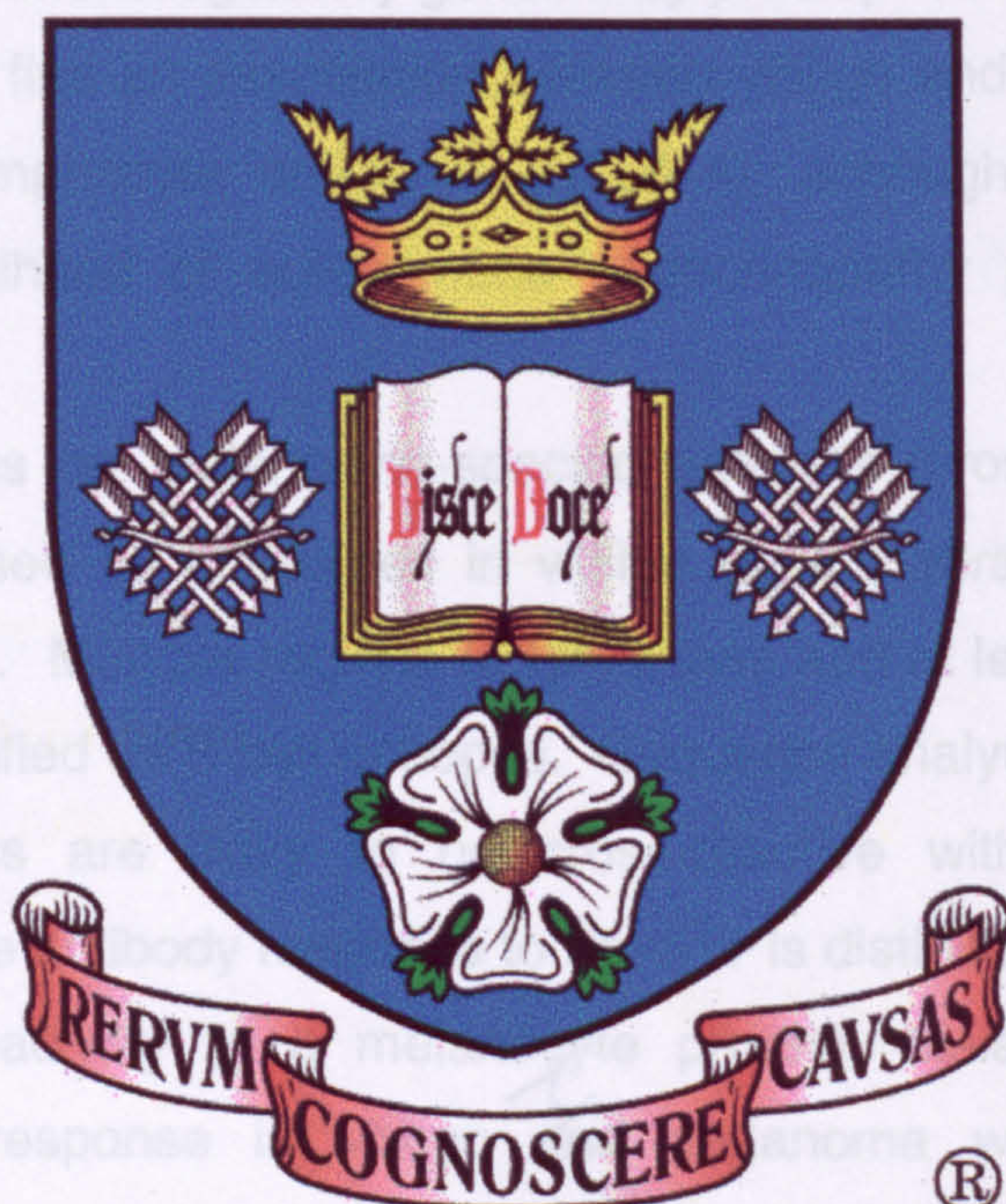
AN INVESTIGATION INTO THE ROLE OF AUTOIMMUNITY IN VITILIGO



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Summary

Vitiligo is an acquired depigmenting disorder characterised by the loss of functional melanocytes from the cutaneous epidermis. A role for autoimmunity is supported by the presence of circulating antibodies and T lymphocytes which react against melanocyte antigens in patients with vitiligo. The identification and characterisation of these autoantigens will improve understanding of the immune response in vitiligo, and may allow development of better therapies and diagnostic tools.

Candidate immunoregulatory genes may predispose to vitiligo. However, this study failed to find an association between vitiligo and a polymorphism of the cytotoxic T lymphocyte antigen-4 (CTLA-4), although the polymorphism increases the likelihood of autoimmune endocrinopathy patients developing vitiligo.

The epitopes on melanocyte-specific antigens tyrosinase and Pmel17 which are recognised by antibodies in vitiligo patient sera were identified by molecular mapping. Multiple regions of tyrosinase and at least two domains on Pmel17 were identified as B cell epitopes. Sequence analysis revealed that the tyrosinase epitopes are likely to be cross-reactive with tyrosinase-related proteins but that the antibody response to Pmel17 is distinct.

Antibody reactivity to a melanocyte protein, MelanA, targeted by a cellular immune response in vitiligo and melanoma was investigated by immunoblotting and radioimmunoassay. No MelanA-specific antibodies were isolated suggesting that either it is not a target of the humoral immune response in vitiligo, or that antibody reactivity was not detectable by the methods used.

To identify novel vitiligo autoantigens, a melanoma cDNA expression library was constructed in a phage-display cloning system and immunoscreened with vitiligo patient IgG. Several possible autoantigens were enriched by this technique, including proteins previously characterised as autoantigens in other disorders. Additionally, humoral reactivity was identified to a protein with a possible role in pigmentation, the melanin-concentrating hormone receptor 1 (MCHR1). MCHR1-specific antibodies were detected in 16.4% (9/55) of vitiligo patients but not in other diseases or healthy control subjects. The study demonstrates the usefulness of phage-display for further autoantigen identification in vitiligo.

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Publications

Papers

"The melanin-concentrating hormone receptor 1, a novel target of autoantibody responses in vitiligo". E. H. Kemp*, E. A. Waterman*, B. E. Hawes, K. O'Neill, R. Gottumukada, D. J. Gawkrodger, A. P. Weetman, and P. F. Watson. (Submitted to J Clin Invest)

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"B cell epitopes on tyrosinase which are recognised by autoantibodies from patients with vitiligo". E. A. Waterman, E. H. Kemp, S. MacNeil, D. J. Gawkrodger, P. F. Watson and A. P. Weetman. Pigment Cell Res (1999) S7: PP152. 17th International Pigment Cell Conference, Nagoya, Japan.

Abbreviations

A	Adenine
Ab	Antibody
ACTH	Adrenal corticotrophin hormone
AD	Addison's disease
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
AIRE	Autoimmune regulator
AMP^R	Ampicillin resistance
APC	Antigen presenting cell
APS1	Autoimmune polyglandular syndrome type 1
bFGF	Basic fibroblast growth factor
bp	Base pairs
BSA	Bovine serum albumin
C	Complement or carboxyl terminus or cytosine
cAMP	Cyclic adenosine monophosphate
CC	Case control
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
cfu	Colony-forming units
CLA	Cutaneous lymphocyte-associated antigen
CMV	Cytomegalovirus
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
cpm	Counts per minute
CTLA-4	Cytotoxic T lymphocyte antigen-4
DEPC	Diethylpyrocarbonate
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2- carboxylic acid quinone
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
ds	Double-stranded (cDNA)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot (assay)
ER	Endoplasmic reticulum

G	Guanine
g	Gravity
g	Gram/s
GAD	Glutamate decarboxylase
GD	Graves' disease
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GST	Glutathione-S-transferase
GTP	Guanine triphosphate
h	Hour/s
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1R-α	Interleukin-1 receptor-alpha
IP	Immunoprecipitation
IPTG	Isopropyl-β-thiogalactopyranoside
kDa	Kilodalton
kHz	Kilohertz
L	Litre/s
LB	Luria-Bertani (medium and agar)
LD PCR	Long-distance polymerase chain reaction
LMP	Low molecular weight protein
mAb	Monoclonal antibody
MAGE-1	Melanoma-associated antigen-1
mA	Milli-ampere/s
MAP	Mitogen-activated protein
MART-1	Melanoma antigen recognised by T cells-1
MCF1	Multicatalytic endopeptidase complex-like 1
MCH	Melanin-concentrating hormone
MCHR1	Melanin-concentrating hormone receptor 1
MCP	Membrane cofactor protein
MCR	Melanocortin receptor
MCS	Multiple cloning site
mg	Milligram/s
MHC	Major histocompatibility complex
mi	Microphthalmia (locus)
min	Minute/s
MITF	Microphthalmia-associated transcription factor
ml	Millilitre/s

M-MLV	Moloney murine leukemia virus
MOPS	3-[N-Morpholino]propanesulphonic acid
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
N	Amino terminus
NGF	Nerve growth factor
NK	Natural killer (cells)
°C	Degrees centigrade
OCA	Oculocutaneous albinism
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	Plaque-forming units
POMC	Propiomelanocortin
PUVA	Psoralen with ultraviolet A
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Si	Silver (locus)
SLE	Systemic lupus erythematosus
ss	Single-stranded (cDNA)
SSC	Saline sodium citrate
ssFv	Single chain variable region of immunoglobulin fragment with antigen binding
T	Thymine
TAE	Tris/acetate/EDTA
TAP	Transporter involved in antigen-processing
Tc	T cytotoxic (lymphocyte)
TCA	Trichloroacetic acid
TDT	Transmission-disequilibrium test
TE	Tris-EDTA (buffer)
TEMED	N, N, N, N'-tetramethylethylenediamine
Th	T helper (lymphocyte)
TNF-α	Tumour necrosis factor alpha

tRNA	Transfer ribonucleic acid
TRP	Tyrosinase-related protein
TSHR	Thyroid-stimulating hormone receptor
TYR	Tyrosinase
UTR	Untranslated region
UV	Ultraviolet
v	Variable (gene segment)
V	Volt/s
VKH	Vogt-Koyanagi-Harada (syndrome)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
yr	Years
α-MSH	Alpha-melanocyte stimulating hormone
$[Ca^{2+}]_i$	Intracellular calcium
μg	Microgram/s
μl	Microlitre/s

1 General Introduction

1.1 Structure and function of the skin

1.1.1 General skin functions

The skin is a complex organ, comprising approximately one-twelfth of total body mass (Millington & Wilkinson 1983), and forming the boundary between the body and its external environment. It performs many essential functions which include the maintenance of internal homeostasis via thermoregulatory secretary activity, protection against chemical and physical assault (Millington & Wilkinson 1983), and the prevention of invasion by pathogenic micro- and macro-organisms (Reeves & Todd 1996).

1.1.2 Ultra-structure of the skin

Human skin is formed of two layers, the epidermis, a cellular, avascular epithelium which derives from the embryonic ectoderm, and the dermis, a dense vascularised connective tissue arising from the embryonic mesoderm. The ultra-structure of the skin is summarised in Figure 1.1. The cutaneous epidermis comprises a multi-layered epithelium, consisting mostly of keratinocytes. Five epithelial layers are described as follows: the basal cell layer (*stratum basale*), prickle cell layer (*stratum spinosum*), granular cell layer (*stratum granulosum*), transitional cell layer (*stratum lucidum*) and the dead squamous cell layer (*stratum corneum*) (Wood & Bladon 1985). The nomenclature is based on the different morphological appearances of the keratinocytes according to their stage in the process of keratinisation. The innermost basal layer consists of a single layer of cylindrically shaped cells attached to a basement membrane that forms the junction between epidermis and dermis. The basal keratinocytes continually proliferate by mitotic division and move outwards through the layers undergoing subsequent differentiation, to be shed eventually at the exterior. The prickle cells are keratinocytes, so called because their protruding desmosomes, filled with keratin filaments, are visible as prickles on the cell surface under a light microscope. Above the prickle cell layer are keratinocytes

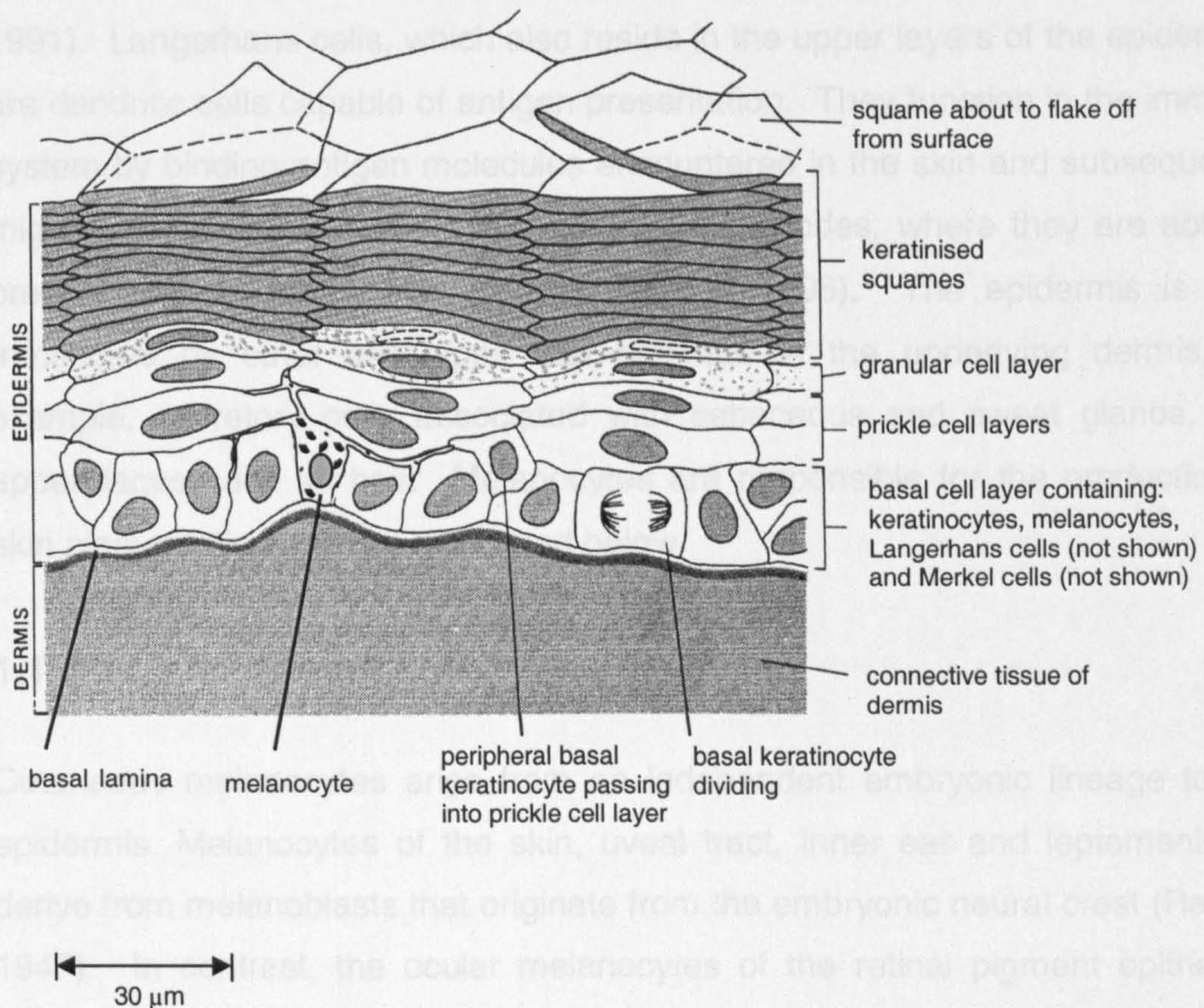


Figure 1.1: A schematic representation of the ultra-structure of the skin.
(Adapted from Alberts *et al.* 1989).

in the penultimate stages of keratinisation. They appear granular because of the aggregation of keratohyaline. The transitional cells are terminally differentiated keratinocytes undergoing loss of their cellular contents. The outermost layer is made up of flattened dead keratinocytes termed squames.

In addition to keratinocytes, the basal epithelial layer contains small numbers of other cell types: Merkel cells, Langerhans cells and melanocytes. Merkel cells are closely associated with nerve terminals of Schwann cells in the epidermis, often lying adjacent to hairs, and are considered to function as sensory receptors or transducers (Montagna & Parakkal 1974; Lacour *et al.* 1991). Langerhans cells, which also reside in the upper layers of the epidermis, are dendritic cells capable of antigen presentation. They function in the immune system by binding antigen molecules encountered in the skin and subsequently migrating from the epidermis to regional lymph nodes, where they are able to present antigen to T cells (Reeves & Todd 1996). The epidermis is also transected by other cell types that originate in the underlying dermis, for example, secretory cells associated with sebaceous and sweat glands, and appendages such as hair. Melanocytes are responsible for the production of skin pigmentation and are discussed below.

1.1.3 The melanocyte

Cutaneous melanocytes arise from an independent embryonic lineage to the epidermis. Melanocytes of the skin, uveal tract, inner ear and leptomeninges derive from melanoblasts that originate from the embryonic neural crest (Rawles 1947). In contrast, the ocular melanocytes of the retinal pigment epithelium arise from neural ectoderm as it invaginates to form the optical cup (Feeny-Burns 1980). Early in human embryonic gestation, considered to be seven weeks, the melanoblasts migrate into the developing epidermis and eventually settle in the basal epithelial layer (Holbrook *et al.* 1989). The control of migration and differentiation of embryonic melanoblasts is not fully understood, but the involvement of several genes, especially c-*kit* proto-oncogene, *pax* gene and stem cell factor gene, and transcription factors, especially microphthalmia-associated transcription factor and SOX10, has been elucidated from mice mutants (Boissy 1995; Ortonne & Ballotti 2000). The melanocytes reside in the basal epithelial layer for the life of an individual at a density of 1000-1500 cells

per mm^2 (Quevedo *et al.* 1987). The primary function of the melanocyte is the synthesis of the pigment melanin which is deposited into the keratinocytes of the upper epidermis to absorb incident ultraviolet (UV) light, thereby protecting the genome of the dividing basal keratinocytes and melanocytes (Boissy 1995). In addition, melanin effectively absorbs oxygen free radicals (Korytowski *et al.* 1987) and therefore might serve to protect the metabolically active keratinocytes from oxidative stress (Boissy 1995). Melanocytes have a roughly pyramidal morphology with numerous fine dendritic processes which can transfer melanin granules to surrounding keratinocytes. The appearance of melanocytes in culture is demonstrated in Figure 1.2. One melanocyte contacts an average of thirty-six keratinocytes in the normal human epidermis, this ratio being termed the 'epidermal melanin unit' (Fitzpatrick *et al.* 1967). The epidermal melanin unit is the same in all ethnic groups. Differences in the rate of melanin synthesis (Iwata *et al.* 1990), the type of melanin produced (Quevedo *et al.* 1974) and the way it is distributed within keratinocytes (Szabo *et al.* 1988), account for the different appearances of skin colour between ethnic groups.

In addition to their principal function of melanin synthesis or melanogenesis (Section 1.1.4), melanocytes also act as accessory cells in the skin immune response (Das *et al.* 2001). They are capable of secreting cytokines which assist in the maturation and migration of antigen presenting cells, such as Langerhans cells, and are involved in the recruitment of immune infiltrates into the skin (Zachariae *et al.* 1991; Swope *et al.* 1994). Additionally, there is evidence to suggest that melanocytes can themselves act as antigen presenting cells (Le Poole *et al.* 1993b; Das *et al.* 2001).

1.1.4 Melanogenesis

The initiation of melanogenesis has two parts: the synthesis of the melanosome, the specialised organelle which carries the melanin and the synthesis of the enzymes responsible for melanin synthesis. Each process occurs independently in the melanocyte in the smooth and rough endoplasmic reticulum, respectively, before convergence in the melanosome. The melanosome is required to enclose safely the pigmentary pathway, many of the intermediates being highly reactive and potentially toxic to the melanocyte (Pawelek *et al.* 1980; Urabe *et al.* 1994).

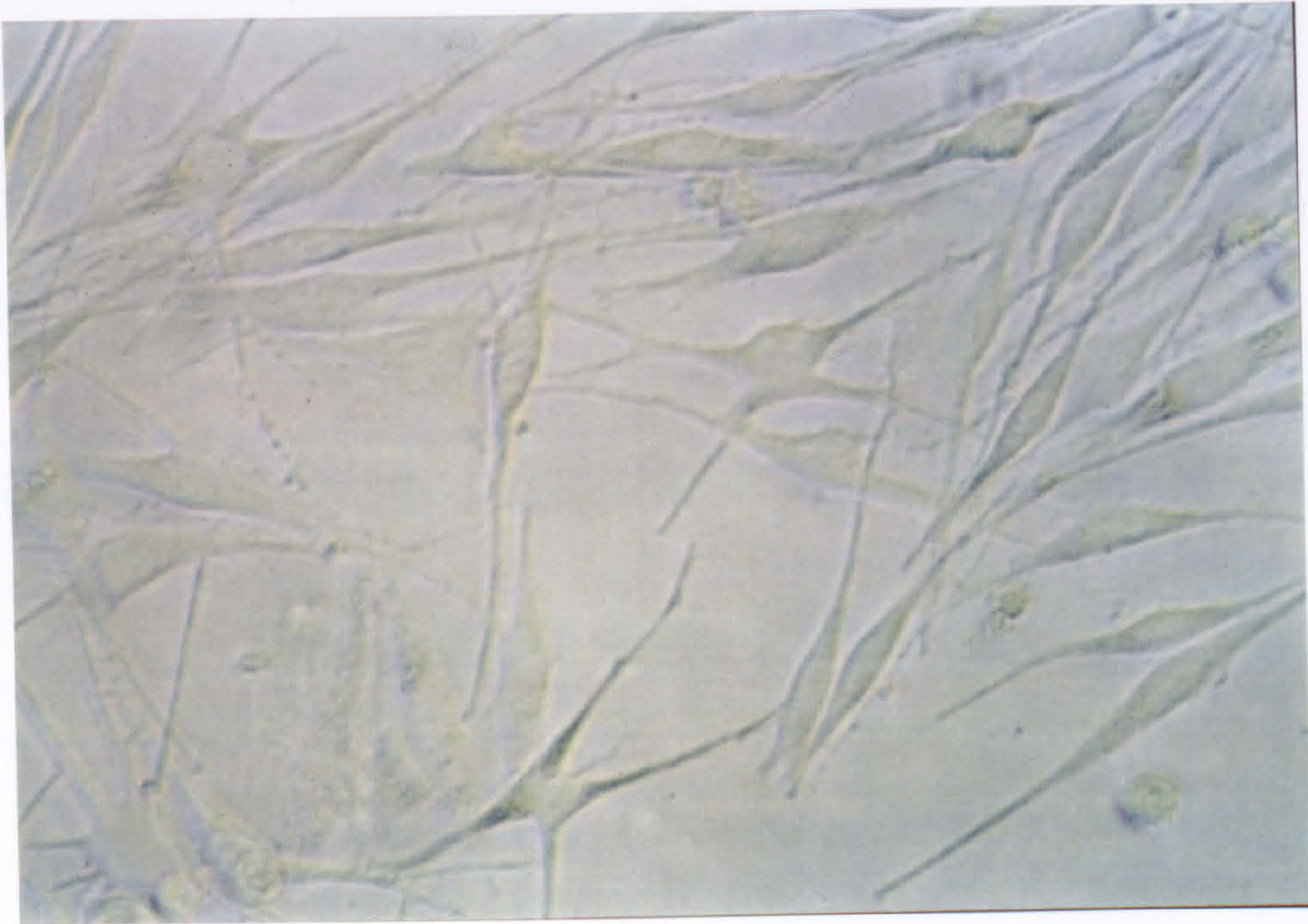
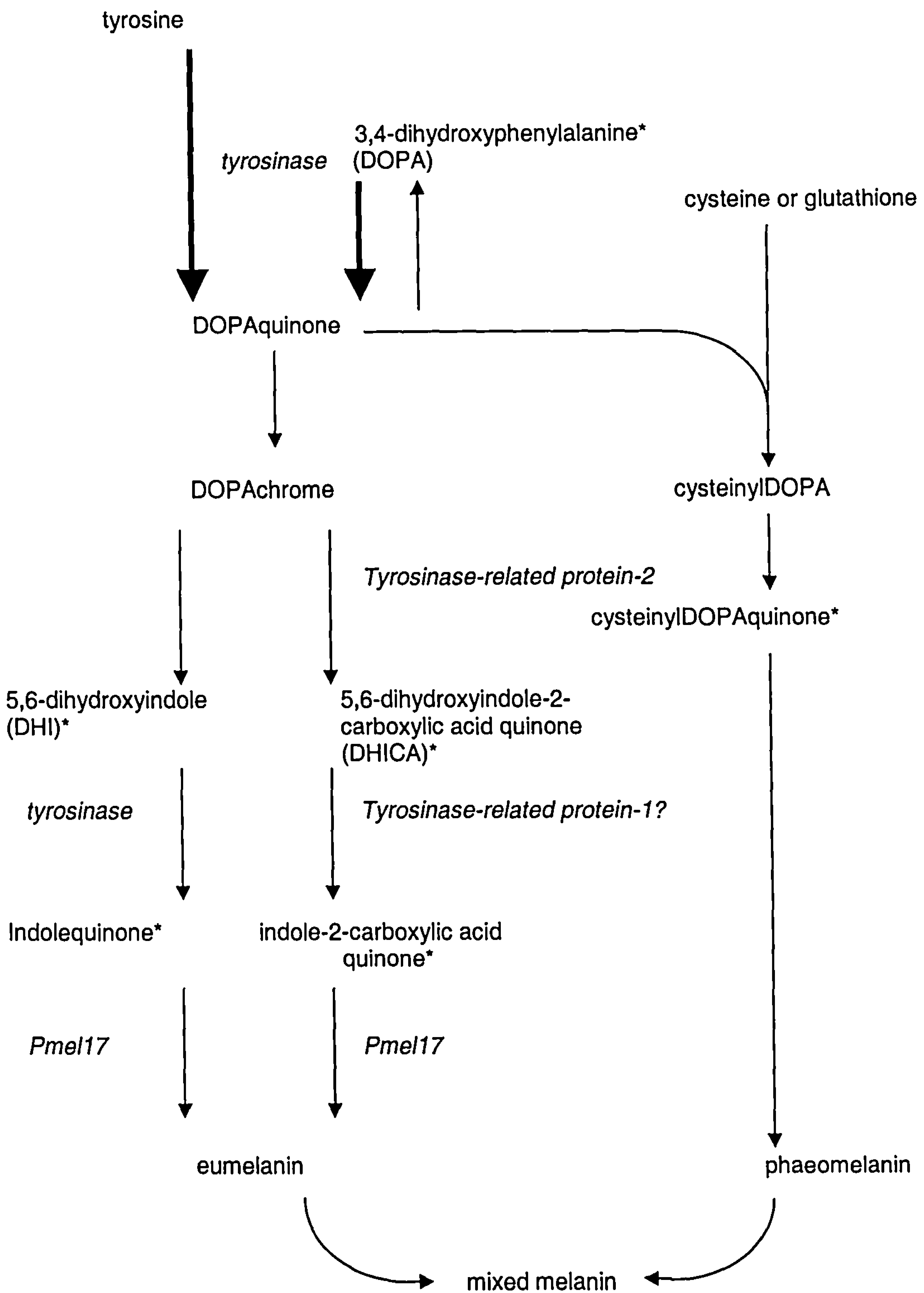


Figure 1.2: Melanocytes grown in culture X200 magnification.

(Provided by Miss C. Balafa and Professor S. MacNeil, Division of Clinical Sciences North, University of Sheffield).

The pathways by which melanin is synthesised are summarised in Figure 1.3. Melanogenesis essentially comprises of the conversion of tyrosine into 3,4-dihydroxyphenylalanine(DOPA)chrome in a two-step reaction controlled by the enzyme tyrosinase. The DOPAchrome is subsequently processed in further reactions, catalysed by tyrosinase, tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2) and Pmel17, into eumelanin. Eumelanins are brown/black melanin polymers and provide protection in response to UV irradiation. A second form of melanin, pheomelanin, a reddish-yellow melanin, can be synthesised in the presence of sulphydryl-containing compounds such as glutathione or cysteine, and appears not to be photoprotective. The melanosome bearing melanin is transported to surrounding keratinocytes via the dendritic processes of the melanocyte. The keratinocytes take up the melanosome by endocytosis of the dendritic ends (Wolff 1973).

In addition to exposure to UV light, several cytokines and growth factors, including tumour necrosis factor alpha (TNF-α), endothelin, stem cell factor (SCF) and basic fibroblast growth factor (bFGF), are known to mediate melanogenesis (Nordlund 1998). Of particular importance in the control of melanogenesis are the melanotrophic peptides alpha-melanocyte stimulating hormone (α-MSH) and adrenal corticotrophin 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 & Ballotti 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 bring about an up-regulation of melanogenesis (Ortonne & Ballotti 2000). However, the complete pathways, and regulation, of melanogenesis have yet to be fully elucidated and the role of many melanocyte-specific proteins in these processes remains to be identified. Table 1.1 summarises some key melansomal proteins and their known function in melanogenesis.



* denotes intermediates of melanogenesis that are ultimately incorporated into the melanin polymers, in addition to eumelanin and phaeomelanin.

Figure 1.3: A diagramic overview of the melanogenesis pathway.

(Adapted from Riley 1997, with additional information from Boissy 1995 and Ortonne & Ballotti 2000).

Table 1.1: Melanosomal proteins.

Protein	Size (kDa)	Function	Gene location	Phenotype of gene mutations	References
Tyrosinase	70	Catalsyes the principle regulatory step in melanogenesis, the conversion of L-tyrosine→DOPAquinone[1].	Chromosome 11	Oculocutaneous albinism type 1 (OCA-1)[2].	1. Oetting 2000 2. King *et al.* 1995
TRP-1 (gp75)	60	Catalyses steps in the eumelanin pathway. May also stabilise tyrosinase[3] and other melanosomal enzymes. Precise role remains unclear.	Chromosome 9	Oculocutaneous albinism type 3 (OCA-3)[4].	3. Kobayashi *et al.* 1994b 4. Hearing 2000
TRP-2	59	Catalyses DOPAchrome→5,6-DHICA[5].	Chromosome 13	Oculocutaneous albinism type 2 (OCA-2)[6].	5. Aroca *et al.* 1990 6. Hearing 2000
Pmel17 (gp100)	73	Controls the deposition of DHI and DHICA into eumelanin[7].	Chromosome 12	Mutations in the mouse Pmel17 gene result in loss of melanocytes and a subsequent greying of the coat [8].	7. Boissy 1995 8. Johnson & Jackson 1992
MelanA/MART-1 (Melanoma Antigen Recognised by T cells-1)	13	Unknown	Chromosome 9	None described	
P-protein	90	Regulation of melanosomal pH[9].	Chromosome 15	OCA-2[10].	9. Puri *et al.* 2000 10. Rinchik *et al.* 1993
Lysosome-associated membrane protein-1	90-120	Purported to protect the melanosomal membrane[11].	Chromosome 13	None described	11. Das *et al.* 2001

Tyrosinase is the rate-limiting enzyme in the melanogenesis pathway through its action of the hydroxylation of tyrosine to DOPAquinone (Oetting 2000). The presence of tyrosinase activity was first described in mammalian skin by Durham, (Durham 1904), who also noted an absence of tyrosinase activity in the skin of albino animals. The tyrosinase protein is a membrane glycoprotein containing 529 amino acids, including a signal protein at the amino end, two copper binding sites and a transmembrane region at the carboxy-end (Oetting 2000). The requirement for two copper atoms in the catalytic site of tyrosinase is demonstrated by the expression of hypopigmentation in the phenotype of Menkes disease, a disorder in which copper transport is deficient (Ortonne & Ballotti 2000). The control of tyrosinase (TYR) gene expression, in response to stimuli such as UV light and α-MSH production, has yet to be fully elucidated, but an important regulatory element contained within the TYR promoter has been identified and termed the 'M' (melanocyte)-Box (Oetting 2000). The 'M'-Box is known to bind the family of transcription factors which include the microphthalmia-associated transcription factor (MITF) (Oetting 2000). Oculocutaneous albinism type 1 (OCA-1), characterised by a complete lack of melanin synthesis in the hair, eyes and skin, is an autosomal recessive disorder caused by mutations of the TYR gene (King *et al.* 1995), of which more than ninety patients have been described (Oetting 2000).

The tyrosinase gene family (Kwon 1993) includes two other structurally homologous glycoproteins: tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein-2 (TRP-2). The primary amino acid sequences of TRP-1 and TRP-2 are overall approximately 45% identical to that of tyrosinase and contain a similar signal sequence and trans-membrane domain (Kwon 1993). However, both proteins differ significantly from tyrosinase at their carboxy-end (Orlow *et al.* 1993) and have distinct catalytic activities. TRP-2 acts as a DOPAchrome tautomerase, catalysing the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid quinone (5,6-DHICA) (Aroca *et al.* 1990). The function of TRP-1 has not been determined in human melanogenesis, despite its role being characterised as DHICA oxidase in murine melanosomes (Hearing 2000). It is thought to stablise tyrosinase activity (Kobayashi *et al.* 1994b) and mutations of TRP-1, which affect its ability to do this, may lead to oculocutaneous albinism type 3 (OCA-3) (Hearing 2000).

Pmel17 is a melanosomal matrix glycoprotein (Kobayashi *et al.* 1994a; Zhou *et al.* 1994) with a limited structural homology to the tyrosinase gene family proteins (Kwon 1993). Indeed, Pmel17 cDNA was originally isolated from a human melanocyte cDNA library using anti-tyrosinase antibodies (Kwon *et al.* 1991). However, the homologous regions are confined to five short regions of amino acids only and it is classified within its own gene family (Kwon 1993). Mutations at the mouse silver (*si*) locus, which encodes Pmel17, result in a loss of melanocytes in the coat hair and subsequent silvering (Johnson & Jackson 1992). Similar mutations in the human Pmel17 gene, *D12S53E*, have been speculated to cause greying of human hair (Ortonne & Ballotti 2000). The Pmel17 protein is thought to function as a stablin, more specifically an indole blocking factor, that regulates the deposition of 5,6-dihydroxyindole (DHI) and DHICA into eumelanin (Boissy 1995).

1.2 Vitiligo

1.2.1 Definition

Vitiligo is an acquired depigmentary disorder characterised by the loss of functional melanocytes from the cutaneous epidermis. Additionally, the disorder may affect pigment cells in the mucous membranes, inner ear and eye. Pigment is lost in circumscribed maculae which have a tendency to enlarge peripherally with disease progression.

1.2.2 Prevalence

Large population surveys have estimated a prevalence ranging from 0.38 (Howitz *et al.* 1977) to 1.13% (Mehta *et al.* 1973). The consensus prevalence is around 1% (Lerner 1971). In approximately 50% of cases the disease presents before the age of 20 years (Majumder *et al.* 1993). The incidence of the disease in both sexes is equal. There is no preponderance of particular racial skin types, although the disease is more visually apparent in darker skin.

1.2.3 Inheritance and genetics

A family history is reported in over 20% of vitiligo patients (Ortonne *et al.* 1983; Majumder *et al.* 1993) and concordance in monozygotic twins has been reported (Mohr 1951; Siemens 1953; Mayenburg *et al.* 1976) but the exact frequency of concordance has not been determined and the reporting of isolated cases is subject to ascertainment bias. Genetic studies of familial cases of vitiligo conclude that the disorder is most likely due to the action of genes at multiple loci (Majumder *et al.* 1993; Kim *et al.* 1998; Acros *et al.* 1999) and is not transmitted by a simple mendelian mechanism. However, some authors propose that an autosomal dominant gene with variable expression and incomplete penetrance may be responsible (Mosher *et al.* 1979; Lorincz 1985). In the Smyth line chicken (Smyth 1989), an animal model of vitiligo, the disorder results from a genetically inherited defect that is expressed in the melanocytes and which appears to be transmitted to offspring as an autosomal recessive trait. Similarly, the depigmentation in a mouse model, strain C57BL/6J Ler-*vit/vit* developed to study vitiligo, results from a genetic mutation that is inherited as an autosomal recessive trait (Lerner *et al.* 1986). The mutation in the *vit/vit* mouse occurs at the microphthalmia (*mi*) locus which encodes microphthalmia-associated transcription factor (MITF), and, therefore, interest arose in the human homologue as a susceptibility gene for vitiligo. However, linkage studies proved negative (Tripathi *et al.* 1999). A mutation in the human *mi* gene is responsible for Waardenburg syndrome type 2, a disease characterised by congenital depigmentation and abnormalities in neural development (Ortonne & Ballotti 2000).

Since vitiligo may have an immune involvement (Sections 1.3.6, 1.4), candidate genes include those that have a regulating role in the immune system. The study of the major histocompatibility complex (MHC) genes has revealed associations of vitiligo with certain human leukocyte antigen (HLA) specificities but there is no consistent association with any of the major MHC class I or class II alleles (Table 1.2), and the reported relative risks have been modest. Additionally, the genes encoding complement components C2 and C4 (MHC class III molecules) have been proposed to influence the development of vitiligo since an increased frequency of heterozygous C4 and C2 deficiency has been found in patients with the disorder (Venneker *et al.* 1992).

Table 1.2: Major histocompatibility complex (MHC) antigen associations with vitiligo.

Origin of population	Number of patients in study	Associated specificity	Reference
Slovakia	67	A2, Dw7	Buc *et al.* 1996
Italy	87	A30, B27, Cw6, DQw3	Finco *et al.* 1991
Holland	42	DR4, C4BQ0	Venneker *et al.* 1992
Holland	50	DR4, DQB1	Zamani *et al.* 2001
Kuwait	40	B21, Cw6	Al-Fouzan *et al.* 1995
Oman	50	Bw6, DR7	Venkataram *et al.* 1995
Hungary	88	DR1, DR3	Poloy *et al.* 1991
America (African)	24	DR4, DQw3	Dunston & Halder 1990
America (Caucasian)	48	DR4	Foley *et al.* 1983
Italy (Northern)	86	A3	Lorini *et al.* 1992
Italy (Northern)	93	A30, Cw6, DQw3	Metzker *et al.* 1980
Morocco (Jewish)	18	B13	Orecchia *et al.* 1992
Yemen	16	Bw35	Metzker *et al.* 1980
Germany (Northern)	102	A2	Schallreuter *et al.* 1993

(Adapted from Kemp *et al.* 2001b).

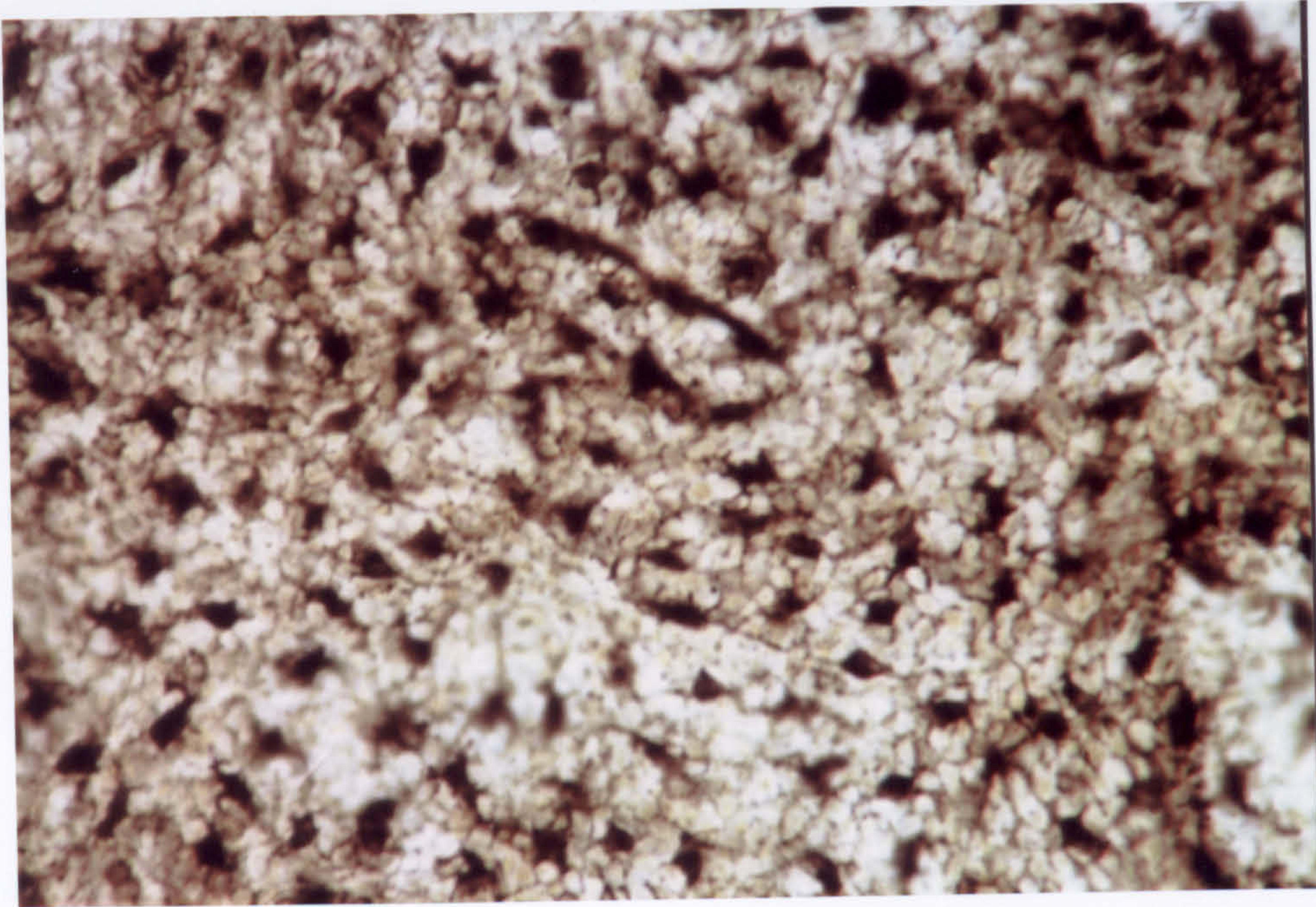
A further candidate is the recently identified *AIRE* (autoimmune regulator) gene which is involved in autoimmune polyglandular syndrome type 1 (APS1), a disorder with which vitiligo can be associated (The Finnish-German APECED Consortium 1997; Nagamine *et al.* 1997). This novel gene encodes a T cell-specific protein that contains motifs indicative of a transcription factor and mutation analysis has identified several mutations in this gene in APS1 patients (Heino *et al.* 2001; Myhre *et al.* 2001). However, this gene is unlikely to be related to the development of vitiligo which is not associated with APS1.

A recent study, involving 552 vitiligo patients and their families, examined polymorphic markers in a panel of twenty-four candidate genes that might confer susceptibility to vitiligo (McCormack *et al.* 2001b). The genes analysed in this study included those encoding tyrosinase, TRP-1, TRP-2, tyrosine hydroxylase, phenylalanine hydroxylase, guanosine triphosphate-cyclohydrolase, the transcription factor nuclear factor 1, and two genes involved in melanocyte embryogenesis, *kit* and *pax*3. No association was found between any of these genes involved in melanocyte function and an increased risk of vitiligo. Genes encoding proteins involved in the immune response, including: MHC class II molecules; low molecular weight protein (LMP)-2 and LMP–7, and transporter involved in antigen-processing (TAP)-1 and TAP–2; the proteosome multicatalytic endopeptidase complex-like 1 (MECL1); CD4; CD59; membrane cofactor protein (MCF); cytokine and cytokine receptors interleukin (IL)-1 β, tumour necrosis factor-alpha (TNFα) and IL-1 receptor-alpha (IL-1R-α); intercellular adhesion molecule-1 (ICAM-1); co-stimulatory lymphocyte activation ligands CD28 and cytotoxic T lymphocyte antigen-4 (CTLA-4), were also examined in these family studies. Polymorphic markers for the immune response LMP/TAP and CD28/CTLA-4 gene regions appeared to be positively associated with vitiligo susceptibility (McCormack *et al.* 2001b). Further details on CTLA-4 and its association with vitiligo will be discussed in Chapter 3.

