloid cells and dendritic cells and binding of

Table 5.1: Properties of IgG subclasses[1]

Property	IgG1	IgG2	IgG3	IgG4
Antibody concentrations				
Adult serum level range (g/l) (mean, g/l)	4.9-11.4 (6.98)	1.5-6.4 (3.8)	0.20-1.10 (0.51)	0.08-1.40 (0.56)
Proportion of total IgG (%)	43-75	16-48	1.7-7.5	0.8-11.7
Antibody response to				
Proteins	++[3]	+/- [3]	++	+/-
Polysaccharides	+[3]	++	-[3]	-
Allergens	+	-	-	++
Complement activation				
C1q binding	++	+	+++[3]	-
Binding to Fcγ receptors				
Fcγ RI[2] (CD64:monocytes, macrophages, neutrophils, dendritic cells)	++	-	+++	+
Fcγ RII (CD32):monocytes, macrophages, neutrophils, eosinophils, platelets, B cells, dendritic cells, endothelial cells)	++	-	+++	-
Fcγ RIIa-H131	++	+++	+++	-
Fcγ RIIa-R131	++	-	++	-
Fcγ RIII (CD16:neutrophils, eosinophils, macrophages, NK cells, subsets of T cells)	++	-	++	-
Fcγ RIIIb-NA1	+++	-	+++	-
Fcγ RIIIb-NA2	++	-	++	-

[1]Information adapted from Roitt 1997.

[2]FcγR, Fc receptors for IgG.

[3]-, negative; -/+, weakly positive; +, positive; ++, strongly positive; +++, very strongly positive.

the Fc part of IgG to a FcγR is instrumental in the induction of the effector cell's function (van de Winkel and Capel 1993; van de Winkel and Capel 1996), which can comprise phagocytosis, endocytosis, antibody-mediated cellular cytotoxicity, release of a range of inflammatory mediators, antigen presentation and clearance of immune complexes.

In autoimmune diseases, the levels of IgG subclasses mostly do not differ from those in healthy individuals, but specific antibodies show variable subclass restrictions. For example, in autoimmune Addison's disease and autoimmune ovarian insufficiency, antibodies against 21-hydroxylase, 17α-hydroxylase and cholesterol side-chain cleavage enzyme are predominantly of the IgG1 subtype (Boe *et al.* 2004; Brozzetti *et al.* 2010). This is also the case for antibody responses against protein tyrosine phosphatase IA-2 and glutamic acid decarboxylase in patients with type I diabetes mellitus (Bonifacio *et al.* 1999; Hawa *et al.* 2000). Comparisons of IgG subclass distributions of anti-thyroid peroxidase antibodies in patients with autoimmune hypothyroidism have indicated a predominance of IgG1 and IgG4 (Kohno *et al.* 1993; Silva *et al.* 2003; Xie *et al.* 2008). In the case of anti-thyroglobulin antibodies in Hashimoto's thyroiditis and Graves' disease, IgG2 and IgG4 were the dominant subtypes, respectively (Caturegli *et al.* 1994).

In autoimmune connective tissue diseases, the IgG subclass distribution of human autoantibodies to Sm-antigen, double-stranded DNA, ribonucleoprotein, SS-B-antigen (also called La), and IgG rheumatoid factor has been determined in patients with systemic lupus erythematosus, Sjögren's syndrome, and rheumatoid arthritis. For anti-Sm-antigen and anti-ribonucleoprotein antibodies, IgG1 was the predominant isotype. For anti-double-stranded DNA and anti-SS-B-antigen antibodies, IgG1 and a lesser contribution of IgG3 was found. In contrast, IgG1 and IgG4 were the predominant isotypes of human IgG rheumatoid factor (Gharavi *et al.* 1988; Yount *et al.* 1988).

Finally, patient antibodies to neutrophil cytoplasmic antigens (ANCA) are predominantly of the IgG1 and IgG4 subclass (Brouwer *et al.* 1991; Mellbye *et al.* 1994). However, in patients with renal involvement, ANCA antibodies of the IgG3 subclass can occur (Mellbye *et al.* 1994).

5.1.3 IgG avidity

Antibody affinity refers to the strength of binding between a single antigenic determinant and an individual antibody combining site (Roitt and Delves 1997). In most practical circumstances, however, avidity (or functional affinity) is used to describe the overall strength of binding between multivalent antigens and antibodies, and this is most applicable to the measurement of the interaction of an antiserum or patient serum with its target antigen (Roitt and Delves 1997). *In vivo*, high avidity antibodies are superior to those of low avidity for a wide variety of immune functions (Roitt and Delves 1997).

It has been suggested that antibody avidity could be an important feature of antibodies in autoimmune disease (Gharavi and Reiber 1996). However, the avidity of autoantibodies has not been sufficiently studied to give decisive answers of their clinical usefulness. In a few cases, high avidity antibodies have been shown to be associated with greater pathogenicity (Takeda *et al.* 2001; Cucnik *et al.* 2004; Cucnik *et al.* 2011). For example, high avidity anti-β2-glycoprotein I antibodies in patients with antiphospholipid syndrome have an increased pathological function compared with low avidity anti-β2-glycoprotein I antibodies (Cucnik *et al.* 2004; Cucnik *et al.* 2011). Furthermore, in systemic lupus erythematosus patients, double-stranded DNA and ribosomal P antibodies which have lower binding avidities are considered less harmful and associated with a lower prevalence of nephropathy (Takeda *et al.* 2001).

5.1.4 IgG titres

An antibody titer is a measurement of how much antibody an organism has produced that recognises a particular epitope, expressed as the greatest dilution that still gives a positive result. Measurement of antibody titres can offer an easy and useful way for determination of disease progression, for example as in autoimmune thyroid disorders (Etienne-Decerf *et al.* 1987)

5.1.5 TH antibodies in vitiligo

Our previous work identified TH antibodies of the IgG class present in vitiligo patient sera. In addition, two main epitopes were determined for TH antibodies. The analysis of TH antibodies in

terms of their subclass, avidity and titres would be valuable characterisations of the humoral immune response against TH.

5.2 Aims

The aims of this part of the study were:

- To determine the titres of antibodies against TH epitopes 1-14 and 61-80 using TH peptide ELISAs.

- To analyse the IgG subclasses of antibodies against TH epitopes 1-14 and 61-80 using TH peptide ELISAs with IgG subtype-specific secondary antibodies.

- To evaluate the avidities of antibodies against TH epitopes 1-14 and 61-80 using TH peptide ELISAs to measure antibody-binding in the presence of increasing NaCl concentrations.

5.3 Experiments and Results

5.3.1 Determination of vitiligo patient antibody titres against TH epitopes 1-14 and 61-80

Patient antibody titres against TH peptides 1-14 and 61-80 (Table 2.9) were determined using TH peptide ELISAs as detailed in Section 2.26. In the TH peptide 1-14 ELISA, serum samples from 17 vitiligo and 10 healthy controls were analysed in duplicate at final dilutions ranging from 1:100 to 1:2000. In the TH peptide 61-80 ELISA, serum samples from 11 vitiligo and 10 healthy controls were analysed in duplicate at final dilutions ranging from 1:100 to 1:2000.

In each assay set, the binding reactivity of each patient and control sera to each TH peptide was expressed as an Ab index calculated as: mean OD_{405} of tested serum/mean OD_{405} of a population of 10 healthy control sera. Each serum was tested in two experiments and the mean Ab index was calculated from the resulting Ab index values. Patient sera with an Ab index greater than the upper limit of normal were regarded as positive for binding to the TH peptide used in the ELISA: an Ab index of 1.69 for TH peptide 1-14, and 1.31 for TH peptide 61-80 (Chapter 4).

The results of the TH peptide ELISAs are shown in Figures 5.1a and b. In Table 5.2, the results are summarised showing the serum dilution in which antibody reactivity could still be detected at levels above the upper limit of normal for the TH peptide ELISA. In general, higher antibody titres were found against the TH peptide representing epitope 1-14. It is unclear why antibody titres against TH peptide 1-14 have a lower measurement at 1:200 dilution compared with 1:500 (Figure 5.1a), or why antibody titres against TH peptide 61-80 have a lower measurement at 1:100 dilution compared with 1:200 (Figure 5.1b).

5.3.2 Analysis of IgG subclasses of vitiligo patient antibodies against TH epitopes 1-14 and 61-80

To determine TH antibody IgG subclasses, sera from vitiligo patients and healthy controls were analysed in ELISAs with TH peptides 1-14 and 61-80 (Table 2.9) as detailed in Section 2.26.

IgG subclass-specific secondary antibodies were used to detect the binding of primary antibodies in the ELISAs (Section 2.26)**.** In the TH peptide 1-14 ELISA, serum samples from 17 vitiligo and 10 healthy controls were analysed in duplicate at a final dilution of 1:100. In the TH peptide 61-80 ELISA, serum samples from 11 vitiligo and 10 healthy controls were analysed in duplicate at a final dilution of 1:100.

In each assay set, the binding reactivity of each patient and control sera to each TH peptide was expressed as an Ab index calculated as: mean OD_{405} of tested serum/mean OD_{405} of a population of 10 healthy control sera. Each serum was tested in two experiments and the mean Ab index was calculated from the resulting Ab index values. The upper limit of normal for each TH peptide ELISA using different secondary antibodies was calculated using the mean Ab index + 3SD of the population of healthy control sera. Patient sera with an Ab index greater than the upper limit of normal were regarded as positive for binding to the TH peptide used in the ELISA.

The Ab indices of the patient and control sera and the upper limit of normal (mean Ab index + 3SD of 10 control sera) for each ELISA are shown in Figures 5.2a and b. The results, which are summarised in Table 5.3, indicated that antibodies against TH epitope 1-14 were exclusively of the IgG1 subclass in 17/17 (100%) vitiligo patients. Predominantly, antibody responses against TH epitope 61-80 were also of the IgG1 subclass and were detected in 9/11 (82%) vitiligo patients. Two of 11 (18%) vitiligo patients had TH epitope 61-80 antibodies of subclass IgG3.