1.2.4 Pathology

Affected skin shows a loss of melanin and decreased numbers of melanocytes in the epidermis. Figure 1.4 demonstrates the difference in density of epidermal melanocytes between normal and vitiligo skin. Immunohistochemical studies and electron microscopic analysis of vitiligo lesions demonstrate the

(a)



(b)

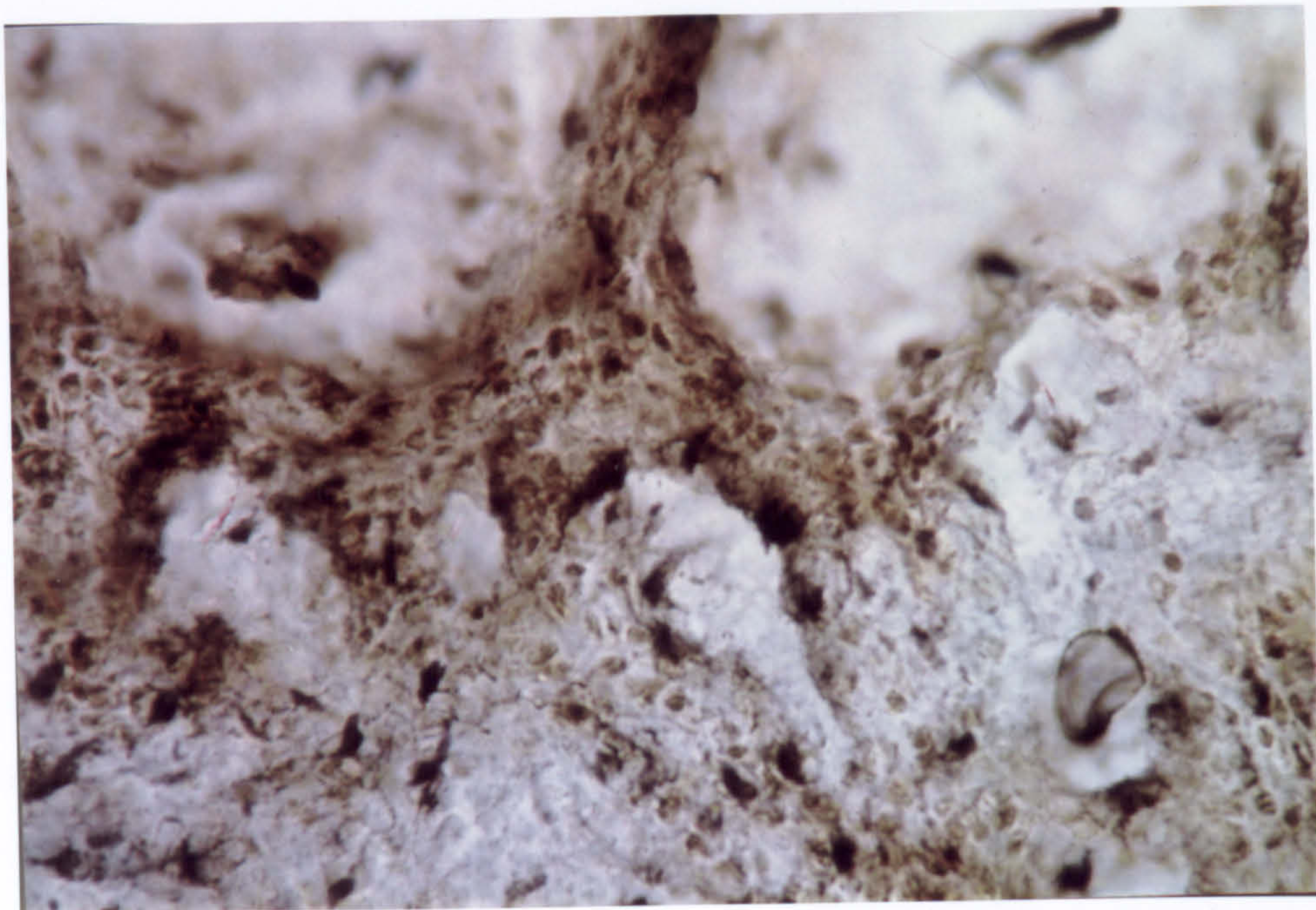


Figure 1.4: (a) Melanocytes in a section of normal epidermis.

(b) Melanocytes in an epidermal section from a patient with vitiligo showing a much reduced cell density.

Both sections are treated with a DOPA immunohistochemical stain, which detects tyrosinase activity and reveals the melanocytes in brown.

(Provided by Dr D. J. Gawkrodger, Royal Hallamshire Hospital, Sheffield).

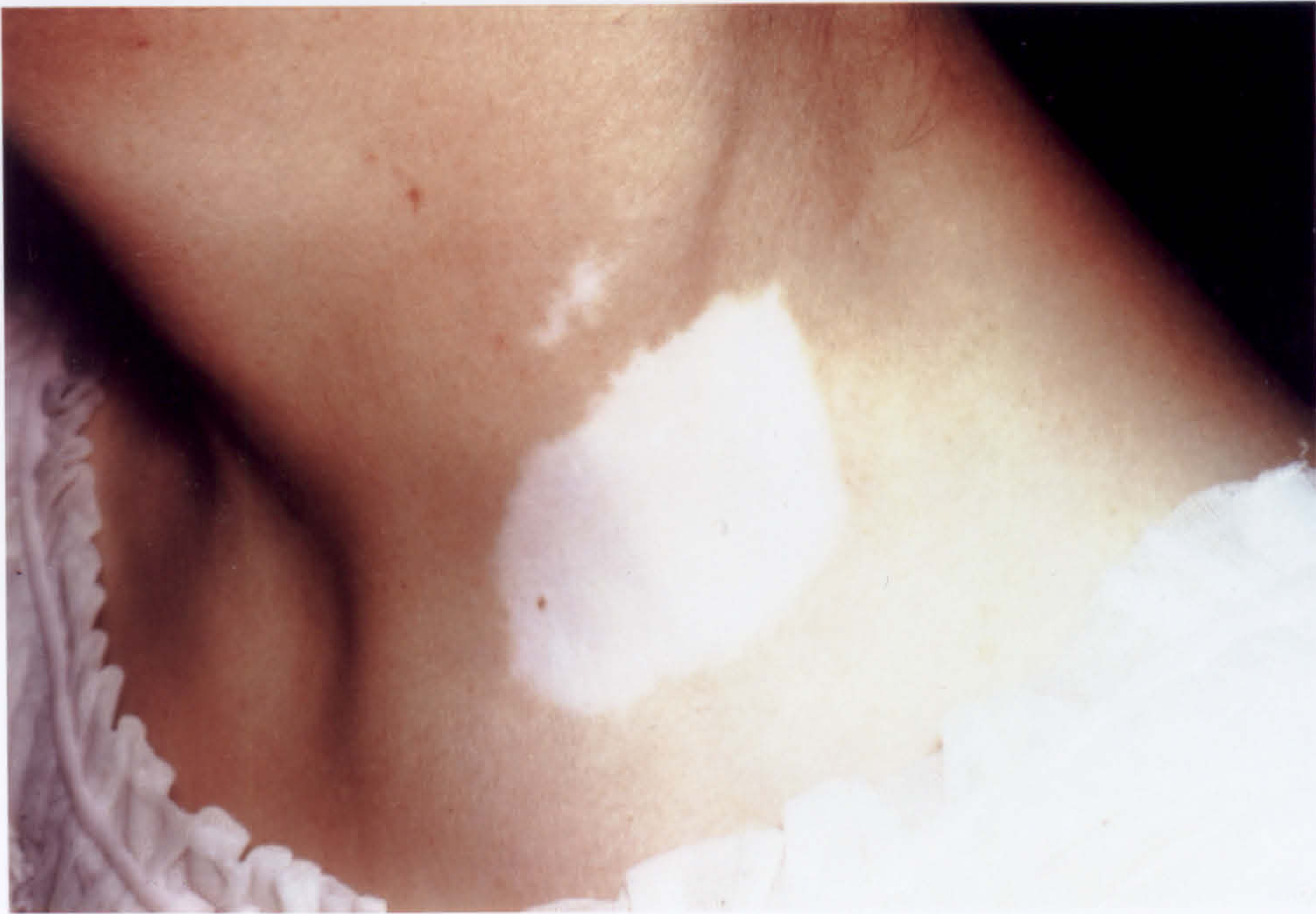
degeneration of melanocytes and also the presence of abnormal keratinocytes (Moellmann *et al.* 1982; Le Poole *et al.* 1993c). In peri-lesional regions, abnormal melanocytes are seen which are fragmented, grossly enlarged or show the appearance of vacuoles (Le Poole *et al.* 1993c; Abdel-Naser *et al.* 1994). Additionally, in the peri-lesional areas of inflammatory vitiligo (Bleehan 1979), and in some cases of generalised vitiligo (Le Poole *et al.* 1993c), a lymphocytic infiltration can be identified in the basal layer of the epidermis in proximity to the melanocytes.

1.2.5 Clinical features

The progression of vitiligo is variable and there are five disease categories dictated by the extent of involvement and distribution of the vitiligo maculae: generalised or symmetrical, segmental, focal, acrofacial and universal (Kovacs 1998). Generalised or symmetrical type vitiligo is characterised by a bilateral, symmetrical depigmentation which can affect the face (especially periorificial areas), neck, torso, the extensor surfaces or bony prominances of the hands, wrists, and legs, axillae and also orifices or mucosal surfaces (Kovacs 1998). Segmental type vitiligo has a dermatomal, asymmetrical distribution of depigmentation (Kovacs 1998). This type of vitiligo often begins in childhood, is rarely associated with autoimmune disorders and has stable results after autologous grafting (Section 1.2.7). In contrast, symmetrical vitiligo has a later age of onset, is frequently associated with autoimmunity and has unstable results with autologous grafting (Kovacs 1998). These contrasts have lead to speculation that symmetrical and segmental vitiligo represent two quite separate disease manifestations (Kovacs 1998; Taieb 2000). Focal type vitiligo is localised and non-dermatomal in distribution (Kovacs 1998), whereas acrofacial vitiligo is characterised by depigmentation of the distal fingers and facial orifices only, the latter being in a circumferential pattern (Kovacs 1998). Finally, patients with universal vitiligo exhibit a loss of pigment over the entire body surface (Kovacs 1998), a type of vitiligo that occurs rarely. The two most common clinical presentations are symmetrical and segmental vitiligo which are shown in Figures 1.5a and 1.5b, respectively.

Trichrome, inflammatory, and occupational vitiligo represent unusual forms of the disease. Trichome vitiligo involves macules in which there are

(a)



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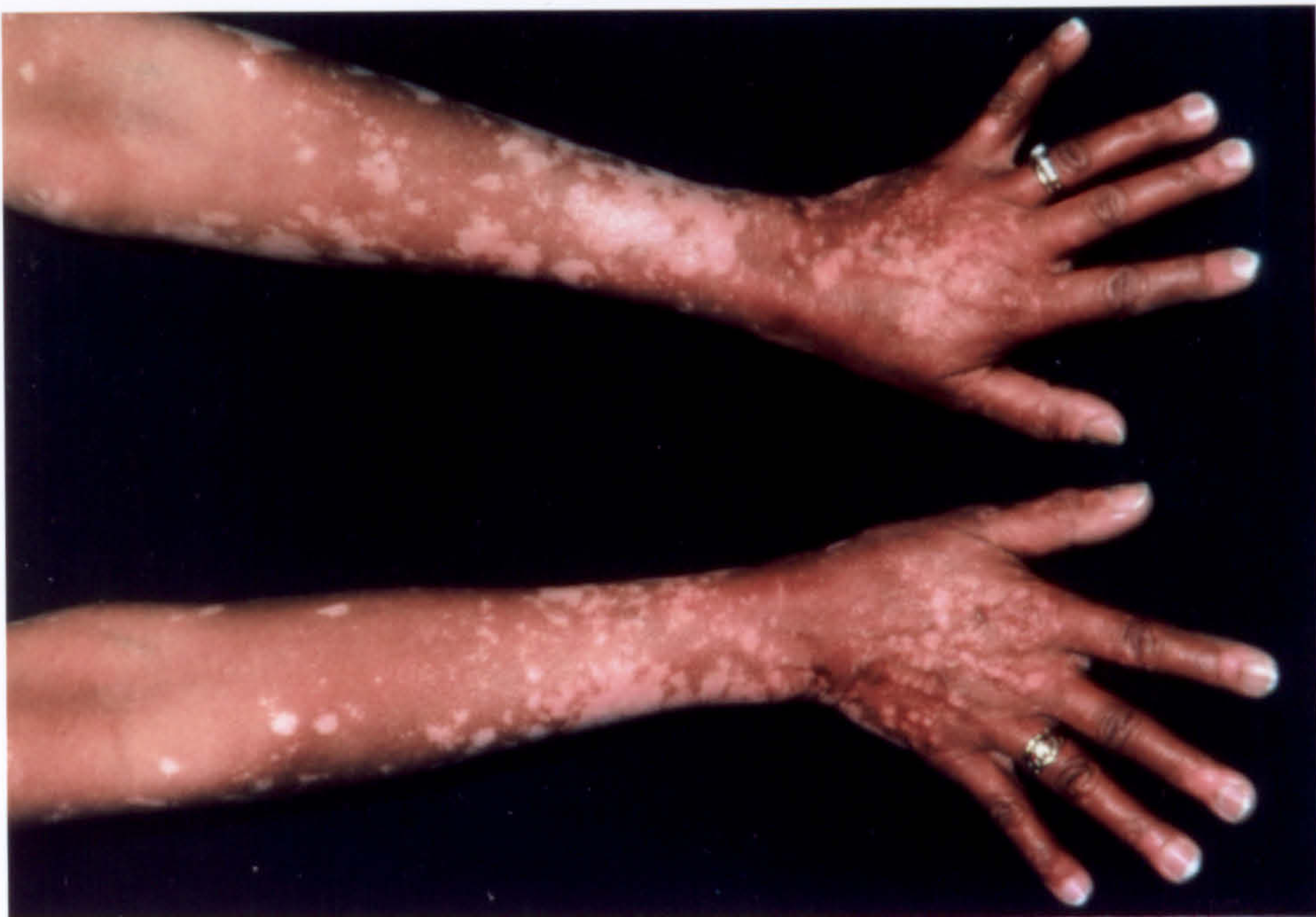


Figure 1.5: (a) **Segmental vitiligo affecting one side of the neck.** (Provided by Professor A. P. Weetman, Division of Clinical Sciences North, University of Sheffield).

(b) **Symmetrical vitiligo on the dorsal aspects of the hands.** (Provided by Dr D. J. Gawkrodger, Royal Hallamshire Hospital, Sheffield).

areas of both complete and partial loss of pigment, forming a pattern of varying hues between the affected and non-affected skin (Fitzpatrick 1964; Gawkrodger 1998). Uniquely, areas of normally pigmented skin adjacent to an area of vitiligo may become hyperpigmented (Gawkrodger 1998). Inflammatory vitiligo is characterised by actively spreading lesions which are inflamed, presenting a raised erythematous peri-lesional border (Grab & Wise 1948; Le Poole *et al.* 1996). Chemically induced occupational vitiligo can occur in workers exposed to compounds such as catechols, phenols, hydroquinone and monobenzene. The depigmentation is usually confined to the parts of the body exposed to the chemical, e.g. the hands and forearms, but can arise in areas distant from the original point of contact, suggestive of systemic exposure through percutaneous adsorption, inhalation or ingestion. Some of the compounds shown to cause depigmentation in occupational vitiligo are now specifically used in depigmenting therapies in idiopathic vitiligo (Table 1.4).

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 by Gauthier (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).

Although vitiligo may be considered a relatively minor disorder, the psychological effects can be considerable, particularly in those with racially darkly pigmented skin in whom the appearance of the lesions is more marked. Detrimental feelings of stress, embarrassment or self-consciousness when in contact with strangers, perception of discrimination and low self-esteem are common, especially in patients with visible lesions (Porter *et al.* 1986; Porter *et al.* 1990; Kent & Al'Abadie 1996).

1.2.6 Associated disorders

Vitiligo is frequently associated with autoimmune disorders (Table 1.3) including autoimmune thyroid disease (Ochi & DeGroot 1969), type 1 diabetes mellitus (Macaron *et al.* 1977), alopecia areata (Sharma *et al.* 1996), pernicious anaemia (Dawber 1969), Addison's disease (Dunlop 1963) and autoimmune polyendocrine syndromes (Neufeld *et al.* 1981; Ahonen *et al.* 1990). Patients with vitiligo are also more likely to suffer from an autoimmune condition than those in the general population. Autoimmune thyroiditis, for example, has a prevalence of up to 30% in vitiligo patients (Cunliffe *et al.* 1968), a figure significantly higher than that of 1% prevalence reported in the general population.

Halo naevi, areas of depigmentation seen surrounding a melanocytic naevus or 'mole', may be associated with vitiligo and sometimes pre-date the onset of the disorder (Lerner 1971). Premature greying of the hair (canities) may also be found in patients with vitiligo (Lerner 1971). Vitiligo can affect all active melanocytes, including those of the retinal pigment epithelium and uveal tract of the eye and the scala vestibuli of the inner ear, which may lead to ocular abnormalities such as iris, conjunctival and chorioretinal depigmentation and uveitis (Albert *et al.* 1979; Cowan *et al.* 1986) or auditory problems including hearing impairment in the 2-8 kHz range (Thurmon *et al.* 1976; Tosti *et al.* 1986; Tosti *et al.* 1987), respectively. The combination of vitiligo with uveitis, canities, auditory anomalies and neurological involvement is known as Vogt-Koyanagi-Harada (VKH) syndrome (Barnes 1988). It has been proposed that VKH syndrome may represent a systemic manifestation of vitiligo, since the neurological involvement may also result from melanocyte destruction in the leptomeninges of the brain (Kovacs 1998).

1.2.7 Treatment

Since the aetiology of vitiligo is yet to be determined, there are no effective treatments available to correct the basic defect/s that lead to destruction of the melanocytes. Repigmenting therapies are, therefore, often unsustained and the patient has to resort to covering the patches with cosmetics. Therapies can be

Table 1.3: Association of vitiligo with autoimmune disorders.

Autoimmune disease	Number of patients in study	Percentage with vitiligo	Reference
Autoimmune polyglandular syndrome type 1	71	8	Neufeld *et al.* 1981
Autoimmune polyglandular syndrome type 1	68	13	Ahonen *et al.* 1990
Autoimmune polyglandular syndrome type 2	224	5	Neufeld *et al.* 1981
Autoimmune polyglandular syndrome type 2	22	4.5	Ahonen *et al.* 1990
Pernicious anaemia	125	9	Dawber 1969
Type 1 diabetes mellitus (juvenile)	300	1.7	Macaron *et al.* 1977
Autoimmune thyroid disease (Graves' disease)	90	7	Ochi & DeGroot 1969
Alopecia areata	808	1.8	Sharma *et al.* 1996

(Adapted from Kemp *et al.* 2001b).

categorised as medical or surgical and are summarised in Table 1.4. On-going psychological support is also important, particularly for patients who have suffered serious psychological affects or social stigma as a result of the disease.

1.3 Theories of vitiligo aetiology

The aetiology of vitiligo is presently unknown. There have been three basic hypotheses proposed to explain the aetiology as follows: (i) autoimmunity, (ii) neurochemical-mediated effects on the melanocyte and (iii) an intrinsic melanocyte defect causing the melanocytes to self-destruct. In the convergence theory, the causal factors of each of the previous three hypotheses act together, or independently, to bring about the destruction of melanocytes (Le Poole *et al.* 1993a). Furthermore, environmental factors, including psychological stress, mechanical stress injury and infections, have been proposed to contribute to vitiligo aetiology (Taieb 2000), in addition to a possible inherent genetic predisposition (Section 1.2.3).

1.3.1 Psychological stress

Many vitiligo patients report that their vitiligo lesions first appeared or expanded under conditions of extreme stress (Lerner 1959; Papadopoulos *et al.* 1999). Stress induces an increase in the production of catecholamines, the possible influence of which in melanocyte destruction is described in Table 1.5.

1.3.2 Physical stress (Koebner's phenomenon)

Vitiligo maculae may appear at the site of mechanical stress or friction injury to the skin (Taieb 2000). Gauthier (Gauthier 1996) demonstrated that the distribution of vitiligo lesions commonly correlates to areas of the body subjected to the most mechanical stresses. A recent study showed that the appearance of experimentally induced Koebner's phenomenon, after small skin biopsy, to some degree correlated with the disease activity in non-segmental type vitiligo patients (Njoo *et al.* 1999).

Table 1.4: Therapies for vitiligo.

Treatment	Reference
I. MEDICAL TREATMENTS	
Cosmetic modalities	Kovacs 1998
Corticosteroids (topical or systemic)	Boissy 1995; Gawkrodger 1998; Kovacs 1998
Psoralen (topical or systemic) and ultraviolet A (PUVA)	Boissy 1995; Gawkrodger 1998; Kovacs 1998
Khellin (topical or systemic) with ultraviolet A or natural sunlight	Gawkrodger 1998
Phenylalanine (topical or systemic) with ultraviolet A	Kovacs 1998
Topical pseudocatalase with ultraviolet B	Gawkrodger 1998
Oral antioxidants (e.g. vitamin E acetate; selenio-methionine)	Gawkrodger 1998
5-Fluorouracil (topical)	Kovacs 1998
Depigmentation with monobenzyl ether of hydroquinone	Kovacs 1998
II. SURGICAL TREATMENTS	
Epidermal grafting	Boissy 1995; Kovacs 1998
Autologous minigrafts	Boissy 1995; Kovacs 1998
Transplantation of cultured epidermis	Boissy 1995; Kovacs 1998
Transplantation of non-cultured melanocytes	Boissy 1995; Kovacs 1998
Tattooing	Boissy 1995

(Adapted from Kemp *et al.* 2001b).

1.3.3 Infections

It is possible that an infection may result in melanocyte destruction by triggering an immune response leading to vitiligo in some patients. The melanocyte may be destroyed as a result of harbouring an infectious agent or because it exposes antigens similar to a micro-organism to immune surveillance (Le Poole *et al.* 1993a). Vitiligo-like hypopigmetation has been reported in some infectious diseases such as leprosy (Shegan 1971), candidiasis (Howanitz *et al.* 1981) and acquired immunodeficiency syndrome (AIDS) (Ivker *et al.* 1994). Cytomegalovirus (CMV) DNA was found in skin biopsies in 11 out of 29 patients with vitiligo, with particular prominence in biopsy specimens of active lesions (Grimes *et al.* 1996), and has therefore been suggested as a trigger for vitiligo. However, the seroprevalance of CMV in the general population is also very high (Hizel *et al.* 1999).

1.3.4 The neuronal theory

Neurochemical-mediated effects on melanocytes would explain the often symmetrical distribution of vitiligo lesions or the dermatomal pattern of segmental-type vitiligo (Lerner 1959). Indeed, immunohistochemical studies of lesional and perilesional vitiligo skin have demonstrated abnormalities in the levels of skin neuropeptides. Al'Abadie *et al.*, (1994), reported that 5 of 10 patients with symmetrical type vitiligo had increased levels of neuropeptide Y in the margins of vitiligo lesions and of these patients, those with active disease also had elevated neuropeptide Y within lesional skin. 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). The secretion of both NGF and calcitonin gene-related peptide is thought to influence normal melanocyte function (Yaar *et al.* 1991; Hara *et al.* 1995b) and could be implicated in vitiligo pathogenesis (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.* 1995), as well as direct cell-cell contact with melanocytes (Morohashi *et al.* 1977), in vitiligous 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 & Misra 1988; Iyengar 1989). Investigations into the levels of neurotransmitters in vitiligo patients are summarised in Table 1.5 and are discussed in relation to the influence of psychological stress (Section 1.3.1).

Several clinical observations provide further evidence for an association of the nervous system with vitiligo. It has 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. 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. Additional indirect evidence is provided by the requirement for innervation for skin transplants to repigment an area. 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 & Lerner 1965; Dutta & Mandal 1982; Merello *et al.* 1993). However, this could be associated with the absence of Merkel cells from the basal layer of vitiligo lesions (Bose & Ortonne 1994) that are thought to interact with nerve endings (Lacour *et al.* 1991).

1.3.5 The melanocyte defect/self-destruction theory

It has been hypothesised that intrinsic melanocytes defects such as the accumulation of toxic intermediate products of melanin synthesis (Pawelek *et al.* 1980; Moellmann *et al.* 1982), the breakdown of free radical defence (Nordlund & Lerner 1982) and the build-up of excessive hydrogen peroxide (Schallreuter *et al.* 1991; Schallreuter *et al.* 1994) might result in the self-destruction of pigment cells in vitiligo. The theory is supported by the clinical observation that certain chemical compounds can produce a pattern of depigmentation indistinguishable from idiopathic vitiligo, e.g., mercaptoamines (Bleehan 1979) and phenolic

agents (Le Poole *et al.* 1999). The suggestion that vitiligo melanocytes show an increased sensitivity to oxidative stress (Schallreuter 1999; Jimbow *et al.* 2001) has also led to the use of the antioxidant pseudocatalase in the treatment of vitiligo (Section 1.2.7). The proposed involvement of some of the biochemicals thought to contribute to vitiligo pathogenesis through melanocyte autocytoxicity is summarised in Table 1.5.

1.3.6 The autoimmune theory

A role for autoimmunity was initially suggested by the frequent clinical association of vitiligo with diseases of an autoimmune origin (Table 1.3) and by the presence of organ-specific autoantibodies (Table 1.7) in vitiligo patients (Brostoff 1969; Betterle *et al.* 1976; Zauli *et al.* 1986; Mandry *et al.* 1996). Subsequently, autoantibodies targeting pigment cell antigens which are capable of causing damage to the melanocyte have been identified in vitiligo patient sera (Section 1.4) (Naughton *et al.* 1983b; Naughton *et al.* 1983a; Norris *et al.* 1988b; Cui *et al.* 1992; Gilhar *et al.* 1995). Autoreactive T lymphocytes targeting melanocyte-specific proteins have also been identified both circulating in the blood and at the margins of advancing lesions of vitiligo patients (Section 1.4) (Ogg *et al.* 1998; van den Wijngaard *et al.* 2000; Lang *et al.* 2001). Furthermore, systemic disorders in vitiligo caused by damage to melanocytes at sites other than the epidermis, e.g., the inner ear and eye, indicate that a progressive immunological response might play a role in the disease development.

The association, albeit weak, of vitiligo with certain HLA specificities (Table 1.2) and other immune response genes (Section 1.2.3) adds credence to an autoimmune hypothesis. All autoimmune endocrinopathies are associated with particular alleles of the MHC class II human leukocyte antigen (HLA)-DR (Dahlberg *et al.* 1981; Platz *et al.* 1981; Maclaren & Riley 1986). Although studies of association of MHC specificities with vitiligo have shown variability (Table 1.2), several studies have reported a significant association of HLA-DR with vitiligo patients in different ethnic populations (Foley *et al.* 1983; Dunston & Halder 1990; Poloy *et al.* 1991; Venneker *et al.* 1992; Venkataram *et al.* 1995; Zamani *et al.* 2001). Additionally, several autoimmune disorders are associated

Table 1.5: Influence of biochemicals implicated in vitiligo aetiology.

Biochemical or enzyme	Normal function	Experimental observations in relation to vitiligo	Proposed contribution to disease aetiology	References
Tetrahydrobiopterin	Regulatory role in melanogenesis since it is a rate-limiting cofactor in tyrosine phenylalanine and tyrosine L-DOPA conversions.[1]	Phenylalanine accumulation seen in epidermis of vitiligo patients.[2] 6-biopterin, metabolite of tetrahydrobiopterin, is highly toxic to cultured melanocytes.[3]	Defective melanogenesis leads to toxic metabolites which cause the melanocytes to self-destruct.[4]	[1] Schallreuter *et al.* 1994 [2] Schallreuter *et al.* 1998 [3] Schallreuter *et al.* 1994 [4] Schallreuter *et al.* 1994
Thioredoxin reductase	A free-radical scavenging enzyme located on melanocyte membrane. Also involved in conversion tyrosine phenylalanine. Action inhibited by calcium.	Vitiligo melanocytes[5] and keratinocytes[6] 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.[7] Inhibition may also affect melanogenesis leading to the build-up of toxic metabolites[4].	[5] Schallreuter *et al.* 1996 [6] Schallreuter & Pittelkow 1988 [7] Ortonne & Bose 1993
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
Glutathione reductase	Anti-oxidant enzyme involved in maintaining intracellular redox status.	Decreased levels of glutathione reductase in vitiligo epidermis.[9]	Imbalance of intracellular redox status renders melanocytes susceptible to damage by toxic free-radicals.[7]	[9] Schallreuter *et al.* 1987
Catecholamines (Adrenalin, Noradrenalin, melatonin, etc.)	Neurotransmitters. Synthesised by adrenal glands but adrenalin and nor-adrenalin also recently shown to be synthesised by epidermal keratinocytes[10] and melanocytes.[11]	Increased catecholamines in vitiligo skin[12] and vitiligo patient urine.[13] Increased synthesis by epidermal keratinocytes in vitiligo patients.[12]	Directly cytotoxic in high levels. Indirectly cytotoxic by causing a build-up of oxidative stress as follows; abnormal release of catecholamines may cause increased vasoconstriction hypoxia-ischaemia reoxygenation increased production of oxidative species.[13]	[10] Schallreuter *et al.* 1992 [11] Iyengar & Misra 1987 [12] Schallreuter *et al.* 1992 [13] Morrone *et al.* 1992

Biochemical or enzyme	Normal function	Experimental observations in relation to vitiligo	Proposed contribution to disease aetiology	References
Melatonin	Control of circadian rhythms.	Met-enkephalin and β-endorphin oscillations no longer circardian in vitiligo patients.[14]		[14] Mozzanica *et al.* 1992
		Melanocytes at the margins of vitiligo lesions are receptive to melatonin, and, therefore, may have melatonin receptors. These melanocytes can become less dendritic in response to melatonin, thereby possibly modulating depigmentation.[15]	Hyperactivation of melatonin receptors on vitiligo melanocytes initiates a dysregulation of melanogenesis by ↑ activity of some of the enzymes involved in melanin synthesis.[16]	[15] Iyengar 1994 [16] Slominski *et al.* 1989
Catechol-O-methyl transferase (COMT)	Enzyme which inactivates catecholamines by methylation.	Increased COMT in lesional vitiligo skin[17], indicative of high levels of catecholamines.	As catecholamines.	[17] Le Poole *et al.* 1994
Melanocyte growth factor/s	Stimulate proliferation of melanocytes.	Cultured vitiligo melanocytes demonstrate growth defects.[18]	Decreased concentrations of melanocyte growth factor/s → melanocytes unable to recover or replenish after damage.[19]	[18] Puri *et al.* 1987 [19] Ramaiah *et al.* 1989

with homozygous or heterozygous deficiencies of the MHC class III molecules, complement components C4 and C2 (Kahl & Atkinson 1988), and a heterozygous deficiency of C4 and C2 has been reported to be associated with vitiligo (Venneker *et al.* 1992). A recent study has also identified significant associations between vitiligo and polymorphisms in immune response genes including those encoding the co-stimulatory lymphocyte activation ligands CD28 and CTLA-4, and the MHC class II molecules LMP and TAP (McCormack *et al.* 2001b) (Section 1.2.3).

The therapeutic response of some vitiligo patients to psoralsen with ultraviolet A radiation (PUVA) (Parrish *et al.* 1976), topical corticosteroids (Kumari 1984) and topical cytotoxic drugs such as fluorouracil (Tsuji & Hamada 1983) is believed to result from immunosuppression of the local immune reactions responsible for damaging melanocytes. Approximately 50% of vitiligo patients will note significant return of pigment in those lesions which retain melanocytes after the application of topical steroids (Boissy 1995).

There are several spontaneous animal models of vitiligo (Boissy & Lamoreux 1988) but how the acquired depigmentation relates to that of the human disease remains to be established. The best documented model, the Smyth line chicken (Smyth 1989), expresses a genetically inherited vitiligo-like depigmentation with considerable autoimmune involvement. Depigmentation of Smyth chicks begins soon after hatching due to loss of melanocytes from the feathers and ocular tissues. An autoimmune response involving both cellular and humoral reactions is initiated by an intrinsic defect of the chicken melanocytes (Boissy *et al.* 1983). An increase of T cells is seen in the feather pulp and in the circulation of Smyth chicks prior to the onset and during development of vitiligo-like depigmentation (Erf *et al.* 1995; Erf & Smyth 1996). In addition, serum antibodies to melanocytes are present before and after presentation of the disease phenotype (Austin & Boissy 1995). These anti-melanocyte antibodies are detected in the sera of 100% of Smyth line chicks but not in the sera of normally pigmented birds (Austin & Boissy 1995). The depigmentation can be decreased by treatment with cyclosporine A, a selective inhibitor of inflammatory T cells, and by performing neonatal bursectomy which causes B cell deficiency (Lamont & Smyth 1981). The vitiligo-like disorder of Smyth chickens is accompanied by autoimmune hypothyroidism and an avian equivalent of alopecia areata (Smyth 1989).

Autoantibodies targeting pigment cell surface antigens have also been identified in several other animal models of vitiligo, including Arabian horses, Siamese cats and Tervuren dogs (Naughton *et al.* 1986a) as well as in the Sinclair pig, an animal model for regressive melanoma with vitiligo-like hypopigmentation (Cui *et al.* 1995a). These antibodies recognise a similar pattern of melanocyte antigens in immunoprecipitation experiments as antibodies in vitiligo patients (Naughton *et al.* 1986a), indicating that similar immunological responses may occur in both animals and humans.

Despite persuasive evidence detailed above for an autoimmune cause of vitiligo aetiology, exactly how the immunological mechanisms, which are described in further detail in Section 1.4, might function in the disease pathogenesis remains open to speculation. It is currently unknown whether the abnormal immune response forms the primary cause of the disease or arises as a secondary phenomenon, resulting from damage to the melanocytes via other mechanisms, and in turn exacerbates the condition. It is also possible that different pathogenic mechanisms account for the different clinical subtypes of vitiligo (Section 1.2.5). For example, associated autoimmunity is rare in patients with segmental vitiligo and, therefore, immune mechanisms might be supposed to play a lesser role than in forms of the disease in which associated autoimmunity is more common.

1.3.7 The convergence theory

The convergence theory (Figure 1.6) proposes that all of the previously described mechanisms can contribute to the development of vitiligo (Le Poole *et al.* 1993a; Taieb 2000). Moreover, the different causal factors can act independently or synergistically to bring about the local destruction of melanocytes and are differentially involved in separate vitiligo patients (Le Poole *et al.* 1993a).

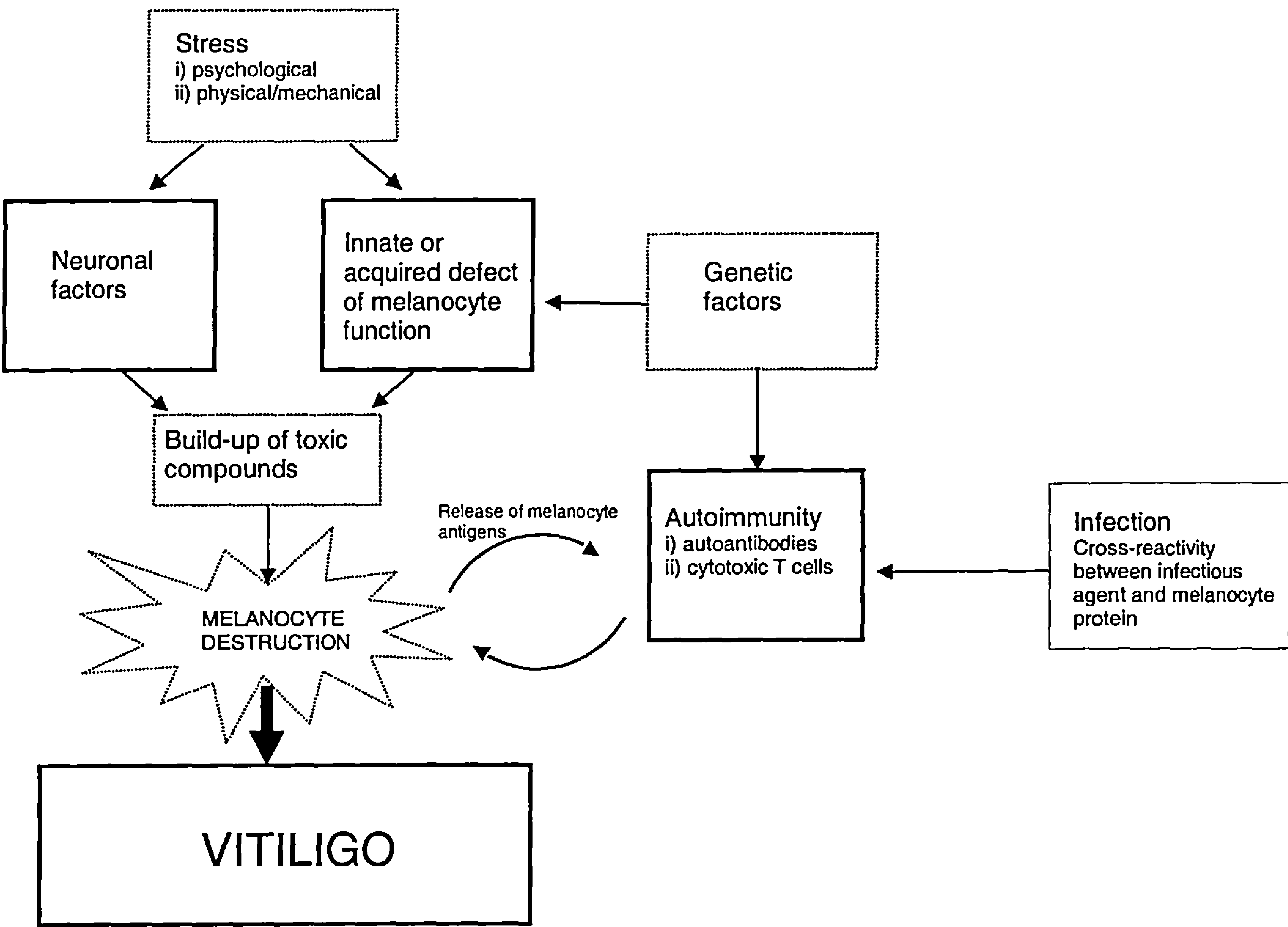


Figure 1.6: A schematic representation of the convergence theory for vitiligo aetiology.

Adapted from Le Poole *et al.* 1993a.

1.4 The immune response in vitiligo

The possible mechanisms by which both cellular and humoral immune reactivities in vitiligo may arise and contribute to its pathogenesis are summarised in Figure 1.6.

1.4.1 Cellular immunity

1.4.1.1 T lymphocytes

The study of circulating T cell subpopulations in vitiligo patients has generated contrasting results. Whilst some groups identified an expansion of peripheral $CD4^+$ T lymphocytes and an increase in the $CD4^+$: $CD8^+$ ratio (Soubiran *et al.* 1985; D'Amelio *et al.* 1990; Al-Fouzan *et al.* 1995), indicative of an autoimmune response (Stites 1994), other groups observed a decrease in $CD4^+$ T lymphocytes and a decreased $CD4^+$: $CD8^+$ ratio (Grimes *et al.* 1986; Halder *et al.* 1986). While such discrepancies may indicate technical differences in the evaluation of these ratios in the circulation, the overall conclusion remains that the circulating T cell populations in vitiligo are rarely normal, although how these changes relate to T cells at the site of the lesion remains speculative. A proportion of activated peripheral T cells, as determined by HLA-DR expression, have been shown in vitiligo patients compared to healthy individuals (Abdel-Naser *et al.* 1992).

Of more functional relevance, a recent study has demonstrated in the circulation of a significant number of patients with vitiligo, the presence of cytotoxic T cells, expressing a skin-homing receptor cutaneous lymphocyte-associated antigen (CLA), that react with the melanocyte antigen MelanA, (Ogg *et al.* 1998). These results were confirmed in a subsequent study, in which the presence of MelanA-specific cytotoxic T cells expressing CLA was also found to be significantly greater in the peripheral blood of vitiligo patients with active, progressive disease (Lang *et al.* 2001). These studies have also demonstrated the presence of $CD8^+$ cytotoxic cells which react with peptides from tyrosinase (Ogg *et al.* 1998; Lang *et al.* 2001) and Pmel17 (Lang *et al.* 2001), when

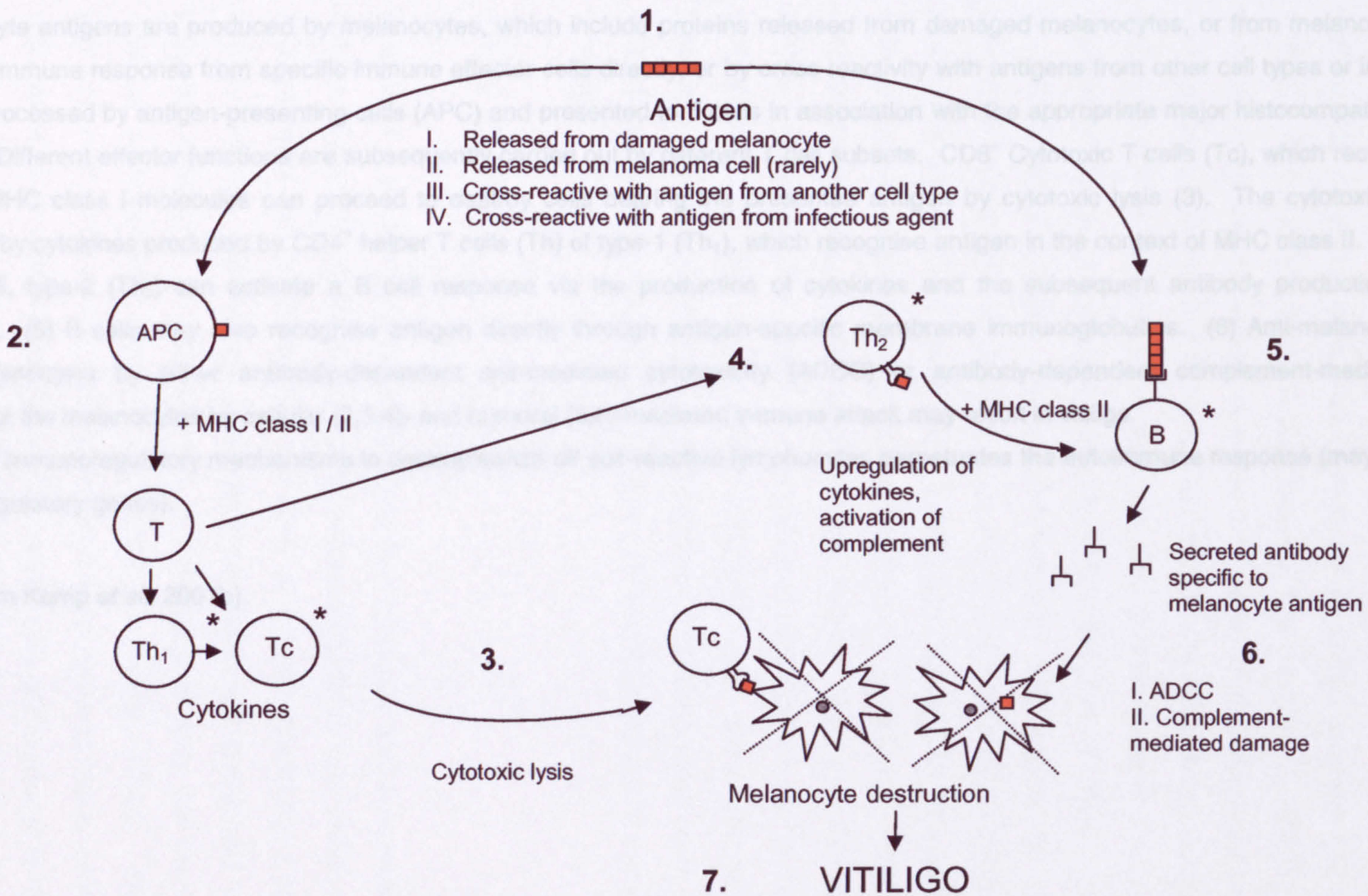


Figure 1.7: A summary of the mechanisms by which cell-mediated and humoral immune responses may arise and bring about vitiligo.

(1) Melanocyte antigens are produced by melanocytes, which include proteins released from damaged melanocytes, or from melanoma cells, which may provoke an immune response from specific immune effector cells directly, or by cross-reactivity with antigens from other cell types or infectious agents. (2) Antigen is processed by antigen-presenting cells (APC) and presented to T cells in association with the appropriate major histocompatibilty complex (MHC) molecules. Different effector functions are subsequently carried out by different T cell subsets. $CD8^+$ Cytotoxic T cells (Tc), which recognise antigen in the context of MHC class I molecules can proceed to destroy cells bearing the presented antigen by cytotoxic lysis (3). The cytotoxic T cell response is upregulated by cytokines produced by $CD4^+$ helper T cells (Th) of type-1 (Th_1), which recognise antigen in the context of MHC class II. (4) A second type of helper T cell, type-2 (Th_2) can activate a B cell response via the production of cytokines and the subsequent antibody production can also activate complement. (5) B cells may also recognise antigen directly through antigen-specific membrane immunoglobulins. (6) Anti-melanocyte antibodies can destroy melanocytes by either antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent complement-mediated damage. (7) Destruction of the melanocytes by cellular (2,3,4)- and humoral (5,6)-mediated immune attack may result in vitiligo.

* A failure of immunoregulatory mechanisms to destroy/switch off self-reactive lymphocytes perpetuates the autoimmune response (may be due to mutations in immunoregulatory genes).

(Adapted from Kemp *et al.*, 2001b).

presented with HLA class I antigen-A2, in the circulation of vitiligo patients. These data supports the role of skin-homing autoreactive melanocyte-specific T cells in causing destruction of melanocytes in vitiligo.

A dermal and epidermal inflammatory infiltrate consisting of $CD3^+$, $CD4^+$ and $CD8^+$ T cells and macrophages, closely associated with areas of melanocyte depletion, has been observed in vitiligo patients (Al Badri *et al.* 1993b). The infiltrate is most prominent at the margins of vitiligo lesions and contains a significant number of activated T cells, as measured by expression of MHC class II antigen HLA-DR and CLA antigen (Al Badri *et al.* 1993b). There is also evidence of interleukin 2 receptor (IL-2R) and interferon γ receptor (IFN-γR) expression by the lymphocytic infiltrate (Abdel-Naser *et al.* 1994). In rare cases of inflammatory vitiligo, similar observations with regard to perilesional, epidermis-infiltrating T cells have been made (Le Poole *et al.* 1996). A recent study isolated MelanA-specific $CD8^+$ T cells expressing CLA antigen from vitiligo skin biopsies, demonstrating the presence of a cytotoxic response to a melanocyte-specific antigen within vitiligo lesions (van den Wijngaard *et al.* 2000). However, the absolute numbers of infiltrating cells in lesional and perilesional skin are small when compared with other inflammatory skin disorders (van den Wijngaard *et al.* 2000) and it remains to be seen whether the infiltrate arises as a result of the disease process as opposed to being the cause of vitiligo.

1.4.1.2 Cytokines

Cytokines mediate many functions of cellular immunity and a number of studies have analysed the levels of various cytokines in patients with vitiligo (Table 1.6). Studies have demonstrated that the level of soluble interleukin-2 receptor (IL-2R) in patients with vitiligo is significantly increased compared with that of controls, indicating an activation of T lymphocytes may be involved in the disease pathogenesis (Caixia *et al.* 1999; Yeo *et al.* 1999). The levels of production of interleukin-6 and interleukin-8 by peripheral mononuclear cells are also elevated in vitiligo patients, both of which can act to recruit lymphocytes to the site of an immune response (Yu *et al.* 1997). In contrast, the production of

Table 1.6: The involvement of cytokines in vitiligo.

Cytokine	Source	Function	Level in vitiligo patients [1]	Method of detection	Number of patients in study	Reference
IL-2 [2]	Activated T cells	Induces growth, differentiation and proliferation of T and B cells and natural killer cells.	Levels of soluble IL-2 receptor are increased, indicating a rise in IL-2	ELISA [8] (serum, skin tissue fluid)	41 79	Caixia *et al.* 1999 Yeo *et al.* 1999
IL-6	T cells, macrophages	Stimulates T and B cell differentiation and proliferation and upregulation of ICAM-1 [6] expression on melanocytes, facilitating lymphocyte-melanocyte interaction.	Increased	ELISA (mononuclear cell supernatants) [9]	12	Yu *et al.* 1997
IL-8	Monocytes, fibroblasts, keratinocytes	Chemotactic to lymphocytes.	Increased	ELISA (mononuclear cell supernatants)	12	Yu *et al.* 1997
GM-CSF [3]	T cells, macrophages	Induces growth of macrophages and granulocytes.	Decreased	ELISA (mononuclear cell supernatants)	12	Yu *et al.* 1997
TNF-α [4]	T cells	Anti-tumour activity. General immunostimulant.	Decreased	ELISA (mononuclear cell supernatants)	12	Yu *et al.* 1997
IFN-γ [5]	T cells	Anti-viral, modulates expression of MHC [7] class I antigens. May inhibit melanocyte growth.	Decreased	ELISA (mononuclear cell supernatants)	12	Yu *et al.* 1997

[1] Compared with healthy individuals.

[2] IL= interleukin.

[3] granulocyte-macrophage colony-stimulating factor.

[4] tumor necrosis factor-alpha.

[5] interferon-gamma.

[6] intercellular adhesion molecule-1.

[7] major histocompatibility complex.

[8] enzyme-linked immunosorbent assay.

[9] obtained from peripheral blood

granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-alpha (TNF-α) by peripheral mononuclear cells in active vitiligo was found to be reduced compared with healthy controls (Yu *et al.* 1997). The expression of cytokines by lymphocytes from the margins of vitiligo lesions has yet to be fully characterised but initial data suggest that cytokines which promote inflammation and cytotoxicity, that is, those of the T helper lymphocyte type-1 (Th1) subset, particularly interferon-gamma (IFN-γ) and TNF-α, are predominantly expressed (Das *et al.* 2001).

1.4.1.3 Adhesion molecules

Adhesion molecules, which allow cell-cell attachments, are required for lymphocyte migration and infiltration through the epidermis and also for interaction with antigen-presenting cells. Several studies have shown increased expression of intercellular adhesion molecule-1 (ICAM-1) by melanocytes and keratinocytes in perilesional skin compared with non-lesional and control skin (Norris 1990; Al Badri *et al.* 1993a; van den Wijngaard *et al.* 2000), which may contribute to the abnormal cellular immune responses seen in vitiligo. Immunohistochemistry of serial sections of vitiligo skin biopsies has shown that the epidermal expression of ICAM-1 coincides with the expression of HLA-DR (van den Wijngaard *et al.* 2000). A similar pattern of ICAM-1 and HLA-DR expression is shown by thyroid follicular cells in autoimmune thyroid disease (Hanafusa *et al.* 1983; Weetman *et al.* 1989; Zheng *et al.* 1990), and is dependent on cytokines, particularly interferon-gamma (IFN- γ).

Vascular expression of E-selectin, the adhesion molecule responsible for capture of leucocytes from the circulation to initiate migration to the site of a local immune response, appears neither to be upregulated nor more widely dispersed in post-capillary venules in vitiligo, when compared to control skin (van den Wijngaard *et al.* 2000). However, immunohistochemical analysis demonstrated that where there is expression of E-selectin in perilesional skin, it is co-localised with T cell infiltration (van den Wijngaard *et al.* 2000). The expression of E-selectin is dependent on cytokines, particularly tumour necrosis factor-alpha (TNF-α) (Pilewski *et al.* 1995).

1.4.1.4 Langerhans cells

The density of Langerhans cells in vitiligo lesions has been reported as normal (Claudy & Rouchouse 1984) or increased (Riley 1967), relative to that of the non-lesional skin and skin from control subjects. These dendritic cells are active in the epidermis, presenting antigen to primed T lymphocytes and, therefore, an increase in their numbers may contribute to immunological responses to melanocytes in vitiligo. Indeed, patients with repigmenting lesions in response to PUVA or Fluocinonide cream show a decrease in epidermal Langerhans cells further supporting a role for these cells in the disease (Kao & Yu 1990).

1.4.1.5 Natural killer cells

It has been shown that the number of natural killer cells (NK cells) present in affected vitiligo skin is no different from that present in the skin of control subjects (Abdel-Naser *et al.* 1992; Hann *et al.* 1993b). Although previous studies have implicated an involvement of these cells in the destruction of pigment cells in vitiligo (Halder *et al.* 1986; Mozzanica *et al.* 1989), a recent study found no significant differences between the level of activity of natural killer cells from vitiligo patients and control subjects, against both normal and malignant melanocytes (Durham-Pierre *et al.* 1995).

1.4.2 Humoral immunity

1.4.2.1 Organ-specific autoantibodies

In addition to the frequent association of vitiligo with autoimmune disorders, previously described (Section 1.2.6 and Table 1.3), a variety of organ-specific autoantibodies are commonly detected in the sera of vitiligo patients (Table 1.7).

Table 1.7: The frequency of organ-specific autoantibodies in vitiligo patients.

Autoantibody reactivity	Number of patients in study	Percentage with antibody reactivity	Reference
Gastric parietal cells	65	17	Zauli *et al.* 1986
Gastric parietal cells	80	21	Brostoff 1969
Gastric parietal cells	96	13.7	Betterle *et al.* 1976
Gastric parietal cells	20	30	Mandry *et al.* 1996
Thyroid cytoplasm	80	28	Brostoff 1969
Thyroid microsomes/peroxidase	96	20	Betterle *et al.* 1976
Thyroid microsomes/peroxidase	20	50	Mandry *et al.* 1996
Thyroglobulin	80	9	Brostoff 1969
Thyroglobulin	20	40	Mandry *et al.* 1996
Adrenal cortex	80	4	Brostoff 1969
Pancreatic islet cells	96	7.2	Betterle *et al.* 1976

(Adapted from Kemp *et al.* 2001b).

1.4.2.2 Anti-melanocyte antibodies

A significant piece of evidence in the debate over a role for autoimmunity in vitiligo is the observation that antibodies to melanocyte antigens are present in the circulation of most patients with the disease (Naughton *et al.* 1983a). In one study, 12 out of 12 of vitiligo patients but none of control subjects were found to have anti-pigment cell antibodies in their sera (Naughton *et al.* 1983b). Furthermore, a correlation has been described between the incidence and level of melanocyte antibodies and disease activity in vitiligo: 8/10 patients with active vitiligo, 0/14 with inactive disease and 0/19 controls were found to have circulating anti-pigment cell antibodies (Harning *et al.* 1991). A decrease in the titre of melanocyte antibodies, correlating with the suppression of vitiligo, with PUVA treatment further suggests that the disease activity is somehow related to incidence of these antibodies (Hann *et al.* 1997). Additionally, the presence of these antibodies is related to the extent of disease: they were detected in 50% of patients with minimal vitiligo (< 2% of skin area involved) compared to 93% of patients with greater depigmentation (covering 5-10% of their skin) (Naughton *et al.* 1986b), although this would fit with either a primary or secondary causation.

Vitiligo antibodies are most commonly directed against pigment cell antigens with molecular weights of 35 kDa, 40-45 kDa, 75 kDa, 90 kDa and 150 kDa which are located on the cell surface, as determined by detection techniques specific to the cell exterior (Cui *et al.* 1992; Cui *et al.* 1995b). Although the proteins have not been specifically identified, some (40-45 kDa, 75 kDa and 150 kDa) appear to be common tissue antigens, while others (35 kDa and 90 kDa) are preferentially expressed on pigment cells (Cui *et al.* 1992). In addition, antibodies to the melanocyte-specific proteins tyrosinase (Song *et al.* 1994a; Baharav *et al.* 1996; Kemp *et al.* 1997a), TRP-1 (Kemp *et al.* 1998c), TRP-2 (Kemp *et al.* 1997b; Okamoto *et al.* 1998) and Pmel17 (Kemp *et al.* 1998b) have been detected in the sera of patients with vitiligo. Although these proteins may be considered cytoplasmic autoantigens, with the exception of TRP-1 which can be expressed on the melanocyte surface (Takechi *et al.* 1996), a *de novo* mechanism may exist whereby antibodies recognise autantigens directly via cell surface or cytoplasmic penetration (Okamoto *et al.* 1998). For example, antibodies have been shown to be taken up by nonhemopoietic cells and react with nuclear antigens *in vivo* (Isenberg *et al.* 1997). Alternatively,

antigens may be released if the melanocyte is damaged by another mechanisms such as cytotoxic T cell lysis. In a recent study, the transcription factors SOX9 and SOX10 have been identified as melanocyte autoantigens related to vitiligo in patients with autoimmune polyglandular syndrome type 1 (Hedstrand *et al.* 2001). A summary of the antibody autoantigens implicated in the disorder is given in Table 1.8.

The capacity for anti-melanocyte antibodies to injure pigment cells has been demonstrated experimentally: vitiligo antibodies are able to destroy melanocytes *in vitro* by complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity (Norris *et al.* 1988b) and *in vivo* following passive immunisation of nude mice grafted with human skin (Gilhar *et al.* 1995). The selective destruction of melanocytes in vitiligo may result from antibody reactivity directed to the antigens preferentially expressed on pigment cells (Cui *et al.* 1992). Alternatively, an antibody response against antigens expressed on a variety of cell types may selectively destroy melanocytes because they have been shown to be intrinsically more sensitive to immune-mediated injury than, for example, keratinocytes or fibroblasts (Norris *et al.* 1988a).

The stimulus for the production of anti-melanocyte antibodies has not been identified and it is unknown whether vitiligo antibodies initiate the development of the disease. It is possible that cross-reacting antigens expressed either on other cell types or on infecting micro-organisms may elicit their production. For example, common antigens have been identified in mycobacteria and malignant melanocytes (Bystryn 1997). Alternatively, vitiligo antibodies might result from a secondary immune response to melanocyte antigens following damage to pigment cells by other mechanisms, and these antibodies might further exacerbate the condition. It is presently unknown if humoral immune responses actually damage melanocytes *in vivo* and it is still possible that they play no direct part in vitiligo aetiology. Nontheless, these antibodies serve as relevant markers to identify potential autoantigens, even if the disease is T cell-mediated.

Table 1.8: Pigment cell antigens recognised by vitiligo autoantibodies.

Antigen	System of detection	Number of patients in study	Percentage with reactivity	Reference
Tyrosinase	Immunoblotting of recombinant human tyrosinase	26	61	Song *et al.* 1994a
Tyrosinase	ELISA[1] of mushroom tyrosinase	18	Not given	Baharav *et al.* 1996
Tyrosinase	Radiobinding assay of recombinant human tyrosinase	46	11	Kemp *et al.* 1997a
TRP-2	Radiobinding assay of recombinant human TRP-2	53	5	Kemp *et al.* 1997b
TRP-2	ELISA of recombinant human TRP-2	30	67	Okamoto *et al.* 1998
TRP-1	Radiobinding assay of recombinant human TRP-1	53	5	Kemp *et al.* 1998c
Pmel17	Radiobinding assay of recombinant human Pmel17	53	5	Kemp *et al.* 1998b
SOX9	Radiobinding assay of recombinant human SOX9	19	47	Hedstrand *et al.* 2001
SOX10	Radiobinding assay of recombinant human SOX10	19	63	Hedstrand *et al.* 2001
40-45 kDa	Immunoprecipitation of human melanocytes	29	72	Cui *et al.* 1995b
75 kDa	Immunoprecipitation of human melanocytes	29	76	Cui *et al.* 1995b
90 kDa	Immunoprecipitation of human melanocytes	29	45	Cui *et al.* 1995b
65 kDa	Immunoprecipitation of human melanocytes	18	44	Park *et al.* 1996
35 kDa	Immunoprecipitation of human melanocytes	23	4	Cui *et al.* 1992
150 kDa	Immunoprecipitation of human melanocytes	23	4	Cui *et al.* 1992

[1]ELISA; enzyme-linked immunosorbent assay. (Adapted from Kemp *et al.* 2001b).

1.4.2.3 Other antibody reactivities

Aside from organ-specific antibodies (Table 1.7), anti-keratinocyte intracellular antibodies that correlate with disease extent and activity have been detected in vitiligo patients (Yu *et al.* 1993) and specific IgA reactivity against human melanoma cells in patients with active vitiligo has been reported (Aronson & Hashimoto 1987). An increased incidence of anti-nuclear, anti-microsomal, and anti-smooth muscle cell antibodies has also been observed in the sera of vitiligo patients (Hann *et al.* 1993a). A significant number of vitiligo patients also have antibodies against compounds containing the benzene ring structure (Wojdani *et al.* 1992; Vojdani & Grimes 1996). These were investigated for immune reactivity because compounds containing the benzene ring, including catechols, phenols, hydroquinone and mono-benzene, can induce cutaneous depigmentation (Section 1.2.5). Although the nature of the action of the anti-benzene ring antibodies is not proven, it has been suggested that exposure to compounds containing this structure may induce aberrant immunological responses in some individuals with vitiligo (Wojdani *et al.* 1992; Vojdani & Grimes 1996). As with melanocyte antibodies, although to a greater degree, it is presently unclear how these antibodies relate temporally and pathogenically to the lesions in vitiligo.

1.5 Melanoma-associated hypopigmentation

Metastatic malignant melanoma is a form of skin cancer caused by uncontrolled growth of melanocytes. It responds poorly to standard treatments, including chemotherapy and radiotherapy, and patients with distant metastases have a poor prognosis (Okamoto *et al.* 1998; Kawakami *et al.* 2000). However, the spontaneous appearance of a vitiligo-like depigmentation, termed melanoma-associated hypopigmentation, in approximately 10% of metastatic melanoma patients (Merimsky *et al.* 1994), during the course of their disease, is thought to be associated with an improved prognosis (Nordlund *et al.* 1983; Bystryn *et al.* 1987; Richards *et al.* 1992). In animal models of melanoma in which hypopigmentation is observed, such as Arabian and Lipizzaner horses and the

Sinclair miniswine, the disease progresses more slowly and can also regress (Lerner & Cage 1973; Oxenhandler *et al.* 1979).

Melanoma-associated hypopigmentation is thought to arise from immune reactivity to the metastatic disease directed against common antigens on both the melanoma cells and normal melanocytes. Indeed, antibodies reactive to tyrosinase (Fishman *et al.* 1997), TRP-1, TRP-2, Pmel17 (Huang *et al.* 1998) and melanoma cells (Naughton *et al.* 1983a) have been identified in some melanoma patient sera. Antibody responses to pigment cell antigens of 40-45 kDa, 75 kDa and 90 kDa, yet to be fully characterised, that are also recognised by patients with vitiligo have also been identified in melanoma patients (Cui & Bystryn 1995). Additionally, some melanoma patients have cytotoxic T lymphocytes specific to tyrosinase (Brichard *et al.* 1993), TRP-1 (Wang *et al.* 1995), and TRP-2 (Wang *et al.* 1996), Pmel17 (Kawakami *et al.* 1995) and MelanA (Coulie *et al.* 1994). These responses to melanocyte-specific antigens and their potential to induce the rejection of melanoma have led to the development of active, specific immunotherapy treatments for melanoma including DNA and peptide vaccines, and adoptive transfer of tumor-infiltrating lymphocytes, in addition to non-specific cytokine therapy using IL-2 to stimulate proliferation of activated T cells.

The clinical efficacy of immune therapy for the treatment of metastatic melanoma is currently being studied. Vaccination with the melanoma-associated antigens; tyrosinase (Robbins *et al.* 1994), Pmel17 (Kawakami *et al.* 1994b), MAGE-1 (Hoon *et al.* 1995), and melanoma cell vaccines (Morton *et al.* 1992; Hayashi *et al.* 1993) has been shown to induce antigen-specific antibodies and cell-mediated immunity in melanoma patients. Melanoma-associated hypopigmentation has been reported in patients responding to immune therapy; for example, IL-2 administration alone induced depigmentation in 20% of patients (Rosenberg & White 1996), and the appearance of vitiligo-like lesions has been reported in patients vaccinated with TRP-2 (Okamoto *et al.* 1998) and MelanA/MART-1 (Yee *et al.* 2000), both at sites of vaccination and distant locations. Recent immunisation strategies have involved vaccination with autologous dendritic cells, pulsed with either tumour lysates or melanoma-associated peptides, to act as antigen presenting cells to acquire tumor-specific cytotoxic T cell reactivity (Nestle *et al.* 1998).

It has been suggested that the immune response in melanoma patients which results in melanocyte destruction is cell-mediated, operating via mechanisms involving $CD8^+$ T cells and natural killer cells, and does not occur by means of a humoral response (van Elsas *et al.* 1999; Nagai *et al.* 2000; Yee *et al.* 2000). It is possible that the melanocyte-specific antibodies in patients with melanoma arise as a secondary immune response following melanocyte damage through cell-mediated effects. Moreover, the serum titres of melanoma-associated antibodies are low, and levels are similar between patients with and without melanoma-associated hypopigmentation. However, the amount of measurable antibody may be decreased due to the formation of immune complexes, the trapping of antibodies by shed antigen or a high number of binding sites on the melanocytes thereby reducing the amount of free antigen (Merimsky *et al.* 1996). Experimental immunotherapy treatments are currently being tried in a mouse model of melanoma, the C57BL/6 strain, which develops the B16 melanoma, and several studies have accomplished immune rejection of B16 melanoma accompanied by associated hypopigmentation (Hara *et al.* 1995a; Weber *et al.* 1998; Bowne *et al.* 1999; Overwijk *et al.* 1999; van Elsas *et al.* 1999; Nagai *et al.* 2000). It has also previously been shown that passive administration of vitiligo patient immunoglobulin G (IgG) to C57BL/6 mice results in the development of fewer metastases than mice injected with normal human IgG (Fishman *et al.* 1993). One author has speculated that vitiligo is "an experiment of nature that accomplishes the goal of melanoma immunotherapy" (Bystryn 1989).

It is not known whether melanoma-associated hypopigmentation and vitiligo are the same disease. Despite a similar clinical appearance and a common association with abnormal immune reactivities to pigment cell antigens, it has yet to be established if they have an identical aetiology. Melanoma patients with associated hypopigmentation have been variously reported to have circulating antibodies with different (Merimsky *et al.* 1994), or shared (Cui & Bystryn 1995) specificities providing evidence that they have distinct or similar aetiologies, respectively. More insight will be gained as the immune response in each disease is further characterised.

1.6 Implications of understanding autoimmune responses in vitiligo

Characterising the autoimmune response in vitiligo has several potential clinical implications and applications. It is possible that elucidating the mechanisms involved in the abnormal immune response in vitiligo may lead to the development of more effective treatments, as well as a greater understanding of the action of current immunosuppressive treatments, such as PUVA, which are effective in some cases of vitiligo. The identification of melanocyte autoantigens recognised by autoantibodies and/or autoreactive T cells in vitiligo patients might be of use in the diagnosis of the disorder and in the development of markers for disease activity and progression. If autoimmune responses could be identified routinely, more appropriate treatments could be applied to individual patients. It may also be possible to establish whether particular clinical subtypes of vitiligo do indeed have separate disease aetiologies. Furthermore, characterisation of pigment cell antigens that can elicit either a humoral or cellular immune response to melanocytes in vitiligo could provide new targets for gene/peptide vaccines in melanoma.

1.7 Aims of this study

The overall aim of this study was to investigate the immune aspects of vitiligo with particular reference to identifying and characterising melanocyte autoantigens recognised by autoantibodies in sera from vitiligo patients. The following specific aims were addressed:

(1) To examine any association of a microsatellite polymorphism of the immunoregulatory gene cytoxic T lymphocyte-associated antigen 4 (CTLA-4) in patients with vitiligo, in order to determine potential subgroups in which autoimmune responses may be more frequent.

(2) To characterise the B cell epitope reactivity of two previously identified vitiligo autoantigens, namely tyrosinase and Pmel17.

(3) To investigate the prevalence of autoantibodies to the melanosomal protein MelanA/MART-1 which forms a significant antigenic target for cytotoxic T lymphocytes in vitiligo.

(4) To construct a phage-display library of melanocyte polypeptides and to apply a novel biopanning technique using vitiligo patient IgG to identify novel disease autoantigens.

(5) To investigate the frequency in vitiligo patient sera of autoantibodies targeting any novel autoantigens identified using the phage-display library.

2 General Materials and Methods

2.1 Patient sera

Sera from patients with vitiligo and other autoimmune diseases were collected from dermatology and endocrinology clinics at the Northern General Hospital, Sheffield, U.K., and the Royal Hallamshire Hospital, Sheffield, U.K. between 1990-2001. Specific patient details are given in the relevant chapters. Control sera were also obtained from healthy laboratory volunteers with no personal or family history of vitiligo or of autoimmune disorders. Serum was separated from whole blood samples (10-20 ml) by centrifugation in a Sorval® RT6000-D centrifuge (3000 revolutions per minute (rpm), 10 min). Sera were kept frozen at –20°C until use.

All work was approved by the Ethics Committee of the Northern General Hospital NHS Trust, and all subjects gave informed consent.

2.2 Chemicals

The majority of chemicals were purchased from either Sigma (Poole, U.K.), or BDH, (Poole, U.K.), and were of either molecular biology grade or 'AnalR' grade, respectively. The source of some chemicals is indicated in the text where appropriate.

2.3 Media

The media used in this study are given below. All media were sterilised either by autoclaving or by filtration using Millipore GS disposable filters (Millipore Corp., Bedford, MA, U.S.A).

2.3.1 Luria Bertani (LB) medium and agar

Luria Bertani (LB) medium was prepared in double distilled water and contained: 1% (w/v) Bacto-tryptone (Sigma), 0.5% (w/v) yeast extract (Sigma) and 1% (w/v) NaCl (Sigma). Luria Bertani agar was made by adding 1.5% (w/v) Bacto-agar

(Sigma) to LB medium. After sterilisation, the agar was left to cool to 40-45°C before the addition of appropriate antibiotics. The agar was then poured into 90 mm petri-dishes, allowed to set and the plates stored at 4°C until required.

2.4 Antibiotics

Antibiotics (Sigma) were prepared as 100x concentrated stocks in double-distilled water, sterilised by filtration with a Millex® Filter Unit (Millipore Corp.) and used in medium at either the final concentration shown below or as stated in the text. Tetracycline was dissolved in 50% (v/v) ethanol/H_2O. The stocks were stored at -20°C.

	Final concentration in medium (µg/ml):
Ampicillin	100
Kanamycin	12
Tetracycline	10

2.5 Bacterial strains

The bacterial strains used in this study were derivatives of *Escherichia coli* K-12 and are given in Table 2.1a. Derivatives of strains carrying various plasmids were constructed by transformation (Section 2.12) and are defined in the text.

2.6 Plasmids

The plasmids used in this study are listed in Table 2.1b. They were kept at –20°C in sterile TE buffer (10 mM Tris-HCl; 1 mM ethylenediaminetetraacetic acid (EDTA); pH 8.0). Maps of the plasmid expression vectors used in DNA cloning experiments are given in the relevant chapters.

Table 2.1: Bacterial Strains and Plasmids used.

a: Bacterial Strains

E. coli K-12 strain	Source	Reference
JM109	Promega (Southampton, U.K.)	Yanisch-Perron *et al.* 1985
DH5α	Clontech Laboratories U.K. Ltd. (Basingstoke, U.K.)	Hanahan 1983
XL-1 Blue	Stratagene (La Jolla, CA, U.S.A)	Bullock *et al.* 1987

b: Plasmids

Plasmid	Characteristics	Source	Reference
pBluescript SK	Multicopy 2.96 kb selectable (amp^R)[1] expression vector. Multiple cloning site flanked by promoters for T3 and T7 polymerases.	Stratagene (La Jolla, CA, U.S.A)	Alting-Mees *et al.* 1992
pcDNA3	Multicopy 5.4 kb selectable (amp^R) expression vector. Contains promoters for T7 and SP6 polymerases flanking a diverse multiple cloning site.	Invitrogen (Abingdon, U.K.)	Akrigg *et al.* 1985 Boshart *et al.* 1985
pBSTYR	pBluescript SK with a 1.9 kb *Xba*I-*Sal*I insert of tyrosinase cDNA	E. H. Kemp (Division of Clinical Sciences North, University of Sheffield, U.K.)	Kemp *et al.* 1997a
pcDNA3TYR	pcDNA3 with a 1.9 kb *Kpn*I-*Xba*I insert of tyrosinase cDNA.	E. H. Kemp	Kemp *et al.* 1997b
pcDNA3Pmel17	pcDNA3 with a 2.0 kb *Eco*RI-*Xho*I insert of Pmel17 cDNA fragment	P. F. Robbins (National Institues of Health, Bethesda, MD, U.S.A.)	–
pSC11MelanA	pSC11 with a 400 bp *Bgl*II-*Nco*I insert of MelanA cDNA	V. Cerundolo (Nuffield Department of Clinical Medicine, University of Oxford, U.K.)	–
pGEX-4T-1	A 4.9 kb glutathione S-transferase fusion vector. Contains a *tac* promoter for chemically inducible high-level prokaryotic expression.	Amersham Pharmacia Biotech (Little Chalfont, U.K.)	Kaelin *et al.* 1992
pJuFo	A 4.28 bp (amp^R) phage-display vector. Contains the leucine zippers Jun and Fos, and the viral coat protein, pIII, of the filamentous phage. Prokaryotic expression under the control of *lac* promoter	R. Crameri (Swiss Institute of Allergy and Asthma Research, Davos, Switzerland)	Crameri & Suter 1993

[1]Amp^R, ampicillin resistance

2.7 Growth and maintenance of bacterial strains

When first obtained, strains were checked for the presence of relevant antibiotic markers by streaking onto selective LB agar. All *E. coli* strains were routinely grown from single colonies on LB agar plates in LB medium, with the appropriate antibiotics, at 37°C in a rotary incubator shaking at 250 rpm.

For storage, bacterial strains were streaked onto LB agar plates, containing appropriate antibiotics, and incubated overnight at 37°C and then placed at 4°C for up to one month. Additionally, 10 ml bacterial cultures were grown overnight at 37°C and then mixed with an equal volume of 50% (v/v) sterile glycerol/H_20 for long-term storage at -20°C.

2.7.1 Small-scale plasmid preparation

The Wizard™ Minipreps DNA Purification System (Promega, Southampton, U.K.) was used to purify plasmid DNA from a 5-10 ml overnight culture of a required bacterial strain, according to the manufacturer's protocol. Briefly, single colonies of the desired bacterial strain were isolated by streaking out 20 µl of frozen bacterial stock onto LB agar containing the appropriate antibiotic. A single colony from a selective plate was then used to inoculate 10 ml of LB containing the relevant antibiotic. This culture was shaken in a rotary incubator at 250 rpm at 37°C overnight. A bacterial cell pellet was obtained by centrifugation at 10,000 *g* for 5 min. The pellet was resuspended in 300 µl of Wizard™ resuspension solution (50 mM Tris, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A), and the cells were lysed by addition of 300 µl of Wizard™ cell lysis solution (0.2 M NaOH; 1% (w/v) SDS) and then neutralised by addition of 300 µl of Wizard™ neutralisation buffer (1.32 M potassium acetate). The resulting solution was mixed gently and centrifuged at 10,000 *g* for 10 min at room temperature. The clear lysate was then mixed with 1 ml of Wizard™ resin and loaded on to a Wizard™ minicolumn via a 2 ml syringe, followed by 2 ml of Wizard™ column wash solution (80 mM potassium acetate; 8.3 mM Tris-HCl, pH 7.5; 40 µM EDTA; 55% (v/v) ethanol). The Wizard™ minicolumn was centrifuged at 10,000 *g* for 2 min to remove excess column wash solution prior to DNA elution. Fifty microlitres of TE buffer, heated to 65°C, was subsequently

added to the column, left for 1 min and then centrifuged at 10,000 *g* for 30 sec. The concentration of the DNA was determined by spectrophotometry at 260 nm.

2.7.2 Large-scale plasmid preparation

Larger scale overnight culture of a desired plasmid was prepared by inoculating 0.5-1 L of LB (containing a relevant antibiotic) and growing as before, shaking, at 37°C. The culture was then centrifuged at 2500 rpm (Sorval® RC-3B) for 30 min and plasmid extracted from the cell pellet using a Qiagen Plasmid DNA Maxiprep Kit (Qiagen Ltd., Crawley, U.K.) as per the kit instructions. The bacterial cell pellet was first resuspended in 10 ml of buffer P1 (50 mM Tris-HCl, pH8.0; 10mM EDTA; 100 µg/ml RNase A). An equal volume of buffer P2 (0.2 M NaOH; 1% (w/v) SDS) was 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 buffer P3 (1.32 M potassium acetate, pH 5.5) and incubated on ice for 20 min. The mixture was subsequently centrifuged at 20,000 *g* for 30 min at 4°C resulting in a clear supernatant. A Qiagen column was equilibrated by adding buffer QBT (750 mM NaCl; 50 mM 3-[N-morpholino]propanesulphonic acid (MOPS), pH 7.0; 15% (v/v) isopropanol and 0.15% (v/v) Triton X100) and was allowed to empty by gravity flow. Clear supernatant was then loaded onto 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 buffer QF (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 isopropanol and centrifugation at 15,000 *g* for 30 min at 4°C. The resulting DNA pellet was washed with 70% (v/v) ethanol, centrifuged at 15,000 *g* for 10 min at 4°C and finally resuspended in 300 µl of TE buffer that had been pre-heated to 65°C. The concentration of the DNA was ascertained by spectrophotometry at 260 nm.

2.8 Restriction enzyme digestion

All restriction endonucleases and restriction endonuclease reaction buffers were supplied by Promega. Restriction enzyme digests of plasmid and polymerase chain reaction (PCR) amplified products were carried out in a volume not normally exceeding 25 µl, containing 10 U of enzyme(s) and 0.1 volumes of an appropriate 10x concentration restriction buffer. Each reaction proceeded for 2 h at 37°C, with the exception of *Sfi*I digestion reactions (Chapter 6) which were carried out at 50°C. A typical reaction comprised:

1 µl 1 µg/µl DNA
16 µl dH_2O
2 µl 10x reaction buffer (Promega)
1 µl 10 U/µl restriction endonuclease (Promega)

2.9 Agarose gel electrophoresis

Agarose gels, 0.8-1% (w/v), were prepared by boiling molecular biology grade agarose (Sigma) in TAE electrophoresis buffer (40 mM Tris-base; 0.1% (v/v) glacial acetic acid; 100 mM EDTA, pH 8.0) for 1-2 min in a microwave oven. One microlitre of ethidium bromide (10 mg/ml) was added for every 50 ml of the gel solution. The molten agarose was cooled and poured into the casting deck of a dedicated gel electrophoresis apparatus. After it had set, the combs were removed, and the electrophoresis tank was filled with TAE as a running buffer. A loading dye (6x: 0.25% (w/v) bromphenol blue; 40% (w/v) sucrose, Sigma) was added to the DNA, acquiring approximately 1/6th of the volume to be loaded, and this was then pipetted into the gel slots. A 'marker' lane was always included which contained *Hind*III-restricted bacteriophage λ DNA (Promega), with which to size the DNA products after they had migrated through the gel. The gels were run at 100 volts (V) and subsequently viewed using an ultraviolet transilluminator.

2.10 Preparation of DNA fragments from agarose gels

When electrophoresis was performed to purify a particular DNA fragment, either from a PCR or a restriction digest, the DNA fragment of interest was recovered from the gel using a Wizard™ PCR Prep DNA Purification Kit (Promega). Briefly, the area of the gel containing the relevant piece of DNA was excised using a clean scalpel and placed in a 1.5 ml eppendorf tube. One millilitre of purification resin was used to dissolve the gel slice and 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, which had been pre-heated to 65°C.

2.11 DNA ligations

Ligation of vector and DNA fragments was performed using T4 DNA ligase (Promega). An estimation of the concentration of each vector and insert was performed by agarose gel electrophoresis next to molecular weight markers of a known concentration. Approximately 200 ng of vector DNA were mixed together with insert, (the amount of which to be added was calculated using a molar ratio, vector: insert, of between 1 and 3), and sterile H_20 added to a final volume of 18 µl in a clean 0.5 ml tube. The mixture was heated to 65°C and gradually cooled to 16°C in a thermal cycler block, to allow DNA to anneal slowly, before addition of 1 µl of T4 DNA ligase enzyme (Promega) and 2 µl of 10x ligase buffer (300 mM Tris-HCl, pH 7.8; 100 mM $MgCl_2$; 100 mM dithiothreitol (DTT); 10 mM ATP) (Promega). The reaction was subsequently incubated at 16°C overnight.

2.12 Bacterial transformation

When required, a 100-µl aliquot of competent *E. coli* JM109 (Promega) cells was thawed. The appropriate DNA sample, usually 0.5-1 µg, was gently mixed with the cells and this was incubated on ice for 10 min. The cells were then heat shocked at exactly 42°C for 45 sec and returned to ice. After 2 min, the cells were transferred to a culture tube containing 990 µl of chilled SOC medium (Life

Technologies Ltd., Paisley, U.K.) which comprises 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose. The culture tube was placed in the rotary incubator 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, and incubated at 37°C overnight. A proportion of the colony forming units, which had selectively grown from successful transformations, were then picked with a sterile pipette tip and streaked on to fresh antibiotic plates for growth overnight at 37°C.

2.13 Polymerase chain reaction

Preceding the PCR reaction, oligonucleotide primers were appropriately designed to amplify regions of a DNA fragment (details are given where relevant in text). Reactions were carried out in 50-μl volumes comprising, unless indicated, 50 ng of template DNA, 0.3 mM of each required (forward and reverse) primer, 1 U of Expand™ High Fidelity *Taq* polymerase (Roche Diagnostics Ltd., Lewes, U.K.), 0.1 mM deoxynucleotides (Promega), in buffer containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 50 mM KCl, 0.1% Tween 20 and 0.1% Nonidet P-40 (Roche Diagnostics Ltd.). Each reaction was overlaid with a drop of mineral oil to prevent evaporation during heating and was subject to PCR amplification in a DNA Thermal Cycler (Perkin-Elmer/Cetus, Norwalk, CT, U.S.A.) using the conditions stated in the text.

2.14 Direct purification of PCR products

PCR amplification products were directly extracted from the PCR constituents using a Wizard™ PCR Prep DNA Purification Kit (Promega). Briefly, one or more PCR reactions assuming a volume of no more than 300 μl, were mixed with 100 μl of Wizard™ direct purification buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.8; 1.5 mM $MgCl_2$; 0.1% (v/v) Triton X-100). One millilitre of Wizard™ PCR Prep DNA Purification resin was added and the mixture vortexed briefly three

times during a period of 1 min. The mixture was then applied to a Wizard™ minicolumn, which was treated as previously described in Section 2.10.

2.15 DNA sequencing

Sequencing of plasmid DNA was performed using a T7 Sequenase® Version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.) by the dideoxy-chain termination method (Sanger *et al.*, 1977) using the appropriate oligonucleotide primer and [α-^{35}S]dATP (NEN, Hounslow, U.K.), as outlined below. Sequencing of PCR amplification products performed according to a Thermo Sequenase Cycle Sequencing Kit (USB Corp., Cleveland, OH, U.S.A.) with [γ-^{32}P]ATP (ICN Pharmaceuticals Ltd., Basingstoke, U.K.) is described separately in Chapter 6.

One-to-three micrograms of plasmid DNA in a volume of 10 µl of TE were denatured by incubation at 37°C with 1 µl of 2 mM EDTA (pH 8.0) and 1 µl of 2 M NaOH for 30 min. One microlitre of 3 M sodium acetate (pH 5.2) and 50 µl of 100% ethanol were added and the DNA was precipitated at -20°C for 1-2 h. A pellet was collected by centrifugation at 12,000 *g* for 15 min and was air-dried, before being resuspended in 10 µl of diluted sequencing primer mixture containing 7 µl sterile dH_20, 2 µl of T7 Sequenase® reaction buffer and 1-5 pmol of the relevant primer (details are given where appropriate in text). The reaction was then heated to 65°C for 2 min, to allow annealing of the primer with its target sequence, prior to slow cooling to 0°C.

Two-point-five microlitres of each of four termination mixes (ddGTP, ddATP, ddTTP, ddCTP; ddGTP contained 80 µM each dGTP, dATP, dTTP, dCTP and 8 µM ddGTP; ddATP as before except ddGTP replaced with 8 µM ddATP; ddTTP as before except with 8 µM ddTTP; ddCTP as before except with 8 µM ddCTP*)* were aliquoted to separate tubes and heated to 37°C. To the annealed DNA, 1 µl DTT, 2 µl diluted labelling mix (7.5 µM dGTP, 7.5 µM dTTP and 7.5 µM dCTP 5x concentrate, diluted to 1x with sterile H_20), 1 µl [α-^{35}S]dATP (1250 Ci/mmol; 12.5 mCi/ml; NEN) and 2 µl diluted T7 Sequenase® polymerase (diluted 1:8 with enzyme dilution buffer) were added. This was incubated at room temperature for 2 min before 3.5 µl was removed from the reaction into each of the four tubes containing dideoxynucleotides. These were

then incubated at 37°C for five min after which 4 µl of stop solution were added. The tubes were subsequently heated at 85°C to denature the DNA and then applied to sequencing gels for electrophoresis as described below.

2.16 Sequence gels and autoradiography

The sequencing reactions were resolved on 6% (w/v) polyacrylamide gels containing 7mM urea, 89 mM Tris-base, 2 mM EDTA (pH 8.0), 89 mM boric acid, and 0.03% (w/v) ammonium persulphate and 0.07% (v/v) of N, N, N, N'-tetramethylethylenediamine (TEMED). A standard non-gradient sequencing gel apparatus, (Bio-Rad Laboratories Ltd., Hemel Hempsted, U.K.), was used in the set up. The gel running buffer contained 89 mM Tris-base, 2 mM EDTA (pH 8.0) and 89 mM boric acid. The gel and buffer were warmed to 50°C prior to electrophoresis by running at 2000 V for one hour. Gel slots were flushed with buffer to remove any urea and unpolymerised acrylamide. DNA sequence reactions were then loaded. Gels were run at a voltage of approximately 1800 V for 2 to 4 h at 50°C depending on the length of the sequence to be studied. After the reactions had run to the desired extent, the current was turned off, apparatus dismantled and the gel fixed in 12% (v/v) methanol, 10% (v/v) glacial acetic acid. The gel was then transferred to a sheet of 3MM blotting paper (Whatman International Ltd., Maidstone, U.K.) and dried (Bio-Rad Gel Dryer 583; Bio-Rad Laboratories Ltd.) at 80°C for 2 h.

The dried gel was subjected to autoradiography by exposure to Fuji RX x-ray film (Genetic Research Instrumentation Ltd., Dunmow, U.K.) in a Hypercassette™ (Amersham Phamarcia Biotech) at room temperature for 24 h. The film was subsequently developed using Photosol CD18 x-ray developer (Photosol Ltd., Basildon, U.K.) for 3 min, rinsed in water and then fixed for 3 min in Photosol CF40 fixer (Photosol Ltd.).

2.17 SDS-PAGE and autoradiography

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples was performed in 12, 12.5, or 15% SDS-polyacrylamide resolving gels. The exact constitution of gels and associated buffers is shown in

Table 2.2. 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 Protean II apparatus (Bio-Rad Laboratories Ltd.).

Briefly, two glass plates (12 cm^2 and 12 x 16 cm) and 1 mm 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, (to a level approximately 5 cm from the top of the smaller plate), and overlaid with 1 ml butan-1-ol. After the gel mixture had polymerised the butanol was poured off, and a comb was inserted before the solution for the stacking gel was poured in on top. Subsequently, the comb was removed and the full apparatus assembled. Laemilli 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 reservoir was full.

Prior to loading, each protein sample was mixed with 2x SDS sample buffer (2x: 4% (w/v) SDS; 20% (v/v) glycerol; 0.002% (w/v) bromophenol blue; 2% (v/v) 2-mercaptoethanol; 25% (v/v) buffer B (Table 2.2)) and heated at 85°C for 5 min. Between ten and thirty microlitres of each sample was then loaded into the wells along with a protein molecular weight marker (Sigma). Protein molecular weight standards used were: myosin, 205 kilodaltons (kDa); β-Galactosidase, 116 kDa; phosphorylase B, 97 kDa; bovine albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa. Gels were run at a current of 35 milli-amperes (mA) 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 transfered to a plastic tray and covered with Coomassie® Blue stain (0.05% (w/v) Coomassie® Brilliant Blue, Bio-rad Laboratories 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/ 25% (v/v) isopropanol, until the protein markers were clearly visible. The destain was discarded and the gel was soaked for a further 30 min in Amplify scintillant (Amersham Pharmacia Biotech), before being dried for 2 h at 60°C onto 3MM Whatman paper.