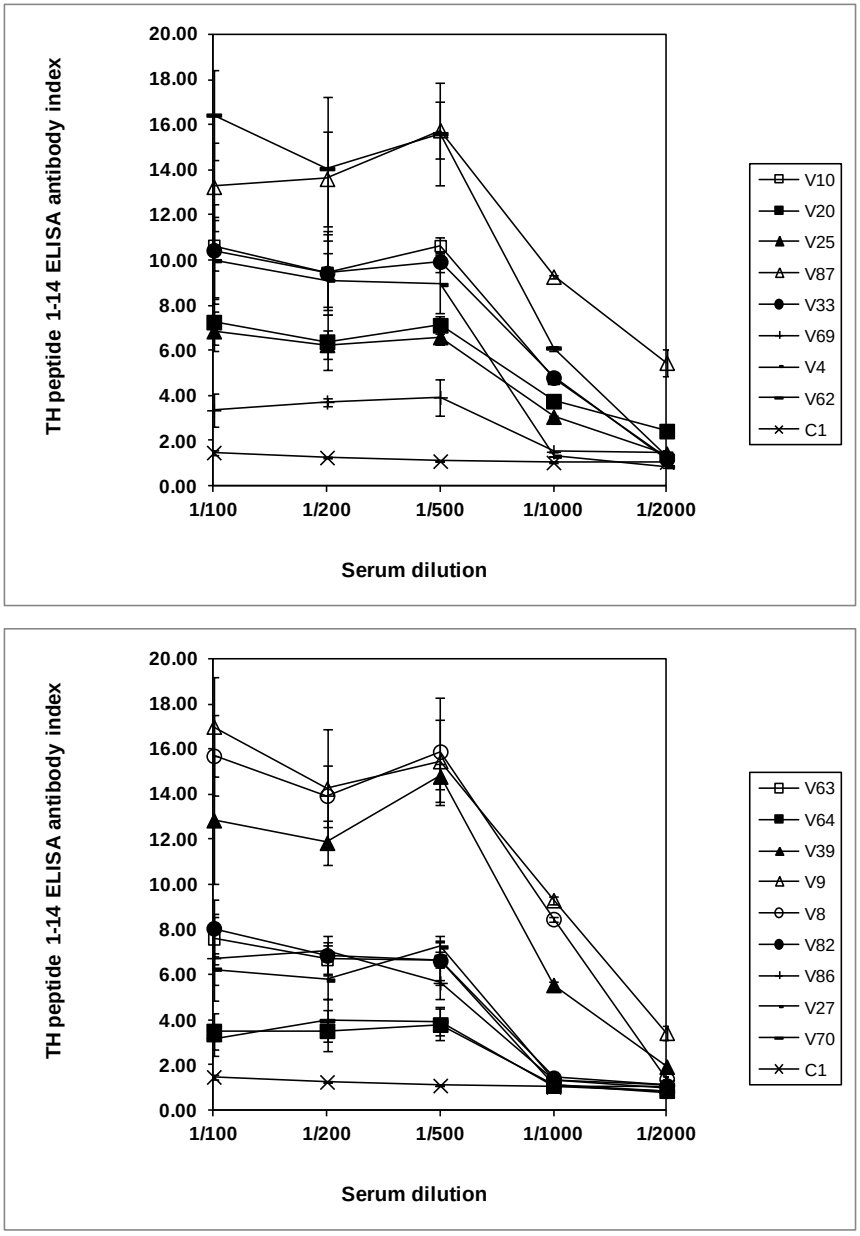


Figure 5.1a: Titres of vitiligo patient antibodies against TH epitope 1-14.

The 17 TH antibody-positive vitiligo (V) sera and 10 healthy control (C) sera were analysed at dilutions of 1:100, 1:200, 1:500, 1:1000, and 1:2000 in TH peptide ELISAs with peptide 1-14. The Ab index (± SD) of each vitiligo patient serum at each dilution is shown and is the mean of two experiments. The results are also shown for 1 control. The upper limit of normal for the TH peptide 1-14 ELISA was an Ab index of 1.69 (Chapter 4).

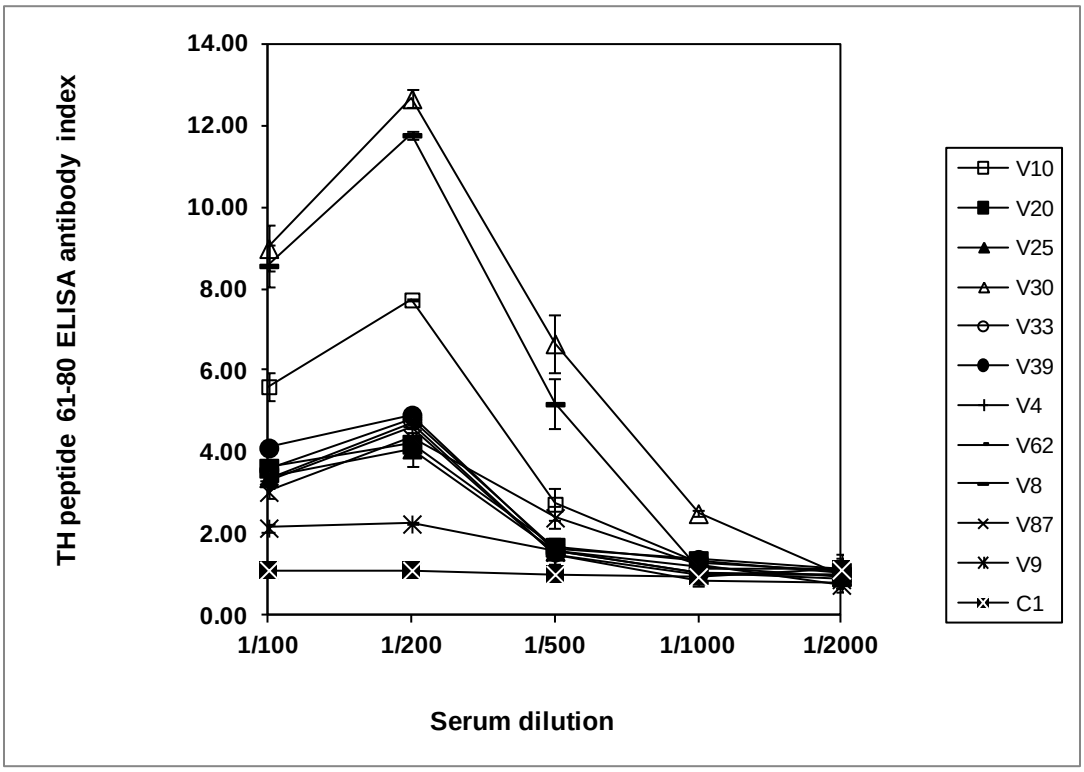


Figure 5.1b: Titres of vitiligo patient antibodies against TH epitope 61-80.

The 11 TH antibody-positive vitiligo (V) sera and 10 healthy control (C) sera were analysed at dilutions of 1:100, 1:200, 1:500, 1:1000, and 1:2000 in TH peptide ELISAs with peptide 61-80. The Ab index (± SD) of each vitiligo patient serum at each dilution is shown and is the mean of two experiments. The results are also shown for 1 control. The upper limit of normal for the TH peptide 61-80 ELISA was an Ab index of 1.31 (Chapter 4).

Table 5.2: Titres of vitiligo patient antibodies against TH epitopes 1-14 and 61-80

Vitiligo patient	TH epitope 1-14 antibodies		TH epitope 61-80 antibodies	
	Antibody reactivity[1]	Antibody titre[2]	Antibody reactivity[1]	Antibody titre[2]
V4	+	1:500	+	1:200
V8	+	1:1000	+	1:500
V9	+	1:2000	+	1:200
V10	+	1:1000	+	1:500
V20	+	1:2000	+	1:200
V25	+	1:1000	+	1:200
V27	+	1:500	-	-
V30	-	-	+	1:1000
V33	+	1:1000	+	1:200
V39	+	1:2000	+	1:200
V62	+	1:1000	+	1:200
V63	+	1:500	-	-
V64	+	1:500	-	-
V69	+	1:500	-	-
V70	+	1:500	-	-
V82	+	1:500	-	-
V86	+	1:500	-	-
V87	+	1:2000	+	1:500

[1]+, denotes positive for antibody reactivity; -, denotes negative for antibody reactivity.

[2]The antibody titre is given as the dilution at which immunoreactivity in the serum sample could still be detected at levels above the upper limit of normal in the TH peptide ELISA: An Ab index of 1.69 for TH peptide 1-14, and 1.31 for TH peptide 61-80 (Chapter 4).

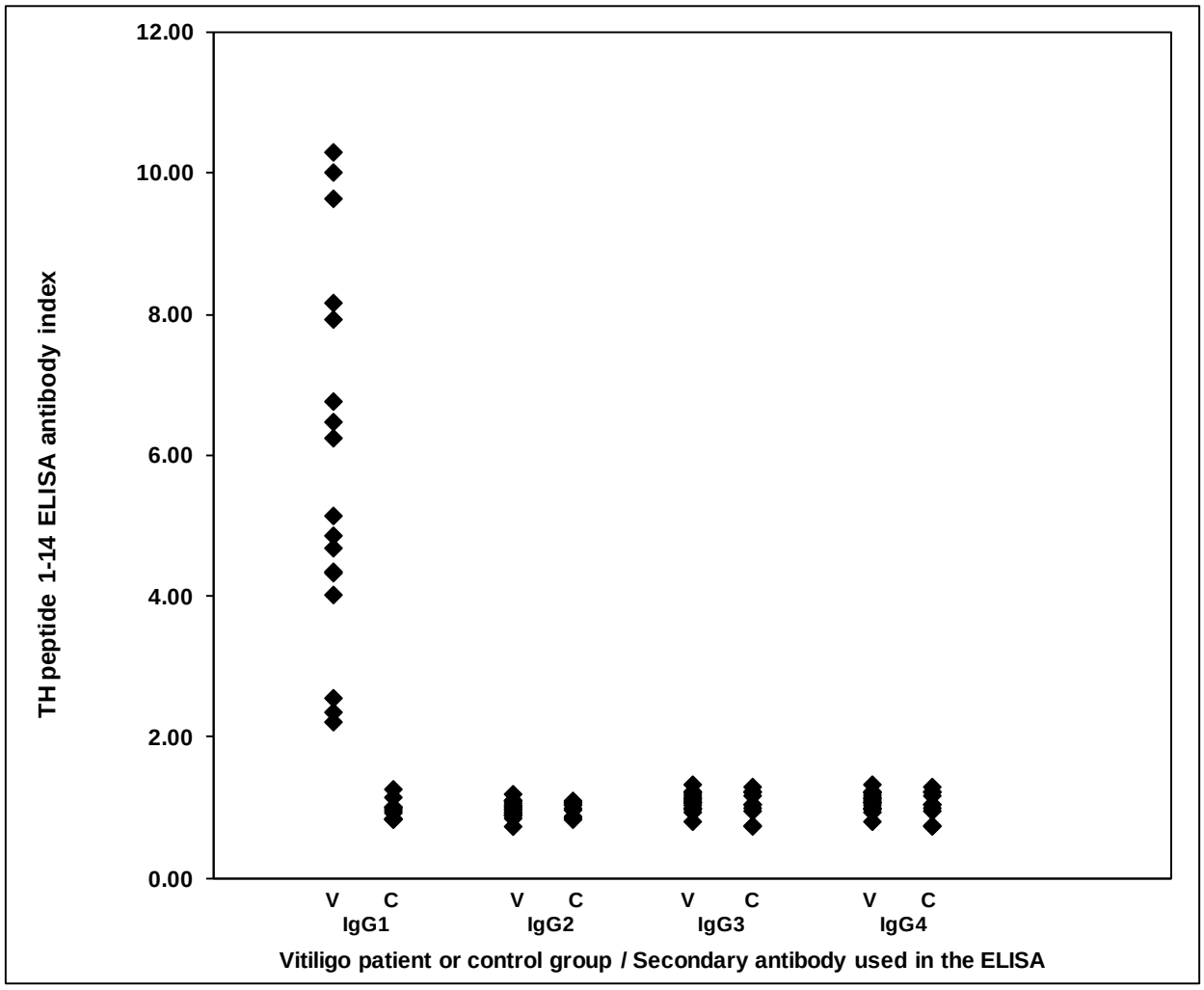


Figure 5.2a: IgG subclasses of vitiligo patient antibodies against TH epitope peptide 1-14.