Table 2.2: Constitution of SDS-PAGE[1] gels.

Resolving gel:

Constituent	Final concentration in gel
Buffer A:	
Tris-Base (BDH)	0.4 M
SDS[2] (Sigma)	0.1% (w/v)
Acrylamide: bisacrylamide (37.5: 1) (Bio-Rad Laboratoriesn Ltd.)	12% (w/v) or 12.5% (w/v) or 15 % (w/v)
Ammonium persulphate (BDH)	0.04% (w/v)
TEMED[3] (Sigma)	0.0004% (v/v)

Stacking gel:

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

Gels containing radiolabelled proteins were exposed to Fuji RX x-ray film at –70°C for the required time (usually overnight) and then developed as in Section 2.16.

3 Analysis of a microsatellite polymorphism of the cytotoxic T lymphocyte antigen-4 gene in patients with vitiligo.

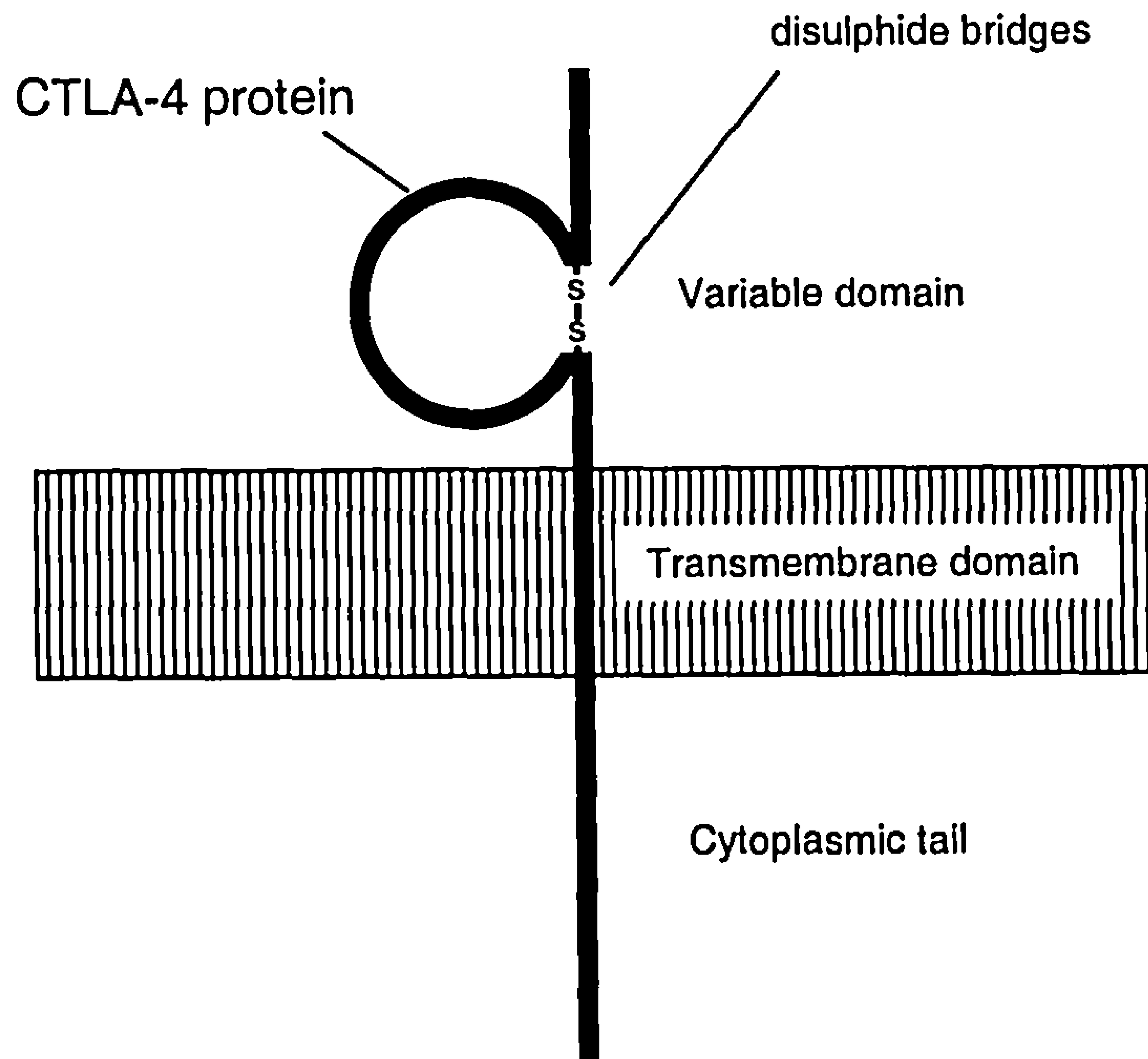
3.1 Introduction

3.1.1 Cytotoxic T lymphocyte antigen-4 structure and function

The cytotoxic T lymphocyte antigen-4 (CTLA-4) is a T cell surface protein that was first identified by differential screening of a murine cytolytic T cell cDNA library (Brunet *et al.* 1987). The protein is classified within the immunoglobulin (Ig) superfamily (Brunet *et al.* 1987) since initial studies have demonstrated that it has an Ig-like structure consisting of a membrane-bound single variable domain (Brunet *et al.* 1987; Dariavach *et al.* 1988; Harper *et al.* 1991). This is illustrated in Figure 3.1a. In addition, the structure of CTLA-4 is very similar to T lymphocyte receptors that are involved in transmembrane signalling, including the CD3 complex (van den Elsen *et al.* 1984; Gold *et al.* 1986) and CD28 (Aruffo & Seed 1987), and is also highly conserved between human and mouse (Dariavach *et al.* 1988; Harper *et al.* 1991).

CTLA-4 is found on the surface of T lymphocytes following T cell activation (Brunet *et al.* 1987; Linsley *et al.* 1992; Alegre *et al.* 1996) induced by the interaction of the T cell receptor with a MHC antigen/peptide complex together with an appropriate costimulatory signal (June *et al.* 1994). CTLA-4 appears to act as a negative regulator of T cell activation (Bluestone 1997; Oosterwegel *et al.* 1999) through engagement with the B7 family of ligands (Green *et al.* 1994) on antigen presenting cells (APC). Functional studies have also shown the receptor to be involved in the negative control of T cell proliferation (Robey&Allison 1995) and in mediating T cell apoptosis (Gribben *et al.* 1995). This is in direct contrast to the role of the structurally homologous T cell receptor CD28 which also acts through B7 costimulation, but which is essential for the proliferation of activated T lymphocytes (Krummel & Allison 1995).

(a)



(b)

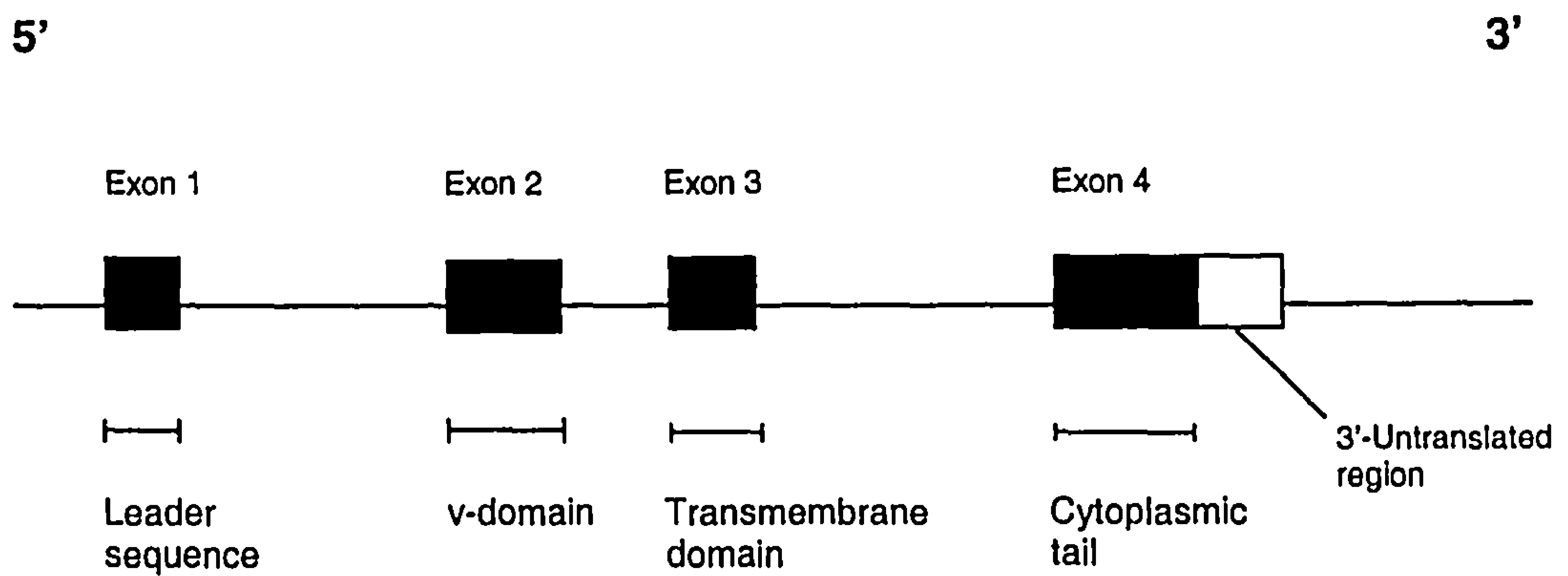


Figure 3.1: A schematic diagram of: (a) the CTLA-4 protein; (b) the CTLA-4 gene.

(Adapted from June *et al.* 1994).

3.1.2 The CTLA-4 gene and its polymorphisms

The CTLA-4 receptor is encoded by the CTLA-4 gene which is located at band q33 of chromosome 2 (Harper *et al.* 1991). Analysis of the preliminary sequence of the human CTLA-4 gene revealed three exon regions encoding the variable, transmembrane, and cytoplasmic domains of the protein, respectively (Dariavach *et al.* 1988). However, subsequent sequence data demonstrated the presence of a fourth exon containing the leader sequence (Harper *et al.* 1991) and the initial nomenclature was altered. A schematic representation of the gene, indicating the different functional domains of the protein, is shown in Figure 3.1b.

Three polymorphisms have been defined within the CTLA-4 gene as follows: a promoter polymorphism with a cytosine to thymine (C/T) substitution at position –318 (Deichmann *et al.* 1996), an exon 1 polymorphism with an adenine to guanine (A/G) substitution at position +49 (Harper *et al.* 1991) and a microsatellite polymorphism with variant lengths of a dinucleotide $(AT)_n$ repeat beginning at position +642 of the 3'-untranslated region (UTR) of exon 4 (Polymeropoulos *et al.* 1991). Figure 3.2 demonstrates the sites described for these polymorphisms in a schematic map of the CTLA-4 gene.

How these various polymorphisms affect the expression or function of CTLA-4 is open to speculation. The microsatellite polymorphism has a possible functional significance to the molecule as differences in the length of the $(AT)_n$ repeat in the 3'-UTR of the CTLA-4 gene could affect mRNA stability (Yanagawa *et al.* 1995). For example, it is well established that AT-rich regions in 3'-UTRs can affect RNA stability, particularly of cytokine genes (Shaw & Kamen 1986). The promoter polymorphism does not affect any known consensus sequence in the regulatory region of the promoter (Deichmann *et al.* 1996) and is therefore unlikely to affect CTLA-4 expression. The A to G transition at position +49 in codon 17 results in an amino acid exchange (threonine to alanine) in the leader sequence of the CTLA-4 gene, but is not expected to affect the function of the leader peptide (Nistico *et al.* 1996). However, recent *in vitro* studies suggest that both the promoter and exon 1 polmorphisms may indeed influence the level of CTLA-4 gene expression and the inhibitory function of the protein. Ligers *et al.* 2001 reported that peripheral blood mononuclear cells (PBMC) from individuals with a thymine at position –318 of the CTLA-4 promoter, who are

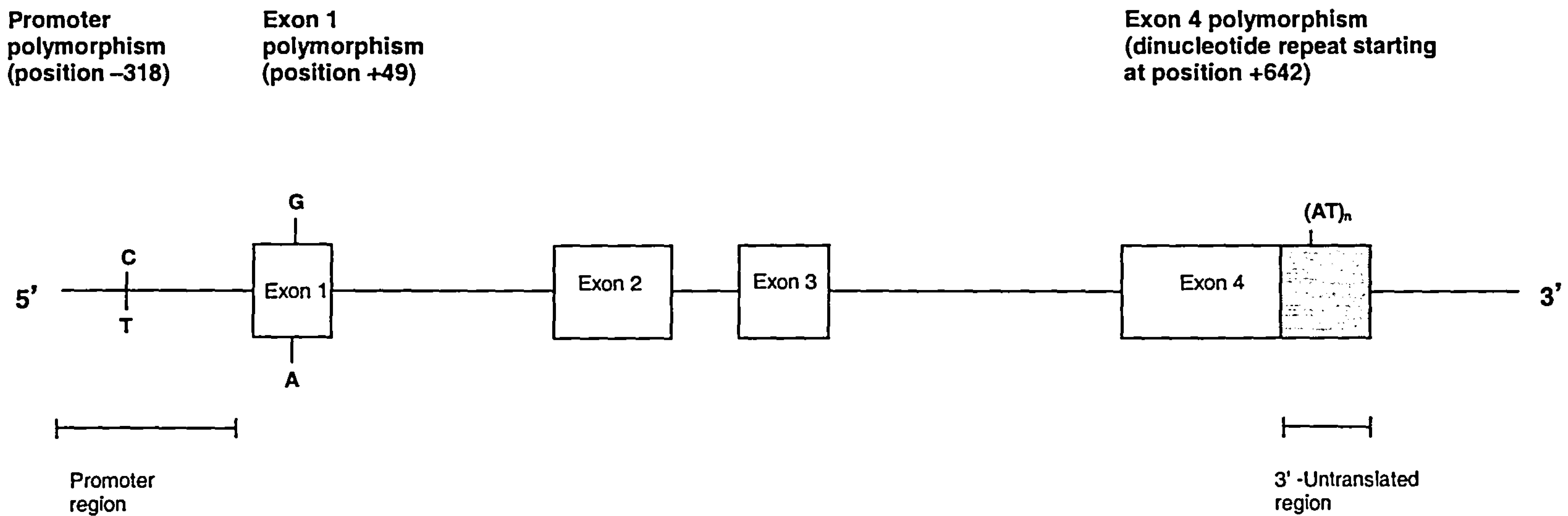


Figure 3.2: Location of three polymorphisms in the CTLA-4 gene.

also homozygous for adenine (A/A) at position +49 of exon 1, show significantly increased expression of CTLA-4 when compared with PBMC from individuals without this genotype. In addition, Kouki *et al.* 2000, demonstrated that peripheral blood T cells cultured from individuals homozygous for guanine (G/G) at position +49 of exon 1 showed greater proliferation in response to stimulation, by incubation with allogenic B cells, than those from A/A individuals. Furthermore, these cells showed reduced augmentation of the same proliferative response when binding of CTLA-4 was prevented with blocking antibodies, therefore suggesting that CTLA-4 protein is less able to negatively regulate T cell proliferation in G/G individuals (Kouki *et al.* 2000). Further experiments are needed to determine how these apparent genotypic affects are mediated.

3.1.3 CTLA-4 and its relationship to autoimmunity

The role of CTLA-4 in autoimmunity has yet to be established but since the receptor plays a part in the regulation of the immune response by modulating T cell reactivity, it is possible that defective CTLA-4 expression or function may predispose to the development of autoimmune disease. This hypothesis is increasingly supported by experimental evidence. For example, a lethal lymphoproliferative disorder in CTLA-4 knockout transgenic mice was characterised by T cell infiltration of multiple organs and destruction of tissue (Waterhouse *et al.* 1995). Recent studies, in which the CTLA-4 protein is manipulated in animal models of autoimmunity, have demonstrated that it is capable of regulating the initiation and progression of autoimmune diseases. The development of experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis, can be accelerated and exacerbated by the blockade of CTLA-4 and its B7 ligand by intra-peritoneal administration of an anti-CTLA-4 antibody (Karandikar *et al.* 1996). The disease can also be prevented by both systemic intra-peritoneal administration and intra-cranial delivery direct to the central nervous system (CNS) of a cross-linking CTLA-4 immunoglobulin (Croxford *et al.* 1998). In addition, the onset of autoimmune disease in diabetic mice can be induced far more rapidly and aggressively by *in vivo* administration of anti-CTLA-4 antibody (Luhder *et al.* 1998).

The experiments described above raise the possibility that polymorphisms in the CTLA-4 gene may modulate the function of the resulting protein, in turn promoting the development of autoimmune disease. Indeed, recent results have revealed associations between polymorphic regions of the CTLA-4 gene and various autoimmune disorders, the details of which are summarised in Table 3.1. In particular the 106 base pair allele of the exon 4 microsatellite polmorphism, the G allele of the exon 1 diallelic polymorphism and the T allele of the diallelic polymorphism in the promoter have been associated with autoimmune disease

3.2 Aim

Since vitiligo is frequently associated with autoimmune disorders and may have an autoimmune origin, the CTLA-4 exon 4 microsatellite polymorphism was analysed in a series of patients with vitiligo to determine whether or not an association exists between this disorder and the gene polymorphism.

Table 3.1: Studies in which polymorphisms of the CTLA-4 gene have been associated with autoimmune disorders.

CTLA-4 gene polymorphism	Disease	Population	Type of study	Reference
1. Microsatellite polymorphism, variant lengths of a dinucleotide $(AT)_n$ repeat more specifically a 106 base pair allele, at position +642 of the 3'-UTR of exon 4[1]	Graves' disease	USA	Case control (CC)	Yanagawa *et al.* 1995
	"	U.K.	CC	Kotsa *et al.* 1997
	"	Japanese	CC	Akamizu *et al.* 2000
	Autoimmune hypothyroidism	U.K.	CC	Kotsa *et al.* 1997
	Thyroid associated ophthalmopathy	U.K.	CC	Vaidya *et al.* 1999a
	Addison's disease	English	CC	Kemp *et al.* 1998a
	Type 1 diabetes mellitus	Italian (sibling pairs)	Sib-pair linkage analysis	Nistico *et al.* 1996
	"	Spanish (families)	Transmission-disequilibrium test (TDT)	Marron *et al.* 1997
	"	French (families)	TDT	Marron *et al.* 1997
	"	Mexican-American (families)	TDT	Marron *et al.* 1997
	"	Chinese (families)	TDT	Marron *et al.* 1997
	"	Korean (families)	TDT	Marron *et al.* 1997
	Systemic lupus erythematosus (SLE)	Japanese	CC	Ahmed *et al.* 2001
(Continued overleaf)				

Table 3.1 *continued*

CTLA-4 gene polymorphism	Disease	Population	Type of study	Reference
2. Point mutation A/G at position +49 of exon 1[2]	Type 1 diabetes mellitus	Italian (families)	TDT	Nistico *et al.* 1996
	"	Spanish (families)	TDT	Nistico *et al.* 1996
	"	Belgian	CC	Nistico *et al.* 1996
	"	Hong Kong Chinese	CC	Nistico *et al.* 1996
	"	French	CC	Djilali-Saiah *et al.* 1998a
	"	Spanish (families)	TDT	Marron *et al.* 1997
	"	French (families)	TDT	Marron *et al.* 1997
	"	Mexican-American (families)	TDT	Marron *et al.* 1997
	"	Chinese (families)	TDT	Marron *et al.* 1997
	"	Korean (families)	TDT	Marron *et al.* 1997
	"	U.K. (families)	TDT	McCormack *et al.* 2001a
	"	German/Canadian[3]	CC	Donner *et al.* 1997b
	"	Belgian	CC	Van der Auwera *et al.* 1997
	"	Chinese children	CC	Lee *et al.* 2000
	Type 1 diabetes mellitus:			
	i) Patients with diabetic ketoacidosis	Japanese	CC	Abe *et al.* 1999
	ii) Patients with islet-specific autoantibody ICAS12	Japanese	CC	Abe *et al.* 1999
	iii) Patients with islet-specific autoantibody GAD	Japanese	CC	Hayashi *et al.* 1999
	iv) Patients with acute onset	Japanese	CC	Awata *et al.* 1998
	Graves' disease	German/Canadian[3]	CC	Donner *et al.* 1997b
	"	Japanese	CC	Yanagawa *et al.* 1997
	"	Korean	CC	Park *et al.* 2000

(Continued overleaf)

Table 3.1 *continued*

CTLA-4 gene polymorphism	Disease	Population	Type of study	Reference
2. Point mutation A/G at position +49 of exon 1[2]	Graves' disease	U.K. (families)	TDT	Vaidya *et al.* 1999b
	"	Japanese	CC	Awata *et al.* 1998
	Hashimoto's thyroiditis	German/Canadian[3]	CC	Donner *et al.* 1997a
	"	Japanese	CC	Awata *et al.* 1998
	Addison's disease	German/Canadian[3]	CC	Donner *et al.* 1997a
	"	English	CC	Vaidya *et al.* 2000
	SLE	Slovakian	CC	Pullmann *et al.* 1999
	"	Japanese	CC	Ahmed *et al.* 2001
	Rheumatoid arthritis	German	CC	Seidl *et al.* 1998
	"	Japanese	CC	Yanagawa *et al.* 2000
	"	Spanish	CC	Gonzalez-Escribano *et al.* 1999
	Multiple sclerosis (MS)	Norwegian	CC	Harbo *et al.* 1999
	MS patients with severe disease	Japanese	CC	Fukazawa *et al.* 1999
	Primary biliary cirrhosis	U.K.	CC	Agarwal *et al.* 2000
	Coeliac disease	French	CC	Djilali-Saiah *et al.* 1998b
	"	Swedish (families)	TDT	Naluai *et al.* 2000
3. Point mutation (C/T) at position –318 of the CTLA-4 promoter[4]	Graves' disease	German/Canadian[3]	CC	Braun *et al.* 1998
	Hashimoto's thyroiditis	German/Canadian[3]	CC	Braun *et al.* 1998
	Type 1 diabetes mellitus	Chinese	Sib-pair linkage analysis	Lee *et al.* 2001
	"	U.K.	TDT	McCormack *et al.* 2001a

[1] All studies demonstrated a significant disease association of the 106 base pair allele. [2] All studies of the exon 1 A/G polymorphism demonstrated a significant disease association of the G allele, with the exception of Djilali-Saiah *et al.* (1998) and Naluai *et al.* (2000) who demonstrated a significant association of the A allele and coeliac disease. [3] Results were analysed as two separate populations and as a whole. [4] All studies demonstrated a significant disease association of the T allele.

3.3 Materials and Methods

3.3.1 Patients and controls

Sixty-four consecutive British vitiligo patients (19 males, 45 females) were included in this study. They had been examined in dermatology and endocrinology clinics in Sheffield between January 1990 and October 1997. A summary of the patient details is presented in Table 3.2. When characterised with respect to the presence of associated autoimmune disorders: 46 patients had no other disease (18 males, 28 females); 18 had one or more autoimmune disorders (1 male, 17 females). The 173 (84 males, 89 females) regionally matched, healthy controls had no clinical evidence of vitiligo or any other autoimmune disorder.

3.3.2 Polymerase chain reaction amplification of the CTLA-4 gene exon 4 microsatellite polymorphism

Briefly, a microsatellite polymorphism in exon 4 of the CTLA-4 gene was analysed by PCR amplification of genomic DNA. The products were resolved on sequencing gels and, due to the inclusion in the PCR amplification reaction of a radiolabelled nucleotide [α-^{32}P]dCTP, could be visualised by autoradiography.

High molecular weight genomic DNA was prepared from heparinized venous blood samples (10-20 ml) using a Gentra Systems Puregene DNA Isolation Kit (Flowgen, Lichfield, U.K.). Approximately 5 µg of DNA was produced per 0.5 ml of blood applying the manufacturer's protocol. The DNA was resuspended in sterile TE buffer and stored at -20°C until use. The primers used were 5'GCCAGTGATGCTAAAGGTTG3' and 5'AACATACGTGCTCTAT GCA3' (R and D Systems, Abingdon, U.K.) and were designed to amplify a polymorphic $(AT)_n$ repeat site beginning at base pair 642 in the 3'-untranslated region of exon 4 of the human CTLA-4 gene. Samples of DNA (50 ng) were subjected to 34 amplification cycles using the following conditions: 94°C, 1 min; 55°C, 1 min; 72°C, 1.5 min; and 72°C for 10 min to terminate the reaction. The composition of each PCR amplification reaction was as previously described (Chapter 2; 2.13), except that each contained deoxynucleotides dATP, dTTP and

dGTP at a concentration of 0.1 mM, but with dCTP at 0.01 mM and 0.5 μCi of [α-^{32}P]dCTP (10 mCi/ml; 3000 Ci/mmol; Amersham Pharmacia Biotech).

Amplified products were resolved in 6% (w/v) polyacrylamide gels (Section 2.16). Gels were dried and autoradiographed at -70°C using Fuji x-ray film (Section 2.16). Genotypes were determined by comparing to ∅X174 DNA/*Hinf*I markers (Promega) and confirmed by comparison with a dideoxy sequencing ladder.

Figure 3.3 shows an autoradiogram executed by Yanagawa *et al.* (1995), that demonstrates all alleles of the CTLA-4 microsatellite polymorphism which are detected by this methodology.

3.3.3 Statistical analysis

The frequency of the CTLA-4 106 base pair allele was compared between vitiligo patients and controls by 2 x 2 contingency tables and chi-squared tests. Yates' correction was applied and *P* values < 0.05 (two-tailed) were regarded as significant. Where sample values were small, for example when comparing data between clinical subclasses of vitiligo and controls, Fisher's exact test was used. Odds ratios associated with the 106 base pair allele were calculated by the method of Woolf (Woolf 1955).

Table 3.2: Summarised details of patients included in this study.

Gender (female: male)	45: 19
Mean age (yr)	46
Age range (yr)	7-78
Mean age of onset (yr)	30
Age range of onset (yr)	<1-70
Mean disease duration (yr)	16
Range of disease duration (yr)	<1-60
Vitiligo type: -	
Symmetrical	26 females, 11 males
Segmental	5 females
Focal	2 females, 2 males
Symmetrical/peri-orificial	8 females, 4 males
Occupational	1 male
Extensive/near total	3 females
Halo naevi	1 male
Autoimmune disorders: -	
Autoimmune thyroid disease	13 females
Autoimmune polyglandular syndrome type 2	2 females
Alopecia areata	2 females, 1 male
Addison's disease	1 female
Type 1 diabetes mellitus	2 females
Systemic lupus erythematosus	1 female
Pernicious anemia	1 female

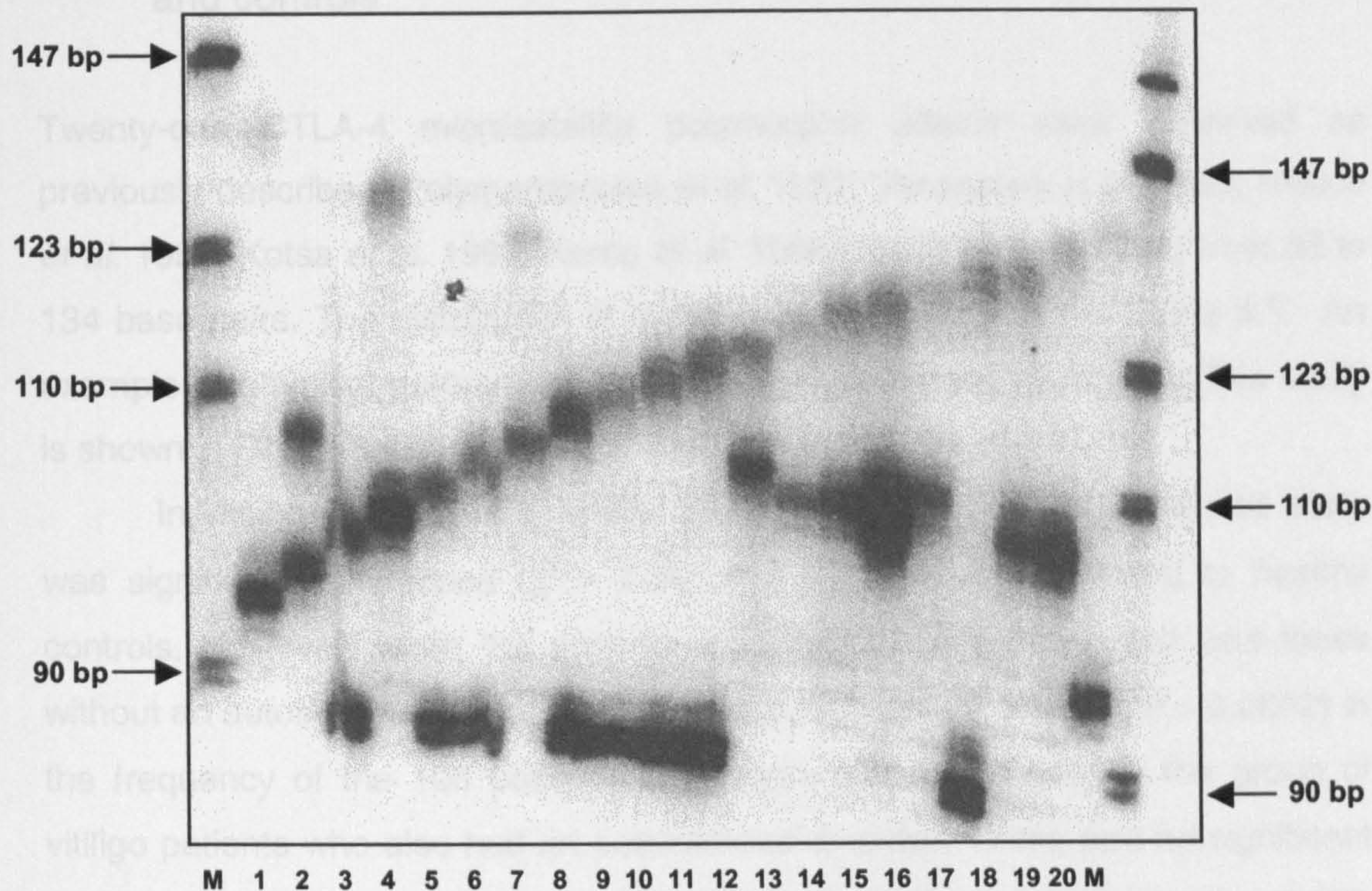


Figure 3.3: An autoradiogram of the CTLA-4 gene microsatellite polymorphism.

(Reproduced from an original figure by Yanagawa *et al.* 1995).

Lane M on both sides contains size markers. The genotype of each sample is as follows: Lane 1, 96/134; 2, 98/108; 3, 100/88; 4, 102/130; 5, 104/88; 6, 106/88; 7, 108/126; 8, 110/88; 9, 112/88; 10, 114/88; 11, 116/88; 12, 118/108; 13, 120/106; 14, 122/106; 15, 124/106; 16, 126/108; 17, 128/88; 18, 130/106; 19, 132/106; 20, 134/96.

3.4 Results

3.4.1 Frequency of the CTLA-4 106 base pair allele in vitiligo patients and controls

Twenty-one CTLA-4 microsatellite polymorphic alleles were observed as previously described (Polymeropoulos *et al.* 1991; Yanagawa *et al.* 1995; Nistico *et al.* 1996; Kotsa *et al.* 1997; Kemp *et al.* 1998a), with sizes ranging from 88 to 134 base pairs. The distribution of these genotypes is shown in Table 3.3. An example of a typical autoradiogram of CTLA-4 genotypes obtained in this study is shown in Figure 3.4.

In vitiligo patients as a whole, the frequency of the 106 base pair allele was significantly increased (χ^2 = 5.96; P = 0.01) when compared to healthy controls. However, when the patients were divided into those with and those without an autoimmune disorder, a significant increase (χ^2 =13.73; P = 0.0002) in the frequency of the 106 base pair genotype was found only in the group of vitiligo patients who also had an autoimmune disorder. There was no significant increase in the frequency of the 106 base pair allele in the group of patients without an associated autoimmune disorder (χ^2 = 1.03; P = 0.31 compared to controls).

3.4.2 Frequency of the 106 base pair allele in vitiligo patient subgroups

3.4.2.1 Clinical subclass

When the vitiligo patients were divided into their clinical subclass (segmental, focal or symmetrical) no significant association of vitiligo type and the 106 base pair allele was revealed (Table 3.4). However, it is interesting to note that two of the three patients with extensive/near total vitiligo were homozygous for the 106 base pair allele. Patients who could not be diagnosed to a single vitiligo type were excluded from this analysis.

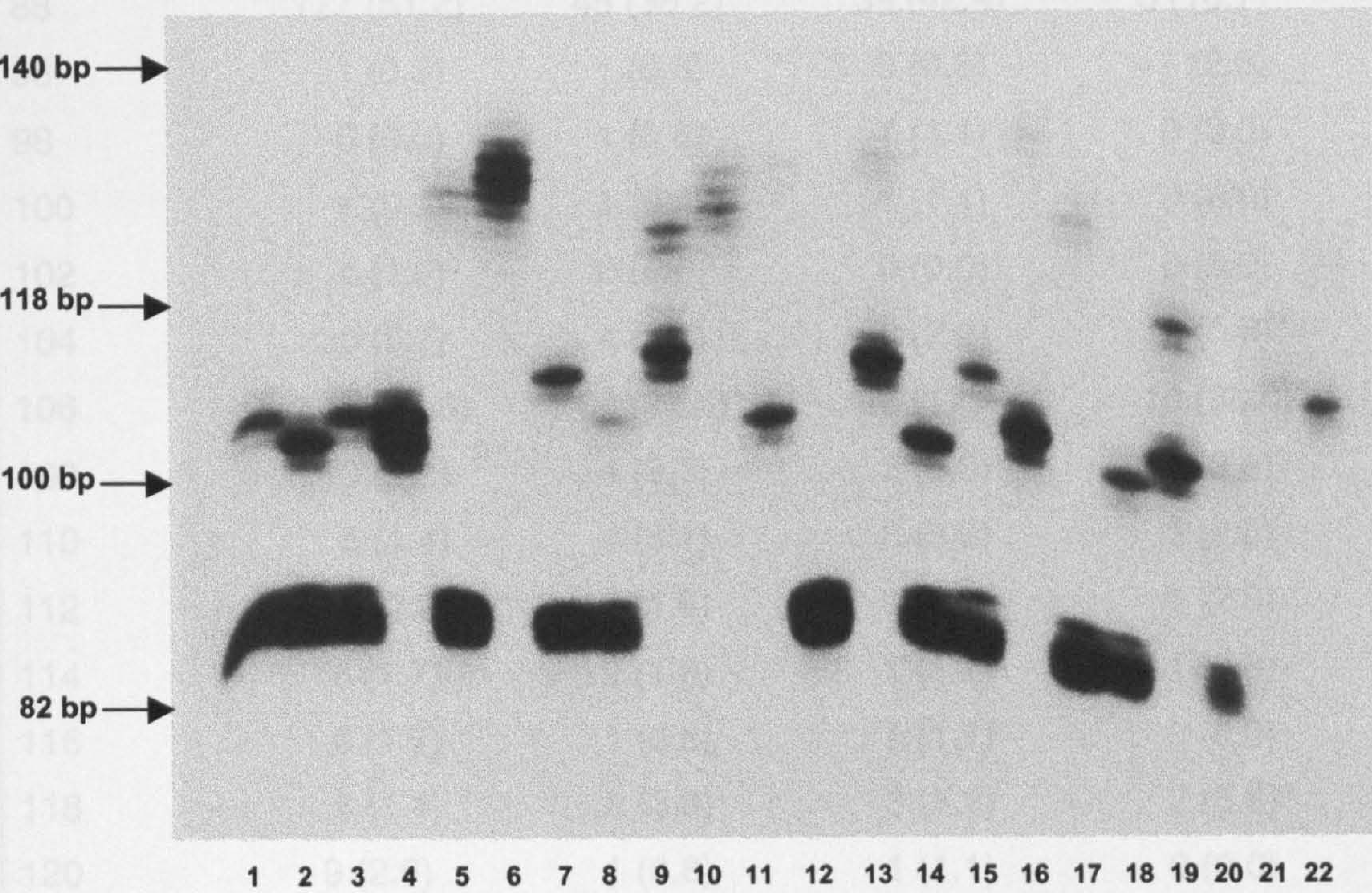


Figure 3.4: A typical autoradiogram of CTLA-4 microsatellite polymorphism genotypes obtained in this study.

The genotype of each sample is as follows: Lane 1, 88/106; 2, 88/104; 3, 88/106; 4, 104/106; 5, 88/126; 6, 126/128; 7, 88/108; 8, 88/104; 9, 110/122; 10, 124/128; 11, 104/128; 12, 88/88; 13, 110/128; 14, 88/104; 15, 88/110; 16, 106/136; 17, 88/128; 18, 88/104; 19, 106/118; 20, 88/106; 21, 114/120; 22, 112/128.

Table 3.3: CTLA-4 microsatellite polymorphic allele genotype frequencies in the investigated groups.[1]

Allele size (base pair)	Controls (n = 346)[2]	Vitiligo[3] (n = 128)	Vitiligo[4] (n = 92)	Vitiligo[5] (n = 36)
88	177 (51.2)	45 (35.2)	39 (42.4)	6 (16.7)
96	1 (0.3)	1 (0.8)	0 (0.0)	1 (2.8)
98	0 (0.0)	1 (0.8)	1 (1.1)	0 (0.0)
100	1 (0.3)	1 (0.8)	1 (1.1)	0 (0.0)
102	4 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)
104	30 (8.7)	4 (3.1)	2 (2.2)	2 (5.6)
106	**47 (13.6)**	**30 (23.4)***	**16 (17.4)****	**14 (38.0)*****
108	10 (2.9)	4 (3.1)	3 (3.3)	1 (2.8)
110	5 (1.4)	4 (3.1)	3 (3.3)	1 (2.8)
112	10 (2.9)	2 (1.6)	1 (1.1)	1 (2.8)
114	6 (1.7)	2 (1.6)	1 (1.1)	1 (2.8)
116	6 (1.7)	1 (0.8)	1 (1.1)	0 (0.0)
118	5 (1.4)	5 (3.9)	3 (3.3)	2 (5.6)
120	9 (2.6)	1 (0.8)	1 (1.1)	0 (0.0)
122	7 (2.0)	5 (3.9)	5 (5.4)	0 (0.0)
124	5 (1.4)	2 (1.6)	1 (1.1)	1 (2.8)
126	8 (2.3)	5 (3.9)	5 (5.4)	0 (0.0)
128	6 (1.7)	9 (7.0)	5 (5.4)	4 (11.1)
130	6 (1.7)	2 (1.6)	2 (2.2)	1 (2.8)
132	1 (0.3)	2 (1.6)	1 (1.1)	1 (2.8)
134	2 (0.6)	2 (1.6)	1 (1.1)	1 (2.8)

[1]Values are the number of alleles positive for each genotype, with the percentage given in parentheses.

[2]n, number of investigated alleles.

[3]All patients with vitiligo.

[4]Patients with vitiligo and no other autoimmune disease.

[5]Patients with vitiligo and one or more autoimmune disease.

*$P = 0.01$; **$P = 0.31$; ***$P = 0.0002$ (compared to controls).

Table 3.4: CTLA-4 106 base pair allele frequency and clinical subclass of vitiligo.

	Controls (n = 173)[1]	Segmental (n = 5)	Focal (n = 5)	Symmetrical (n = 39)
Number of alleles investigated	346	10	10	78
Number of 106 base pair alleles	47	2	1	17
Frequency of the 106 base pair allele	13.6%	20.0%	10.0%	22.0%
P value[2]	-	0.63	1.0	0.08

[1]n, number of patients.

[2]Compared to controls.

3.4.2.2 Age of onset

No association was found between the age of onset of vitiligo and the 106 base pair genotype (Table 3.5). Fisher's exact test was applied due to small sample values.

3.4.2.3 Gender

When the data was analysed with respect to gender (Table 3.6), a significant ($\chi^2 = 12.5$; $P = 0.0004$) increase in the frequency of the 106 base pair genotype was found only in the female patients. However, this significance is probably conveyed by the higher proportion of patients in this subgroup with an associated immune disorder, rather than by gender alone, thus demonstrating the problem of multiple comparisons.

3.4.2.4 Antibody status

Some of the patients in this study had previously been tested for autoantibodies against the melanocyte antigens tyrosinase, TRP-1, TRP-2 and Pmel17 (Kemp *et al.* 1997a; Kemp *et al.* 1997b; Kemp *et al.* 1998b; Kemp *et al.* 1998c) but no correlation was apparent between those patients with humoral autoreactivity and the 106 base pair allele.

3.4.3 Odds ratios associated with the CTLA-4 106 base pair allele

The odds ratio conferred by the 106 genotype was 1.8 for patients with vitiligo, 4.3 for vitiligo patients with an associated autoimmune disorder and 1.2 for vitiligo patients without an associated autoimmune disorder.

Table 3.5: CTLA-4 106 base pair allele frequency and age of onset.

	Controls (n = 173)[1]	Vitiligo 0-10 yr (n = 11)	Vitiligo 11-20 yr (n = 12)	Vitiligo 21-40 yr (n = 23)	Vitiligo >40 yr (n = 18)
Number of alleles investigated	346	22	24	46	36
Number of 106 base pair alleles	47	5	6	11	8
Frequency of the 106 base pair allele	13.6%	22.7%	25.0%	23.9%	22.2%
P value[2]	-	0.22	0.13	0.08	0.21

[1]n, number of patients.

[2]Compared to controls.

Table 3.6: CTLA-4 106 base pair allele frequency in males and females in the investigated groups.

	Male		Female	
	Controls (n=84)[1]	Vitiligo (n=19)	Controls (n=89)	Vitiligo (n=45)
Number of alleles investigated	168	38	178	90
Number of 106 base pair alleles	20	3	27	27
Frequency of the106 base pair allele	12.0%	8.0%	15.2%	30.0%
P value[2]	-	0.46	-	0.0004
χ-square	-	0.54	-	12.5

[1]n, number of patients.

[2]Compared to controls.

3.5 Discussion

These results indicate that there is no association between the 106 base pair allele and vitiligo in patients without an associated autoimmune disorder (P = 0.31). The odds ratio conferred by the 106 allele in this patient group was 1.2, a significantly lower value than those previously published for autoimmune diseases: Graves disease, 2.1; autoimmune hypothyroidism, 2.2 (Kotsa *et al.* 1997). In vitiligo patients with one or more associated autoimmune disorder, the frequency of the 106 base pair allele was significantly increased, (P = 0.0002), when compared to healthy controls. However, 15 out of 18 of these patients had immune disorders that have been demonstrated to be associated with the presence of the 106 base pair allele (e.g., Graves disease, autoimmune hypothyroidism, autoimmune Addison's disease, and type 1 diabetes mellitus). A disease genotype predisposing to other autoimmune disorders may, therefore, have created the apparent association of vitiligo and the 106 base pair allele in this patient group. It was found, however, that the odds ratio conferred by the 106 base pair allele in this group of patients was significantly greater than that found for patients with only Graves disease (Kotsa *et al.* 1997), autoimmune hypothyroidism (Kotsa *et al.* 1997) or autoimmune Addison's disease (Kemp *et al.* 1998a), an odds ratio of 4.3 versus values of 2.1, 2.2 and 2.2, respectively. This suggests that multiple autoimmunity may be determined in part by the CTLA-4 polymorphism, and autoimmune endocrinopathy patients with the 106 base pair allele may be more likely to develop vitiligo.

It would appear from this study that vitiligo, at least without an associated autoimmune disorder, is not influenced by the CTLA-4 exon 4 microsatellite polymorphism. However, this does not rule out an autoimmune basis for the disease nor the involvement of T cells in its pathogenesis. The initiation of autoimmunity may be caused by the action of diverse cell types. The role of T cells in the disease pathogenesis may be to drive antibody formation in a T helper 2-type activity, in response to B cell-recognised autoantigen/s. Furthermore, active proliferation of a cytotoxic T cell response may be caused by an imbalance of signaling through the cytokine network, rather than by faulty negative regulation via the CTLA-4 receptor.

There are difficulties related to attempting to identify a vitiligo susceptibility gene through a population-based, case-control association study.

It is a very sensitive technique, providing the control population is carefully genetically matched, and can detect minor genetic associations which are not necessary for disease expression but which represent an increased risk for the disease (Barbesino *et al.* 1998). Despite this sensitivity, genetic heterogeneity between and within population groups may prevent significant differences in the frequency of a particular allele from being detected. It is possible that such variations have accounted for the failure of several studies to detect significant associations between CTLA-4 polymorphisms and autoimmune disease including, post-partum thyroiditis (Waterman *et al.* 1998), and bullous and cicatrical pemphigoid (Drouet *et al.* 2000), and chronic inflammatory disorders such as asthma and atopy (Heinzmann *et al.* 2000). Population-based, case-control studies lack specificity (Lander & Schork 1994). They cannot distinguish between a disease allele at a disease loci and a nearby allele, which does not affect disease expression, but is in linkage disequilibrium (transmitted with) the disease causing allele. Therefore the association reported here between CTLA-4 and vitiligo with associated autoimmunity may in fact represent an association with a polymorphism/s in a gene/s located in close proximity to CTLA-4 on the chromosomal region 2q33, for example, CD28 or the newly identified Inducible T-cell co-stimulator (ICOS) gene (Hutloff *et al.* 1999).

Small data sets generate a risk of bias or chance probabilities revealing falsely positive associations and to test thoroughly the association of CTLA-4 with vitiligo, a larger data set and transmission studies of the allele in family groups are required. A recent case-control study, in which a larger cohort of vitiligo patients was analysed for association with CTLA-4 polymorphisms, also suggests genetic association with vitiligo (Kristensen *et al.* 2000). Furthermore, a family study involving 552 vitiligo patients and their families (Section 1.2.3) also indentified polymorphic markers for the CD28/CTLA-4 gene region, including the CTLA-4 microsatellite marker, that are positively associated with vitiligo susceptibility (McCormack *et al.* 2001b). In both of these studies, genetic association was suggested in all vitiligo patients, not only in those patients with other autoimmunity (Dr. W. T. McCormack; University of Florida, personal communication). Further experiments, including family-based genome screening, are needed to map the genetic susceptibility markers in greater detail.

The failure to find a significant association with a single gene polymorphism in the vitiligo patients, as a whole, most likely reflects the complex spectrum of the disease itself. Previous genetic studies have concluded that vitiligo is most likely due to the action of genes at multiple loci (Majumder *et al.* 1993; Kim *et al.* 1998; Acros *et al.* 1999), which therefore makes the effect of an individual locus difficult to establish. Other genes whose dysfunction affects immune regulation may control the development of autoimmune vitiligo for example, those encoding complement components C2 and C4, or the autoimmune regulator (AIRE) gene described previously (Section 1.2.3). It is also possible that environmental factors, such as stress (Sections 1.3.1 and 1.3.2) or infections (Section 1.3.3), interact with genes to contribute significantly to disease development.

4 Molecular mapping of the B cell epitopes of melanocyte-specific autoantigens tyrosinase and Pmel17

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 & 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 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 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 & 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 of B cell epitope mapping

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. If 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 enzyme-linked immunosorbent assay (ELISA) (Petterson 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 (Petterson 1992). However, 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).

Table 4.1: A summary of techniques for mapping linear and conformational B cell epitopes.

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

Study	Antigen	Disease	Techniques
Burch *et al.* 1993	Thyroid stimulating hormone-receptor	Graves' disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria. Immunoprecipitation (IP) experiments with patient sera.
Peterson & Krohn 1994	Steroid 17α-hydroxylase	Autoimmune Addison's disease	Subcloning of endonuclease restricted cDNA fragments and exonuclease digestion. Expressed in bacteria and immunoblotted with patient sera.
Wedlock *et al.* 1993	Steroid 21-hydroxylase	Autoimmune Addison's disease	Subcloning of endonuclease restricted cDNA fragments. Expressed in bacteria and immunoblotted with patient sera.
Song *et al.* 1994b	Steroid 21-hydroxylase	Autoimmune Addison's disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria and immunoblotted with patient sera.
Volpato *et al.* 1998	Steroid 21-hydroxylase	Autoimmune Addison's disease	Subcloning of endonuclease restricted cDNA fragments. Site-directed mutagenesis. Expressed in bacteria. IP experiments with patient sera.
Nikoshkov *et al.* 1999	Steroid 21-hydroxylase	Autoimmune Addison's disease	Site-directed mutagenesis. Expressed in bacteria. IP experiments with patient sera. Also expressed in mammalian cells and immunoblotted with patient sera.
von Mikecz *et al.* 1995	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 IP experiments with patient sera.
Syren *et al.* 1996	Glutamate decarboxylase	Type 1 diabetes mellitus	Subcloning of endonuclease restricted cDNA fragments and cDNA amplified by PCR. Expressed in bacteria. IP experiments with patient monoclonal antibodies (mAbs). Also expressed in mammalian cells and immunoblotted with mAbs.
Daw *et al.* 1996	Glutamate decarboxylase	Type 1 diabetes mellitus	Subcloning of endonuclease restricted cDNA fragments and cDNA amplified by PCR. Expressed in bacteria. IP experiments with patient sera.
Lin *et al.* 1999	'BP 180' (collagen XVII)	Bullous pemphigoid	Subcloning of cDNA amplified by PCR. Expressed in bacteria and immunoblotted with patient sera.
Kemp *et al.* 2001a	Sodium iodide symporter	Autoimmune thyroid disease	Subcloning of cDNA amplified by PCR. Expressed in bacteria. IP experiments with patient sera.

4.1.3 The role of B cell epitopes in autoimmunity

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 (Hoedemaekers *et al.* 1997). Similarly, the autoantigen epitopes in systemic lupus erythematosus (SLE) have been found to reside in highly conserved regions of proteins and can thus inhibit functions of both structural proteins and enzymes (Casiano & 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). Futhermore, epitope mapping may provide insight 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, an epitope on the bacterium *Yersinia entercolitica* has homology with the extracellular domain of human thyroid-stimulating hormone receptor, which could cross-react with autoantibodies in Graves' disease (Tomer & Davies 1993). In addition, similarities between epitopes on the parasite *Trypanosoma cruzi* and the cardiac muscle protein myosin cause the aberrant immune response in Chagas' disease (Roitt 1997).

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

A further application of epitope mapping lies in the development of specific-active immunotherapies: the ability to determine a single relevant antigenic site within a target protein is the basis for the development of synthetic vaccines for the prevention of and protection against disease (Atabani 2001). There is a need for vaccines to be epitope specific to eliminate the possibility of cross-reactivity with self-protein or related antigens and also to make the production of the synthetic peptides economical. Specific, active immunotherapy with respect to the present study is discussed in Section 4.5.

4.2 Aim

In this study, the previously identified autoantibody reactivity to the melanocyte-specific autoantigens tyrosinase (Song *et al.* 1994a; Baharav *et al.* 1996; Kemp *et al.* 1997a) and Pmel17 (Kemp *et al.* 1998b) in vitiligo patient sera was further characterised, with respect to the B cell epitopes involved, since this may give further clues into the nature of the immune response in vitiligo. This was undertaken by the construction of deletion derivatives of tyrosinase cDNA using both exonuclease III treatment and PCR amplification. Truncated derivatives of Pmel17 cDNA were created by subcloning of specific cDNA fragments generated by endonuclease restriction and PCR amplification. Full-length tyrosinase and its deletion derivatives, and full-length Pmel17 and its truncated derivatives, were then be translated *in vitro* with [^{35}S]methinonine to produce [^{35}S]-labelled intact and modified proteins, which were employed for testing antibody reactivity in vitiligo patient sera in radioimmunoassays. The experiments performed on tyrosinase and Pmel17 are presented separately in Sections 4.3 and 4.4, followed by an overall discussion in Section 4.5.

4.3 Mapping the B cell epitopes of tyrosinase

4.3.1 Materials and Methods

4.3.1.1 Patients and controls

Sera from five vitiligo patients (one male, four females; mean age: 50 years; range: 24-64 years (yr); disease duration: 3-22 yr; vitiligo type: one segmental, one symmetrical/periorificial, three symmetrical), which were previously shown to contain tyrosinase antibodies (Kemp *et al.* 1997a), were analysed in these experiments. With respect to autoimmune disorders, the two patients with the lowest levels of tyrosinase antibodies had no personal or family history of associated autoimmune diseases. The three patients with the highest levels of tyrosinase antibodies also had an associated autoimmune disorder: Graves' disease in one and autoimmune hypothyroidism in two. Sera from 20 healthy individuals (nine male and 11 female; age range: 23-47 yr; mean age: 31 yr), with no history of either vitiligo or autoimmune disorders, were used as controls.

4.3.1.2 Generation of tyrosinase cDNA deletion derivatives by exonuclease III treatment

This technique involves plasmid containing the cDNA of interest initially being linearised by the use of two restriction endonucleases, one to create a 5' overhanging end, which is susceptible to exonuclease digestion, and one to create a 3' overhanging end, which is resistant to exonuclease digestion. In this way, subsequent deletions produced by exonuclease III digestion can only proceed in one direction (Putney *et al.* 1981; Henikoff 1984). The extent of exonuclease III deletions is controlled by the removal of timed aliquots during the digestion and the rate of the enzyme's activity can be slowed down by allowing the reaction to proceed with a sub-optimal NaCl concentration. A schematic diagram summarising the procedure of creating nested deletions is given in Figure 4.1.

Deletion derivatives of tyrosinase cDNA were generated using a Nested Deletion Kit according to the manufacturer's instructions (Amersham Pharmacia

Biotech). Full-length tyrosinase cDNA, cloned as a *Sal*I-*Xba*I fragment in pBluescript SK (Stratagene, La Jolla, CA, U.S.A) (Kemp *et al.* 1997a), was digested with *Sac*I restriction endonuclease (Promega) at a *Sac*I site residing in a 3' direction from the tyrosinase cDNA. The plasmid was then digested with enzyme *Xba*I (Promega), the site for which is located upstream of the *Sac*I site. Exonuclease III (Amersham Pharmacia Biotech) was then used to create unidirectional deletions from the 3' end of the cDNA fragment. Exonuclease III digestion was carried out in a 40 µl reaction volume comprising 2 µg *Xba*I/*Sac*I digested pBSTYR DNA, 90-130 U of exonuclease III, in buffer containing 66.67 µM Tris-HCl (pH 8.0), 0.67 mM $MgCl_2$ and 50 µM NaCl (Amersham Pharmacia Biotech), the reaction was allowed to proceed at 37°C for 12 min. Two-microlitre samples were removed from the reaction at 3 min intervals after which S1 nuclease treatment of the linear DNA was carried out as follows: 1.8-2.7 U of S1 nuclease (Amersham Pharmacia Biotech), in buffer containing 150 mM potassium acetate (pH 4.6), 1.25 M NaCl, 5 mM $ZnSO_4$ and 25% (v/v) glycerol (Amersham Pharmacia Biotech), was added to each of the 2-µl exonuclease III-digested samples which were then incubated for 30 min at room temperature. The reaction was stopped by the addition of 1 µl S1 stop solution comprising 303 mM Tris base and 50 mM EDTA (Amersham Pharmacia Biotech) followed by 10 min incubation at 65°C. Half of each S1-treated sample was then subjected to electrophoretic analysis on 0.8% (w/v) agarose gels (Section 2.12) and the other half re-ligated in 20-µl reaction volumes comprising 0.5-0.7 U T4 DNA Ligase, 1.25% (w/v) polyethylene glycol (PEG) and T4 ligase buffer (Amersham Pharmacia Biotech).

The recircularised plasmids were used to transform *Escherichia coli* JM109 (Promega) as detailed elsewhere (Section 2.12). Plasmid DNA was purified from individual transformants using a Wizard Minipreps DNA Purification System (Promega) (Section 2.7.1) and analysed by gel electrophoresis using 0.8% (w/v) agarose gels (Section 2.9) to assess which constructs still contained a fragment of tyrosinase cDNA.

Appropriate plasmids were sequenced by the dideoxy chain termination method, as previously described (Section 2.15), with M13 reverse primer

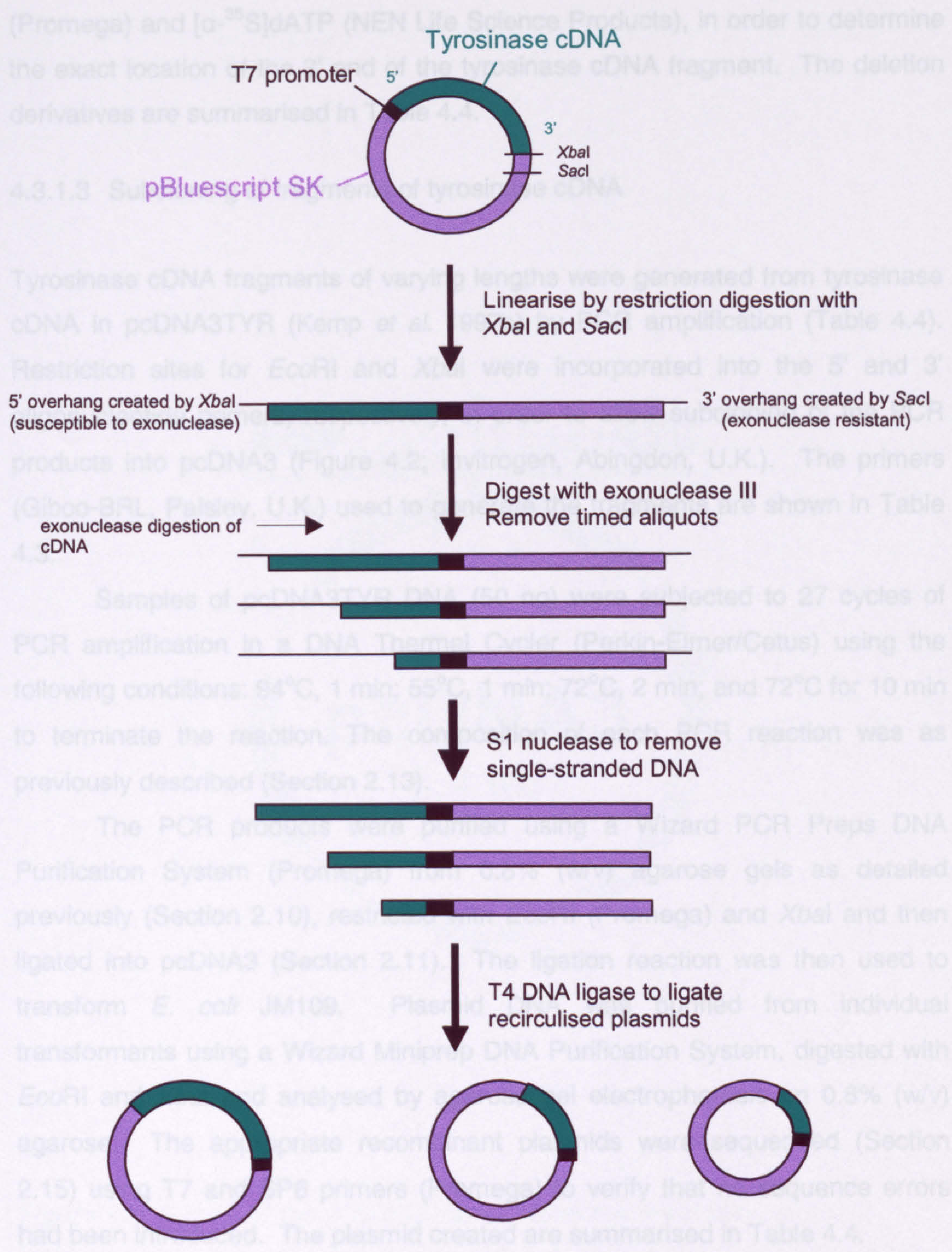


Figure 4.1: A schematic diagram of the procedure for creating nested deletions of tyrosinase.

Tyrosinase deletion derivatives were generated from full-length tyrosinase cDNA in the expression plasmid pBluescript SK by the method of Henikoff (1984). Adapted from a figure in the 'Promega Protocols & Applications Guide' (Promega).

(Promega) and [α-^{35}S]dATP (NEN Life Science Products), in order to determine the exact location of the 3' end of the tyrosinase cDNA fragment. The deletion derivatives are summarised in Table 4.4.

4.3.1.3 Subcloning of fragments of tyrosinase cDNA

Tyrosinase cDNA fragments of varying lengths were generated from tyrosinase cDNA in pcDNA3TYR (Kemp *et al.* 1997b) by PCR amplification (Table 4.4). Restriction sites for *Eco*RI and *Xba*I were incorporated into the 5' and 3' oligonucleotide primers, respectively, in order to allow subcloning of the PCR products into pcDNA3 (Figure 4.2; Invitrogen, Abingdon, U.K.). The primers (Gibco-BRL, Paisley, U.K.) used to generate the fragments are shown in Table 4.3.

Samples of pcDNA3TYR DNA (50 ng) were subjected to 27 cycles of PCR amplification in a DNA Thermal Cycler (Perkin-Elmer/Cetus) using the following conditions: 94°C, 1 min; 55°C, 1 min; 72°C, 2 min; and 72°C for 10 min to terminate the reaction. The composition of each PCR reaction was as previously described (Section 2.13).

The PCR products were purified using a Wizard PCR Preps DNA Purification System (Promega) from 0.8% (w/v) agarose gels as detailed previously (Section 2.10), restricted with *Eco*RI (Promega) and *Xba*I and then ligated into pcDNA3 (Section 2.11). The ligation reaction was then used to transform *E. coli* JM109. Plasmid DNA was purified from individual transformants using a Wizard Miniprep DNA Purification System, digested with *Eco*RI and *Xba*I and analysed by agarose gel electrophoresis on 0.8% (w/v) agarose. The appropriate recombinant plasmids were sequenced (Section 2.15) using T7 and SP6 primers (Promega) to verify that no sequence errors had been introduced. The plasmid created are summarised in Table 4.4.

4.3.1.4 Coupled *in vitro* transcription and translation and electrophoretic analysis

The plasmid constructs (Table 4.4) were used in a TnT® T7 Coupled Reticulocyte Lysate System (Promega) to produce and label tyrosinase and its deletion derivatives with [^{35}S]methionine *in vitro*. Each tyrosinase cDNA was

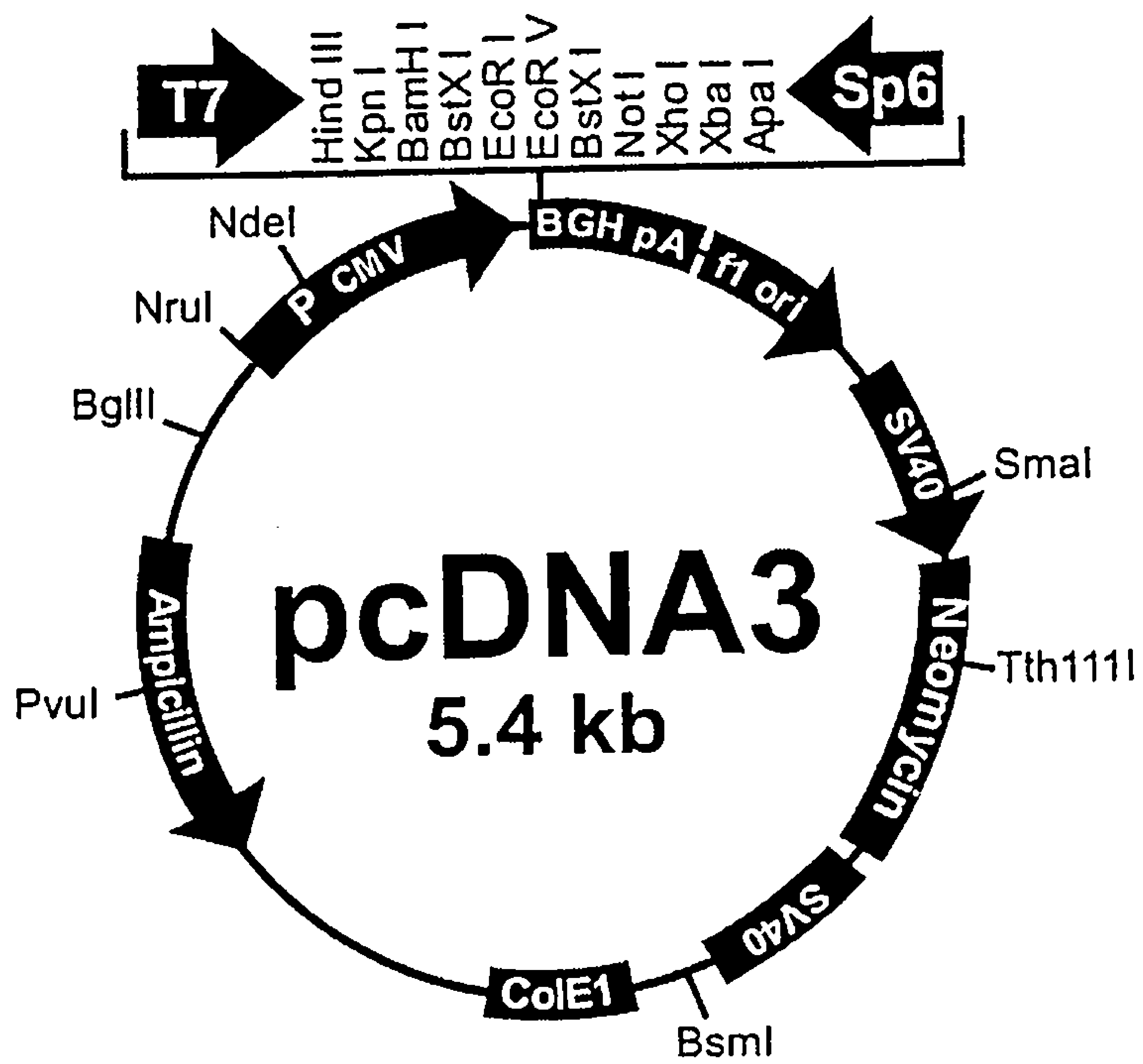


Figure 4.2: A map of the pcDNA3 vector detailing restriction sites.
(Invitrogen).

Tyrosinase cDNA fragments were cloned into the *Eco*RI-*Xba*I sites of this vector.

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

Base pairs[1]	Primers	Primer sequences[2]
1-831	TYR1	5'TTGAATTCGCCGCC**ATG**CTCCTGGCTGTT3'
	TYR2	5'AATCTAGACGC**CTA**GCTACAGACAATCTGCA3'
1-675	TYR1	
	TYR225	5'AATCTAGACGC**CTA**CAGCTTCTGGATTTCTTG3'
1-717	TYR1	
	TYR240	5'AATCTAGACGC**CTA**CCGCCAGTCCCAATATGG3'
1-765	TYR1	
	TYR255	5'AATCTAGACGC**CTA**CTGACCTCCCATGTACTC3'
1-849	TYR1	
	TYR283	5'AATCTAGACGC**CTA**GTTGTACTCCTCCAATCG3'
1-867	TYR1	
	TYR289	5'AATCTAGACGC**CTA**GCATAAAGACTGATGGCT3'
1-885	TYR1	
	TYR295	5'AATCTAGACGC**CTA**TCCCTCGGGCGTTCCATT3'
1-1305	TYR1	
	TYR435	5'AATCTAGACGC**CTA**ATTTCTGTACAGTGGTAT3'
1-1344	TYR1	
	TYR448	5'AATCTAGACGC**CTA**GTCATAGCCCAGATCTTT3'
1-1383	TYR1	
	TYR461	5'AATCTAGACGC**CTA**TTGAAAAGAGTCTGGGTC3'

[1] The numbers correspond to bp of tyrosinase cDNA included in the construct with the A residue of the initiating ATG codon assigned as bp number one.

[2] The *Eco*RI and *Xba*I restriction sites are underlined. The ATG translation initiation codon in forward primer TYR1 and the TAG translation termination codon in all reverse primers are shown in bold-type face.

inserted in the correct orientation to allow expression from the T7 promoter, and each template contained appropriate start and stop codons to ensure accurate translation. The TnT® T7 Coupled Reticulocyte Lysate System (Promega) is 'cell-free'; purified reticulocyte lysate contains ribosomes, tRNA, and all the initiation, elongation and termination factors necessary for protein synthesis, but contains little endogenous mRNA, which would produce unwanted background protein.

A standard reaction mix contained: rabbit reticulocyte lysate, 25 µl; TnT® T7 RNA polymerase, 1 µl; TnT® reaction buffer, 2 µl; amino acids minus methionine, 1 µl; RNasin (Promega), 40 U; plasmid template, 2 µg; [^{35}S]methionine (1000 Ci/mmol; 10 mCi/ml; Amersham), 4 µl; sterile dH_20 to a final volume of 50 µl. The reaction was incubated for 120 min at 30°C and then stored at –20°C until needed.

SDS-PAGE of *in vitro* translated products was performed in 12.5% SDS-polyacrylamide resolving gels and 4% SDS-polyacrylamide stacking gels (Section 2.17) which were stained, dried and autoradiographed as described elsewhere (Section 2.17).

4.3.1.5 Immunoprecipitation assays

Vitiligo sera were tested for binding to [^{35}S]-tyrosinase and its labelled deletion derivatives in immunoprecipiation experiments as follows. For each assay, an aliquot of the *in vitro* translation reaction mixture (equivalent to 12,000- 20,000 counts per minute (cpm) of trichloroacetic acid (TCA)-precipitable material) was suspended in 50 µl of immunoprecipitation buffer containing 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100 and 10 µg/ml aprotinin (Bayer, Newbury, U.K.). Serum was then added to a final dilution of 1:10. After incubation overnight with gentle rotation at 4°C, 50 µl of protein G Sepharose 4 Fast Flow slurry (Amersham Pharmacia Biotech), prepared according to the manufacturer's directions, was added and incubated for 1 h at 4°C. The protein G Sepharose-antibody complexes were then collected by centrifugation and washed six times with immunoprecipitation buffer at 4°C. Immunoprecipitated radioactivity was then evaluated in a Wallac 1217 Rackbeta liquid scintillation analyser (Wallac U.K., Milton Keynes, U.K.).

The binding reactivity of each of the sera to full-length tyrosinase and each deletion derivative was expressed as an antibody (Ab) index calculated as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 20 healthy control sera. Each serum was analysed in at least two experiments and the mean Ab index was calculated from these. The upper level of normal for each assay with a different ligand was calculated using the mean Ab index + 3 SD of 20 control sera. Patient sera with an Ab index greater than the upper level of normal were regarded as positive for binding to the radiolabelled ligand used in the assay.

4.3.1.6 Computer analysis

Predictions of B cell epitopes were carried out using a computer program based on a scale of hydrophilicity (Parker *et al.* 1986) by Dr. Part Peterson (Institute of Biomedical Sciences, University of Tampere, Finland). Genbank homology searches were performed using the Sequence Analysis Software package, GCG (University of Wisconsin, WN, U.S.A.).

4.3.2 Results

4.3.2.1 *In vitro* translation of tyrosinase cDNA and its deletion derivatives

For identification of autoepitopes on tyrosinase, the cDNA in pBSTYR was deleted with exonuclease III treatment. As the deletions were carried out from the 3' end of the tyrosinase cDNA, the correct translational reading frame was maintained. The exact location of the 3' end of each of the tyrosinase cDNA deletion derivatives, as determined by sequencing, is shown in Table 4.4.

PCR amplification was used to generate tyrosinase cDNA fragments of varying lengths which were then cloned into pcDNA3 (Table 4.4). DNA sequencing of the fragments was carried out to verify that no sequence errors had been introduced.

Products generated from *in vitro* translation of tyrosinase cDNA and its deletion derivatives were evaluated by SDS-PAGE and autoradiography (Figure 4.3a and 4.3b). Major bands representing the intact and modified [^{35}S]-labelled tyrosinase proteins were found in each case (Table 4.4).

4.3.2.2 Immunoprecipitation of tyrosinase deletion derivatives with sera from vitiligo patients

Sera from five vitiligo patients were tested for their ability to immunoprecipitate [^{35}S]-labelled tyrosinase and its modified derivatives. The results of the reactivity of the vitiligo sera with the tyrosinase deletion derivatives are summarised in Table 4.5.

One patient serum did not react when tyrosinase containing a C-terminal deletion of amino acids 462-529 was used. Another serum studied failed to react with any derivative containing amino acid deletions beyond residue 447. The remaining three vitiligo sera reacted with derivatives deleted up to amino acid residues 254, 294, and 300, respectively.

Table 4.4: Deletion derivatives used in determining the B cell epitopes on tyrosinase which are recognised by vitiligo sera.

Tyrosinase construct	Amino acids encoded[1] (bp[2])	Predicted molecular weight of expressed protein[3] (kDa)	Estimated molecular weight of expressed protein[4] (kDa)
pcDNA3TYR[5]	1-529 (1-1587)	58	58
pTYR9.8	1-500 (1-1502)	55	56
pTYR9.10	1-480 (1-1441)	53	52
pTYR9.2	1-426 (1-1279)	47	47
pTYR10.7	1-398 (1-1194)	44	44
pTYR9.6	1-362 (1-1088)	40	42
pTYR9.1	1-354 (1-1063)	39	38
pTYR10.4	1-313 (1-940)	34	30
pTYR10.6	1-301 (1-903)	33	27
pTYR1.2	1-277 (1-831)	30	24
pTYR10.10	1-209 (1-632)	23	17
pTYR225	1-225 (1-675)	25	20
pTYR240	1-240 (1-717)	26	21
pTYR255	1-255 (1-765)	28	23
pTYR283	1-283 (1-849)	31	25
pTYR289	1-289 (1-867)	32	26
pTYR295	1-295 (1-885)	32	27
pTYR435	1-435 (1-1305)	48	45
pTYR448	1-448 (1-1344)	49	48
pTYR461	1-461 (1-1383)	51	50

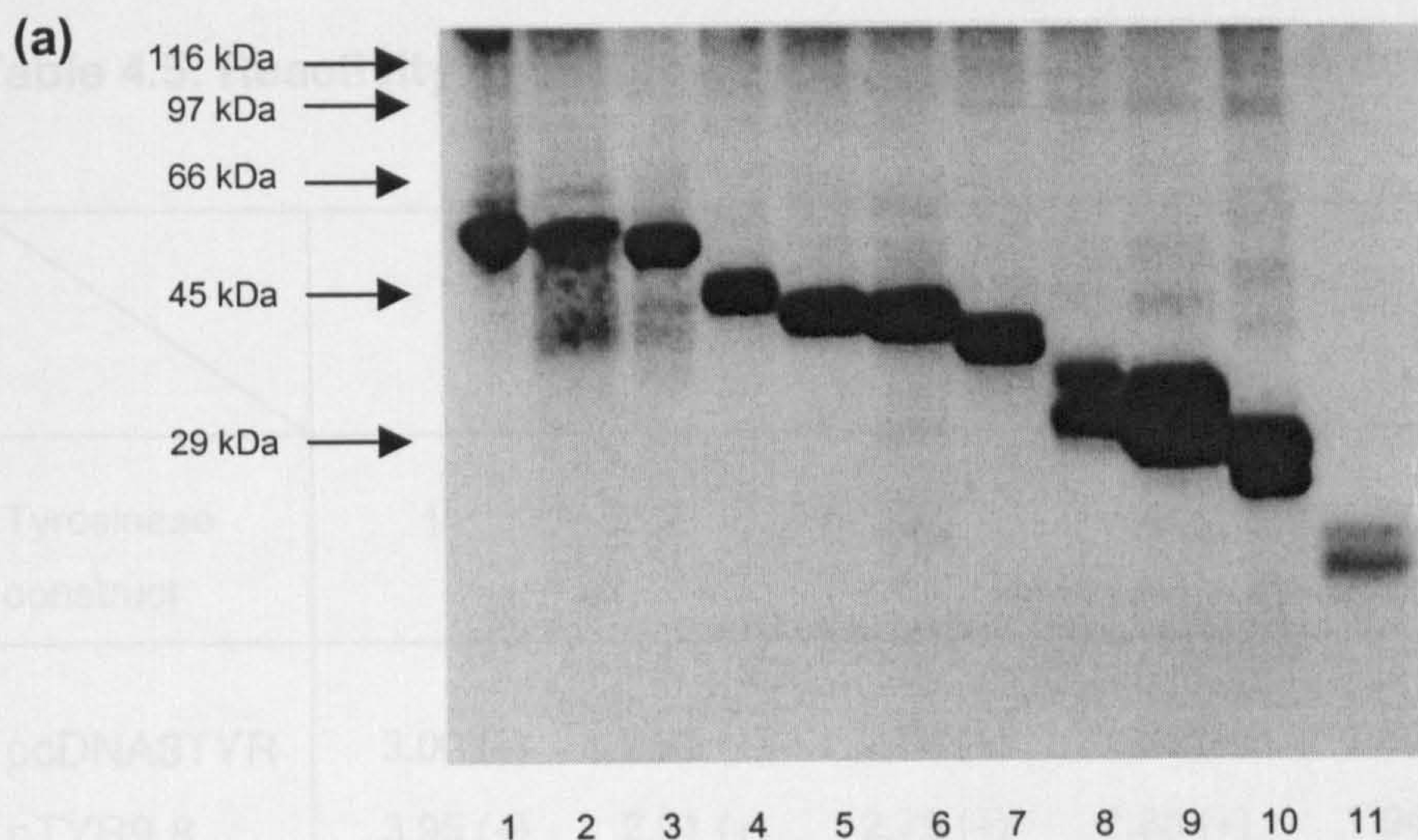
[1] Numbers correspond to the amino acid residues of tyrosinase with the intiating methionine as residue one.

[2] Numbers correspond to bp of tyrosinase cDNA with the A residue of the intiating ATG codon assigned as bp number one.

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

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

[5] Encodes full-length tyrosinase.



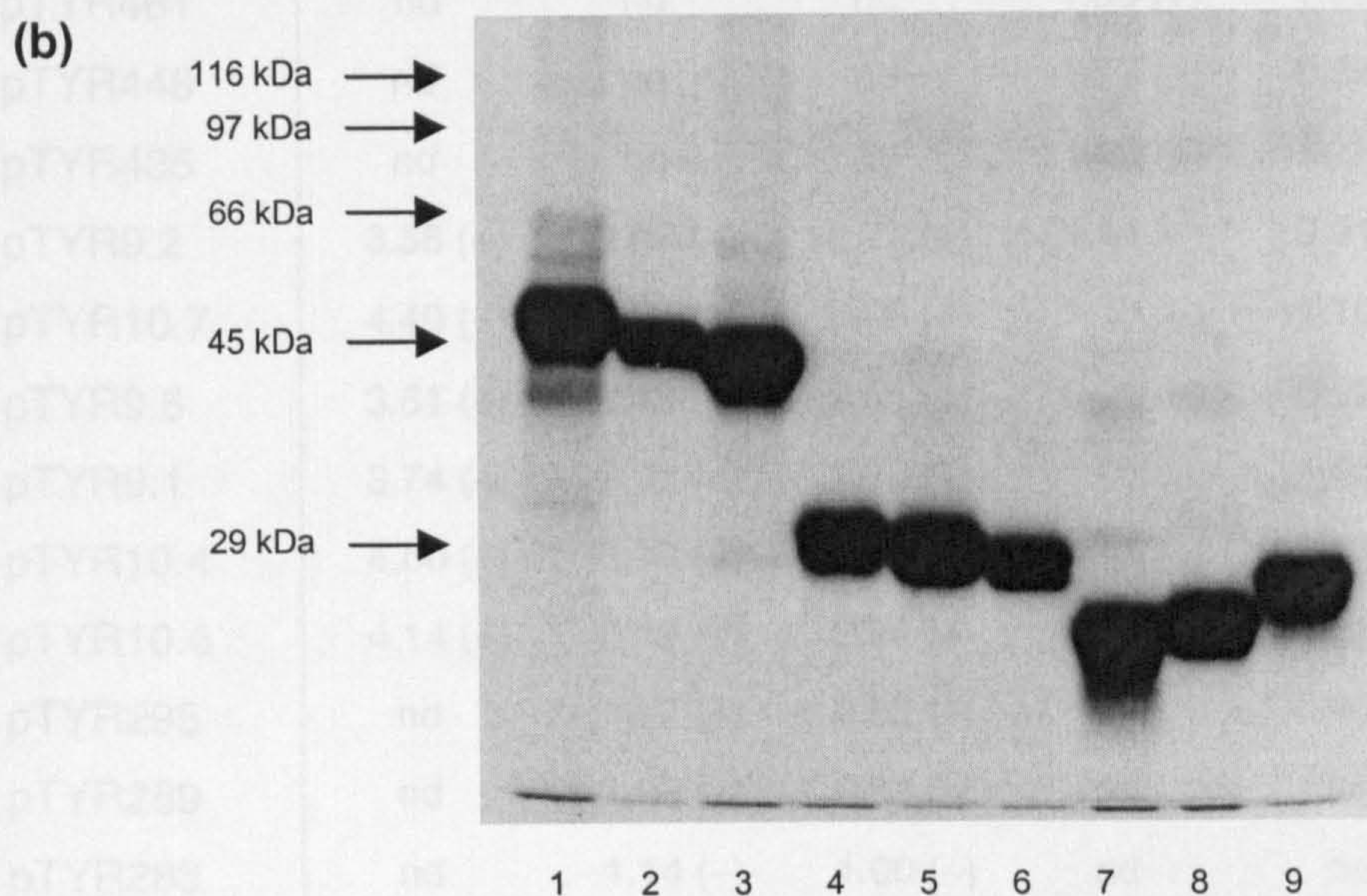


Figure 4.3: SDS-polyacrylamide gel electrophoresis and autoradiography of products arising from *in vitro* translation of tyrosinase cDNA and its deletion derivatives.

Tyrosinase cDNA and its deletion derivatives were translated *in vitro* in a TnT® T7 Coupled Reticulocyte Lysate System as described in Section 4.3.1.4. Subsequently, 5 μl of the reaction mixture 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 on a 12.5% gel. (a) Full-length tyrosinase *in vitro* translated from pcDNA3TYR (lane 1); deletion derivatives of tyrosinase *in vitro* translated from: pTYR9.8 (lane 2); pTYR9.10 (lane 3); pTYR9.2 (lane 4); pTYR10.7 (lane 5); pTYR9.6 (lane 6); pTYR9.1 (lane 7); pTYR10.4 (lane 8); pTYR10.6 (lane 9); pTYR1.2 (lane 10); pTYR10.10 (lane 11). (b) pTYR461 (lane 1); pTYR448 (lane 2); pTYR435 (lane 3); pTYR295 (lane 4); pTYR289 (lane 5); pTYR283 (lane 6); pTYR225 (lane 7); pTYR240 (lane 8); pTYR255 (lane 9).

Table 4.5: Reactivity of vitiligo sera to tyrosinase deletion derivatives.

	Reactivity of sera[1]					Upper level of normal for the assay[2]
Tyrosinase construct	1	2	3	4	5	
pcDNA3TYR	3.00 (+)	2.95 (+)	2.68 (+)	1.65 (+)	1.80 (+)	1.36
pTYR9.8	3.95 (+)	2.91 (+)	2.79 (+)	1.83 (+)	1.94 (+)	1.41
pTYR9.10	3.94 (+)	2.88 (+)	2.86 (+)	1.41 (+)	2.04 (+)	1.19
pTYR461	nd[3]	nd	nd	1.80 (+)	1.09 (–)	1.13
pTYR448	nd	nd	nd	1.72 (+)	0.99 (–)	1.30
pTYR435	nd	nd	nd	1.22 (–)	1.23 (–)	1.26
pTYR9.2	3.58 (+)	2.82 (+)	2.77 (+)	1.14 (–)	0.91 (–)	1.23
pTYR10.7	4.40 (+)	3.54 (+)	3.06 (+)	1.12 (–)	1.10 (–)	1.24
pTYR9.6	3.61 (+)	2.49 (+)	2.81 (+)	0.90 (–)	0.92 (–)	1.35
pTYR9.1	3.74 (+)	3.02 (+)	2.74 (+)	1.02 (–)	1.00 (–)	1.21
pTYR10.4	4.00 (+)	3.10 (+)	2.77 (+)	0.95 (–)	0.88 (–)	1.13
pTYR10.6	4.14 (+)	3.19 (+)	2.94 (+)	1.04 (–)	0.99 (–)	1.47
pTYR295	nd	2.7 (+)	0.92 (–)	nd	nd	1.33
pTYR289	nd	1.06 (+)	0.98 (–)	nd	nd	1.08
pTYR283	nd	1.14 (–)	1.00 (–)	nd	nd	1.25
pTYR1.2	3.76 (+)	1.18 (–)	1.06 (–)	1.04 (–)	0.98 (–)	1.22
pTYR255	3.23 (+)	nd	nd	nd	nd	1.26
pTYR240	1.24 (–)	nd	nd	nd	nd	1.25
pTYR225	1.16 (–)	nd	nd	nd	nd	1.16
pTYR10.10	1.24 (–)	1.04 (–)	1.00 (–)	1.02 (–)	0.89 (–)	1.25

[1] The binding reactivity of each of the sera to the full-length tyrosinase and each deletion derivative was expressed as an antibody index calculated as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 20 healthy control sera. Each serum was analysed in at least two experiments and the mean antibody index was calculated from these.
[2]The upper level of normal for each assay with a different ligand was calculated using the mean antibody index + 3 SD of 20 control sera. Patient sera with an antibody index greater than the upper level of normal were regarded as positive for binding to the radiolabelled ligand used in the assay (+). (–) denotes negative for binding to the radiolabelled ligand used in the assay. [3] nd, not determined.

The results of the immunoprecipitation experiments indicate that multiple epitope domains exist on tyrosinase: between amino acids 240-254 (DEAKCDICTDEYMGG), 289-294 (CNGTPE) and 295-300 (GPLRRN) located centrally in the tyrosinase molecule, and between 435-447 (NGDFFISSKDLGYD) and 461-479 (QDYIKSYLEQASRIWSWLL) found towards the C-terminal of the protein. The reactivity of each serum to each epitope is shown in Table 4.6.

The 3' end deletion procedure predominantly reveals those antigenic regions which are closer to the N-terminus of the polypeptide. The reactivity of three of the sera with the C-terminal antigenic regions was therefore unable to be confirmed because of the presence of reactivity with epitope regions located nearer to the N-terminal domain of tyrosinase.

4.3.2.3 Computer prediction of putative antigenic regions within tyrosinase

The computer prediction analysis identified several putative antigenic regions within tyrosinase (Figure 4.4). All the epitope regions defined by the vitiligo sera were found in these putative antigenic areas: antigenic domains 289-294 and 295-300 were within the predicted epitope region 275-315, antigenic domains 435-447 and 461-479 were located in the predicted epitope region 430-470 and antigenic domain 240-254 was located in the predicted epitope region 230-260.

4.3.2.4 Comparison of identified epitope regions with the amino acid sequences of TRP-1 and TRP-2

The amino acid sequence of two of the identified epitope regions (amino acids 289-294 and 295-300) had homology to an amino acid sequence present in both TRP-1 (Kwon 1993) and TRP-2 (Yokoyama *et al.* 1994): a tyrosinase peptide of 12 residues (amino acids 291-302) included eight identical and two conservatively changed amino acids when compared to an amino acid sequence present in TRP-1 (amino acids 305-318), and eight identical residues when compared to an amino acid sequence in TRP-2 (amino acids 301-315). This is illustrated in Figure 4.5. Additionally, no homology was found between

Table 4.6: Epitope regions on tyrosinase recognised by vitiligo sera[1].

	Patient				
Amino acids	1	2	3	4	5
240-254	+	-	-	-	-
289-294	NA	+	-	-	-
295-300	NA	NA	+	-	-
435-447	NA	NA	NA	+	-
461-479	NA	NA	NA	NA	+

[1] Reactivities with epitope regions are indicated: + denote reactivity with the specified epitope region; - denotes unreactive with specific epitope region; NA denotes reactivity with the specified epitope region unknown.