The 17 TH antibody-positive vitiligo (V) sera and 10 healthy control (C) sera at a dilution of 1:100 were analysed in TH peptide ELISAs with peptide 1-14 and IgG-specific subclass secondary antibodies. The Ab index is shown for each serum and is the mean of two experiments. The upper limits of normal for the TH peptide 1-14 ELISA were Ab indices of 1.41, 1.29, 1.63 and 1.63 for secondary antibodies IgG1, IgG2, IgG3 and IgG4, respectively. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

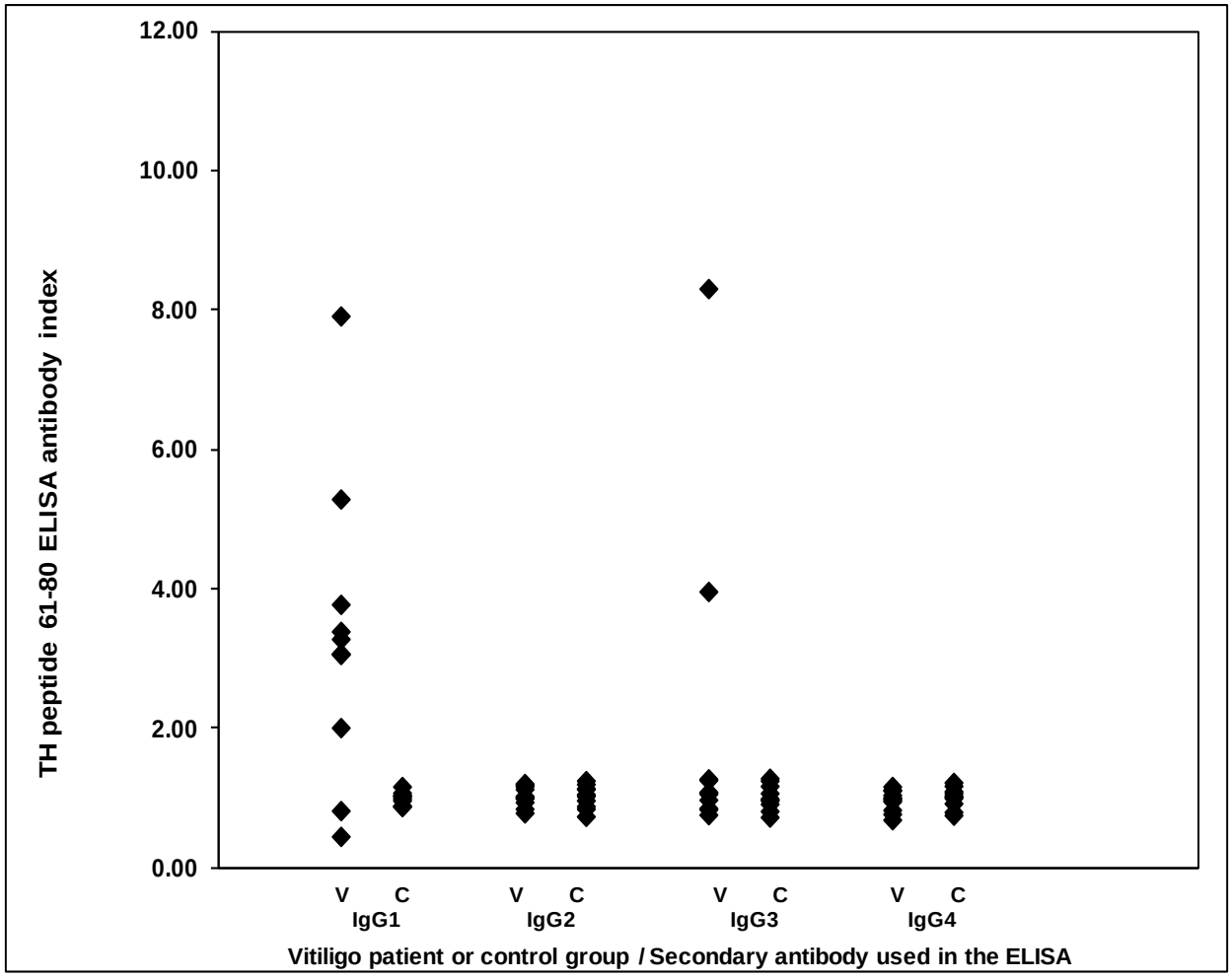


Figure 5.2b: IgG subclasses of vitiligo patient antibodies against TH epitope peptide 61-80.

The 11 TH antibody-positive vitiligo (V) sera and 10 healthy control (C) sera at a dilution of 1:100 were analysed in TH peptide ELISAs with peptide 61-80 and IgG-specific subclass secondary antibodies. The Ab index is shown for each serum and is the mean of two experiments. The upper limits of normal for the TH peptide 61-80 ELISA were Ab indices of 1.27, 1.52, 1.56 and 1.46 for secondary antibodies IgG1, IgG2, IgG3 and IgG4, respectively. Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

Table 5.3: IgG subclasses of vitiligo patient antibodies against TH epitopes 1-14 and 61-80

Vitiligo patient	TH epitope 1-14 antibodies		TH epitope 61-80 antibodies	
	Antibody reactivity[1]	IgG subclass	Antibody reactivity[1]	IgG subclass
V4	+	IgG1	+	IgG1
V8	+	IgG1	+	IgG1
V9	+	IgG1	+	IgG1
V10	+	IgG1	+	IgG1
V20	+	IgG1	+	IgG1
V25	+	IgG1	+	IgG1
V27	+	IgG1	-	-
V30	-	-	+	IgG3
V33	+	IgG1	+	IgG1
V39	+	IgG1	+	IgG1
V62	+	IgG1	+	IgG1
V63	+	IgG1	-	-
V64	+	IgG1	-	-
V69	+	IgG1	-	-
V70	+	IgG1	-	-
V82	+	IgG1	-	-
V86	+	IgG1	-	-
V87	+	IgG1	+	IgG3

[1]+, denotes positive for antibody reactivity; -, denotes negative for antibody reactivity.

5.3.3 Evaluation of avidities of vitiligo patient antibodies against TH epitopes 1-14 and 61-80

To determine TH antibody avidity, sera from vitiligo patients, and healthy controls were incubated with NaCl at final concentrations of 0.15, 0.5, 1.5 and 3.0 M (with account taken of the concentration of NaCl in the PBS which was used as the diluent) to give chaotropic conditions (Takeda *et al.* 2001; Cucnik *et al.* 2004; Cucnik *et al.* 2011) prior to analysis in TH peptide ELISAs with peptides 1-14 and 61-80 (Table 2.9) as detailed in Section 2.26.

In each assay set, the binding reactivity of each patient and control sera to each TH peptide was expressed as an Ab index calculated as: mean OD_{405} of tested serum/mean OD_{405} of a population of 10 healthy control sera. Each serum was tested in two experiments and the mean Ab index was calculated from the resulting Ab index values. Patient sera with an Ab index greater than the upper limit of normal were regarded as positive for binding to the TH peptide used in the ELISA: An Ab index of 1.69 for TH peptide 1-14, and 1.31 for TH peptide 61-80 (Chapter 4).

The results of the TH peptide ELISAs are shown in Figures 5.3a and b. In Table 5.4, the results are summarised and show the molar NaCl concentration at which antibody reactivity could still be detected at levels above the upper limit of normal for the TH peptide ELISA. TH antibodies against TH epitope 1-14 in 15/17 (88%) vitiligo patients retained binding activity, as measured by a positive Ab index, at a NaCl concentration of 0.5 M. Loss of binding activity was evident in a further 8 vitiligo patient sera at 1.5 M NaCl, as indicated by a negative Ab index. At 3.0 M NaCl, 7 vitiligo patient sera remained positive for TH antibodies in the TH peptide 1-14 ELISA. For TH antibodies against epitope 61-80, immunoreactivity was still detected in 8/11 (73%) vitiligo patients at a NaCl concentration of 0.5 M. Loss of binding activity was evident in a further 6 vitiligo patient sera at 1.5 M NaCl. Two vitiligo patient sera remained positive for TH antibodies against TH peptide 61-80 at 3.0 M NaCl. Overall, the results suggested that TH antibodies were of variable avidity towards their antigenic TH peptide target.

5.3.4 Comparison of patient details with TH antibody titres, IgG subclasses and avidities

The demographic details, clinical features, MCHR1 and tyrosinase antibody reactivities, and TH antibody epitopes of the vitiligo patients are given in Table 5.5 along with TH antibody titres,

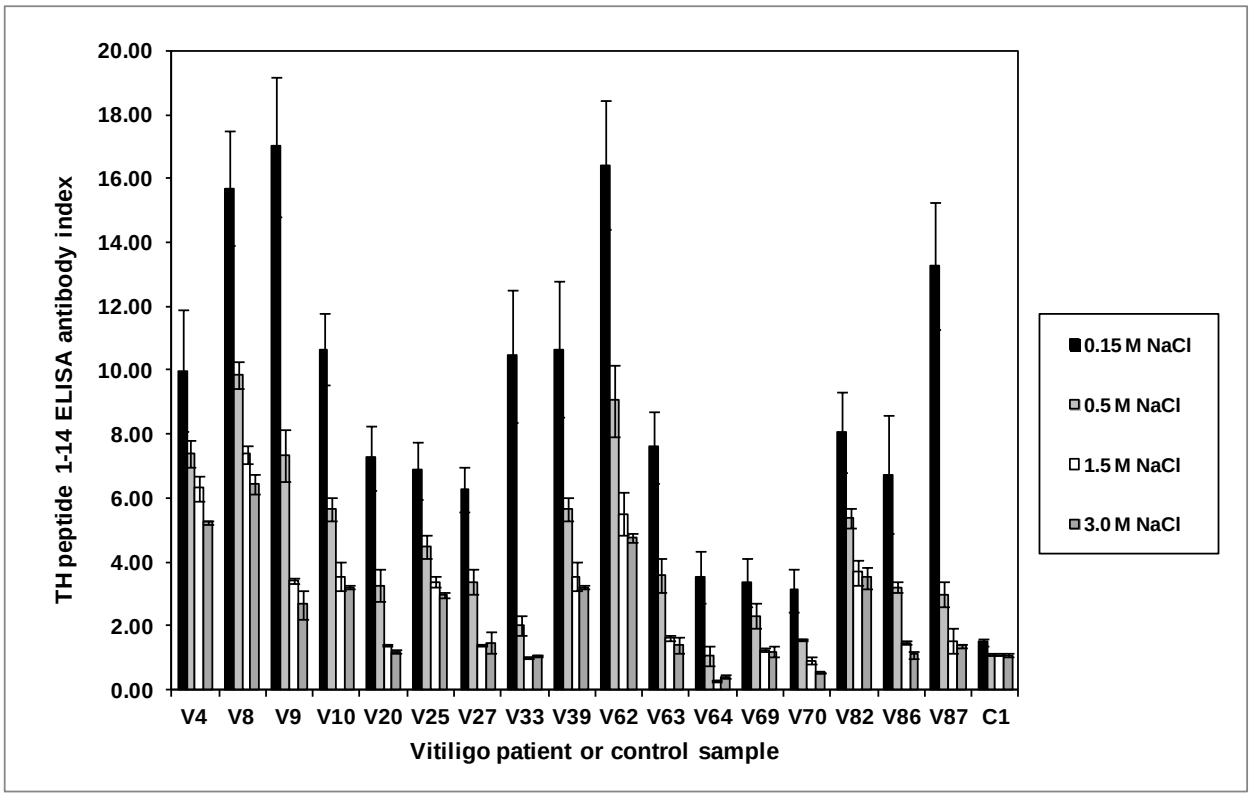


Figure 5.3a: Avidities of vitiligo patient antibodies against TH epitope peptide 1-14.