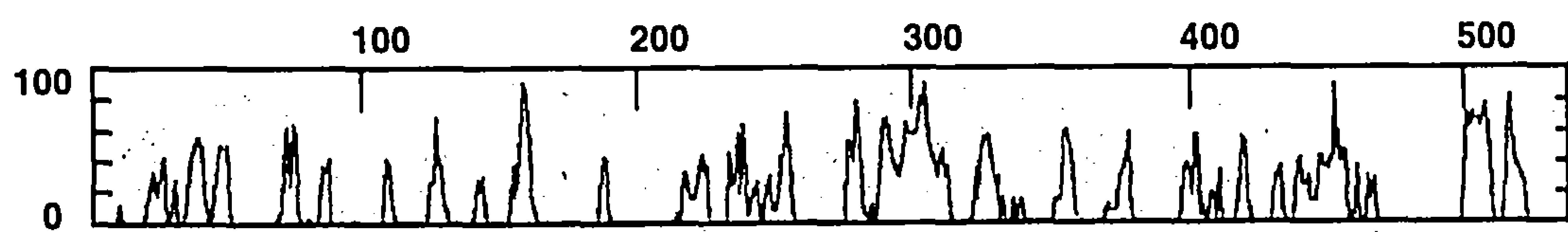


Figure 4.4: Antigenicity plot of the deduced amino acid sequence of tyrosinase.

Local antigenicity values, calculated as detailed previously (Parker *et al.* 1986), are plotted against the amino acid residues of tyrosinase. The *y*-axis scale from 0 to 100 reflects increasing antigenicity.

Tyrosinase	291	G **T** P E **G P** L **R R N P** - - **G**	302
TRP-1	305	S **T** E D **G P** I **R R N P** - A **G**	317

Tyrosinase	291	**G T** P **E G** P **L R R N** P - - G	302
TRP-2	301	**G T** Y **E G** L **L R R N** Q M G R	314

Figure 4.5: Amino acid sequence homology between an autoantigenic region of tyrosinase and a peptide sequence of TRP-1 and of TRP-2.

Numbers represent the amino acid residues of the protein with the intiation methionine being assigned as number one. Identical amino acids are indicated by bold type-face and conservative amino acid changes are underlined.

the epitope regions described here and any other proteins including those of viral and bacterial origin.

4.3.3 Results summary

The construction and expression of several deletion mutants of tyrosinase enabled the characterisation of multiple B cell epitope domains between amino acids 240-254 (DEAKCDICTDEYMGG), 289-294 (CNGTPE) and 295-300 (GPLRRN) located centrally in the tyrosinase molecule, and between 435-447 (NGDFFISSKDLGYD) and 461-479 (QDYIKSYLEQASRIWSWLL) found towards the C-terminal of the protein. Since the 3' deletion method predominantly reveals those antigenic regions which are closer to the N-terminus of the polypeptide, the reactivity of three of the sera with the C-terminal antigenic regions was unable to be confirmed because of the presence of reactivity with epitope regions located nearer to the N-terminus of tyrosinase. In order to examine the reactivity of these sera with the C-terminus of tyrosinase, further deletion derivatives would need to be constructed which exclude the central regions of the protein.

Since tyrosinase shows amino acid sequence homology with TRP-1 (Kwon 1993) and TRP-2 (Yokoyama *et al.* 1994), the identified tyrosinase autoantigenic regions were compared for amino acid homology with both the tyroinase-related enzymes to locate possible cross-reactive domains. The amino acid sequence of two of the identified epitope regions (amino acids 289-294 and 295-300) had homology to an amino acid sequence in both TRP-1 (Kwon 1993) and TRP-2 (Yokoyama *et al.* 1994): a tyrosinase peptide of 12 residues (amino acids 291-302) included eight identical and two conservatively changed amino acids when compared to an amino acid sequence present in TRP-1 (amino acids 305-318), and eight identical residues when compared to an amino acid sequence in TRP-2 (amino acids 301-315). This finding is consistent with previous studies in which three vitiligo patients were identified whose sera contained tyrosinase antibodies which cross-reacted with both TRP-1 and TRP-2 (Kemp *et al.* 1997a; Kemp *et al.* 1997b; Kemp *et al.* 1998c). In this present work, two of these patients had tyrosinase antibodies which reacted

with the epitope region 289-294 and 295-300, respectively. The third patient had tyrosinase antibodies which recognised the 240-254 antigenic domain. However, reactivity of this patient sera with the 289-294 and 295-300 epitope regions may have been masked by antibody binding to the site closer to the N-terminus of tyrosinase. The sera from the remaining two patients, which were found to contain tyrosinase antibodies but not antibodies to TRP-2 or TRP-1 (Kemp *et al.* 1997a; Kemp *et al.* 1997b; Kemp *et al.* 1998c), reacted with epitopes 435-447 and 461-479, respectively, which did not appear to be homologous to amino acid sequences in either TRP-1 or TRP-2.

The present results indicate that multiple regions of tyrosinase are epitopes for autoantibodies from the vitiligo patients studied and regions 289-294 and 295-300 are likely to contain epitopes which are cross-reactive with both TRP-1 and TRP-2.

4.4 Mapping the B cell epitopes of Pmel17

4.4.1 Materials and Methods

4.4.1.1 Patients and controls

Sera from three vitiligo patients (three females; mean age: 50 yr; age range: 43-59 yr), which were previously shown to contain Pmel17 antibodies (Kemp *et al.* 1998b), were analysed in these experiments. In addition to symmetrical type vitiligo, the three patients had an associated autoimmune disorder: Graves' disease in one and autoimmune hypothyroidism in two. A further set of 20 sera from vitiligo patients (12 male and eight female; age range; 30-77 yr; mean age; 55 yr), previously untested for Pmel17 antibodies, were also examined. Nineteen patients had symmetrical type vitiligo and one presented with segmental vitiligo. An associated autoimmune disease was also diagnosed in three of the 20 patients: alopecia areata in one and autoimmune hypothyroidism in two.

Sera from 20 healthy individuals, with no history of either vitiligo or autoimmune disorders, were used as controls (Section 4.3.1.1). As a further two sets of controls, sera from 10 patients (eight female and two male; age range: 21-84 yr; mean age: 43 yr) with Graves' disease and nine patients (nine female; age range: 24-65 yr; mean age: 45 yr) with Hashimoto's thyroiditis, all without clinical signs of vitiligo, were analysed.

4.4.1.2 Specific antiserum

Anti-Pmel17 rabbit polyclonal antiserum AZN-LAM (Schreurs *et al.* 1997) was a gift from Dr. Marco Schreurs (Department of Tumour Immunology, University Hospital Nijmegen, Nijmegen, The Netherlands). This antiserum was generated against a synthetic peptide corresponding to the C-terminal sixteen amino acids of Pmel17.

4.4.1.3 Generation of Pmel17 deletion constructs by PCR amplification

Full-length human Pmel17 cDNA, cloned as an *Eco*RI-*Xho*I fragment in pcDNA3, was a gift from Dr. Paul Robbins (National Institutes of Health, Bethesda, MD, U.S.A.) and was used to generate Pmel17 cDNA fragments of varying lengths by PCR amplification. Restriction sites for *Eco*RI and *Xba*I were incorporated into the 5' and 3' oligonucleotide primers, respectively, in order to allow subcloning of the PCR products into pcDNA3. The primers (Gibco-BRL) used to generate the Pmel17 cDNA fragments are listed in Table 4.7.

Samples of plasmid DNA containing Pmel17 cDNA (50 ng) were subjected to 27 cycles of PCR amplification in a DNA Thermal Cycler as described in Section 4.3.1.3 above. The PCR amplification products were purified and subcloned into pcDNA3 using materials and methods outlined previously (Section 4.3.1.3). Appropriate recombinant plasmids were sequenced by the dideoxy chain termination method with T7 and SP6 primers, as before (Section 4.3.1.3), in order to verify that no sequence errors had been introduced. The deletion derivatives are summarised in Table 4.8.

4.4.1.4 Generation of Pmel17 deletion constructs by subcloning of Pmel17 cDNA fragments

Endonuclease restriction was used to generate fragments of Pmel17 cDNA which were subsequently subcloned into vector pcDNA3 (Figure 4.6). Briefly, plasmid pcDNA3 containing full-length Pmel17 cDNA was restricted initially with the appropriate enzymes. The required cDNA fragments were then purifed from 0.8% (w/v) agarose gels using a Wizard PCR Preps DNA purification kit (Promega; Section 2.10) and ligated into pcDNA3 which had been restricted with the necessary endonuclease(s) (Promega). Plasmid pPMEL17-582 was constructed by the initial subcloning of a 1139 base pair (bp) *Hin*dIII-*Bgl*II fragment into the *Hin*dIII-*Bam*HI site of pcDNA3 followed by insertion of a 512 bp *Hin*dIII fragment into the *Hin*dIII site of the recombinant plasmid. The required recombinant plasmids were identified from transformants of *E. coli* JM109 by endonuclease digestion and agarose gel electrophoresis. DNA sequencing with T7 primer was used to determine the orientation of the Pmel17 cDNA fragment

Table 4.7: Oligonucleotide primers used to generate Pmel17 cDNA fragments by PCR amplification.

Base pairs[1]	Primers	Primer sequences[2]
1-1983	Pmel17-1	5'TTGAATTCGCCGCC**ATG**GATCTGGTGCTA3'
	Pmel17-661	5'AATCTAGACGC**CTA**GGGGCTATTCTCACC3'
1-1962	Pmel17-1	
	Pmel17-654	5'AATCTAGACGC**CTA**ACAAGAGCAGAAGAT3'
1-1932	Pmel17-1	
	Pmel17-644	5'AATCTAGACGC**CTA**CCAGTGACTGCTGCT3'
1-1902	Pmel17-1	
	Pmel17-1900	5'AATCTAGACGC**CTA**TACGGAGAAGTCTTGCTT3'
1-1728	Pmel17-1	
	Pmel17-1750	5'AATCTAGACGC**CTA**GCTGTTGGTATCAGC3'
1-1381	Pmel17-1	
	Pmel17-1400	5'AATCTAGACGC**CTA**ACCATCCAGCAGGGGGCC3'
1-1230	Pmel17-1	
	Pmel17-1250	5'AATCTAGACGC**CTA**AAGCACCACAATTGATAC3'
1-1113	Pmel17-1	
	Pmel17-371	5'AATCTAGACGC**CTA**GCTCTCTGCAGTTGG3'
1-1068	Pmel17-1	
	Pmel17-356	5'AATCTAGACGC**CTA**AGTGGTTGGCACCTG3'
1-1023	Pmel17-1	
	Pmel17-341	5'AATCTAGACGC**CTA**TGGCGCCTGACCAGG3'
1-978	Pmel17-1	
	Pmel17-1000	5'AATCTAGACGC**CTA**TTGGCCAGCTGTGGTGTT3'
1-829	Pmel17-1	
	Pmel17-850	5'AATCTAGACGC**CTA**CACCACAAGTGCCCG3'

[1] The numbers correspond to base pairs of Pmel17 cDNA included in the construct with the A residue of the initiating ATG codon assigned as base pair number one.

[2] The *Eco*RI and *Xba*I restriction sites are underlined. The ATG translation initiation codon in forward primer Pmel17-1 and the TAG translation termination codon in all reverse primers are shown in bold-type face.

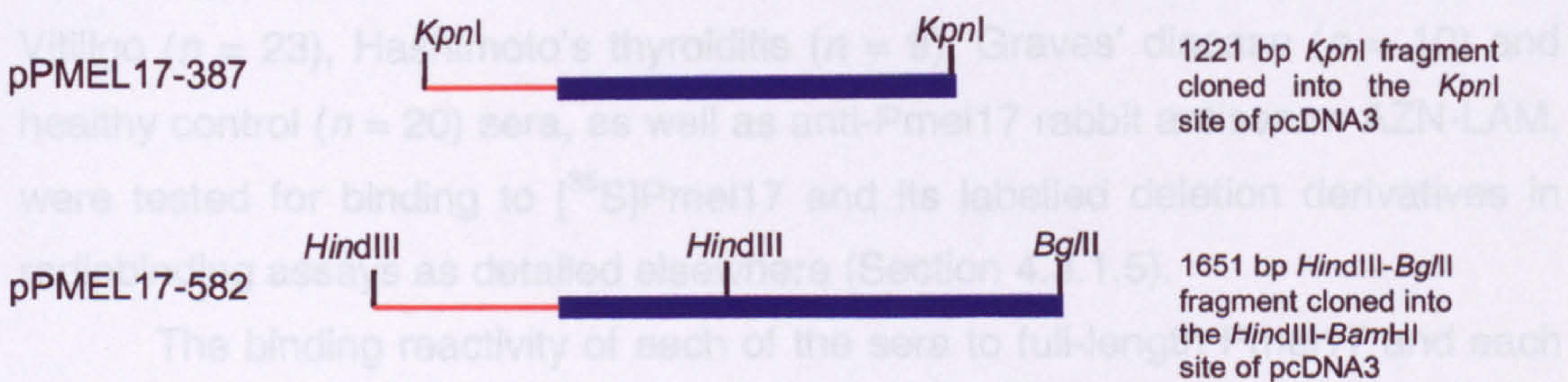


Figure 4.6: Schematic diagram of full-length Pmel17 cDNA in pcDNA3 and its truncated derivatives constructed by subcloning of restriction fragments.

The blue lines represent Pmel17 cDNA and the red lines pcDNA3. This representation is not to scale.

with respect to the T7 promoter in pcDNA3. The deletion derivatives are summarised in Table 4.8.

4.4.1.5 Coupled *in vitro* transcription and translation and electrophoretic analysis

The plasmid constructs (Table 4.8) were used in a TnT® T7 Coupled Reticulocyte Lysate System, with [^{35}S]methionine, to synthesise and radiolabel Pmel17 and its deletion derivatives *in vitro*, as previously described (Section 4.3.1.4). SDS-PAGE of *in vitro* translated products was performed in either 10% or 15% SDS-polyacrylamide resolving gels and 4% SDS-polyacrylamide stacking gels as detailed previously (Sections 2.17 and 4.3.1.4) which were stained, dried and autoradiographed as described elsewhere (Section 2.17).

4.4.1.6 Radiobinding assays

Vitiligo (n = 23), Hashimoto's thyroiditis (n = 9), Graves' disease (n = 10) and healthy control (n = 20) sera, as well as anti-Pmel17 rabbit antiserum AZN-LAM, were tested for binding to [^{35}S]Pmel17 and its labelled deletion derivatives in radiobinding assays as detailed elsewhere (Section 4.3.1.5).

The binding reactivity of each of the sera to full-length Pmel17 and each deletion derivative was expressed as an antibody (Ab) index calculated as previously described (Section 4.3.1.5). Each serum was analysed in at least two experiments and the mean Ab index was calculated from these. The upper level of normal for each assay with a different ligand was calculated, as previously described (Section 4.3.1.5), and those patient sera with an Ab index greater than the upper level of normal were regarded as positive for binding to the radiolabelled ligand used in the assay.

4.4.1.7 Computer analyses

Predictions of B cell epitopes were carried out as previously described for tyrosinase epitope mapping experiments (Section 4.3.1.6) by Dr. Part Peterson (Institute of Biomedical Sciences, University of Tampere, Finland. Genbank

homology searches were performed using the Sequence Analysis Software package as before (Section 4.3.1.6).

4.4.2 Results

4.4.2.1 *In vitro* translation of Pmel17 cDNA and its deletion derivatives

For identification of autoepitopes on Pmel17, PCR amplification was used to generate Pmel17 cDNA fragments of varying lengths which were then cloned into pcDNA3. DNA sequencing of the fragments was carried out to verify that no sequence errors had been introduced. In addition, subcloning of various Pmel17 cDNA fragments was undertaken (Figure 4.6). All the constructs used in this study are listed in Table 4.8.

Products generated from *in vitro* translation of Pmel17 cDNA and its deletion derivatives were evaluated by SDS-PAGE and autoradiography (Figure 4.7 a, b, c). In each case, major bands representing the intact and modified [^{35}S]-labelled Pmel17 proteins were found (Table 4.8).

4.4.2.2 Radiobinding assay of Pmel17 deletion derivatives with sera from patients and controls

Of the sera analysed, none from patients with either Graves' disease (n = 10) or Hashimoto's thyroiditis (n = 9) was positive in the radiobinding assays when either full-length Pmel17 or any of its deletion derivatives were used as the radiolabelled antigen. Five out of twenty vitiligo patient sera were reactive against full-length Pmel17 (Table 4.9). Of these, four reacted with all derivatives deleted up to amino acid residue 341. One vitiligo patient serum failed to react with any derivative containing amino acid deletions beyond residue 644. The remaining 18 vitiligo patient sera analysed did not bind to full-length Pmel17 or any of its deleted derivatives. The positive control serum AZN-LAM only reacted with full-length Pmel17, indicating that the last seven amino acids at the C-terminal of the protein are required for antibody binding.

4.4.2.3 Identification of the antibody binding sites on Pmel17

The results of the radiobinding assays indicate that at least two epitope domains on Pmel17 are recognised by sera from patients with vitiligo: one between

Table 4.8: Deletion derivatives used in determining the B cell epitopes on Pmel17 which are recognised by vitiligo sera.

Pmel17 construct	Amino acids encoded[1] (bp[2])	Predicted molecular weight of expressed protein[3] (kDa)	Estimated molecular weight of expressed protein[4] (kDa)
pcDNA3PMEL17[5]	1-668 (1-2004)	73	71
pPMEL17-661	1-661 (1-1983)	73	69
pPMEL17-654	1-654 (1-1962)	72	69
pPMEL17-644	1-644 (1-1932)	71	68
pPMEL17-634	1-634 (1-1902)	70	68
pPMEL17-576	1-576 (1-1728)	63	64
pPMEL17-528	1-528 (1-1584)	58	61
pPMEL17-460	1-460 (1-1381)	51	52
pPMEL17-410	1-410 (1-1230)	45	48
pPMEL17-387	1-387 (1-1161)	43	47
pPMEL17-371	1-371 (1-1113)	41	42
pPMEL17-356	1-356 (1-1068)	39	39
pPMEL17-341	1-341 (1-1023)	38	36
pPMEL17-326	1-326 (1-978)	36	39
pPMEL17-276	1-276 (1-829)	30	32
pPMEL17-194	1-194 (1-582)	21	28
pPMEL17-148	1-148 (1-446)	16	22

[1] Numbers correspond to the amino acid residues of Pmel17 included in the construct with the initiating methionine as residue number one.

[2] Numbers correspond to base pairs of Pmel17 cDNA included in the construct with the A residue of the intiating ATG codon assigned as base pair number one.

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

[4] Estimated from the mobility of the protein in SDS-acrylamide gels.

[5] Encodes full-length Pmel17.

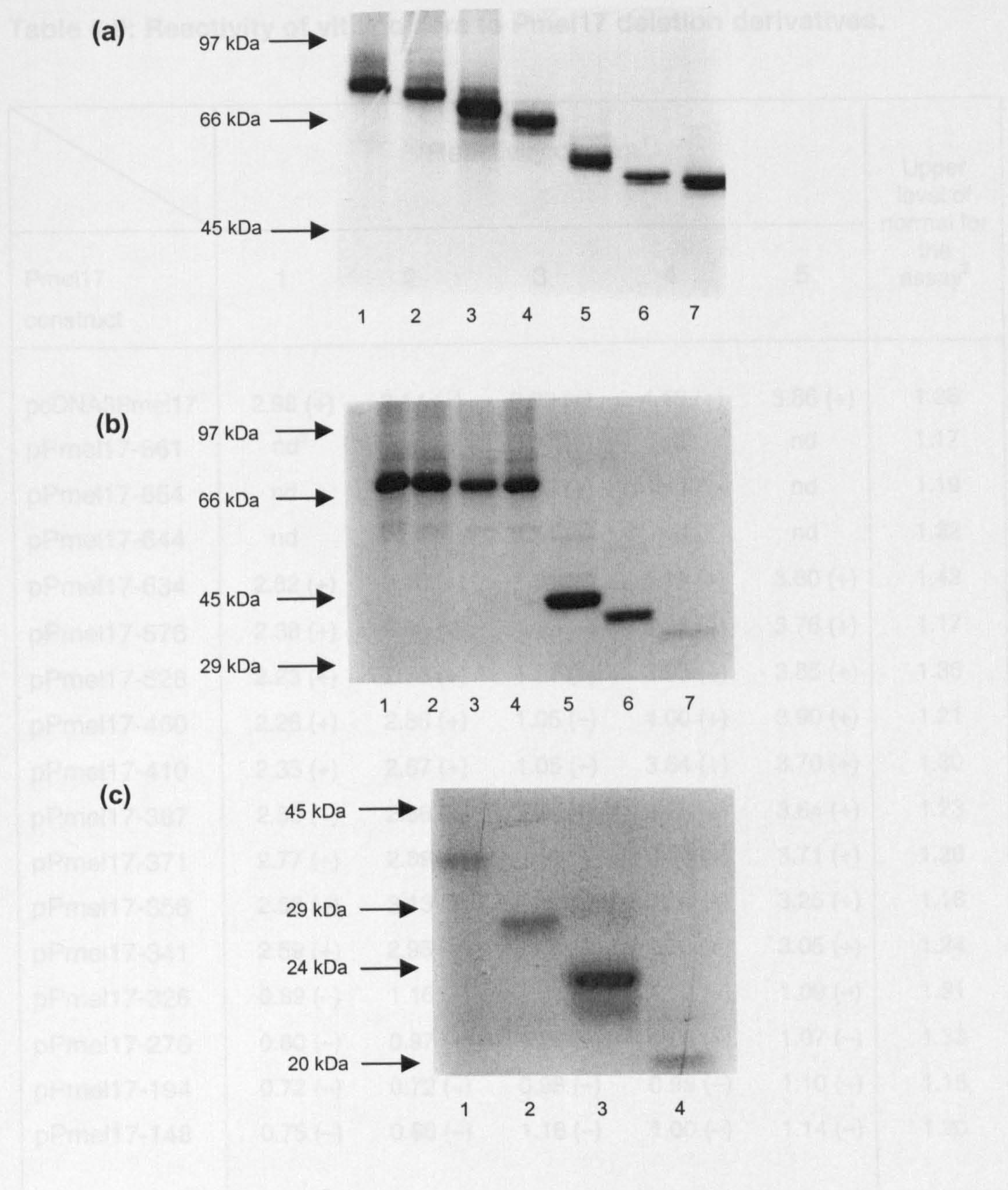


Figure 4.7: SDS-PAGE and autoradiography of products arising from *in vitro* translation of Pmel17 cDNA and its deletion derivatives.

Pmel17 cDNA and its deletion derivatives were translated *in vitro* in a TnT® T7 Coupled Reticulocyte Lysate System as described in Section 4.3.1.4. Subsequently, an aliquot of each reaction mixture was added to SDS-sample buffer, boiled for 5 min and then analysed by SDS-PAGE. (a) A 10% gel with [^{35}S]-labelled products from *in vitro* translation of: Full-length Pmel17 (lane 1); pPMEL17-634 (lane 2); pPMEL17-576 (lane 3); pPMEL17-528 (lane 4); pPMEL17-460 (lane 5); pPMEL17-410 (lane 6); pPMEL17-387 (lane 7). (b) A 10% gel with [^{35}S]-labelled products from *in vitro* translation of: Full-length Pmel17 (lane 1); pPMEL17-661 (lane 2); pPMEL17-654 (lane 3); pPMEL17-644 (lane 4); pPMEL17-371 (lane 5); pPMEL17-356 (lane 6); pPMEL17-341 (lane 7). (c) A 15% gel with [^{35}S]-labelled products from *in vitro* translation of: pPMEL17-326 (lane 1); pPMEL17-276 (lane 2); pPMEL17-194 (lane 3); pPMEL17-148 (lane 4).

Table 4.9: Reactivity of vitiligo sera to Pmel17 deletion derivatives.

	Reactivity of sera[1]					Upper level of normal for the assay[2]
Pmel17 construct	1	2	3	4	5	
pcDNA3Pmel17	2.98 (+)	3.14 (+)	3.62 (+)	4.10 (+)	3.86 (+)	1.26
pPmel17-661	nd[3]	nd	3.37 (+)	nd	nd	1.17
pPmel17-654	nd	nd	3.33 (+)	nd	nd	1.19
pPmel17-644	nd	nd	3.54 (+)	nd	nd	1.32
pPmel17-634	2.62 (+)	3.10 (+)	1.31 (–)	4.11 (+)	3.80 (+)	1.42
pPmel17-576	2.38 (+)	2.89 (+)	1.12 (–)	4.24 (+)	3.76 (+)	1.17
pPmel17-528	2.23 (+)	2.76 (+)	1.27 (–)	3.98 (+)	3.85 (+)	1.36
pPmel17-460	2.26 (+)	2.86 (+)	1.05 (–)	4.00 (+)	3.90 (+)	1.21
pPmel17-410	2.35 (+)	2.67 (+)	1.05 (–)	3.64 (+)	3.70 (+)	1.30
pPmel17-387	2.50 (+)	2.86 (+)	1.04 (–)	3.26 (+)	3.64 (+)	1.23
pPmel17-371	2.77 (+)	2.89 (+)	nd	3.44 (+)	3.71 (+)	1.26
pPmel17-356	2.56 (+)	3.13 (+)	nd	3.25 (+)	3.25 (+)	1.16
pPmel17-341	2.59 (+)	2.95 (+)	nd	2.98 (+)	3.05 (+)	1.24
pPmel17-326	0.89 (–)	1.16 (–)	1.01 (–)	1.12 (–)	1.09 (–)	1.31
pPmel17-276	0.80 (–)	0.97 (–)	1.27 (–)	0.98 (–)	1.07 (–)	1.33
pPmel17-194	0.72 (–)	0.72 (–)	0.98 (–)	0.99 (–)	1.10 (–)	1.18
pPmel17-148	0.75 (–)	0.66 (–)	1.16 (–)	1.00 (–)	1.14 (–)	1.20

[1] The binding reactivity of each of the sera to the full-length Pmel17 and each deletion derivative was expressed as an antibody index calculated as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by 20 healthy control sera. Each serum was analysed in at least two experiments and the mean antibody index was calculated from these.
[2]The upper level of normal for each assay with a different ligand was calculated using the mean antibody index + 3 SD of 20 control sera. Patient sera with an antibody index greater than the upper level of normal were regarded as positive for binding to the radiolabelled ligand used in the assay (+). (–) denotes negative for binding to the radiolabelled ligand used in the assay.
[3] nd, not determined.

amino acids 634 and 644 (VPQLPHSSSHW) located at the C-terminal of the protein and a second between amino acids 326 and 341 (QVPTTEVVGTTPGQAP) found towards the centre of the Pmel17 molecule (Table 4.10). Of the vitiligo sera examined, four reacted with the same epitope at amino acids 326-341 and one reacted with the epitope at the C-terminal domain (amino acids 634-644). As deletion from the 3' end of a molecule reveals predominantly antigenic regions which are nearer to the N-terminus of the protein, the reactivity of four of the sera with the C-terminal epitope domain was not confirmed because of the presence of reactivity with the antigenic region found closer to the N-terminal end of Pmel17.

4.4.2.4 Antigenicty predictions for Pmel17

Computer prediction analysis identified several putative antigenic regions within Pmel17 (Figure 4.8). One extensive putative antigenic domain (amino acids 290-340) encompassed the identified epitope amino acids 326-341. The epitope located at the C-terminal of Pmel17 (amino acids 634-644), however, was within a region of the protein predicted to have low antigenicity.

4.4.2.5 Protein homology searches

The amino acid sequences of the identified Pmel17 epitopes were compared to the amino acid sequences of the related melanogenic enzymes tyrosinase (Takeda *et al.* 1989), tyrosinase-related protein-1 (TRP-1) (Cohen *et al.* 1990) and tyrosinase-related protein-2 (TRP-2) (Yokoyama *et al.* 1994). No sequence homology was found between either of the Pmel17 epitopes and the aforementioned proteins.

Table 4.10: Epitope regions on Pmel17 recognised by vitiligo sera[1].

	Patient				
Amino acids	1	2	3	4	5
326-341	+	+	-	+	+
634-644	NA	NA	+	NA	NA

[1] Reactivities with epitope regions are indicated: + denote reactivity with the specified epitope region; - denotes unreactive with specific epitope region; NA denotes reactivity with the specified epitope region unknown.

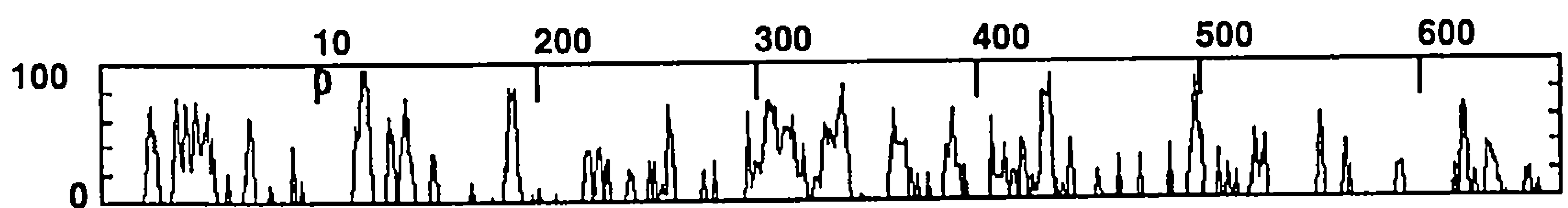


Figure 4.8: Antigenicity profile of the deduced amino acid sequence of Pmel17.

Local antigenicity values, calculated as previously detailed (Parker *et al.* 1986), are plotted against the amino acid residues of Pmel17. The *y*-axis scale from 0-100 reflects increasing antigenicity.

4.4.3 Results summary

These results indicate that at least two epitope domains are present in Pmel17: one between amino acids 326 and 341 (QVPTTEVVGTTPGQAP) and a second between amino acids 634 and 644 (VPQLPHSSSHW). These were located centrally and towards the C-terminal, respectively, of the Pmel17 protein. Four vitiligo patient sera reacted with the same epitope at amino acids 326-341 and one reacted with the epitope at the C-terminal domain (amino acids 634-644). The reactivity of four of the sera with the C-terminal antigenic domain could not be confirmed because of the presence of reactivity with the epitope region located nearer to the N-terminus of the Pmel17 molecule. In order to examine the reactivity of these sera with the C-terminus of Pmel17, further deletion derivatives would need to be constructed which exclude the central regions of the protein.

Computer analysis of the potential B cell epitopes on Pmel17 revealed that the epitope domain encompassing amino acids 326-341 was located in an area of the protein which was predicted to be highly antigenic. In contrast, the epitope identified at the C-terminal of Pmel17 (amino acids 634-644) was located in a region of the protein predicted to have low antigenicity. Computer predictions of putative antigenic regions may not always correlate, therefore, with epitopes found experimentally.

There is considerable amino acid sequence homology between Pmel17 (Kwon *et al.* 1991) and other melanogenic enzymes, for example, tyrosinase (Takeda *et al.* 1989), TRP-1 (Cohen *et al.* 1990) and TRP-2 (Yokoyama *et al.* 1994). We therefore compared the Pmel17 autoantigenic regions for amino acid homology with these proteins to locate possible cross-reactive domains. However, no sequence homology was found between either of the Pmel17 epitopes and the melanogenic proteins analysed. This finding is consistent with a previous study from this laboratory in which no Pmel17 antibodies were identified that were cross-reactive with either tyrosinase, TRP-1 or TRP-2 (Kemp *et al.* 1998b). It also suggests that the IgG response to Pmel17 is distinct from the antibody response to the other melanocyte-specific antigens.

Although only two regions of Pmel17 were recognised as epitopes for the autoantibodies in the vitiligo patients studied, it is possible that if a larger cohort

of vitiligo patient sera contaning Pmel17 antibodies was tested, more autoreactive epitopes may be identified.

4.5 Discussion

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.* 2001a). In this chapter the technique was used to radiolabel full-length and deletion derivatives of both tyrosinase and Pmel17, which were then used in radiobinding assays to identify antigenic regions on both proteins. 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 (Petterson 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 (Table 4.1; Scott 1992; Williams *et al.* 2001) to future epitope mapping studies of tyrosinase and Pmel17. 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 & Finlay 1998).

Tyrosinase and Pmel17 may be considered cytoplasmic autoantigens, since they are associated with the melanosomal membrane within melanocytes,

and are not thought to be expressed on the melanocyte cell surface. However, since the amino acid sequence of two of the identified epitope regions in tyrosinase had homology to an amino acid sequence present in TRP-1, and TRP-1 can be expressed on the melanocyte surface (Takechi *et al.* 1996), cross-reactivity with surface epitopes may be responsible for anti-tyrosinase antibody reactivity. In addition a transport protein, the melansome transport signal (MTS), has been implicated in directing melanosomal glycoproteins to the endocytic pathway leading to MHC class II-mediated antigen presentation on the melanocyte surface (Wang *et al.* 1999). It is possible that damage to melanocytes, by mechanisms which could include cytotxic T cell lysis, might cause release and degradation of intracellular proteins thereby exposing epitopes to autoreactive antibodies. Alternatively, autoantibodies might encounter these protein epitopes by directly penetrating the cytoplasm. Cellular uptake of anti-nuclear antibodies has previously been demonstrated in non-hemopoietic cells (Isenberg *et al.* 1997).

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 insulin-dependent 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; Table 4.2). 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 tyrosinase and Pmel17 autoantibodies.

The potential applications of the molecular characterisation of B cell epitopes in disease include: (i) a greater understanding of the association of an autoantigen with autoimmune pathogenesis, (ii) possible insights into the initiation of the autoimmune process, (iii) the establishment of novel and more specific assays for measuring autoantibodies in patient sera and (iv) the development of specific-active immunotherapies. The ways in which these criteria were met by the study undertaken will be discussed below.

Firstly, an association of the autoantigens tyrosinase and Pmel17 with vitiligo pathogenesis may be revealed by examining the location of the identified epitopes with respect to the functional domains of each protein. For example, if the epitopes reside in, or contribute to, the structure of the active sites of tyrosinase/Pmel17, autoantibodies targeting these epitopes might inhibit melanogenic activity. However, none of the identified tyrosinase B cell epitopes maps to the two copper-binding regions (amino acid residues 154-220 and 343-385) which are located in the catalytic site of the enzyme (Tomita *et al.* 1989). The role of Pmel17 in melanogenesis, and therefore its functionally significant domains, has yet to be fully elucidated (Section 1.1.4). However, the centrally positioned epitope identified in Pmel17 (amino acids 326-341) lies within a region of the protein containing an amino acid repeat motif (amino acid residues 315-392) thought to be functionally important (Kwon 1993).

Secondly, no apparent homology was identified between the specified epitope regions and any microbial proteins, this would suggest that molecular mimicry does not initiate the production of tyrosinase and Pmel17 autoantibodies in vitiligo patients. However, as yet uncharacterised B cell epitopes on tyrosinase and Pmel17, which may be revealed by a study employing a larger cohort of vitiligo patients or conformational epitope mapping techniques, may demonstrate cross-reactivity. Moreover, the current computer databases are unlikely to represent all microbial proteins.

Thirdly, since the both tyrosinase and Pmel17 had multiple epitope regions and the antibody response provoked in vitiligo patients was heterogenous, it is unlikely that a more specific assay for measuring reactivity to either protein, limited to the epitope regions identified here, would be suitable for screening vitiligo patients.

Finally, since the specific destruction of melanocytes is the goal of melanoma therapy and is a consequence of vitiligo, the identification of epitopes on both tyrosinase and Pmel17 could be used in the development of specific immunotherapies for treatment of this disease. In a recent study, it has been shown that antibody reactivity to Pmel17 and to tyrosinase is present in 48% and 57% of melanoma patients, respectively (Huang *et al.* 1998). It would, therefore, be of interest to determine the epitopes on Pmel17 and tyrosinase which elicit the IgG responses in these melanoma patients and to ascertain if they are the same epitopes as those reported here for vitiligo. As expected since B and T lymphocytes often recognise different autoepitopes, no precise homology was apparent between reported cytotoxic T cell epitopes on Pmel17 (Kawakami *et al.* 1995; Tsomides *et al.* 1997) and tyrosinase (Brichard *et al.* 1993; Wolfel *et al.* 1994; Kang *et al.* 1995; Tsomides *et al.* 1997) in melanoma patients and the B cell epitopes reported here for vitiligo. Cytotoxic T cell epitopes have recently been identified in vitiligo for tyrosinase and Pmel17 (Lang *et al.* 2001; Palermo *et al.* 2001) and these also showed no homology with the B cell epitopes characterised in this study.

5 An investigation of autoantibody responses to the melanocyte-specific antigen MelanA in vitiligo patients

5.1 Introduction

5.1.1 Structure and function of MelanA

The MelanA gene was first isolated from a human melanoma cell line cDNA library by its reactivity with autologous cytotoxic T cells (Coulie *et al.* 1994). The same gene was independently cloned by analysis of the antigenic targets of melanoma tumour-infiltrating lymphocytes and named MART-1 (Melanoma Antigen Recognised by T cells-1) (Kawakami *et al.* 1994a). Analysis of normal human cells and tissues for expression of MelanA mRNA demonstrate that it is melanocyte-specific and can, therefore, be termed a melanocyte differentiation antigen (Coulie *et al.* 1994; Kawakami *et al.* 1994a). The MelanA protein is a relatively short polypeptide of 118 amino acids in length with a single highly hydrophobic transmembrane domain. Subcellular fractionation analysis has suggested that MelanA is concentrated in melanosomes and in the endoplasmic reticulum (ER) (Kawakami *et al.* 1997). It does not, however, share any homology with other melanosomal proteins and, unlike tyrosinase and Pmel17, does not contain a leader sequence. Rather, it is a type III membrane protein with a signal-anchor region that functions as both an ER targeting sequence and as a transmembrane domain, and is orientated in the membrane with the C-terminal exposed to the cytosol (Rimoldi *et al.* 2001). The role of MelanA in the melanosome and in melanogenesis has yet to be elucidated.

5.1.2 The immune response to MelanA in vitiligo

Several recent studies have demonstrated specific cytotoxic T cell responses against MelanA in patients with vitiligo (Ogg *et al.* 1998; Lang *et al.* 2001; Palermo *et al.* 2001). In the first of these studies, a MelanA peptide bound to an appropriate MHC class I antigen, HLA-A2, in tetrameric complexes, was

incubated with peripheral blood lymphocytes from both vitiligo patients and normal subjects, and the frequency of MelanA-specific cytotoxic T lymphocytes quantified by flow cytometric analysis (Ogg *et al.* 1998). MelanA-specific $CD8^+$ cytotoxic T lymphocytes were observed at a significantly high frequency in 7 out of 9 (78%) HLA-A2 positive vitiligo patients and only 1 out of 6 (17%) HLA-A2 positive normal control subjects. Furthermore, a high proportion of the MelanA-specific T cells from the vitiligo patients were shown to be expressing the skin-homing receptor cutaneous lymphocyte-associated antigen (CLA), in contrast to those of the one positive control subject, and their frequency correlated with the extent of depigmentation. The same technique was applied by a second study to detect MelanA-specific cytotoxic T lymphocytes in the circulation of vitiligo patients and healthy controls with similar findings: 9 out of 9 (100%) vitiligo patients and 2 out of 6 (33%) healthy controls (Palermo *et al.* 2001) had such T cells. A third study used an enzyme-linked immunospot (ELISPOT) assay for detection of HLA-A2 restricted, MelanA-specific cytotoxic T cells using IFN-γ-specific antibody, and reported that the presence of these cells correlated with vitiligo disease activity (Lang *et al.* 2001). Among the 10 patients in this study with actively progressing disease, 7 (70%) had cytotoxic T cell reactivity to MelanA, compared with 4 out of 22 (18%) with only moderately progressing disease, and 1 out of 17 (6%) control subjects. The results of these studies are consistent with a role for skin-homing melanocyte-specific T lymphocytes in the pathogenesis of vitiligo.

Since MelanA is targeted by cytotoxic T cells (Ogg *et al.* 1998; Lang *et al.* 2001; Palermo *et al.* 2001) in vitiligo, it is possible that autoantibodies that target MelanA are also present in vitiligo patient sera. Moreover, the detection of MelanA-specific autoantibodies may prove valuable for the development of diagnostic and therapeutic tools for vitiligo.

5.2 Aim

The aim of the present study was to examine vitiligo patient sera for the presence of autoantibodies to MelanA. For this analysis, both Western blotting with a glutathione S-transferase (GST)-MelanA fusion protein and a radiobinding assay with [^{35}S]-labelled MelanA were used.

5.3 Material and Methods

5.3.1 Sera

Serum samples from 51 patients with vitiligo (28 female, 23 male; mean age: 45 yr; age range, 14-77 yr) were analysed in this study. Of these patients, 13 had an associated autoimmune disease: alopecia areata, 2; autoimmune thyroid disease, 7; systemic lupus erythematosus, 2; autoimmune thyroid disease and pernicious anaemia, 1; scleroderma, 1. The vitiligo patients were diagnosed with the following subtypes: peri-orificial, 1; symmetrical, 37; symmetrical and peri-orificial, 6; symmetrical and segmental, 1; segmental, 2; focal, 2; universal, 1; occupational, 1. Sera from 26 normal individuals (9 male, 17 female; mean age: 36 yr; age range: 22-59 yr), all without clinical signs of vitiligo or autoimmunity, were used as controls.

5.3.2 Specific antibodies

Monoclonal anti-MelanA antibody A103 was purchased from Novocastra Laboratories (Newcastle-upon-Tyne, U.K.). Anti-GST antibody was from Amersham Pharmacia Biotech. Anti-goat, anti-mouse and anti-human IgGs conjugated to horse-radish peroxidase were from Sigma.

5.3.3 Cloning of MelanA cDNA into pcDNA3

MelanA cDNA cloned between the *Bgl*II-*Nco*I sites of pSC11 was a gift from Professor Vincenzo Cerundolo (Institute of Molecular Medicine, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, U.K.). The cDNA was subcloned into plasmid pcDNA3 in order to allow expression of MelanA from the T7 promoter in the vector. Briefly, 5 µg of pSC11/MelanA plasmid was restricted with enzymes *Bgl*II (Promega) and *Nco*I (Promega) (Section 2.8) and the resulting DNA fragments resolved in a 0.8% (w/v) agarose gel (Section 2.9). The 400 bp full-length MelanA cDNA fragment was excised from the gel and purified according to a Wizard PCR Prep DNA Purification System (Promega). The 5'-protuding termini of the MelanA cDNA fragment, created by *Bgl*II and

*Nco*I digestion, were end-filled using a DNA Polymerase I Large (Klenow) Fragment Mini Kit (Promega). The end-filling reaction consisted of 1 µg of the purified *Bgl*II-*Nco*I restricted MelanA cDNA fragment, 1 x Klenow buffer (50 mM Tris-HCl, pH 7.2; 10 mM $MgSO_4$; 1 mM DTT), 10 mM of each dNTP, 0.1 mg/ml bovine serum albumin (BSA), 1 U Klenow polymerase and dH_20 to a final volume of 10 µl. The reaction was allowed to proceed for 20 min at 37°C, after which time the Klenow polymerase was inactivated by heating to 65°C for 15 min. The end-filled MelanA cDNA fragment was ligated into *Eco*RV-restricted pcDNA3 (Section 2.11), which was then used to transform *E. coli* JM109 (Section 2.12). Plasmid DNA was purified from individual transformants using a Wizard Minipreps DNA Purification System (Section 2.7.1), digested with *Hin*dIII and *Xba*I and analysed by agarose gel electrophoresis. One appropriate recombinant plasmid was sequenced by the dideoxy chain termination method as previously described (Section 2.15), using T7 primer with [α-^{35}S]dATP to verify the clone. The recombinant plasmid, pcMelanA, was then purified with a Qiagen Plasmid Maxi Kit (Qiagen Ltd.) (Section 2.7.2).

5.3.4 Cloning of MelanA cDNA into pGEX-4T-1

MelanA cDNA was cloned into pGEX-4-T-1 (Amersham Pharmacia Biotech) (Figure 5.1) in order to create a glutathione S-transferase (GST)-MelanA fusion protein which could be purified using a glutathione-linked affinity resin (Smith & Johnson 1988). A full-length fragment of MelanA cDNA was generated from pcMelanA by PCR amplification. Restriction sites for *Eco*RI and *Xho*I were incorporated into the 5' and 3' oligonucleotide primers (Life Technologies), respectively, in order to allow subcloning of the PCR product into pGEX-4T-1. The primers used are listed in Table 5.1.