The 17 TH antibody-positive vitiligo (V) sera and 10 healthy control (C) sera were analysed at a dilution of 1:100 in TH peptide ELISAs with peptide 1-14 following incubation with NaCl at concentrations of 0.15, 0.5, 1.5 and 3.0 M. The Ab index (± SD) is shown for 17 vitiligo patient sera and 1 control serum and is the mean of two experiments. The upper limit of normal for the TH peptide 1-14 ELISA was an Ab index of 1.69 (Chapter 4). Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

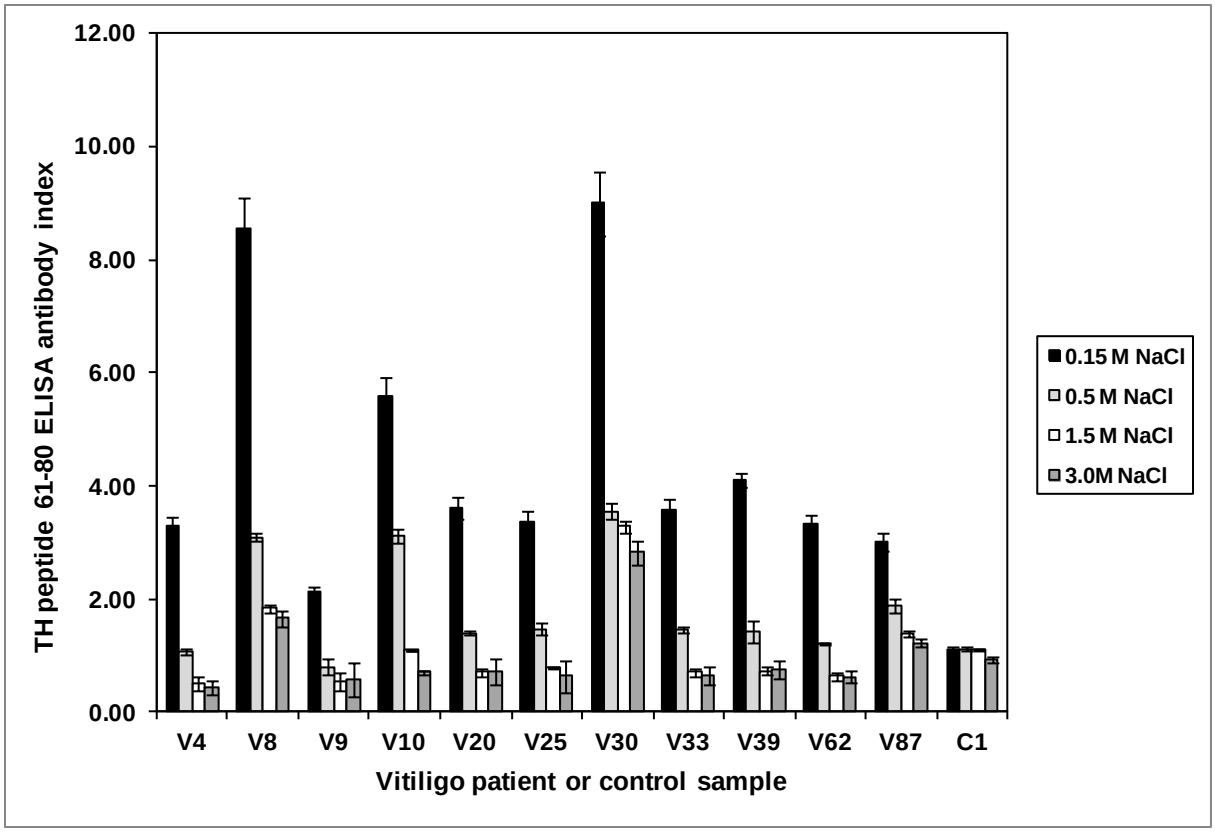


Figure 5.3b: Avidities of vitiligo patient antibodies against TH epitope peptide 61-80.

The 11 TH antibody-positive vitiligo (V) sera and 10 healthy control (C) sera were analysed at a dilution of 1:100 in TH peptide ELISAs with peptide 61-80 following incubation with NaCl at concentrations of 0.15, 0.5, 1.5 and 3.0 M. The Ab index (± SD) is shown for 11 vitiligo patient sera and 1 control serum and is the mean of two experiments. The upper limit of normal for the TH peptide 61-80 ELISA was an Ab index of 1.31 (Chapter 4). Taken with kind permission from John Wiley & Sons Ltd. (The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK) from (Rahoma *et al.* 2012).

Table 5.4: Avidities of vitiligo patient antibodies against TH epitopes 1-14 and 61-80

Vitiligo patient	TH epitope 1-14 antibodies		TH epitope 61-80 antibodies	
	Antibody reactivity[1]	Antibody avidity[2]	Antibody reactivity[1]	Antibody avidity[2]
V4	+	3.0 M	+	0.15 M
V8	+	3.0 M	+	3.0 M
V9	+	3.0 M	+	0.15 M
V10	+	3.0 M	+	0.5 M
V20	+	0.5 M	+	0.5 M
V25	+	3.0 M	+	0.5 M
V27	+	0.5 M	-	-
V30	-	-	+	3.0 M
V33	+	0.5 M	+	0.5 M
V39	+	0.5 M	+	0.5 M
V62	+	3.0 M	+	0.15 M
V63	+	0.5 M	-	-
V64	+	0.15 M	-	-
V69	+	0.5 M	-	-
V70	+	0.15 M	-	-
V82	+	3.0 M	-	-
V86	+	0.5 M	-	-
V87	+	0.5 M	+	0.5 M

[1]+, denotes positive for antibody reactivity; -, denotes negative for antibody reactivity.

[2]The antibody avidity is given as the highest molar (M) NaCl concentration at which immunoreactivity of the serum sample could still be detected at levels above the upper limit of normal in the TH peptide ELISAs; An Ab index of 1.69 for TH peptide 1-14, and 1.31 for TH peptide 61-80 (Chapter 4).

Table 5.5: Summary of TH antibody epitope reactivity, titres, IgG subclass and avidity with demographic details, clinical features and antibody responses of TH antibody-positive vitiligo patients

Vitiligo patient	Sex	Autoimmune disease[1]	Onset age (years)	Vitiligo duration (years)	Antibodies[2]		TH epitope 1-14 antibodies				TH epitope 61-80 antibodies			
					MCHR1	TYR	Antibody reactivity[3]	Antibody titre[4]	IgG subclass	Antibody avidity[5]	Antibody reactivity[3]	Antibody titre[4]	IgG subclass	Antibody avidity[5]
V4	M	None	70	7	-	-	+	1:500	IgG1	3.0 M	+	1:200	IgG1	0.15 M
V8	F	None	10	27	-	-	+	1:1000	IgG1	3.0 M	+	1:500	IgG1	3.0 M
V9	F	None	40	36	+	-	+	1:2000	IgG1	3.0 M	+	1:200	IgG1	0.15 M
V10	M	None	56	4	-	+	+	1:1000	IgG1	3.0 M	+	1:500	IgG1	0.5 M
V20	M	AA	20	50	-	-	+	1:2000	IgG1	0.5 M	+	1:200	IgG1	0.5 M
V25	F	None	6	13	-	-	+	1:1000	IgG1	3.0 M	+	1:200	IgG1	0.5 M
V27	F	None	19	4	-	+	+	1:500	IgG1	0.5 M	-	-	-	-
V30	F	None	52	1	-	-	-	-	-	-	+	1:1000	IgG3	3.0 M
V33	F	None	51	3	-	-	+	1:1000	IgG1	0.5 M	+	1:200	IgG1	0.5 M
V39	M	None	13	19	-	-	+	1:2000	IgG1	0.5 M	+	1:200	IgG1	0.5 M
V62	F	ATD	7	20	+	-	+	1:1000	IgG1	3.0 M	+	1:200	IgG1	0.15 M
V63	M	None	44	1	-	+	+	1:500	IgG1	0.5 M	-	-	-	-
V64	M	Psoriasis	5	42	-	-	+	1:500	IgG1	0.15 M	-	-	-	-
V69	F	None	39	31	-	-	+	1:500	IgG1	0.5 M	-	-	-	-
V70	F	ATD	20	28	-	-	+	1:500	IgG1	0.15 M	-	-	-	-
V82	F	None	6	1	-	-	+	1:500	IgG1	3.0 M	-	-	-	-
V86	M	None	35	41	+	+	+	1:500	IgG1	0.5 M	-	-	-	-
V87	M	None	19	4	-	-	+	1:2000	IgG1	0.5 M	+	1:500	IgG3	0.5 M

[1]AA, alopecia areata; ATD, autoimmune thyroid disease.

[2]Tyrosinase (TYR) and MCHR1 antibody data are taken from Chapter 3.

[3]+, denotes positive for antibody reactivity; -, denotes negative for antibody reactivity.

[4]The antibody titre is given as the dilution at which immunoreactivity of the serum sample could still be detected at levels above the upper limit of normal in the TH peptide ELISA.

[5]The antibody avidity is given as the highest molar (M) NaCl concentration at which immunoreactivity of the serum sample could still be detected at levels above the upper limit of normal in the TH peptide ELISA.

IgG subclasses and avidities. There was no apparent association of patient gender, disease duration, age of disease onset, the presence of tyrosinase or MCHR1 antibodies or the presence of autoimmune disease with TH antibody titres, avidities or IgG subclasses.

5.4 Summary of Results

The results indicated that:

- In general, higher antibody titres were found against the TH peptide representing epitope 1-14.
- Antibodies against TH epitope 1-14 were exclusively of the IgG1 subclass.
- Antibody responses against TH epitope 61-80 were also predominantly of the IgG1 subclass with IgG3 subtype antibodies in a minority of patients.
- TH antibodies were of variable avidity towards their antigenic TH peptide target with antibody binding detected at 3 M NaCl in several patient sera.
- There was no apparent association of patient gender, disease duration, age of disease onset, the presence of tyrosinase or MCHR1 antibodies or the presence of autoimmune disease with TH antibody titres, avidities or IgG subclasses.

5.5 Discussion

In this part of the study, particular characteristics of TH antibodies in vitiligo patients were examined including titres, subclasses and avidities. Overall, the titres, subclasses and avidities of TH antibodies were not associated with the clinical features of the vitiligo patients.

Like many reports on IgG subclass determination in autoimmune disease (Boe *et al.* 2004; Brozzetti *et al.* 2010), TH antibodies against both identified epitopes were predominantly of the IgG1 subclass. Antibodies against epitope 61-80 were of the IgG3 subtype in a minority of patients. No TH antibodies representing subclasses IgG2 or IgG4 were detected. Previously, anti-melanocyte antibodies have also been characterised as IgG (Naughton *et al.* 1983a; Naughton *et al.* 1983b; Cui *et al.* 1992; Cui *et al.* 1995; Hann *et al.* 1996a; Hann and Lee 1996; Rocha *et al.* 2000; Farrokhi *et al.* 2005), and as belonging to subclasses IgG1, IgG2 and IgG3 (Xie *et al.* 1991). Interestingly, IgG1 and IgG3 subclass antibodies have the capacity to activate complement and to induce cellular effector mechanisms (Flanagan and Rabbitts 1982; van Loghem 1986), both of which have been reported to damage melanocytes in vitiligo patients (Norris *et al.* 1988). Such possible functional effects of TH antibodies in vitiligo patients have yet to be studied.