A 50 ng sample of pcMelanA was subjected to 36 cycles of PCR amplification (Section 2.13) using an Expand™ High Fidelity PCR System (Roche Diagnostics Ltd.) and the following conditions: 94°C, 1 min; 55°C, 1 min; 72°C, 2 min; and 72°C for 10 min for termination. The PCR product was restricted with *Eco*RI and *Xho*I and purified according to a Wizard PCR Prep DNA Purification System (Section 2.10). Two-micrograms of pGEX-4T-1 vector was restricted with *Eco*RI and *Xho*I and purified, following agarose gel

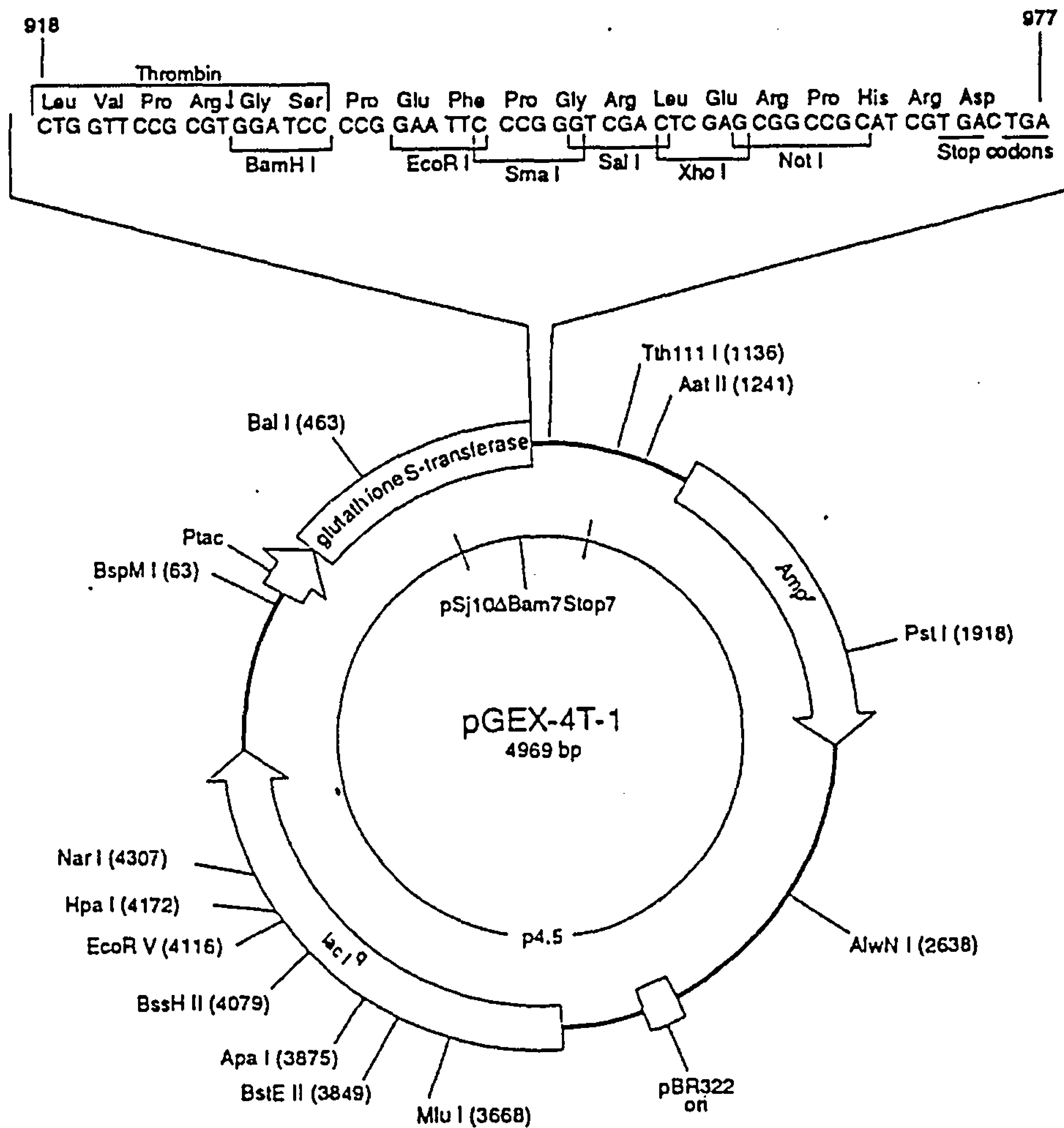


Figure 5.1: A map of the pGEX-4T-1 vector detailing restriction sites.
(Amersham Pharmacia Biotech)

Full-length MelanA cDNA was cloned into the *Eco*RI-*Xho*I sites of this vector to create an in-frame fusion with glutathione S-transferase (GST).

Table 5.1: Oligonucleotide primers used to generate MelanA cDNA by PCR amplification.

Base pairs[1]	Primer	Primer sequence[2]
1-354	MelanA/EcoRI	5'GGGAATTC**ATG**CCAAGAGAAGATGCT3'
	MelanA/XhoI	5'CCCTCGAGTCATTA**AGG**TGAATAAGGTGGTGG3'

[1]The numbers correspond to base pair of MelanA cDNA included in the construct with the A residue of the initiating ATG codon assigned as base pair number one.

[2]*Eco*RI and *Xho*I restriction sites are underlined. The first and last codons of MelanA cDNA in the forward and reverse primers, respectively, are shown in bold-type face.

electrophoresis, as previously described (Section 2.9). The MelanA cDNA fragment was then ligated into the *Eco*RI and *Xho*I sites of pGEX-4T-1 (Section 2.11) and used to transform *E. coli* DH5α (Clontech Laboratories U.K. Ltd., Basingstoke, U.K.) (Section 2.12). Plasmid DNA was purified from individual transformants using a Wizard Minipreps DNA Purification System, digested with *Eco*RI and *Xho*I and analysed by agarose gel electrophoresis. One appropriate recombinant plasmid was sequenced using a 5' pGEX sequencing primer (Amersham Pharmacia Biotech), as previously detailed (Section 2.15), to verify that no sequence errors had been introduced. The recombinant plasmid, pGST-MelanA, was then purified with a Qiagen Plasmid Maxi Kit (Qiagen Ltd.) (Section 2.7.2)

5.3.5 Coupled *in vitro* transcription and translation of MelanA cDNA

Plasmid pcMelanA was used in a TnT® T7 Coupled Reticulocyte Lysate System (Promega) to produce MelanA *in vitro* and concomitantly label the protein with [^{35}S]methionine (Amersham Pharmacia Biotech) as detailed elsewhere (Section 4.3.2.1).

5.3.6 Radiobinding assays

For each assay, an aliquot of the *in vitro* translation reaction mixture (equivalent to 50,000-100,000 counts per minute of trichloroacetic acid-precipitable material) was suspended in 50 μl of immunoprecipitation buffer. Serum was then added to a final dilution of 1:50. Radiobinding assays were carried out as previously described (Section 4.3.1.5).

5.3.7 Expression and affinity purification of GST-MelanA

Escherichia coli DH5α containing either pGST-MelanA or pGEX-4T-1 was cultured overnight in 20 ml of LB with 100 μg/ml ampicillin (Sigma). Each culture was then inoculated into 1 L of fresh LB containing 100 μg/ml ampicillin and incubated at 37°C until an OD_{600} of 0.6 was reached. Isopropyl-β-thiogalactopyranoside (IPTG; Promega) was subsequently added to a final

concentration of 1 mM and the cultures incubated for a further 3 h to allow expression of either GST or GST-MelanA protein. The cultures were harvested by centrifugation and resuspended in 10 ml of ice cold phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20, 1 μM PepstatinA (CN Biosciences, Nottingham, U.K.), 1 μM Nα-tosyl-phenylalanyl chloromethyl ketone (CN Biosciences) and 10 μg/ml aprotinin. Cells were lysed using sonication followed by the addition of Triton X-100 to a final concentration of 1% (w/v). The bacterial lysates were centrifuged at 5000 *g* for 10 min to remove unlysed cells and the supernatants retained on ice.

For affinity purification of GST and GST-MelanA, 2 ml of 50% (w/v) glutathione-agarose beads (Sigma), prepared as detailed bellow, were added to the bacterial supernatants. Briefly, lyophilised glutathione-agarose beads were swelled in dH_2O at 200 μl/g for 2 h. A 50% (w/v) bead-slurry was prepared by collecting the glutathione-agarose beads by centrifugation at 1000 *g* and gently resuspending in an equal volume of PBS/0.1% (v/v) Tween 20 with protease inhibitors as detailed above. Following overnight incubation of the bacterial supernatants and glutathione-agarose beads with gentle agitation at 4°C, the beads were washed four times with 10 ml of ice cold PBS/0.1% (v/v) Tween 20 with protease inhibitors and collected by centrifugation at 1000 *g*. After the final wash, the beads were resuspended in 2 ml of SDS sample buffer and bound proteins were eluted by boiling for 5 min before storing at -20°C. Thirty-microlitre aliquots were subjected to subsequent electrophoretic and western blot analysis.

5.3.8 Electrophoretic analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of GST and GST-MelanA was performed in 12% (w/v) polyacrylamide resolving gels and 4% (w/v) polyacrylamide stacking gels as described elsewhere (Section 2.17). After electrophoresis, gels were stained with 0.05% (w/v) Coomassie blue in 10% (v/v) glacial acetic acid/25% (v/v) isopropanol and destained with 10% (v/v) glacial acetic acid/25% (v/v) isopropanol, 1 h each at room temperature, in order to visualise protein bands.

SDS-PAGE of *in vitro* translated and radiolabelled MelanA was also performed in a 12% (w/v) polyacrylamide gel and a 4% (w/v) stacking polyacylamide gel. Five-microlitres of *in vitro* translated and radiolabelled MelanA, mixed with 10 µl of SDS sample buffer, was loaded on to the gel. Following staining and destaining as above, the gel was soaked in Amplify fluorographic reagent (Amersham Pharmacia Biotech) for 30 min at room temperature before drying under vacuum at 60°C for 2 h onto 3MM chromatography paper. The gel was autoradiographed at -70°C.

5.3.9 Western blot analysis

Following SDS-PAGE, proteins were transferred electrophoretically to nitrocellulose (Bio-Rad Laboratiries Ltd.) overnight at 40 volts in 20% (v/v) methanol, 25 mM Tris-HCl, 192 mM glycine (pH 8.3) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad laboratories Ltd.). Membranes were then soaked overnight at 4°C in PBS containing 5% (w/v) skimmed milk. Nitrocellulose strips were subsequently incubated for 2 h at room temperature with either patient sera, anti-GST antibody or MelanA monoclonal A103 at a final dilution of 1:100 in PBS with 5% (w/v) skimmed milk. Washing of the strips, 4 times each for 15 min, was carried out in PBS containing 0.05% (w/v) Tween 20. Subsequently, the strips were probed with the appropriate horse-radish peroxidase conjugated IgG used at a final dilution of 1:1000 in PBS/5% (w/v) skimmed milk. After washing 4 times for 15 min in PBS/0.05% (v/v) Tween 20, the blots were processed using ECL™ Western blotting detecting reagent (Amersham Pharmacia Biotech) as described below. Equal volumes of ECL™ detection solutions 1 and 2 were mixed to give ECL™ Western blotting detecting reagent in a sufficient quantity to cover the nitrocellulose strips. The washed strips were drained of excess PBS/0.05% (v/v) Tween 20 and arranged protein side up on an acetate sheet. The detection reagent was added to the surface of the strips and held, by surface tension, for 1 min at room temperature without agitation. Excess detection reagent was then drained and the acetate sheet and strips wrapped in Saran Wrap (Scientific Laboratory Supplies Ltd., Hessle, U.K.) and exposed to Fuji RX x-ray film.

5.4 Results

5.4.1 *In vitro* transcription-translation of MelanA

In vitro transcription-translation of MelanA was evaluated by SDS-PAGE and autoradiography which revealed a protein product with an estimated molecular weight of 22 kDa (Figure 5.2). This agrees well with the molecular weight of 20-22 kDa previously reported (Chen *et al.* 1996) but is larger than the mass of 13 kDa predicted from the amino acid sequence of the protein (Kawakami *et al.* 1994a). The difference in size has been attributed to post-translation modification of the protein, not applicable here, or to aberrant protein mobility in the SDS-PAGE system (Chen *et al.* 1996).

5.4.2 Radiobinding assays

Sera from 51 vitiligo patients and 20 healthy controls were tested for their ability to immunoprecipitate [^{35}S]MelanA in the radiobinding assay. For each serum a MelanA antibody index was assigned, this being the mean MelanA antibody index of at least two experiments. The upper level of normal for the radiobinding assay (mean MelanA antibody index + 3 SD of 20 healthy controls) was estimated as a MelanA antibody index of 1.59 (Figure 5.3).

None of the healthy individuals was positive for MelanA autoantibodies (mean MelanA antibody index ± SD = 1.02 ± 0.19). Of the 51 vitiligo patients tested, none had a MelanA antibody index above 1.59 (Figure 5.3) and all were considered negative for MelanA autoantibodies. The mean ± SD of the vitiligo patient group was 0.84 ± 0.26.

5.4.3 Expression and purification of GST-MelanA

Following expression of GST-MelanA in *E. coli* DH5α and affinity purification using glutathione agarose, the fusion protein was resolved by electrophoresis in a 12% SDS-polyacrylamide gel and visualised by Coomassie blue staining (Figure 5.4). This demonstrated a protein band with an apparent molecular weight of 45 kDa consistent with the size of the MelanA polypeptide, previously

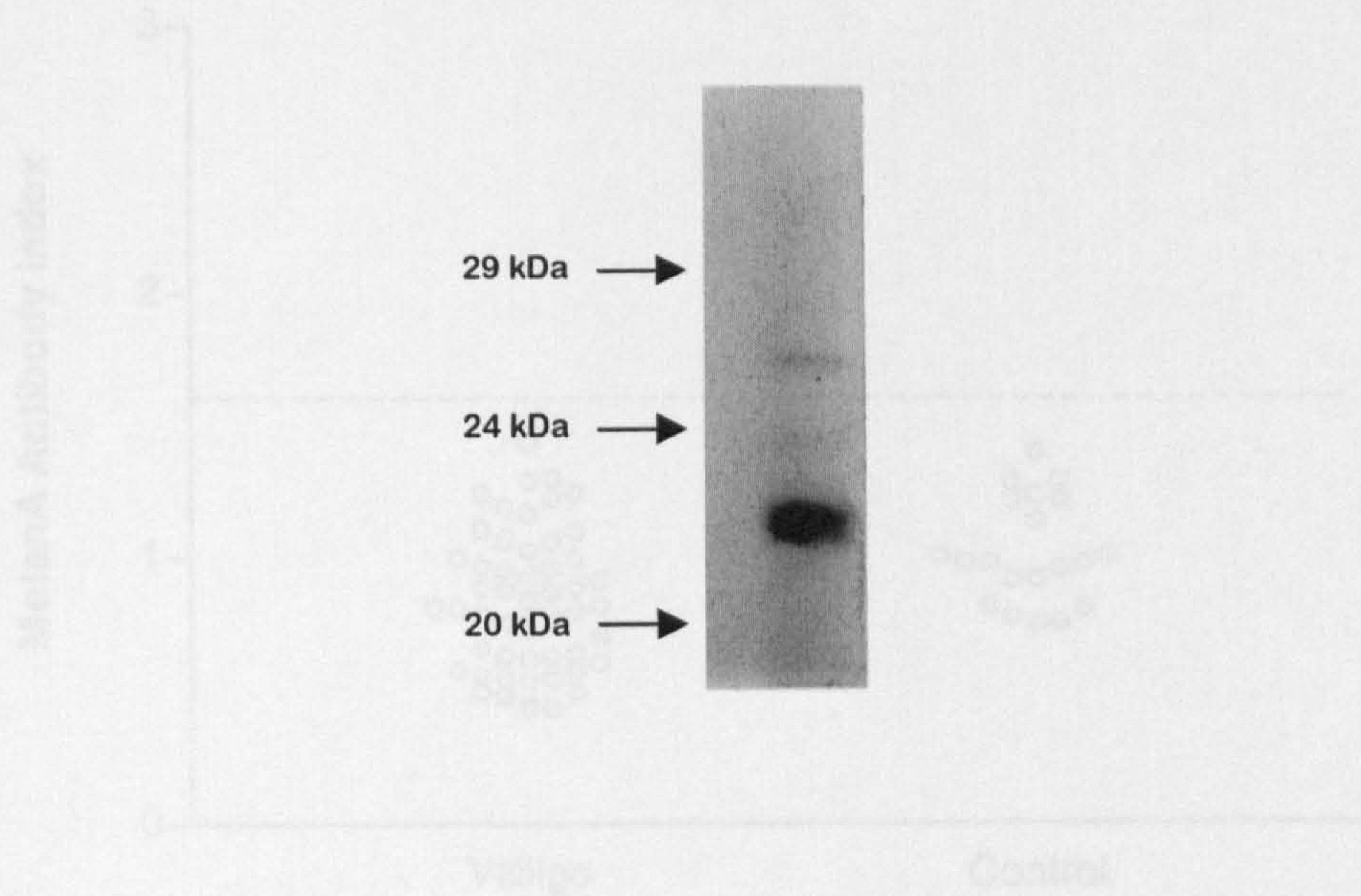


Figure 5.2: SDS-PAGE and autoradiography of *in vitro* translated [^{35}S]MelanA.

Lane 1, *in vitro* translated MelanA radiolabelled with [^{35}S]methionine.

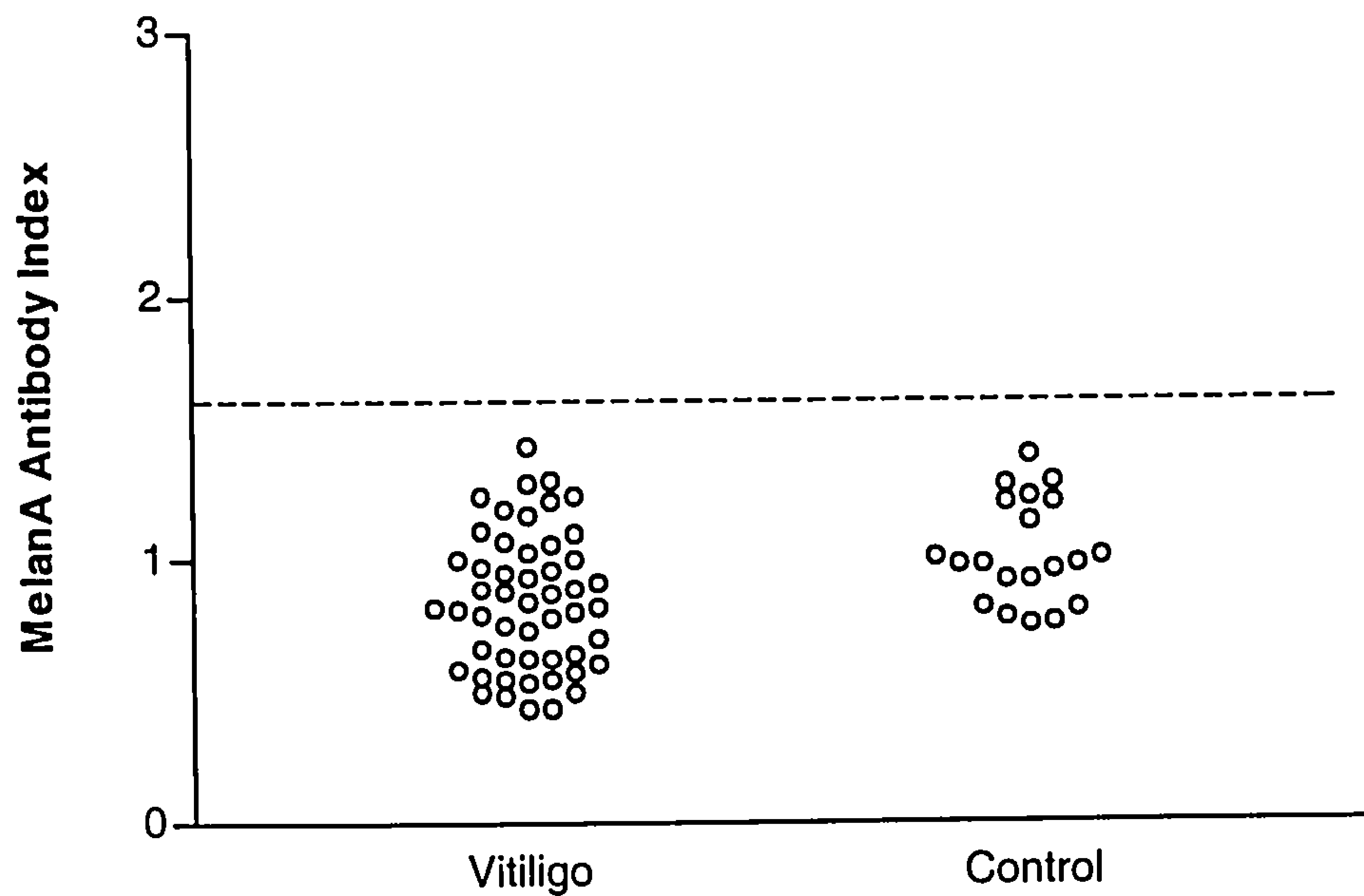


Figure 5.3: MelanA antibody indices of patient sera in the radiobinding assay.

The sera used were: vitiligo (*n* = 51), and healthy controls (*n* = 20). The MelanA antibody index shown for each serum is the mean MelanA antibody index of at least two experiments. The dotted line shows the upper level of normal (mean MelanA antibody index + 3 SD of 20 healthy control sera) for the radiobinding assay.

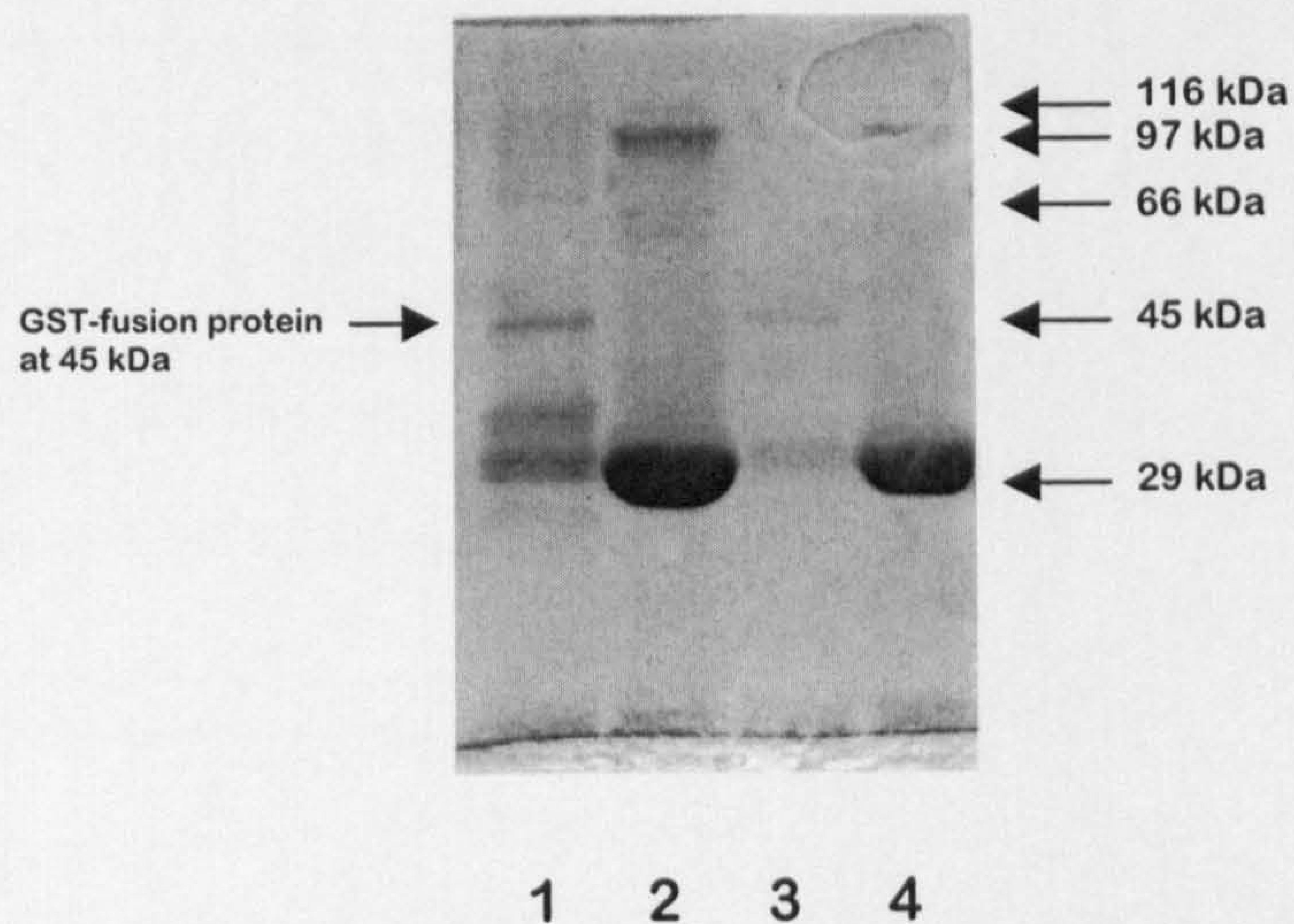


Figure 5.4:. SDS-polyacylamide gel of purified of GST and GST-MelanA fusion protein.

GST and GST-MelanA were affinity purified with glutathione agarose beads from *E. coli* DH5α bacterial cell sonicates and resolved by SDS-PAGE in a 12% gel. Protein bands were visualised using Coomassie blue staining. Lane 1, GST-MelanA purified from bacterial sonicate prepared following treatment of a culture with IPTG; lane 2, GST purified from bacterial sonicate prepared following treatment of a culture with IPTG; lane 3, GST-MelanA purified from bacterial sonicate prepared from a culture previously untreated with IPTG; lane 4, GST purified from bacterial sonicate prepared from a culture previously untreated with IPTG.

reported as 20-22 kDa, plus the 26 kD contributed by the GST leader sequence. The results in Figure 5.4 also imply a limited recovery of GST-MelanA. This is probably, in part, due to a decrease in the solubility of the fusion protein relative to the GST leader polypeptide alone. The gel also shows that a basal level of expression of GST and GST-MelanA occurs in cells not treated with IPTG.

To identify the fusion protein specifically, a Western blot of purified GST and GST-MelanA was made and probed using the anti-MelanA monoclonal antibody A103. The antibody reacted with the fusion protein at 45 kDa but not with the GST polypeptide at 26 kDa (Figure 5.5). Both GST and GST-MelanA were found to react with anti-GST antibody in immunoblotting experiments (data not shown).

5.4.4 Western blotting analysis

To examine the reactivity of vitiligo sera with GST-MelanA, a Western blot of GST-MelanA was made onto nitrocellulose. Strips of membrane were probed with vitiligo patient (n = 51) and control sera (n = 20) and the anti-MelanA monoclonal antibody A103 followed by either anti-human or anti-mouse horse-radish peroxidase conjugated IgG, as appropriate. None of the vitiligo patient sera were found to react with GST-MelanA and a sample of the results is shown in Figure 5.6.

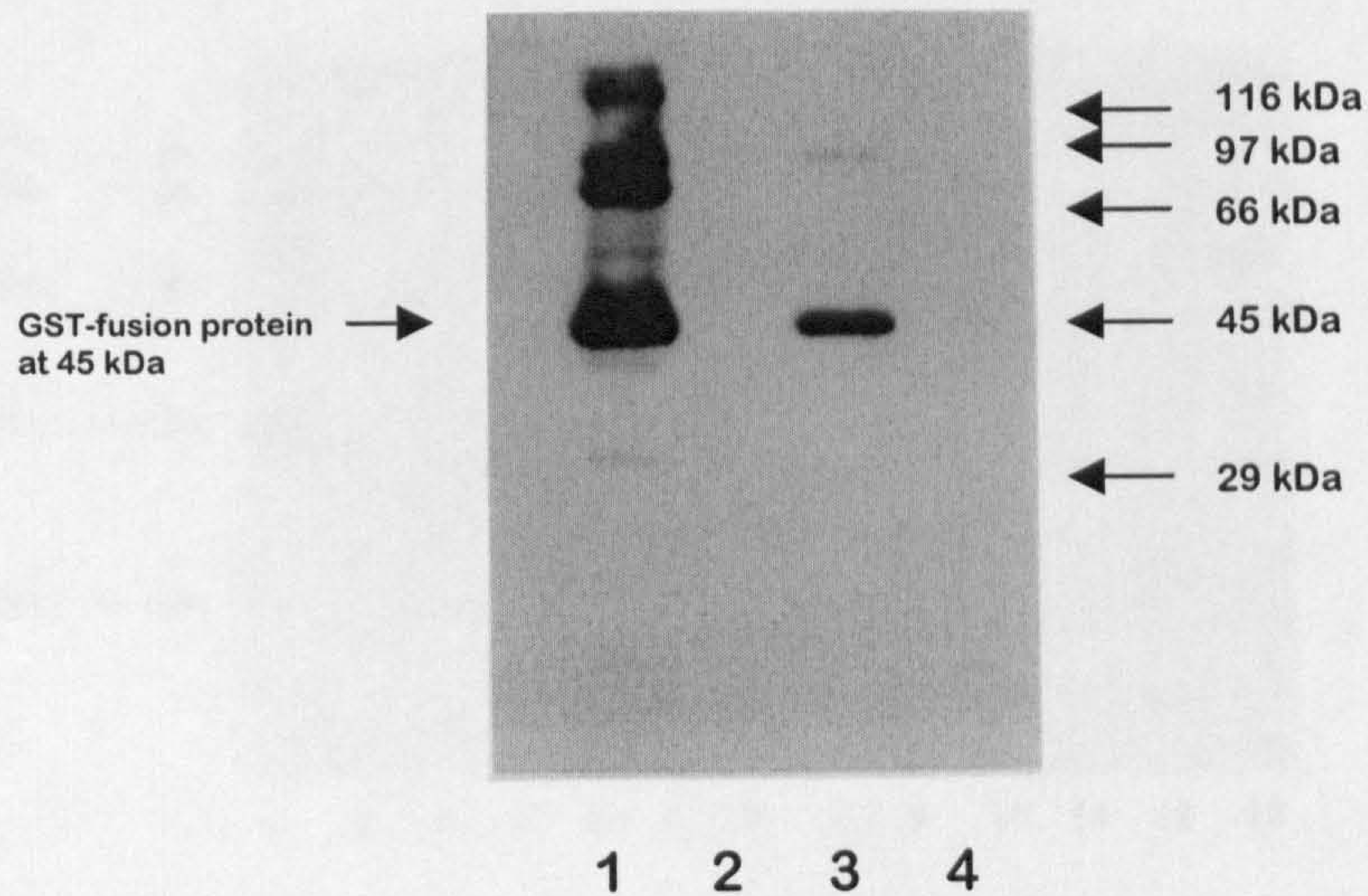


Figure 5.5: Western blot of purified of GST and GST-MelanA fusion protein probed with anti-MelanA monoclonal antibody A103.

GST and GST-MelanA were affinity purified with glutathione agarose beads from *E. coli* DH5α bacterial cell sonicates, resolved by SDS-PAGE in a 12% gel and transferred to nitrocellulose. The membrane was probed with anti-MelanA monoclonal antibody A103 at a 1:100 dilution followed by anti-mouse IgG horse-radish peroxidase conjugate at a 1:1000 dilution. After treatment with ECL™ Western blotting detecting reagents, the blot was exposed to x-ray film. Lane 1, GST-MelanA purified from bacterial sonicate prepared following treatment of a culture with IPTG; lane 2, GST purified from bacterial sonicate prepared following treatment of a culture with IPTG; lane 3, GST-MelanA purified from bacterial sonicate prepared from a culture previously untreated with IPTG; lane 4, GST purified from bacterial sonicate prepared from a culture previously untreated with IPTG.

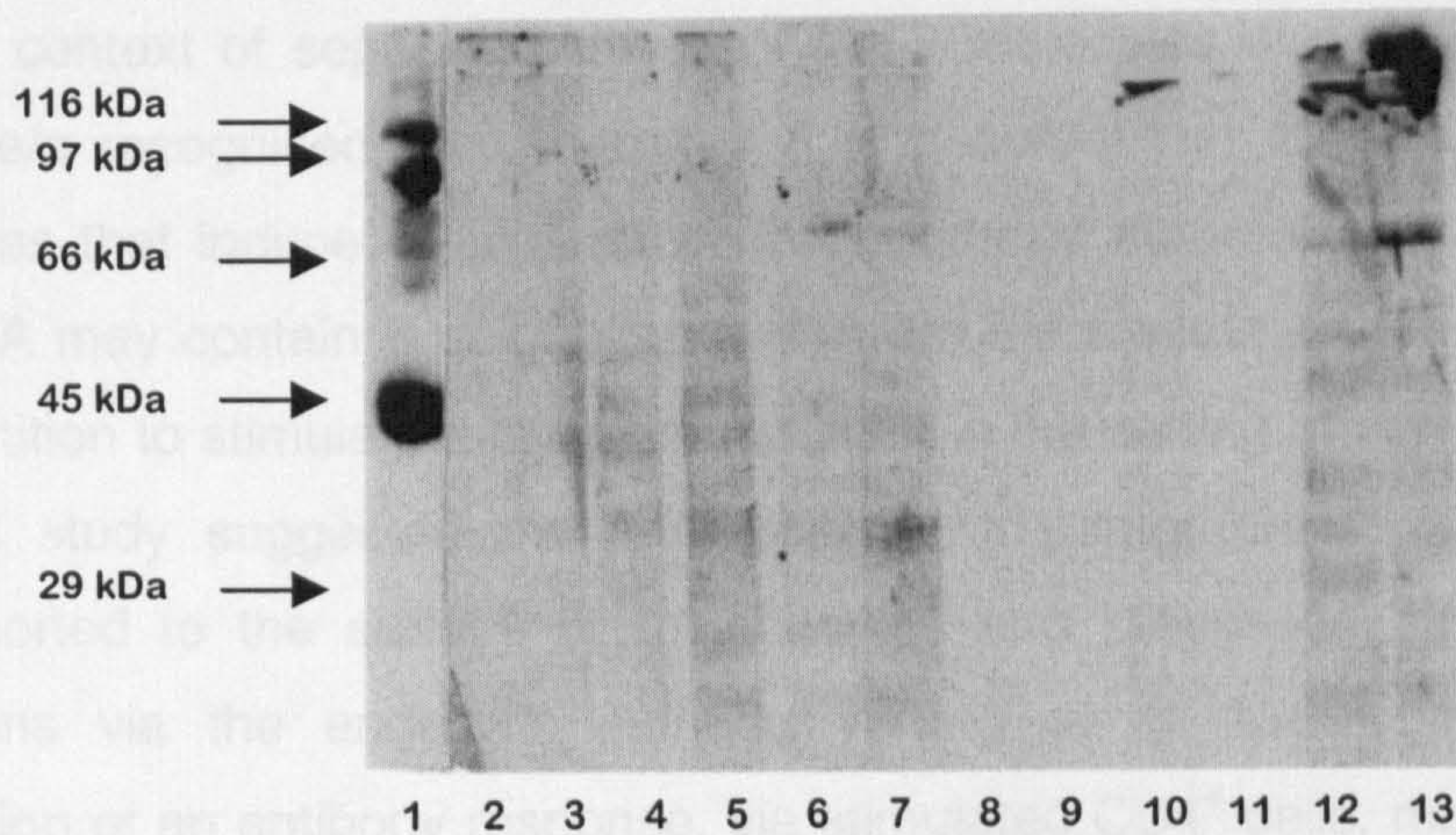


Figure 5.6: Western blot of purified GST-MelanA fusion protein probed with anti-MelanA monoclonal antibody A103, vitiligo patient sera and healthy control sera.

GST-MelanA was affinity purified with glutathione agarose beads from an *E. coli* DH5 α bacterial cell sonicate, resolved by SDS-PAGE in a 12% gel and transferred to nitrocellulose. Strips of membrane were probed with either vitiligo sera or healthy control sera at a dilution of 1:100 followed by anti-human IgG horse-radish peroxidase conjugate at a dilution of 1:1000. One strip was probed with anti-MelanA monoclonal antibody A103 at 1:100 dilution followed by anti-mouse IgG horse-radish peroxidase conjugate at 1:1000 dilution. After treatment with ECL™ Western blotting detecting reagents, the blot was exposed to x-ray film. Lane 1, anti-MelanA monoclonal antibody A103; lanes 2-7, vitiligo patient sera; lanes 8-13, healthy control sera.

5.5 Discussion

Autoantibodies to the melanocyte-specific protein MelanA could not be detected in the sera of vitiligo patients by either of the assay methods applied in this study, although previous studies have detected a specific cytotoxic T lymphocyte response against MelanA in vitiligo patients (Ogg *et al.* 1998; Lang *et al.* 2001; Palermo *et al.* 2001). B cells and cytotoxic T lymphocytes encounter antigen differently, both in the appropriate presentation of the antigen in the context of separate classes of MHC molecules and in the structure of epitope/s recognised, and therefore it is possible that MelanA only contains epitopes that induce cellular rather than humoral autoreactivity. Alternatively, MelanA may contain B cell epitopes that are inadequately exposed to immune recognition to stimulate a response, at least in the setting of vitiligo. Although a recent study suggested that melanosome transmembrane proteins can be transported to the surface of melanocytes and processed as MHC class II antigens via the endocytic pathway (Wang *et al.* 1999), the subsequent induction of an antibody response, via stimulated $CD4^+$ cells, may depend on a sufficient level of surface expression and the expression of appropriate MHC class II molecules by the melanocyte. Additionally, temporal aspects may be relevant when looking for antibodies against MelanA in vitiligo, since they may be produced after chronic disease progression. The observed immunogenicity of MelanA to cytotoxic T cells may arise through cross-reactivity with viral antigens; a cytotoxic T cell epitope on MelanA is homologous to glycoprotein C from the herpes simplex virus (Loftus *et al.* 1996). It would be of interest to study a cohort of vitiligo patients with sustained T cell reactivity against MelanA as antibody production may be observed by determinant spreading in these patients.

An immune response to MelanA was first characterised in melanoma patients, and indeed the protein was identified by its reactivity with melanoma patient T lymphocytes (Coulie *et al.* 1994; Kawakami *et al.* 1994a). MelanA contains several immunodominant epitopes for both cytotoxic (Kawakami *et al.* 1994c; Castelli *et al.* 1995; Romero *et al.* 1997; Pittet *et al.* 1999) and helper T cells (Zarour *et al.* 2000) in melanoma patients and has, therefore, been used in specific immunotherapy strategies (Nestle *et al.* 1998; Rosenberg *et al.* 1998).

In a recent immunotherapy study, a patient developed melanoma-associated hypopigmentation (Section 1.5) as a result of adoptive transfer of MelanA-specific autologous cytotoxic T cells and the depigmentation was attributed directly to the destruction of melanocytes by these cells (Yee *et al.* 2000). The humoral immune response to MelanA has been investigated in melanoma patients using recombinant MelanA protein in enzyme-linked immunosorbent assay (ELISA), and no MelanA-specific antibodies were detected in the sera of 127 melanoma patients or 70 control subjects (Stockert *et al.* 1998), which fits with data found in the present series of vitiligo patients.

The results of this study indicate that either MelanA is not a target of the humoral response in vitiligo patients or that any antibody reactivity to the protein is not detectable even when using a sensitive radiobinding assay. It is possible that MelanA-specific antibodies would be identified if a mammalian expression system was used to produce MelanA protein with native conformation. The bacterial expression systems used in this study are only appropriate for the discovery of antibodies recognising linear epitopes. Additionally, the amount of free, measurable antibody in patient sera might be too low for detection due to sequestering of antibody at sites of vitiligo lesions (Merimsky *et al.* 1996). Alternatively, the immune response to MelanA in vitiligo may be analogous to that of melanoma, in that cellular reactivity is predominant, and a humoral response apparently absent.

6 Identification of putative vitiligo autoantigens using cDNA phage-display technology

6.1 Introduction

6.1.1 Phage-display technology

Filamentous phage, so called because of their narrow rod-shaped appearance (Figure 6.1), comprise three families of bacterial viruses that depend on the F-pilus for infection of their *E. coli* host (Wilson & Finlay 1998). Each phage particle consists of a single-stranded DNA genome packaged in a tube made up of 2700 copies of a major coat protein, pVIII, closed at the ends by four or five copies of each of four species of minor coat proteins, including pIII (Figure 6.1) (Wilson & Finlay 1998). The discovery that insertion of foreign DNA fragments into the filamentous phage gene gIII results in the display of the encoded foreign polypeptide as a fusion protein with the minor coat protein pIII on the phage surface (Smith 1985), led to the development of phage-display technology. Phage-display allows the construction of diverse libraries of fusion proteins that can be selected on the basis of their binding properties, and which are physically linked to the genetic material that encodes them. The technique has become a powerful tool for studying protein-ligand interactions and has been applied to the study of receptor-ligand (Bass *et al.* 1990; Jacobsson & Frykberg 1996), enzyme-substrate (Matthews & Wells 1993; Redl *et al.* 1999), transcription factor-ligand (Butteroni *et al.* 2000), and antibody-antigen (Barbas 1993; Winter *et al.* 1994; Bartoli *et al.* 1998; Burgoon *et al.* 2001) interactions.

6.1.2 Identification of antigens using cDNA phage-display

Previous immunological applications of phage-display include immunoscreening of phage libraries displaying antibody fragments with a known, immobilised antigen (Barbas 1993), and epitope mapping by using specific antibodies to screen a library displaying random peptide sequences generated

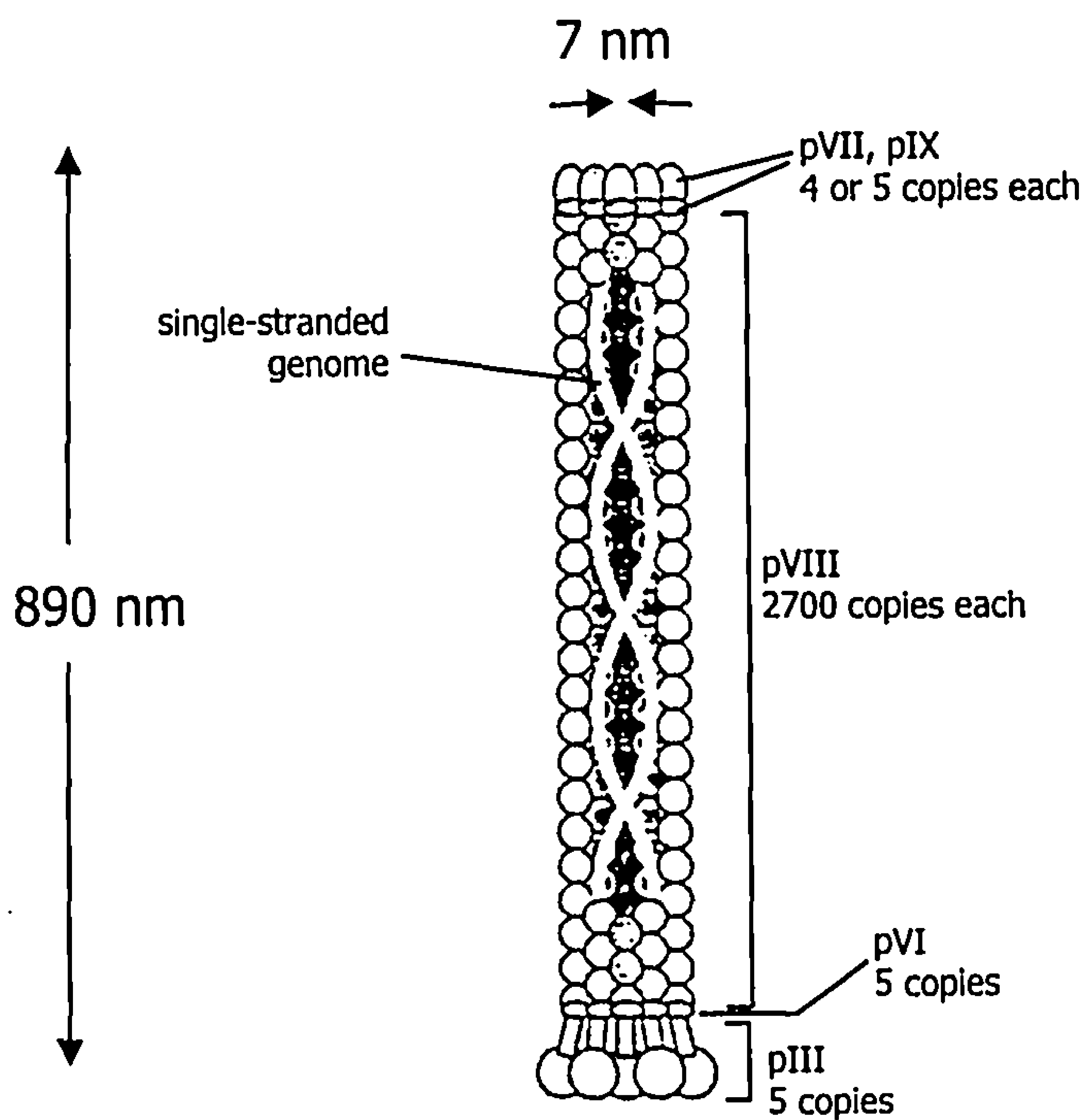


Figure 6.1: The filamentous phage.