The profile of IgG isotypes is influenced by the T helper cell Th1/Th2 balance of the immune response (Snapper and Paul 1987; Finkelman *et al.* 1990). T helper cells differentiate into functionally distinct subsets termed Th1 and Th2 which produce distinct immune responses associated by distinct patterns of cytokine secretion (Romagnani 1995; Mosmann and Sad 1996). The Th1 subset is characterised by the production of IFN-γ, IL-2, and TNF-β, and Th1 responses induce macrophage and cytotoxic T lymphocyte activation and immunoglobulin IgG subclass switching to favor complement fixation and opsonisation (Romagnani 1995; Mosmann and Sad 1996). Th1 responses favor effective clearance of intracellular pathogens and are likely to be important in organ-specific autoimmune diseases (Romagnani 1995; Mosmann and Sad 1996). In contrast, Th2 cells, defined by their propensity to secrete IL-4, IL-5, and IL-10, are important in allergy, mast cell/IgE-mediated immediate type hypersensitivity responses, and helminth infections, in which protective responses are mediated by eosinophils (Romagnani 1995; Mosmann and Sad 1996). In humans, the levels of IgG1 and IgG3 have been related to a Th1 predominance of immune responses, whereas a Th2 dominant response promotes an antibody

selection of the IgG4 subclass (Snapper and Paul 1987; Finkelman *et al.* 1990). In vitiligo, there is an immunopolarisation of CD4+ and CD8+ T cells to a Th1-type cytokine profile associated with melanocyte loss, which would fit with the finding that TH antibodies are mainly of the IgG1 subclass (Wankowicz-Kalinska *et al.* 2003). Interestingly, the SL chicken model of vitiligo also appears to be a Th1-polarised autoimmune disease (Section 1.3.9.3) (Shi and Erf 2012).

More accurate identification of IgG subclasses of the TH antibodies could have been made by using affinity chromatography to purify the different IgG subtypes from vitiligo patient sera prior to using in the ELISA (Soundararajan *et al.* 2005). Furthermore, purified IgG subclass antibodies could have been used to immunoprecipitate TH in the TH antibody RIA. This might have enabled the identification of other IgG subclasses of TH antibodies that were not against the two dominant linear TH epitopes used as the antigen in the IgG subclass ELISA experiments.

The avidities of the TH antibodies were found to vary between the vitiligo patient sera analysed. It has been suggested that antibody avidity could be an important feature of antibodies in autoimmune disease (Gharavi and Reiber 1996). High avidity antibodies have been shown to be associated with greater pathogenicity as in the case of anti-β2-GPI antibodies in patients with antiphospholipid syndrome (Cucnik *et al.* 2004; Cucnik *et al.* 2011). However, further studies will need to be undertaken with respect to TH antibodies in vitiligo to ascertain, in the first instance, if they have any pathogenic properties, and secondly to determine if any pathogenic properties are related to antibody avidity.

The method used here to determine TH antibody avidity is relatively simple and measures the ionic interactions between the antibody and antigen, which can be disrupted by changing NaCl concentrations. A better measure of avidity could be gained from other solid phase assays including competitive inhibition assays, and elution assays (Pullen *et al.* 1986; Goldblatt *et al.* 1999; Zhang *et al.* 2010). In both of them the antigen is attached to a solid support. In the competitive inhibition assay, antibody is added in solution at a fixed concentration, together with free antigen in different concentrations, and the amount of antigen which inhibits solid phase binding in an ELISA by 50% is determined (Zhang *et al.* 2010). The less antigen needed, the stronger the avidity.

In elution assays, the antibody is added in solution. After obtaining a state of equilibrium, a chaotrope or denaturant agent (e.g., isothiocyanate, urea, or diethylamine) is added in different

concentrations to disrupt antibody/antigen interactions. The amount of antibody resisting elution is determined thereafter with an ELISA. The higher the avidity, the more chaotropic agent is needed to elute a certain amount of antibody. The relative avidity of a heterogeneous mixture of antibodies can be expressed as the avidity index, equal to the concentration of eluting agent needed to elute 50% of the bound antibody. Refined analysis of data can be performed by determining percentages of eluted antibody at different concentrations of the eluting agent. However, the avidity index is not an absolute but only a relative measure, and for identical pairs of antibody/antigen, it strongly varies as a function of the precise conditions of the assay. Moreover, the coating antigen may be different from antigen found in physiological conditions with respect to density and conformation (Goldblatt *et al.* 1999). Information on low-avidity antibodies might be lost, as on reaching the equilibrium before addition of the eluting agent, lower proportions of low-avidity antibody are bound to the coating antigen than of higher-avidity antibody, which means that higher-avidity antibodies are preferentially detected and analysed (Butler 2000)

The TH antibody titres varied between the different vitiligo patients. Further studies are required to determine if TH antibody titres alter during the course of the disease and whether or not they have any prognostic significance.

CHAPTER 6

6. General Discussion

6.1 Discussion of the Results

Various hypotheses have been suggested to explain the mechanisms involved in vitiligo pathogenesis such as genetic susceptibility, biochemical and neural abnormalities, melanocyte dysfunction and both the cellular and humoral immunity (Le Poole *et al.* 1993a; Schallreuter *et al.* 2008a; Boissy and Spritz 2009). There are several lines of evidence for the involvement of the immune system in the destruction of cutaneous melanocytes in vitiligo:

- Vitiligo is frequently associated with a variety of autoimmune disorders such as autoimmune thyroiditis, Addison's disease, pernicious anaemia and type 1 diabetes mellitus (Taïeb 2000; Alkhateeb *et al.* 2003).
- Detection in vitiligo patients of autoreactive T lymphocytes and autoantibodies against melanocyte proteins (Kemp *et al.* 1997a; Kemp *et al.* 1997b; Kemp *et al.* 1998b; Kemp *et al.* 1998c; Lang *et al.* 2001; Palermo *et al.* 2001; Kemp *et al.* 2002; van den Boorn *et al.* 2009).
- Non-segmental vitiligo responds less well to autologous melanocyte grafting than segmental vitiligo (Gauthier and Surleve-Bazeille 1992; Taïeb 2000) and is responsive to treatment with immunosuppressive agents such as steroids and calcineurin inhibitors (Lepe *et al.* 2003).
- The presence of allelic variations in genes that are involved in the regulation of the immune response (Spritz 2006; Spritz 2007; Spritz 2008; Spritz 2010b; Spritz 2011; Spritz 2012). For example, the association between certain allelic variations of HLA genes and vitiligo susceptibility (Metzker *et al.* 1980; Orecchia *et al.* 1992; Buc *et al.* 1996; Yang *et al.* 2005; Singh *et al.* 2012).

Characterisation of the immune response against melanocytes in vitiligo will help in designing therapeutic and diagnostic tools and in a better understanding of the aetiology of the disease.

Tyrosine hydroxylase catalyses the initial step in catecholamine biosynthesis through conversion of L-tyrosine to L-dopa (Lewis *et al.* 1993; Nagatsu 1995). Previously, the enzyme was identified as a potential vitiligo-associated autoantigen after being enriched by vitiligo patient IgG from a melanocyte cDNA phage-display library in panning experiments (Kemp *et al.* 2002; Waterman *et al.* 2010). Furthermore, antibodies directed against TH were identified in patients with APS1, including those patients where vitiligo was a part of their clinical manifestations (Hedstrand *et al.* 2000). The aims of my study were: (i) to investigate the frequency of TH antibodies in vitiligo patients using a RIA specific for TH antibodies; (ii) to determine if there are any associations between the presence of TH antibodies and the clinical features of vitiligo; (iii) to analyse the vitiligo patient TH antibody binding sites (epitopes) on TH using deletion derivatives of TH in RIAs; and (iv) to evaluate the characteristics of TH antibodies in terms of titres, subclasses and avidities.

Initially, the frequency of immunoreactivity against TH in vitiligo patient sera was investigated using a RIA specific for TH antibodies (Kemp *et al*. 2011). TH antibodies were detected in 23% of those patients with the non-segmental vitiligo. Although this frequency appears to be low, earlier studies have detected antibodies against several autoantigens in vitiligo patients at a similar prevalence. For example, antibodies against tyrosinase were present in 11% of tested patients (Kemp *et al.* 1997a), TYRP1 in 5% (Kemp *et al.* 1998c), DCT in 5% (Kemp *et al.* 1997b), PMEL in 5% (Kemp *et al.* 1998b), transcription factor SOX10 in 3% (Hedstrand *et al.* 2000) and MCHR1 in 16% (Kemp *et al.* 2002).

In contrast to non-segmental vitiligo, none of the segmental vitiligo patient sera showed immunoreactivity against TH (Kemp *et al.* 2011a). This supports the concept that segmental vitiligo has an aetiology that does not involve autoimmune processes (Taïeb 2000). However, serum samples from only eight segmental vitiligo patients were available for testing, so we cannot draw any firm conclusions as to the prevalence of TH antibodies in this disease sub-type and analysis of a larger number of segmental vitiligo patients would be required to confirm this finding.

The results also demonstrated that the frequency of TH antibodies was significantly increased in the group of non-segmental vitiligo patients with active disease (Kemp *et al.* 2011a). Furthermore, the frequency of active vitiligo was elevated significantly in the group of patients

who were positive for TH antibody responses (Kemp *et al.* 2011a). However, only 15 patients with stable disease were available for study, so further verification of this result is also necessary in a larger series.

Interestingly, TH was an antibody target in both patients with and without concomitant autoimmune disease (Kemp *et al.* 2011a). This finding contrasts to previous studies in which antibodies directed against melanocyte-specific proteins PMEL, TYRP1 and DCT were only detected in vitiligo patients who had other autoimmune diseases (Kemp *et al.* 1997b; Kemp *et al.* 1998b; Kemp *et al.* 1998c). Tyrosinase and SOX10 antibodies were also predominantly detected in vitiligo patients with additional autoimmune disorders. Whether this reflects a distinct difference in the pattern of TH antibody production would require a very large series of patients tested for all autoantibodies.

There are four isoforms of human TH (TH1-TH4), which differ in the regulatory N-terminal region responsible for the enzyme activity (Lewis *et al.* 1993; Nakashima *et al.* 2009). In this study, immunoreactivity against TH enzyme was observed against the TH2 isoform (Kemp *et al.* 2011a). Nevertheless, it is possible that vitiligo patient TH antibodies react against other TH isoforms since they exhibit a high similarity of amino acid sequence (Hedstrand *et al.* 2000). In APS1 patients, antibodies have also been demonstrated against two enzymes that have significant amino acid sequence homology with TH at the N-terminus, namely TPH and PAH (Ekwall *et al.* 1999; Ekwall *et al.* 2000). In the present study, antibodies against these two enzymes were not detected in vitiligo patients (Kemp *et al.* 2011a). Furthermore, TH antibodies were not cross-reactive with either PAH or TPH (Kemp *et al.* 2011a).

Part of my study was concerned with the molecular analysis of the B cell epitopes on the TH autoantigen. The mapping of B cell epitopes can provide an understanding of the association of an autoantigen with a particular autoimmune disease (Morgenthaler *et al.* 1999; Gora *et al.* 2004) and may give an insight into the mechanisms involved in the initiation and pathogenesis of the disease as well as it can be useful as diagnostic and therapeutic tool (Routsias *et al.* 2006; Mahler and Fritzler 2010).

Molecular mapping of the B cell epitopes on TH protein was performed by using recombinant proteins in RIA coupled with absorption experiments (Rahoma *et al.* 2012). In RIAs, two regions of TH were found to be targets for vitiligo patient TH antibodies: between

amino acid residues 1 and 14 and between amino acid residues 61 and 80 (Rahoma *et al.* 2012). Subsequently, the antibody-binding sites were confirmed in ELISAs with the relevant TH peptide representing the epitope (Rahoma *et al.* 2012). The immunoreactivity to both antibody-binding sites was demonstrated in most of TH antibody-positive vitiligo patients (56%), indicating that the humoral response to TH in vitiligo is heterogeneous in nature (Rahoma *et al.* 2012). Similar results have been obtained from characterisation of autoantigens in autoimmune thyroid disorders and in type 1 diabetes mellitus, where multiple epitopes have been detected on thyroid peroxidase (Zanelli *et al.* 1992) and tyrosine phosphatase-like IA-2 autoantigen (Lampasona *et al.* 1996), respectively. This has been explained in the case of in type 1 diabetes mellitus by expansion or 'spreading' of autoimmune reactivity against a single or a few epitopes from early childhood to multiple and different epitopes with the advancement of age (Naserke *et al.* 1998). In the current study, there was no follow-up sampling, so the possibility of epitope spreading could not be investigated.

The TH epitopes identified in this study were linear in nature, so further studies need to be undertaken to characterise antibodies that may recognise conformational epitopes which depend on the native structure of the protein. Such antibodies are usually important in disease pathogenesis (Pettersson 1992; Morgenthaler *et al.* 1999). In Graves' disease, for example, it has been reported that the autoantibodies to the thyrotropin receptor, which are responsible for disease activity, recognise a number of different conformational epitopes (Morgenthaler *et al.* 1999).

It has been speculated that the autoimmune responses may arise from cross-reactivity between self-proteins and those of infecting micro-organisms in so-called molecular mimicry (Wucherpfennig 2001). For example, bacterial and viral proteins have homology with endothelial cell components which can be damaged by cross-reacting antibodies resulting from infections (Luo *et al.* 2010; Liu *et al.* 2011). However, no similarity was apparent between the amino acid sequences of the identified epitope regions and those of microbial proteins and so no evidence for molecular mimicry in causing the humoral immune response to TH was apparent. Because of the presence of multiple TH epitope and the heterogeneity of the TH antibody response it is unlikely that TH antibody that a specific assay for measuring TH antibody

immunoreactivity for either of the epitopes demonstrated in this study would be of diagnostic usefulness in screening vitiligo patients.

The IgG isotype of vitiligo patient TH antibodies was also determined in this study. The majority of TH antibodies against the two identified antibody-binding sites were of the IgG1 subclass (Rahoma *et al.* 2012). Few vitiligo patient sera contained TH antibodies of the IgG3 subtype and these were against the epitope between amino acids 61 and 80 (Rahoma *et al.* 2012). No TH antibodies representing subclasses IgG2 or IgG4 were detected (Rahoma *et al.* 2012). The IgG subclass distribution suggested a Th1 type of immune response in vitiligo. Indeed, in vitiligo, there is an polarisation of CD4+ and CD8+ T cells towards a Th1-type cytokine profile associated with melanocyte loss, which would fit with the finding that TH antibodies are mainly of the IgG1 subclass (Wankowicz-Kalinska *et al.* 2003). Interestingly, the SL chicken model of vitiligo also appears to be a Th1-polarised autoimmune disease (Shi and Erf 2012). In addition, it is well known that antibodies of the IgG1 and IgG3 subclasses are very effective in activating the complement system and inducing cellular effector mechanisms (Flanagan and Rabbitts 1982; van Loghem 1986; Bindon *et al.* 1988). Both of these pathogenic mechanisms, which can induce lysis of human melanocytes, have been demonstrated in the sera of vitiligo patients (Norris *et al.* 1988). More studies are required to investigate the possible pathogenic effects of TH antibodies in vitiligo patients.

6.2 TH Antibodies in Vitiligo: Possible Implications

6.2.1 TH antibodies in diagnosis

Although TH is an intracellular protein, serum antibodies against intracellularly located antigens can be of clinical importance with respect to diagnosis and prognosis. For example, the serum titre of antibodies can be correlated with disease activity as with anti-DNA antibodies in systemic lupus erythematosus (Riboldi *et al.* 2005). The presence of specific antibodies can also be of prognostic value as in the case of anti-Scl70 and anti-centromere protein B antibodies in systemic sclerosis (Nihtyanova and Denton 2010). Particular antibodies or their higher incidence can also indicate different organ involvement in disease such as anti-Sm antigen antibodies in

systemic lupus erythematosus (Rahman and Isenberg 2008). With respect to TH antibodies, they appear to occur at a greater frequency in active vitiligo. However, larger numbers of patients would need to be tested to confirm these initial results.

6.2.2 Possible origin of TH antibodies

The origin of antibodies against intracellular antigens is not fully understood. It has been observed that apoptotic cells can release apoptotic blebs or microbodies that contain several intracellular antigens (Casciola-Rosen *et al.* 1999). With defective clearance reported in various autoimmune diseases, the subsequent accumulation of these microbodies, along with activation of dendritic cells and the triggering of the immune response, may result in the generation of antibodies to their content (Fehr *et al.* 2009; Fransen *et al.* 2009; Munoz *et al.* 2010).

In addition, increasing evidence indicates that alterations in epigenetic modification including DNA methylation, histone acetylation and microRNA expression can contribute to B cell autoreactivity and generation of antibodies to intracellular antigens (Renaudineau *et al.* 2010). For example, it has been observed that a systemic lupus erythematosus-like syndrome develops in some patients treated with drugs such as hydralazine or procainamide. This was due to the reaction of these drugs with thymidine and deoxycytidine which prevented DNA methylation and thus initiated the disease (Dubroff and Reid 1980). Furthermore, in vasculitides patients with ANCA, epigenetic modifications due to gene silencing are responsible for improper intracellular expression of both of myeloperoxidase and proteinase-3, which are the main targets of ANCA in patients (Ciavatta *et al.* 2010).

Several studies have indicated that antibodies can be generated by molecular mimicry due to homology between the actual autoantigen and ‘foreign’ proteins (Routsias *et al.* 2006; Racanelli *et al.* 2011). In this context, viral, bacterial or fungal protein can induce antibodies that can cross-react with intracellular antigens. For example, a SSA-antigen epitope, targeted by antibodies in systemic lupus erythematosus, has a similar antigenic amino acid sequence to anti-latent viral protein Epstein-Barr virus nuclear antigen-1. Mice immunised with Epstein-Barr virus nuclear antigen-1 produced antibodies that cross-reacted with multiple epitopes on Ro60 antigen (McClain *et al.* 2005). The animals ultimately developed systemic lupus erythematosus-like symptoms such as leucopenia and renal dysfunction (McClain *et al.* 2005), supporting the

hypothesis of molecular mimicry as one of the causes of the generation of antibodies against intracellular antigens.

With respect to the development of TH antibodies, this is most likely a result of melanocyte destruction initiated primarily by non-antibody-dependent mechanisms such oxidative stress or cytotoxic T cells (Soubiran *et al.* 1985; D'Amelio *et al.* 1990; Abdel-Naser *et al.* 1992; Lang *et al.* 2001) (Ogg *et al.* 1998; Kroll *et al.* 2005; Schallreuter *et al.* 2005; Dell'Anna and Picardo 2006; Shalbaf *et al.* 2008). There have been several suggestions to account for immune responses against intracellular melanocyte proteins, including the formation of neo-antigens, the exposure of cryptic epitopes and the modification of proteins during apoptosis (Namazi 2007; Westerhof and d'Ischia 2007). Following processing by mature Langerhans cells, antigenic peptides could be presented to autoreactive T cells which have escaped clonal deletion or to naïve T lymphocytes which have not been tolerised against cryptic epitopes (Namazi 2007; Westerhof and d'Ischia 2007). Following stimulation of autoreactive B cells by activated autoreactive $CD4^+$ T lymphocytes, antibodies could then be produced (Namazi 2007).

6.2.3 Possible pathogenic effects of TH antibodies

Although TH is intracellularly located, there is evidence that antibodies can mediate their adverse effects inside the cell, for example, anti-SSA-antigen antibodies in systemic autoimmune disease (Mallery *et al.* 2010; Racanelli *et al.* 2011). Furthermore, TH antibodies that inhibit the function of the enzyme have been detected in patients with APS1 (Hedstrand *et al.* 2000). Expression of TH has been detected in cutaneous melanocytes in association with tyrosinase on the membrane of the melanosome, where it has been suggested to play a direct role in melanogenesis by providing L-dopa to tyrosinase as its activating substrate (Marles *et al.* 2003). However, to date, the function of TH in melanogenesis is not clearly defined, so it is difficult to speculate on how antibodies that might affect TH function could affect melanin synthesis and ultimately melanocyte behaviour.

Other possible pathogenic effects of TH antibodies include complement binding and ADCC, but it is difficult to imagine how these would interact with their intracellular target antigen to cause melanocyte damage by a cytotoxic mechanism. It is also possible that TH

antibodies are an epiphenomenon and play no part in vitiligo aetiology, but rather indicate the presence of autoreactive anti-TH T lymphocytes that are cytotoxic to pigment cells.

6.3 Future Work

The study could be improved by the analysis of further vitiligo patient serum samples for TH antibodies. More non-segmental vitiligo patient sera are already being collected for testing in a replication study. In addition, more patients with stable vitiligo and also individuals with the segmental clinical subtype should be analysed for the presence of TH antibodies to extend the initial results. However, these categories of patients are less likely to be available in great enough numbers to make this possible in the short-term. Further future work that could be carried out also includes:

6.3.1 Identification of TH antibodies which recognise conformation-dependent epitopes

Antibodies that bind to conformational epitopes are usually important in autoimmune disease pathogenesis. In Graves' disease, it is well-documented that the antibodies against the thyrotropin receptor, which are responsible for disease activity, recognise a number of different conformational epitopes (Morgenthaler *et al.* 1999). This is also the case for pathogenic antibodies against thyroid peroxidase in autoimmune hypothyroidism (Gora *et al.* 2004). The TH antibodies so far detected in vitiligo bind to linear determinants (Rahoma *et al.* 2012), and so it is possible that the prevalence of the humoral immune response against the enzyme has been underestimated (Kemp *et al.* 2011a; Kemp *et al.* 2011c). Future studies could identify antibodies that bind to conformational epitopes by employing mammalian expression systems to produce TH protein with native conformation which could be used in immunoprecipitation assays. This technique has been used in our laboratory to detect antibody binding to the calcium-sensing receptor (Gavalas *et al.* 2007).

6.3.2 Examination of the possible effects of TH antibodies on TH function

Antibodies that affect TH function are more likely to have a role in disease pathogenesis than antibodies that simply bind TH. For example, thyroid-stimulating antibodies activate the thyrotropin receptor and elicit hyperthyroidism in Graves' disease by mimicking the binding of thyroid-stimulating hormone (Weetman and McGregor 1994), and antibodies directed against the acetylcholine receptor block the acetylcholine-binding site and provoke accelerated receptor degradation causing myasthenia gravis (Hoedemaekers *et al.* 1997). Antibody-mediated deleterious effects on the function of TH could result in impaired melanocyte or keratinocyte behavior in vitiligo skin, a preliminary event in the development of disease. Although TH is located intracellularly, there is evidence that antibodies can mediate their adverse effects inside the cell, for example, anti-SSA-antigen antibodies in systemic autoimmune disease (Mallery *et al.* 2010; Racanelli *et al.* 2011). Furthermore, as a precedent, TH antibodies that inhibit the function of the enzyme have been detected in patients with APS1 (Hedstrand *et al.* 2000). Therefore, patient and control IgG samples should be tested for their effects upon TH function which can be done using an *in vitro* assay (Reinhard *et al.* 1986; Daubner and Fitzpatrick 1993; Lou *et al.* 2010).

6.3.3 Examination of vitiligo patient lesions for TH antibodies

The TH antibodies detected so far in vitiligo patients have been circulating serum antibodies (Kemp *et al.* 2011a; Kemp *et al.* 2011c). It would be of interest to determine if patient TH antibodies are also found in vitiligo lesions. Furthermore, the co-localisation within the same skin tissue sample of purified vitiligo patient TH antibodies and expressed TH could be evaluated using double indirect immunofluorescence.

6.3.4 Investigation of cytotoxicity of TH antibodies

It has been shown that vitiligo-associated antibodies have the ability to destroy melanocytes *in vitro* by complement-mediated damage and ADCC (Norris *et al.* 1988) and, *in vivo,* following passive immunisation of nude mice grafted with human skin (Gilhar *et al.* 1995). It would be

useful to investigate whether TH antibodies can have destructive effects on melanocytes by either of these mechanisms.

6.3.5 Investigation of T cell responses against TH

Several studies have detected the presence in vitiligo patients of circulating melanocyte-specific CD8+ cytotoxic T lymphocytes that target melanocyte differentiation antigens, including MelanA (MART-1), PMEL and tyrosinase (Ogg *et al.* 1998; Lang *et al.* 2001; Le Gal *et al.* 2001; Palermo *et al.* 2001; Mandelcorn-Monson *et al.* 2003; Le Poole *et al.* 2004; Garbelli *et al.* 2005; Le Poole and Luiten 2008; van den Boorn *et al.* 2009). Such cytotoxic T cells express high levels of the skin-homing receptor cutaneous lymphocyte-associated antigen and their frequency correlates with both the extent and activity of the disease supporting a crucial role of the cell-mediated immune response in vitiligo (Lang *et al.* 2001). With regard to this, the investigation of T cell-mediated immune responses against the TH protein would be of importance, as TH antibodies may be markers of anti-TH T lymphocyte reactivity in vitiligo patients.

6.4 Overall Conclusion

This thesis has concentrated on characterising TH antibody reactivity in vitiligo patients. However, the relationship between TH antibodies, and indeed all vitiligo antibodies, and the pathogenesis of the disease has yet to be established. The possibility that TH antibodies play no part in vitiligo aetiology but rather indicate the presence of autoreactive anti-TH T lymphocytes that are cytotoxic to pigment cells should be investigated. Even if they prove not to be directly pathogenic, TH antibodies may serve as useful markers of cellular disruption in the epidermis of vitiligo patients so further characterisation of them will continue.

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APPENDICES

Appendix I: DNA sequence of TH cDNA and TH cDNA deletion derivatives

```
pcDNA3-TH    ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH480       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH440       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH400       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH360       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH320       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH280       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH240       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH200       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH170       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH140       ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60
pTH1-80      ATGCCCACCCCCGACGCCACCACGCCACAGGCCAAGGGCTTCCGCAGGGCCGTGTCTGAG   60

pcDNA3-TH    CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH480       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH440       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH400       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH360       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH320       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH280       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH240       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH200       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH170       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH140       CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120
pTH1-80      CTGGACGCCAAGCAGGCAGAGGCCATCATGTCCCCGCGGTTCATTGGGCGCAGGCAGAGC   120

pcDNA3-TH    CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH480       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH440       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH400       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH360       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH320       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH280       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH240       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH200       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH170       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH140       CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180
pTH1-80      CTCATCGAGGACGCCCGCAAGGAGCGGGAGGCGGCGGTGGCAGCAGCGGCCGCTGCAGTC   180

pcDNA3-TH    CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH480       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH440       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH400       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH360       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH320       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH280       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH240       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH200       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH170       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH140       CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240
pTH1-80      CCCTCGGAGCCCGGGGACCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGAGGGGAAGGCC   240

pcDNA3-TH    GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG   300
pTH480       GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG   300
pTH440       GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG   300
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pTH400        GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG   300
pTH360        GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG   300
pTH320        GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG   300
pTH280         GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG  300
pTH240         GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG  300
pTH200         GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG  300
pTH170         GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG  300
pTH140         GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG  300
pTH81-140      GTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCCTCGGCGCTGTCCCGAGCTGTG  300

pcDNA3-TH     AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH480        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH440        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH400        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH360        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH320        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH280        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH240        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH200        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH170        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH140        AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360
pTH81-140     AAGGTGTTTGAGACGTTTGAAGCCAAAATCCACCATCTAGAGACCCGGCCCGCCCAGAGG   360

pcDNA3-TH     CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH480        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH440        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH400        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH360        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH320        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH280        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH240        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH200        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH170        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH140        CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420
pTH81-140     CCGCGAGCTGGGGGCCCCCACCTGGAGTACTTCGTGCGCCTCGAGGTGCGCCGAGGGGAC   420

pcDNA3-TH     CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH480        CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH440        CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH400        CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH360        CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH320        CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH280        CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH240        CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH200        CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH170        CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480
pTH141-497    CTGGCCGCCCTGCTCAGTGGTGTGCGCCAGGTGTCAGAGGACGTGCGCAGCCCCGCGGGG   480

pcDNA3-TH     CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540
pTH480        CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540
pTH440        CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540
pTH400        CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540
pTH360        CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540
pTH320        CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540
pTH280        CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540
pTH240        CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540
pTH200        CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540
pTH170        CCCAAGGTCCCCTGGTTCCCAAGAAAAGTG                                 540
pTH141-497    CCCAAGGTCCCCTGGTTCCCAAGAAAAGTGTCAGAGCTGGACAAGTGTCATCACCTGGTC   540

pcDNA3-TH     ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC   600
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pTH480        ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC   600
pTH440        ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC   600
pTH400        ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC   600
pTH360         ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC  600
pTH320         ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC  600
pTH280         ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC  600
pTH240         ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC  600
pTH200         ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC  600
pTH141-497     ACCAAGTTCGACCCTGACCTGGACTTGGACCACCCGGGCTTCTCGGACCAGGTGTACCGC  600

pcDNA3-TH      CAGCGCAGGAAGCTGATTGCTGAGATCGCCTTCCAGTACAGGCACGGCGACCCGATTCCC  660
pTH480         CAGCGCAGGAAGCTGATTGCTGAGATCGCCTTCCAGTACAGGCACGGCGACCCGATTCCC  660
pTH440         CAGCGCAGGAAGCTGATTGCTGAGATCGCCTTCCAGTACAGGCACGGCGACCCGATTCCC  660
pTH400         CAGCGCAGGAAGCTGATTGCTGAGATCGCCTTCCAGTACAGGCACGGCGACCCGATTCCC  660
pTH360         CAGCGCAGGAAGCTGATTGCTGAGATCGCCTTCCAGTACAGGCACGGCGACCCGATTCCC  660
pTH320         CAGCGCAGGAAGCTGATTGCTGAGATCGCCTTCCAGTACAGGCACGGCGACCCGATTCCC  660
pTH280         CAGCGCAGGAAGCTGATTGCTGAGATCGCCTTCCAGTACAGGCACGGCGACCCGATTCCC  660
pTH240         CAGCGCAGGAAGCTGATTGCTGAGATCGCCTTCCAGTACAGGCACGGCGACCCGATTCCC  660
pTH141-497     CAGCGCAGGAAGCTGATTGCTGAGATCGCCTTCCAGTACAGGCACGGCGACCCGATTCCC  660

pcDNA3-TH      CGTGTGGAGTACACCGCCGAGGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAG  720
pTH480         CGTGTGGAGTACACCGCCGAGGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAG  720
pTH440         CGTGTGGAGTACACCGCCGAGGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAG  720
pTH400         CGTGTGGAGTACACCGCCGAGGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAG  720
pTH360         CGTGTGGAGTACACCGCCGAGGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAG  720
pTH320         CGTGTGGAGTACACCGCCGAGGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAG  720
pTH280         CGTGTGGAGTACACCGCCGAGGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAG  720
pTH240         CGTGTGGAGTACACCGCCGAGGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAG  720
pTH141-497     CGTGTGGAGTACACCGCCGAGGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAG  720

pcDNA3-TH      GGCCTCTACGCCACGCACGCCTGCGGGGAGCACCTGGAGGCCTTTGCTTTGCTGGAGCGC  780
pTH440         GGCCTCTACGCCACGCACGCCTGCGGGGAGCACCTGGAGGCCTTTGCTTTGCTGGAGCGC  780
pTH400         GGCCTCTACGCCACGCACGCCTGCGGGGAGCACCTGGAGGCCTTTGCTTTGCTGGAGCGC  780
pTH360         GGCCTCTACGCCACGCACGCCTGCGGGGAGCACCTGGAGGCCTTTGCTTTGCTGGAGCGC  780
pTH320         GGCCTCTACGCCACGCACGCCTGCGGGGAGCACCTGGAGGCCTTTGCTTTGCTGGAGCGC  780
pTH280         GGCCTCTACGCCACGCACGCCTGCGGGGAGCACCTGGAGGCCTTTGCTTTGCTGGAGCGC  780
pTH141-497     GGCCTCTACGCCACGCACGCCTGCGGGGAGCACCTGGAGGCCTTTGCTTTGCTGGAGCGC  780

pcDNA3-TH      TTCAGCGGCTACCGGGAAGACAATATCCCCCAGCTGGAGGACGTCTCCCGCTTCCTGAAG  840
pTH480         TTCAGCGGCTACCGGGAAGACAATATCCCCCAGCTGGAGGACGTCTCCCGCTTCCTGAAG  840
pTH440         TTCAGCGGCTACCGGGAAGACAATATCCCCCAGCTGGAGGACGTCTCCCGCTTCCTGAAG  840
pTH400         TTCAGCGGCTACCGGGAAGACAATATCCCCCAGCTGGAGGACGTCTCCCGCTTCCTGAAG  840
pTH360         TTCAGCGGCTACCGGGAAGACAATATCCCCCAGCTGGAGGACGTCTCCCGCTTCCTGAAG  840
pTH320         TTCAGCGGCTACCGGGAAGACAATATCCCCCAGCTGGAGGACGTCTCCCGCTTCCTGAAG  840
pTH320         TTCAGCGGCTACCGGGAAGACAATATCCCCCAGCTGGAGGACGTCTCCCGCTTCCTGAAG  840
pTH141-497     TTCAGCGGCTACCGGGAAGACAATATCCCCCAGCTGGAGGACGTCTCCCGCTTCCTGAAG  840

pcDNA3-TH      GAGCGCACGGGCTTCCAGCTGCGGCCTGTGGCCGGCCTGCTGTCCGCCCGGGACTTCCTG  900
pTH480         GAGCGCACGGGCTTCCAGCTGCGGCCTGTGGCCGGCCTGCTGTCCGCCCGGGACTTCCTG  900
pTH440         GAGCGCACGGGCTTCCAGCTGCGGCCTGTGGCCGGCCTGCTGTCCGCCCGGGACTTCCTG  900
pTH400         GAGCGCACGGGCTTCCAGCTGCGGCCTGTGGCCGGCCTGCTGTCCGCCCGGGACTTCCTG  900
pTH360         GAGCGCACGGGCTTCCAGCTGCGGCCTGTGGCCGGCCTGCTGTCCGCCCGGGACTTCCTG  900
pTH320         GAGCGCACGGGCTTCCAGCTGCGGCCTGTGGCCGGCCTGCTGTCCGCCCGGGACTTCCTG  900
pTH141-497     GAGCGCACGGGCTTCCAGCTGCGGCCTGTGGCCGGCCTGCTGTCCGCCCGGGACTTCCTG  900

pcDNA3-TH      GCCAGCCTGGCCTTCCGCGTGTTCCAGTGCACCCAGTATATCCGCCACGCGTCCTCGCCC  960
pTH480         GCCAGCCTGGCCTTCCGCGTGTTCCAGTGCACCCAGTATATCCGCCACGCGTCCTCGCCC  960
pTH440         GCCAGCCTGGCCTTCCGCGTGTTCCAGTGCACCCAGTATATCCGCCACGCGTCCTCGCCC  960
pTH400         GCCAGCCTGGCCTTCCGCGTGTTCCAGTGCACCCAGTATATCCGCCACGCGTCCTCGCCC  960
pTH360         GCCAGCCTGGCCTTCCGCGTGTTCCAGTGCACCCAGTATATCCGCCACGCGTCCTCGCCC  960
pTH320         GCCAGCCTGGCCTTCCGCGTGTTCCAGTGCACCCAGTATATCCGCCACGCGTCCTCGCCC  960
pTH141-497     GCCAGCCTGGCCTTCCGCGTGTTCCAGTGCACCCAGTATATCCGCCACGCGTCCTCGCCC  960
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pcDNA3-TH    ATGCACTCCCCTGAGCCGGACTGCTGCCACGAGCTGCTGGGGCACGTGCCCATGCTGGCC 1020

pTH480       ATGCACTCCCCTGAGCCGGACTGCTGCCACGAGCTGCTGGGGCACGTGCCCATGCTGGCC 1020
pTH440       ATGCACTCCCCTGAGCCGGACTGCTGCCACGAGCTGCTGGGGCACGTGCCCATGCTGGCC 1020
pTH400       ATGCACTCCCCTGAGCCGGACTGCTGCCACGAGCTGCTGGGGCACGTGCCCATGCTGGCC 1020
pTH360       ATGCACTCCCCTGAGCCGGACTGCTGCCACGAGCTGCTGGGGCACGTGCCCATGCTGGCC 1020
pTH1412-497  ATGCACTCCCCTGAGCCGGACTGCTGCCACGAGCTGCTGGGGCACGTGCCCATGCTGGCC 1020


pcDNA3-TH    GACCGCACCTTCGCGCAGTTCTCGCAGGACATTGGCCTGGCGTCCCTGGGGGCCTCGGAT 1080
pTH480       GACCGCACCTTCGCGCAGTTCTCGCAGGACATTGGCCTGGCGTCCCTGGGGGCCTCGGAT 1080
pTH440       GACCGCACCTTCGCGCAGTTCTCGCAGGACATTGGCCTGGCGTCCCTGGGGGCCTCGGAT 1080
pTH400       GACCGCACCTTCGCGCAGTTCTCGCAGGACATTGGCCTGGCGTCCCTGGGGGCCTCGGAT 1080
pTH360       GACCGCACCTTCGCGCAGTTCTCGCAGGACATTGGCCTGGCGTCCCTGGGGGCCTCGGAT 1080
pTH141-497   GACCGCACCTTCGCGCAGTTCTCGCAGGACATTGGCCTGGCGTCCCTGGGGGCCTCGGAT 1080

pcDNA3-TH    GAGGAAATTGAGAAGCTGTCCACGCTGTCATGGTTCACGGTGGAGTTCGGGCTGTGTAAG 1140
pTH480       GAGGAAATTGAGAAGCTGTCCACGCTGTCATGGTTCACGGTGGAGTTCGGGCTGTGTAAG 1140
pTH440       GAGGAAATTGAGAAGCTGTCCACGCTGTCATGGTTCACGGTGGAGTTCGGGCTGTGTAAG 1140
pTH400       GAGGAAATTGAGAAGCTGTCCACGCTGTCATGGTTCACGGTGGAGTTCGGGCTGTGTAAG 1140
pTH141-497   GAGGAAATTGAGAAGCTGTCCACGCTGTCATGGTTCACGGTGGAGTTCGGGCTGTGTAAG 1140

pcDNA3-TH    CAGAACGGGGAGGTGAAGGCCTATGGTGCCGGGCTGCTGTCCTCCTACGGGGAGCTCCTG 1200
pTH480       CAGAACGGGGAGGTGAAGGCCTATGGTGCCGGGCTGCTGTCCTCCTACGGGGAGCTCCTG 1200
pTH440       CAGAACGGGGAGGTGAAGGCCTATGGTGCCGGGCTGCTGTCCTCCTACGGGGAGCTCCTG 1200
pTH400       CAGAACGGGGAGGTGAAGGCCTATGGTGCCGGGCTGCTGTCCTCCTACGGGGAGCTCCTG 1200
pTH141-497   CAGAACGGGGAGGTGAAGGCCTATGGTGCCGGGCTGCTGTCCTCCTACGGGGAGCTCCTG 1200

pcDNA3-TH    CACTGCCTGTCTGAGGAGCCTGAGATTCGGGCCTTCGACCCTGAGGCTGCGGCCGTGCAG 1260
pTH480       CACTGCCTGTCTGAGGAGCCTGAGATTCGGGCCTTCGACCCTGAGGCTGCGGCCGTGCAG 1260
pTH440       CACTGCCTGTCTGAGGAGCCTGAGATTCGGGCCTTCGACCCTGAGGCTGCGGCCGTGCAG 1260
pTH141-497   CACTGCCTGTCTGAGGAGCCTGAGATTCGGGCCTTCGACCCTGAGGCTGCGGCCGTGCAG 1260

pcDNA3-TH    CCCTACCAAGACCAGACGTACCAGTCAGTCTACTTCGTGTCTGAGAGCTTCAGTGACGCC 1320
pTH480       CCCTACCAAGACCAGACGTACCAGTCAGTCTACTTCGTGTCTGAGAGCTTCAGTGACGCC 1320
pTH440       CCCTACCAAGACCAGACGTACCAGTCAGTCTACTTCGTGTCTGAGAGCTTCAGTGACGCC 1320
pTH141-497   CCCTACCAAGACCAGACGTACCAGTCAGTCTACTTCGTGTCTGAGAGCTTCAGTGACGCC 1320

pcDNA3-TH    AAGGACAAGCTCAGGAGCTATGCCTCACGCATCCAGCGCCCCTTCTCCGTGAAGTTCGAC 1380
pTH480       AAGGACAAGCTCAGGAGCTATGCCTCACGCATCCAGCGCCCCTTCTCCGTGAAGTTCGAC 1380
pTH141-497   AAGGACAAGCTCAGGAGCTATGCCTCACGCATCCAGCGCCCCTTCTCCGTGAAGTTCGAC 1380

pcDNA3-TH    CCGTACACGCTGGCCATCGACGTGCTGGACAGCCCCCAGGCCGTGCGGCGCTCCCTGGAG 1440
pTH480       CCGTACACGCTGGCCATCGACGTGCTGGACAGCCCCCAGGCCGTGCGGCGCTCCCTGGAG 1440
pTH141-497   CCGTACACGCTGGCCATCGACGTGCTGGACAGCCCCCAGGCCGTGCGGCGCTCCCTGGAG 1440

pcDNA3-TH    GGTGTCCAGGATGAGCTGGACACCCTTGCCCATGCGCTGAGTGCCATTGGC 1491
pTH141-497   GGTGTCCAGGATGAGCTGGACACCCTTGCCCATGCGCTGAGTGCCATTGGC 1491
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Appendix II: Protein sequence of TH and TH deletion derivatives

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TH              MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH480           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH440           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH400           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH360           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH320           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH280           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH240           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH200           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH170           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH140           MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60
TH1-80          MPTPDATTPQAKGFRRAVSELDAKQAEAIMSPRFIGRRQSLIEDARKEREAAVAAAAAAV 60

TH              PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH480           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH440           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH400           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH360           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH320           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH280           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH240           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH200           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH170           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH140           PSEPGDPLEAVAFEEKEGKAVLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120
TH1-80          PSEPGDPLEAVAFEEKEGKA                                          120
TH81-140                          VLNLLFSPRATKPSALSRAVKVFETFEAKIHHLETRPAQR 120

TH              PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180
TH480           PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180
TH440           PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180
TH400           PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180
TH360           PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180
TH320           PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180
TH280           PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180
TH240           PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180
TH200           PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180
TH170           PRAGGPHLEYFVRLEVRRGDLAALLSGVRQVSEDVRSPAGPKVPWFPRKV            180
TH140           PRAGGPHLEYFVRLEVRRGD                                          180
TH81-140        PRAGGPHLEYFVRLEVRRGD                                          180
TH141-497                         LAALLSGVRQVSEDVRSPAGPKVPWFPRKVSELDKCHHLV 180

TH              TKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYRHGDPIPRVEYTAEEIATWKEVYTTLK 240
TH480           TKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYRHGDPIPRVEYTAEEIATWKEVYTTLK 240
TH440           TKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYRHGDPIPRVEYTAEEIATWKEVYTTLK 240
TH400           TKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYRHGDPIPRVEYTAEEIATWKEVYTTLK 240
TH360           TKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYRHGDPIPRVEYTAEEIATWKEVYTTLK 240
TH320           TKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYRHGDPIPRVEYTAEEIATWKEVYTTLK 240
TH280           TKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYRHGDPIPRVEYTAEEIATWKEVYTTLK 240
TH240           TKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYRHGDPIPRVEYTAEEIATWKEVYTTLK 240
TH141-497       TKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYRHGDPIPRVEYTAEEIATWKEVYTTLK 240

TH              GLYATHACGEHLEAFALLERFSGYREDNIPQLEDVSRFLKERTGFQLRPVAGLLSARDFL 300
TH480           GLYATHACGEHLEAFALLERFSGYREDNIPQLEDVSRFLKERTGFQLRPVAGLLSARDFL 300
TH440           GLYATHACGEHLEAFALLERFSGYREDNIPQLEDVSRFLKERTGFQLRPVAGLLSARDFL 300
TH400           GLYATHACGEHLEAFALLERFSGYREDNIPQLEDVSRFLKERTGFQLRPVAGLLSARDFL 300
TH360           GLYATHACGEHLEAFALLERFSGYREDNIPQLEDVSRFLKERTGFQLRPVAGLLSARDFL 300
TH320           GLYATHACGEHLEAFALLERFSGYREDNIPQLEDVSRFLKERTGFQLRPVAGLLSARDFL 300
TH280           GLYATHACGEHLEAFALLERFSGYREDNIPQLEDVSRFLK                      300
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TH141-497       GLYATHACGEHLEAFALLERFSGYREDNIPQLEDVSRFLKERTGFQLRPVAGLLSARDFL 300

TH              ASLAFRVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASD 360
TH480           ASLAFRVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASD 360
TH440           ASLAFRVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASD 360
TH400           ASLAFRVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASD 360
TH360           ASLAFRVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASD 360
TH320           ASLAFRVFQCTQYIRHASSP                                          360
TH141-497       ASLAFRVFQCTQYIRHASSPMHSPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASD 360

TH              EEIEKLSTLSWFTVEFGLCKQNGEVKAYGAGLLSSYGELLHCLSEEPEIRAFDPEAAAVQ 420
TH480           EEIEKLSTLSWFTVEFGLCKQNGEVKAYGAGLLSSYGELLHCLSEEPEIRAFDPEAAAVQ 420
TH440           EEIEKLSTLSWFTVEFGLCKQNGEVKAYGAGLLSSYGELLHCLSEEPEIRAFDPEAAAVQ 420
TH400           EEIEKLSTLSWFTVEFGLCKQNGEVKAYGAGLLSSYGELL                      420
TH141-497       EEIEKLSTLSWFTVEFGLCKQNGEVKAYGAGLLSSYGELLHCLSEEPEIRAFDPEAAAVQ 420

TH              PYQDQTYQSVYFVSESFSDAKDKLRSYASRIQRPFSVKFDPYTLAIDVLDSPQAVRRSLE 480
TH480           PYQDQTYQSVYFVSESFSDAKDKLRSYASRIQRPFSVKFDPYTLAIDVLDSPQAVRRSLE 480
TH440           PYQDQTYQSVYFVSESFSDA                                          480
TH141-497       PYQDQTYQSVYFVSESFSDAKDKLRSYASRIQRPFSVKFDPYTLAIDVLDSPQAVRRSLE 480

TH              GVQDELDTLAHALSAIG 497
TH141-497       GVQDELDTLAHALSAIG 497
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