A schematic representation of the structure of a filamentous phage particle reproduced from (Wilson & Finlay 1998). A single-stranded circular genome is surrounded by ~2700 copies of the major coat protein pVIII, and 4 or 5 copies of each of four types of minor coat proteins, including pIII. Part of the coat sheath, formed by copies of the major coat protein, is not shown in order to reveal the phage genome.

from a selected antigen (Scott 1992). More recently, immunoscreening of proteins translated from a cDNA expression library on phage has been utilised to identify novel antigens (Burgoon *et al.* 2001). Used in this way, phage-display technology provides several advantages over conventional immunoscreening of plasmid or lambda-phage cDNA libraries performed after transfer of phage plaques or bacterial colonies to nitrocellulose membrane. Firstly, by screening the cDNA library in a fluid-phase, denaturation of proteins displayed on the surface of the phage is avoided. In addition, immobilisation of the IgG instead of the cDNA library allows the specific enrichment of phage particles that interact with the antibody immobilised onto a solid-phase by repeated rounds of selection. Moreover, as many as 10^{10}-10^{11} individual clones can be screened in a single experiment since this number of phage particles occupies only a small volume.

6.1.3 The pJuFo vector

The phage-display expression vector pJuFo (Crameri & Suter 1993; Crameri *et al.* 1994) was designed to circumvent problems associated with the translation of cDNA libraries on phage. In particular, the presence of translational stop codons or non-coding regions at the 3' end of cDNAs which can prevent the expression of N-terminal pIII fusion proteins. This cannot be avoided by expression of C-terminal pIII fusions, since the attachment of a protein to the C-terminus of pIII would impair its incorporation into the phage coat. The vector pJuFo overcomes the limitations of direct fusions to the pIII protein, by expressing the cDNA insert fused to the C-terminus of a Fos protein and a Jun protein as an N-terminal fusion to pIII (Crameri & Suter 1993; Crameri *et al.* 1994) (Figure 6.2). The Jun and Fos proteins have a high protein-protein affinity leading to formation of a Jun/pIII-Fos/cDNA heterodimer, which is then incorporated into the phage surface during phage morphogenesis. The strategy therefore permits both the expression of complex cDNA library repertoires and the covalent attachment of the expressed products as Fos-fusion proteins on the surface of filamentous phage particles. The pJuFo system is outlined in greater detail in Figure 6.2. Phage displaying IgG-binding peptides can be isolated by interaction with immobilised patient antibody, a procedure referred to as biopanning (Figure 6.3).

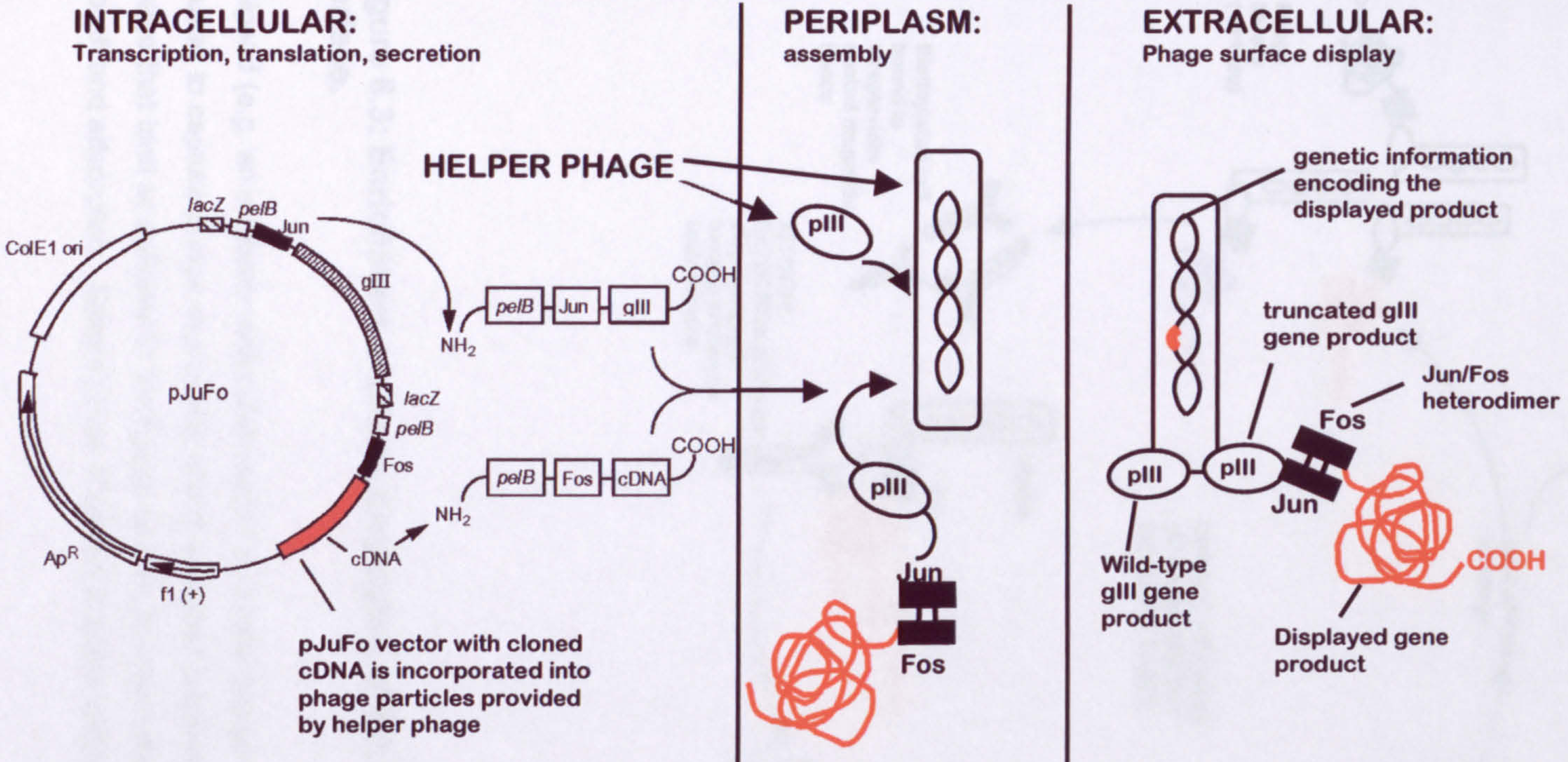


Figure 6.2: The pJuFo phage-display system.

Within a bacterial host cell, the Jun leucine zipper flanked by cysteine residues is expressed and secreted as an N-terminal fusion to a truncated gIII gene product of the phagemid. The Fos leucine zipper flanked by cysteine residues is used to produce the Fos-decorated cDNA fusions secreted into the periplasm of the bacterial host. Generation of phage surface display libraries occurs by Jun/pIII-Fos/cDNA heterodimerisation and incorporation into the phage surface together with the wild-type pIII of the helper phage. The physical linkage of the gene product displayed on the phage surface to the genetic information required for its production, achieved by the heterodimerisation of the Jun and Fos leucine zippers, allows specific isolation of genes by gene-product/ligand interaction. Adapted from (Crameri & Suter 1993).

Figure 6.3: Enrichment of phage displaying cDNA on products on their surface.

A ligand (e.g. an antibody molecule) coated to a solid-phase surface (e.g. a magnetic bead) is used to capture phage expressing cDNA-encoded products by ligand-product interaction. Phage that bind specifically to the ligand can be isolated after consecutive rounds of phage growth and adsorption. Adapted from (Crameri & Suter 1993).

The cDNAs encoding immunoreactive peptides can be recovered from phage particles by infection of bacterial cultures and then identified by DNA sequencing and database searches.

Phage-display based on the pJuFo cloning system (Crameri & Suter 1993; Crameri *et al.* 1994) has been used to demonstrate that the large subunit of human immunodeficiency virus-1 reverse transcriptase interacts with β-actin (Hottiger *et al.* 1995), and to identify allergens that bind to human IgE antibodies using yeast and fungal cDNA phage-display libraries (Crameri & Blaser 1996; Crameri *et al.* 1996; Lindborg *et al.* 1999).

6.2 Aim

The aim of the present study was to identify novel melanocyte autoantigens in vitiligo exploiting the benefits of phage-display based on the pJuFo cloning system (Crameri & Suter 1993; Crameri *et al.* 1994).

6.3 Materials and methods

6.3.1 Library construction

The construction and cloning of a human melanoma cDNA expression library, employing a SMART™ cDNA Library Construction Kit (Clontech Laboratories U.K. Ltd.), is schematically represented in Figure 6.4 and outlined in detail below. The cloning of the cDNA library in a λ-phage expression vector, λTriplEx2, and filamentous phage-display vector, pJuFo, was used to allow future conventional immunoscreening of library inserts as well as the selective enrichment by phage-display techniques.

6.3.1.1 RNA extraction

Cultured human Skmel23 melanoma cells were a gift from Professor Sheila MacNeil (Division of Clinical Sciences (North), University of Sheffield). RNA was prepared from 2 x 10^6 cells using TRIZOL®LS Reagent (Life Technologies Ltd.). Briefly, cells were pelleted by centrifugation and lysed directly in l ml TRIZOL®LS Reagent by repetitive pipetting. The homogenised sample was incubated for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. The organic and aqueous phases were then separated by the addition of 250 µl of chloroform (BDH) followed by centrifugation of the mixture for 15 min at 12,000 rpm in a Microcentaur centrifuge (Sanyo Gallenkamp plc, Loughborough, U.K.). The upper aqueous layer was carefully removed to a fresh 1.5 ml eppendorf tube and total RNA recovered by precipitation with an equal volume of isopropyl alcohol (BDH). The RNA precipitate was pelleted by centrifugation for 10 min at 12,000 rpm, washed with 1 ml 70% (v/v) ethanol and air-dried for 10-15 min. The RNA pellet was then dissolved in 400 µl sterile RNase-free water and allowed to rehydrate for 30 min at 4°C. RNase-free water was prepared by the addition of diethylpyrocarbonate (DEPC) (Sigma) to 0.01% (v/v). The quality of the RNA was assessed by agarose gel electrophoresis of a 5-µl aliquot of the preparation and the quantity measured by photometry at 260 nm.

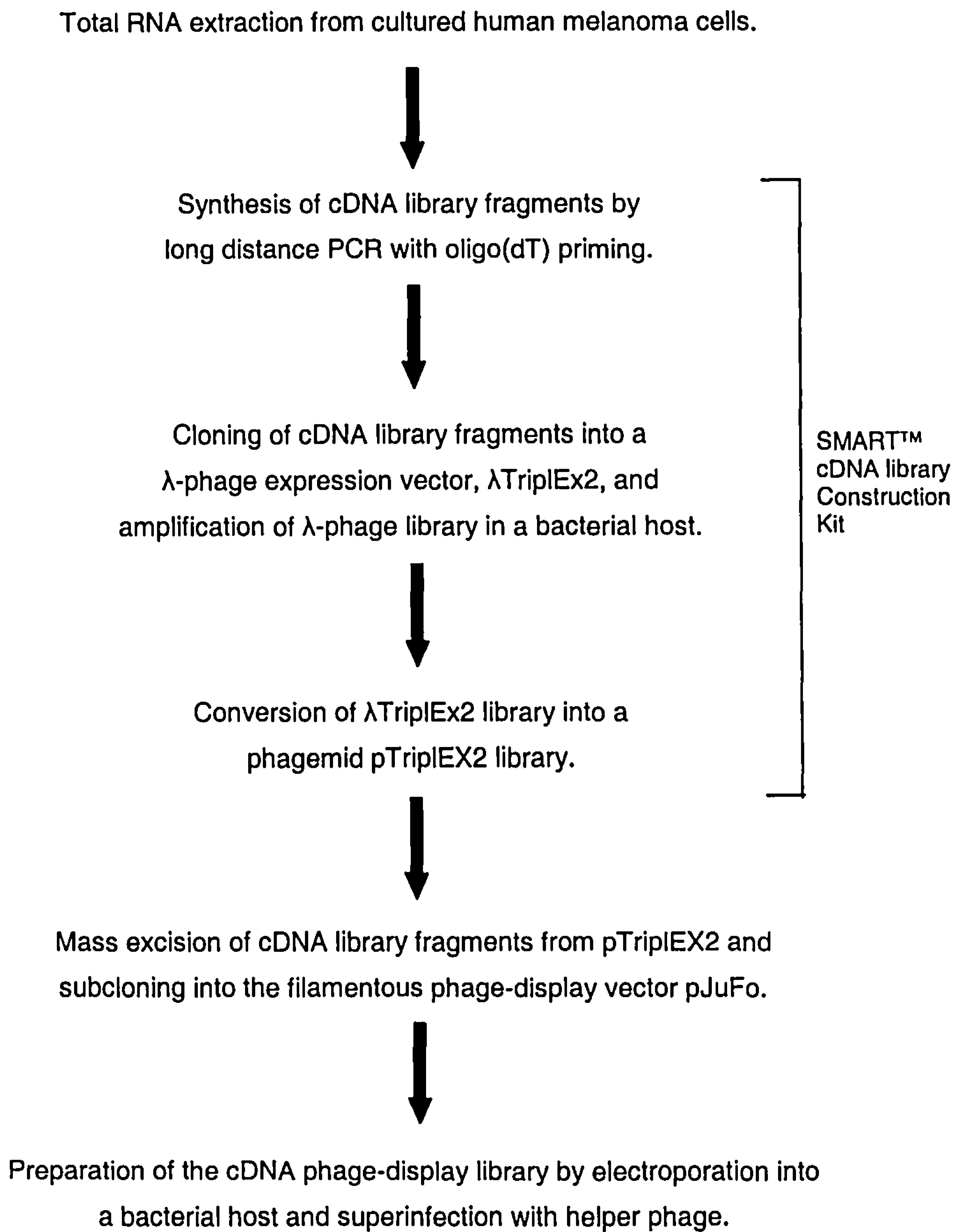


Figure 6.4: A brief overview of the melanoma cDNA library construction.

6.3.1.2 Synthesis of cDNA library fragments

Total RNA was then processed in a SMART™ cDNA Library Construction Kit (Clontech Laboratories U.K. Ltd.), as detailed below, to prepare melanoma cDNA fragments restricted with endonuclease *SfiI* ready for cloning into a λ-phage library expression vector, λTriplEx2. All reagents were supplied by the SMART™ cDNA Library Construction Kit, unless otherwise stated. Firstly, single-stranded (ss) cDNA was reverse transcribed from 3 µg of prepared total RNA in a 10-µl reaction containing 1 µM SMART III™ oligonucleotide, 1 µM CDS III oligo(dT) primer, 2 µl 5x First-Strand buffer (250 mM Tris, pH8.3; 30 mM $MgCl_2$; 375 mM KCl), 2 mM DTT, 1 mM dNTPs and 600 U Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (RT; Promega) by incubation for 1 h at 42°C. The first-strand ss cDNA was placed on ice until needed.

Secondly, double-stranded (ds) cDNA was synthesised by long-distance (LD) PCR using primers designed to both selectively amplify full-length ss templates and introduce *SfiI* restriction sites to the resulting ds cDNA products. The 50-µl LD PCR reaction comprised 2 µl of the previously prepared ss cDNA, 10 µl 10x Advantage® 2 PCR buffer, 2 µl 50x dNTP mix, 0.2 µM of each of the 5' and 3' PCR primers, 2 µl of 50x Advantage® 2 Polymerase mix and 80 µl sterile dH_20. After initial denaturation by heating to 95°C for 1 min, the reaction mixture was subjected to 20 cycles of LD PCR amplification in a DNA Thermal Cycler (Perkin-Elmer/Cetus) using the following conditions: 95°C, 15 sec; 68°C, 6 min. When cycling was complete, the LD PCR reaction, containing amplified ds cDNA, was placed on ice. A 5-µl aliquot was analysed by electrophoresis on a 1.1% (w/v) agarose gel and the approximate yield and size range of cDNA products was compared with a figure illustrating the expected result provided in the maufacturer's protocol.

The Advantage® 2 Polymerase was subsequently removed from the LD PCR reaction by Proteinase K digestion. Briefly, 2 µl of Proteinase K (20 µg/µl) was added to 50 µl of the amplified ds cDNA, mixed and heated to 42°C for 20 min. The ds cDNA was then extracted from the mixture using phenol: chloroform (1:1 v/v) as follows; an equal volume of phenol: chloroform (1:1 v/v) (BDH) was mixed with the reaction by continuous gentle inversion for 1-2 min and then centrifuged at 13,000 rpm in a Microcentaur centrifuge (Sanyo) for 5 min to separate the organic and aqueous phases. The aqueous phase was

carefully removed, transferred to a fresh tube and an equal volume of chloroform added. The mixture was centrifuged at 13,000 rpm after which the aqueous phase removed to a fresh tube and the organic phase and interface discarded. The cDNA was recovered from the aqueous phase by precipitation with 2.5 volumes of 95% (v/v) ethanol, 1.3 µl glycogen (20 mg/ml) and 0.1 volumes of 3 M sodium acetate. The cDNA precipitate was washed with 100 µl of 80% (v/v) ethanol, air-dried and resuspended in 79 µl sterile dH_2O.

Finally, the cDNA was subjected to digestion with *Sfi*I endonuclease. The digestion was performed in a 100-µl reaction comprising 79 µl of cDNA, 10 µl (10x) *Sfi*I buffer, 1 µl BSA, and 200 U *Sfi*I, at 50°C for 2 h. The *Sfi*I-restricted cDNA fragments were then size fractionated according to the kit, using a CHROMA SPIN™-400 Column, to select fragments of 500-2000 bp for ligation into a λ-phage library vector, λTriplEx2 (Figure 6.5a).

6.3.1.3 Construction of a melanoma cDNA library in λTriplEx2 phage vector

The cDNA fragments were ligated into λTriplEx2 vector in a reaction comprised of 1x ligation buffer (50 mM Tris-HCl, pH 7.8; 10 mM $MgCl_2$; 10 mM DTT; 0.5 mg/ml BSA), 1 mM ATP, 200 U T4 DNA ligase, 1 µl λTriplEx2 vector (500 ng) with 1 volume of melanoma cDNA (approximately 50 ng) and dH_2O to a final volume of 5 µl. The ligation reaction was incubated overnight at 16°C.

The ligation was subsequently packaged into λ-phage using Gigapack III® Gold packaging extract (Stratagene) as per the manufacturer's instructions. Briefly, 1 µl of the ligation reaction was added to a 25-µl aliquot of the packaging extract, which had been rapidly thawed from storage at −80°C immediately prior to use. The tube was gently mixed and then incubated at room temperature for 2 h. Five hundred mircolitres of 1x lambda dilution buffer (100 mM NaCl; 10 mM $MgSO_4$; 35 mM Tris-HCl; 0.01% (w/v) gelatin) and 20 µl of chloroform were subsequently added to the tube, which was spun briefly to collect debris before transfer of the supernatant to a clean tube for storage at 4°C.

The titre of the resulting library was determined by infection of *E. coli* XL1-Blue cells as detailed below. Fifteen millilitres of LB medium (Section 2.3), supplemented with 10 mM $MgSO_4$ and 0.2 % (w/v) maltose, was inoculated with a single colony of *E. coli* XL1-Blue grown on LB agar containing 10 µg/ml tetracycline and 10 mM $MgSO_4$. The culture was shaken at 200 rpm overnight,

at 37°C, until a culture OD_{600} of 2.0 was reached. The cells were then pelleted by centrifugation and resuspended in 7.5 ml of 10 mM $MgSO_4$. A range of dilutions (I: 5 - 1: 20) of the packaging extract was prepared in 1x lambda dilution buffer and used to infect *E. coli* XL1-Blue. One-microlitre aliquots of each phage dilution were added to 200-µl aliquots of *E. coli* XL1-Blue and allowed to adsorb at 37°C for 15 min. Four millilitres of molten (45°C) LB overlay, containing 0.8% (w/v) agar and 10 mM $MgSO_4$, was added to each infection, mixed and poured over 90-mm LB agar plates (Section 2.3.1) containing 10 mM $MgSO_4$. Once the overlay had hardened the plates were inverted and incubated at 37°C overnight until lysis plaques were visible. The number of plaques on each plate was counted and the titre (plaque forming units (pfu)/ml) calculated for the packaged ligation. An approximate total of 1×10^6 independent library clones was estimated from the titre (pfu/ml) of the packaged ligation.

Since the multiple cloning site of λTriplEx2 is embedded within the coding sequence of the α-polypeptide of beta-galactosidase (*lacZ*) (Figure 6.5), it was possible to determine the percentage of recombinant clones by blue-white screening on medium containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Promega) and IPTG (Sambrook *et al.* 1989). Screening was performed on plates containing 500-1000 plaques prepared as previously described for the titration except that 2.5 mM of each X-gal and IPTG was additionally added to the LB overlay. No blue (i.e., non-recombinant) plaques were apparent indicating that the library was 100% recombinant.

The library was amplified by scaling-up the infection of bacterial host cells previously described for the titration experiment. An aliquot of log-phase *E.coli* XL1-Blue was infected with a sufficient quantity of the λTriplEx2 library to yield approximately 145,000 plaques on a large (22 cm x 22 cm) petri dish, containing LB agar supplemented with 10 mM $MgSO_4$. This was repeated on a further six large dishes to yield an approximate total of 1×10^6 plaques, each representing an independent library clone. The plates were incubated overnight at 37°C until the lysis plaques were confluent, 30 ml of 1x lambda dilution buffer was then added to the surface of each plate and they were stored for a further 3 h at 4°C. The surface overlay from each plate was then scraped and poured into a separate 50 ml polypropylene screw-top tube and allowed to stand for an h at room temperature to produce a λ-phage lysate. The amplified library lysate was

then cleared of agar and cell debris, and any remaining intact bacterial cells lysed, by the addition of 0.1 volumes of chloroform followed by vortexing for 2 min and centrifugation at 5000 *g* for 10 min. The supernatant from each 50 ml tube was pooled in a single fresh sterile 50-ml tube and stored at 4°C. One-millilitre aliquots for long-term storage at –70°C were transfered to sterile 1.5-ml eppendorf tubes and dimethyl sulphoxide (DMSO; Sigma) was added to each tube to a final concentration of 7% (v/v).

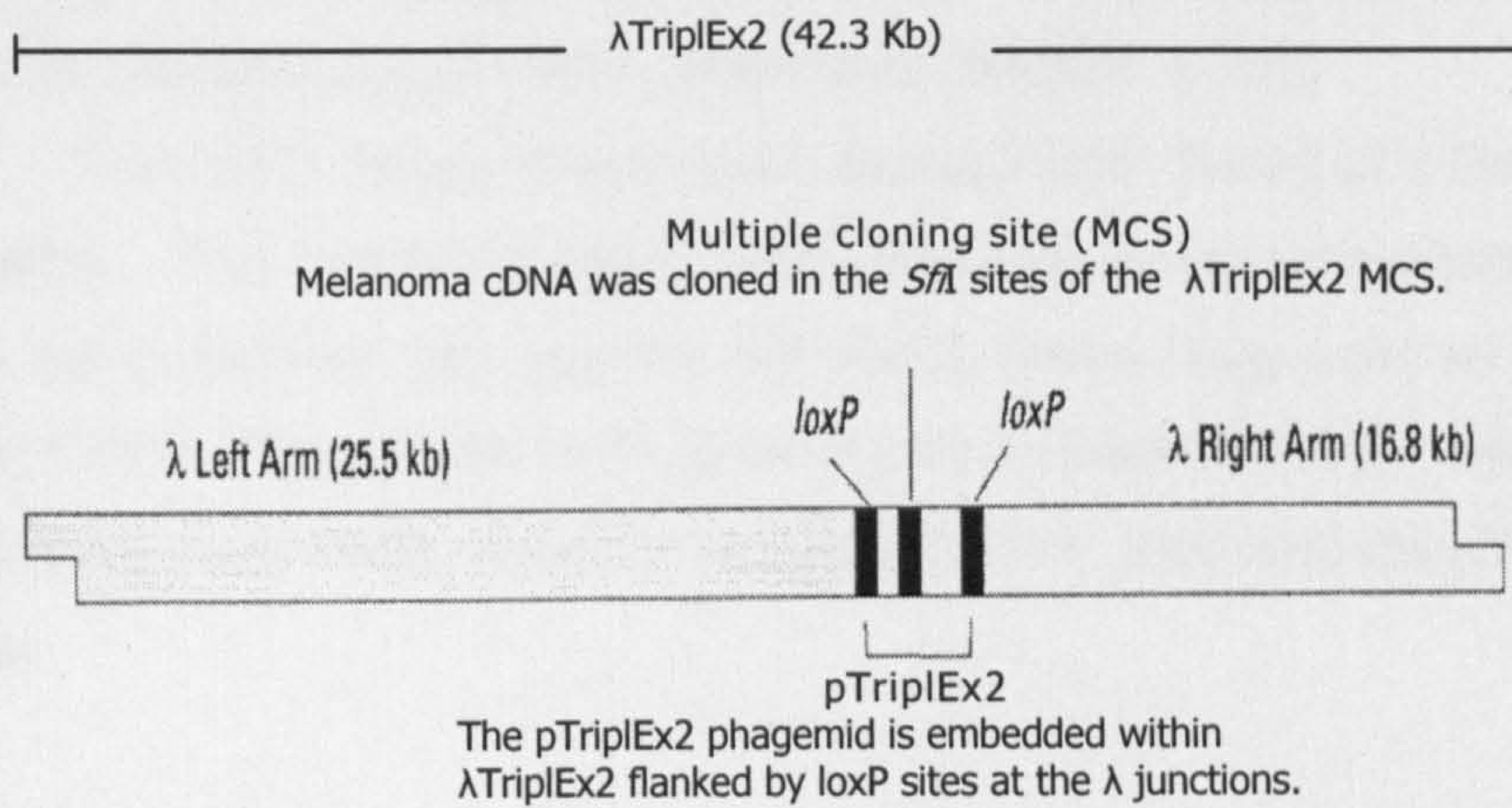
The titre of the final amplified library was measured as previously described for the unamplified library and estimated at 1×10^{10} pfu/ml.

6.3.1.4 Conversion of the λTriplEx2 library to a phagemid pTriplEx2 library

The conversion of the λTriplEx2 library to a phagemid pTriplEx2 library was carried out by transduction of the recombinant λ-phage into a bacterial host in which Cre recombinase is being expressed. The pTriplEx2 phagemid (Figure 6.5 b) is embedded within *lox*P sites in λTriplEx2, Cre recombinase activity promotes *in vivo* excision and circularisation of pTriplEx2 phagemid from the recombinant phage at the *lox*P sites. A large-scale preparation of the pTriplEx2 phagemid was prepared so that the melanoma cDNA inserts could be excised for cloning into pJuFo.

Escherichia coli strain BM25.8, grown at 31°C, provided the necessary Cre recombinase activity and a stock was maintained on LB agar containing 50 µg/ml kanamycin and 34 µg/ml ampicillin. For large-scale culture, a single colony of *E. coli* BM25.8 was picked from the stock plate and used to inoculate 10 ml of LB medium, supplemented with 50 µg/ml kanamycin, 0.2% maltose and 10 mM $MgCl_2$, which was shaken at 150 rpm at 31°C overnight. The following day, a further 10 mM $MgCl_2$ was added to the culture prior to infection with a 1-µl aliquot of the λTriplEx2 library at 31°C for 15 min without shaking. The infected culture was then supplemented with 100 µg/ml ampicillin and shaken at 150 rpm at 31°C for 2½ h, before sub-culture into 100 ml of fresh LB medium containing 100 µg/ml ampicillin and growth at 31°C, as before, for a further 4 h. Finally, the library culture was subbed into 500 ml of fresh LB medium containing 100 µg/ml ampicillin and maintained, shaking at 150 rpm, at 31°C for 24 h. The presence of the excised phagemid pTriplEx2 was detected in a 10-ml aliquot of the culture by use of a Wizard Minipreps DNA Purification System (Promega)

(a)



(b)

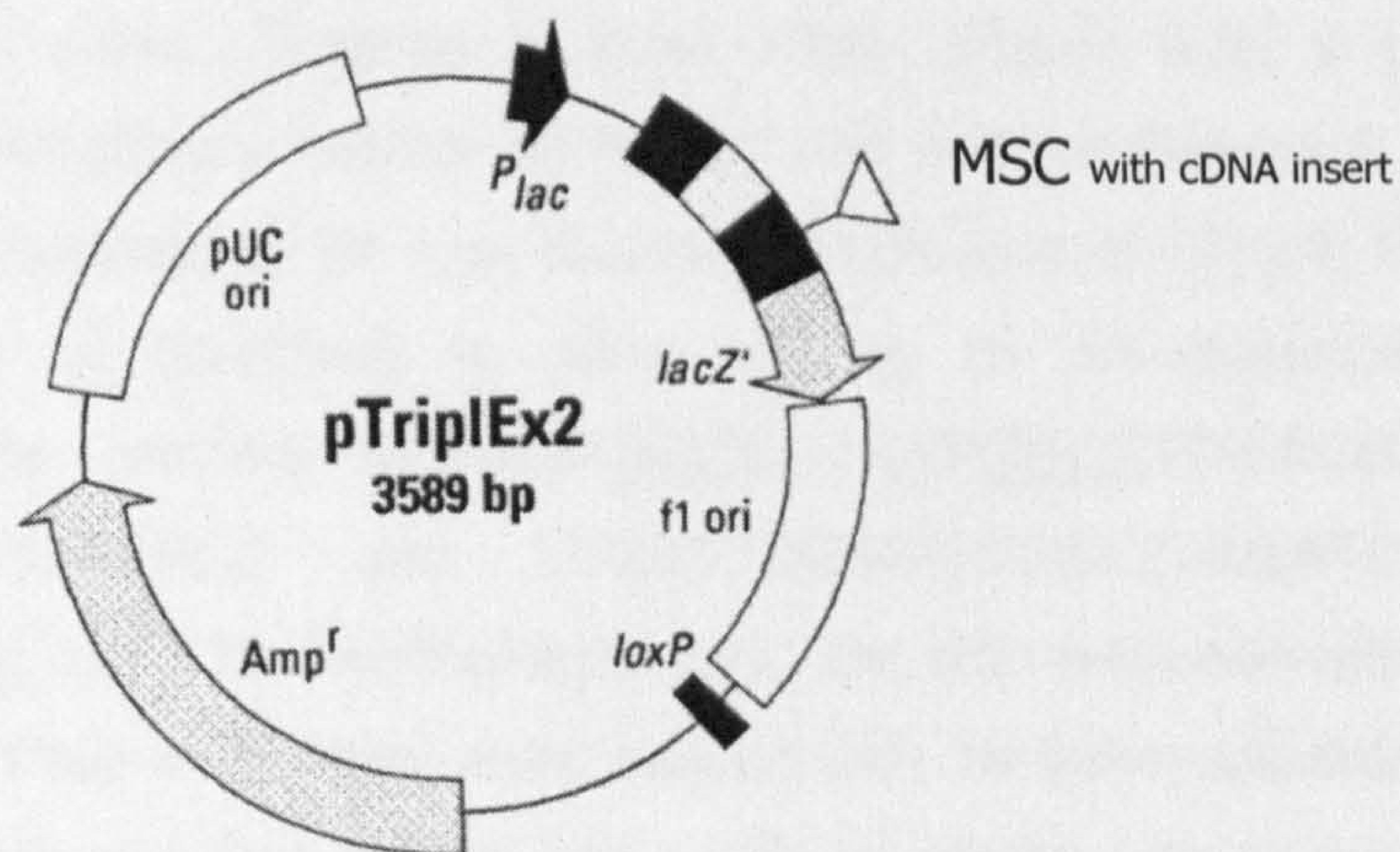


Figure 6.5: A schematic diagram of the λTriplEx2 vector (a) and pTriplEx2 phagemid (b).

Adapted from a figure in the SMART™ cDNA Library Construction Kit User Manual (Clontech).

Melanoma cDNA was cloned into the *Sfi*I sites of λTriplEx2 to create a λTriplEx2 library. Subsequent transduction of the λTriplEx2 library into *E. coli* strain BM25.8 promoted *in vivo* excision and circularisation of pTriplEx2 phagemid at the *lox*P sites. The phagemid pTriplEx2 library was then purified ready for mass excision of the library inserts to allow subcloning into the phage-display vector pJuFo-Sfi.

(Section 2.7.1). A large bacterial pellet was harvested by centrifugation of the culture at 5000 *g* for 30 min, and stored at –20°C. A large-scale phagemid preparation was subsequently made from the pellet using a Qiagen Plasmid Maxi Kit (Qiagen) as previously described (Section 2.7.2).

The cDNA library inserts were excised from 20 µg of pTriplEx2 using *Sfi*I digestion. The restricted vector was subjected to agarose electrophoresis in a 0.8% (w/v) agarose gel, and the *Sfi*I cDNA library fragments recovered using a Wizard PCR Preps DNA Purification System (Section 2.10). Recovered library fragments were dried down in a vacuum drier and resuspended in 10 µl TE buffer.

6.3.1.5 Subcloning of cDNA library fragments into the phage-display vector pJuFo-Sfi

Initially, the pJuFo vector (Crameri & Suter 1993) (Figure 6.6), a gift from Professor R. Crameri (Swiss Institute of Allergy and Asthma Research, Davos, Switzerland), was modified by Mr. Lee Shunburne (Division of Clinical Sciences (North), University of Sheffield) to allow cloning of *Sfi*I-restricted cDNA fragments. Briefly, primers 5'CTAGAGGCCATTATGGCCTGCAGGATCCG GCCGCCTCGGCCGGTAC3' and 5'CGGCCGAGGCGGCCGGATCCTGCA GGCCATAATGGCCT3' (Life Technologies Ltd.; the *Sfi*I restriction site coding sequence is underlined in the text) were treated with T4 polynucleotide kinase (Promega), annealed and then ligated into pJuFo restricted with enzymes *Xba*I and *Kpn*I.

To construct the melanoma phage-display cDNA library, approximately 0.5 µg of *Sfi*I-restricted cDNA inserts were ligated to 3 µg of *Sfi*I-digested pJuFo-Sfi phagemid in a 50-µl reaction at 16°C overnight (Section 2.11). A 2-µl aliquot of the ligation reaction was electroporated into electrocompetent *E. coli* XL1-Blue cells (Stratagene) (Table 2.1a) as described by the manufacturer. Briefly, a 50-µl aliquot of *E. coli* XL1-Blue cells was thawed, on ice, and added to a pre-chilled 0.1 cm electroporation cuvette (Invitrogen). The 2-µl aliquot of the ligation reaction was added to the cells and mixed by gentle pipetting. The cuvette was then placed into the electroporator ('*E. coli* pulser', Bio-Rad Laboratories Ltd.) and a charge of 1800 V applied. After electroporation, 2 ml of SOC medium (Life Technologies Ltd.) were added to the cells and the culture

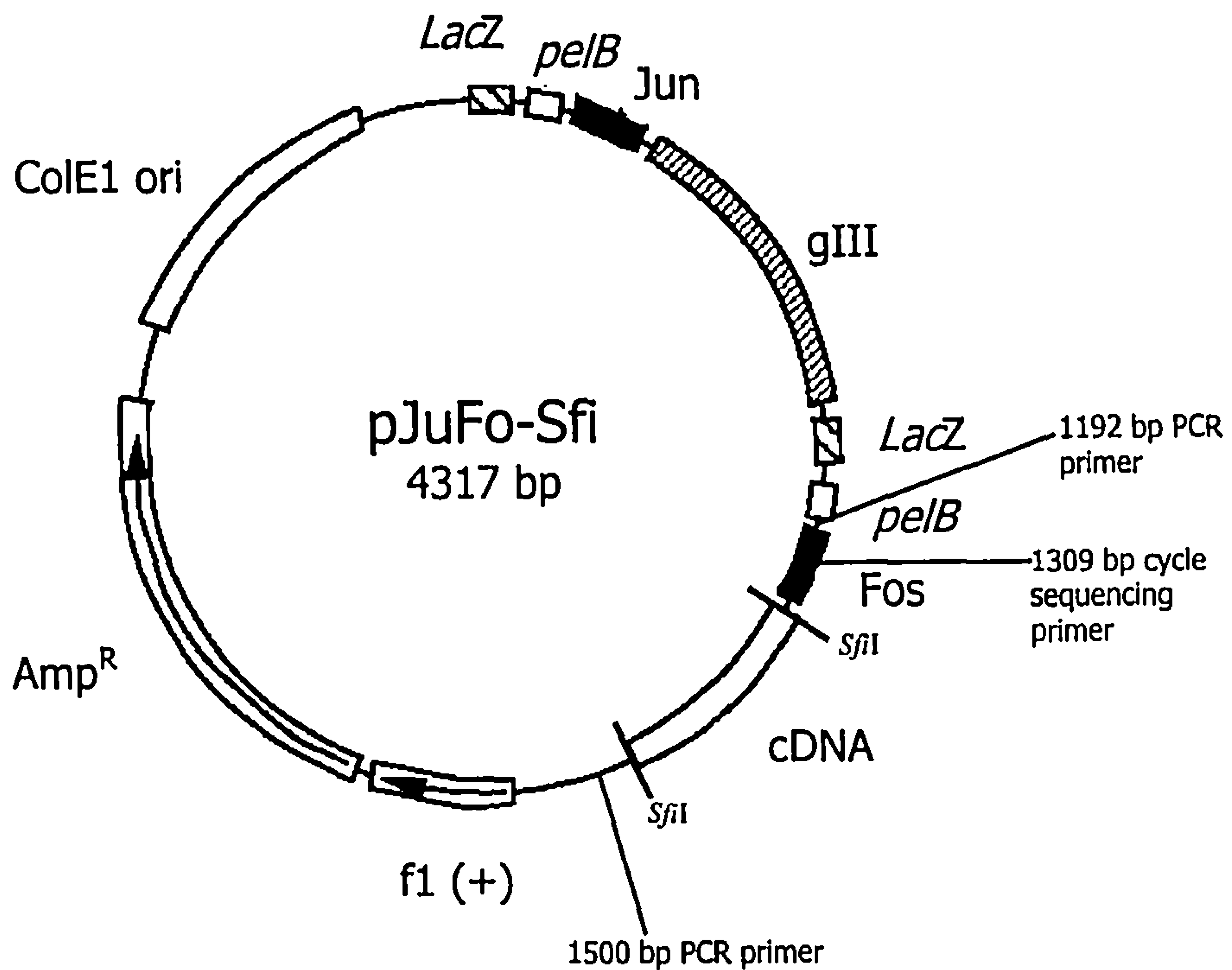


Figure 6.6: A schematic diagram of the pJuFo-Sfi vector.
(Adapted from Crameri & Suter 1993; Crameri *et al.* 1994).

The melanoma cDNA library inserts were cloned into the *Sfi*I sites of this vector to create a fusion with Fos. The annealing positions for the cycle sequencing primer and flanking PCR primers used for the analysis of recombinant phagemid cDNA inserts are indicated.

shaken at 200 rpm for 1 h at 37°C. Ligation efficiency was determined by plating aliquots of the culture onto LB agar containing 50 µg/ml ampicillin and 10µg/ml tetracycline. Five separate electroporations were performed and an approximate total of 1 x 10^6 ampicillin/tetracycline resistant independent clones was obtained.

6.3.1.6 Preparation of the cDNA phage-display library

In order to prepare the melanoma cDNA phage-display library, 100 ml of fresh LB medium supplemented with 50 µg/ml ampicillin and 10 µg/ml tetracycline were added to the pooled electroporated cells. The culture was then shaken at 200 rpm for 1 h at 37°C before superinfection with 1 x 10^{12} pfu of VCMS13 helper phage (Stratagene) at room temperature for 30 min. The culture was subsequently transferred to 400 ml of LB medium supplemented with 50 µg/ml ampicillin, 10 µg/ml tetracycline and 10 µg/ml kanamycin. After overnight incubation at 200 rpm and 37°C, the culture was centrifuged at 5000 rpm in an MSE Centaur 1 centrifuge (Sanyo Gallenkamp plc) for 20 min and phage particles precipitated from the supernatant by the addition of 0.2 volumes of 2.5 M NaCl/20% (w/v) polyethylene glycol 4000 at 4°C for 1 h. Phage were harvested by centrifugation at 5000 rpm for 20 min and resuspended in 2-3 ml of PBS containing 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, (pH 7.4). The phage titre was determined by infecting 2 ml of log-phase *E. coli* XL1-Blue (Stratagene) with an aliquot of the phage-display library, incubating at room temperature for 30 min and then plating out samples of the culture onto LB agar containing 50 µg/ml ampicillin and 10 µg/ml tetracycline. A titre of 1 x 10^{11} colony-forming units (cfu) was estimated.

6.3.2 Qualification of the cDNA library

6.3.2.1 Colony transfer of library-infected *E. coli* host to nylon membrane

In order to assess the quality of the melanoma cDNA library, transfer of library DNA from infected *E.coli* host to nylon membrane was performed. Briefly, 2 ml of log-phase *E. coli* XL1-Blue was infected with an aliquot of the phage-display library and incubated at room temperature for 30 min. A sample of the culture,

equivalent to 25,000 cfu, was then plated out onto LB agar containing 50 µg/ml ampicillin and 10 µg/ml tetracycline in a 20 cm^2 petri dish. Infected bacterial colonies were visualised after overnight incubation at 37°C. After pre-cooling the dish to 4°C, to ease subsequent removal of the nylon membrane without smearing of the colonies, a 20-cm^2 piece of Hybond N^+ nylon membrane (Amersham Pharmacia Biotech) was positioned above the dish and gently lowered onto the agar, allowing the middle of the membrane to touch the surface before the outer edges. The membrane was carefully removed after 1 min and placed, colony side up, on to a stack of 3MM chromatography paper soaked in denaturing solution (1.5 M NaCl; 0.5 M NaOH) for 7 min. The membrane was then placed on to a stack of 3MM chromatography paper soaked in neutralising solution (1.5 M NaCl; 0.5 M Tris HCl, pH 7.2; 0.1 mM EDTA), colony side up, for 3 min. The neutralisation step was repeated with fresh neutralisation solution for a further 3 min. The membrane was then washed vigorously in 2x saline sodium citrate (SSC; 30 mM NaCl; 33 mM Na-citrate) to remove bacterial debris, and air-dried on a piece of 3MM paper.

6.3.2.2 DNA hybridisation

Two nylon colony-lift filters were hybridised with a [^{32}P]-labelled cDNA probe for either a ubiquitous gene, β-actin, or for the melanocyte-specific gene tyrosinase, in order to assess the frequency of cDNAs encoding these proteins in the library.

The β-actin (full-length cDNA available in the laboratory) and tyrosinase (full-length cDNA fragment excised from pcDNA3TYR (Kemp *et al.* 1997b) with *Kpn*I and *Xba*I), cDNAs were 5'-end labelled with [γ-^{32}P]ATP (ICN Pharmaceuticals Ltd.) using T4 polynucleotide kinase. The 20-µl labelling reaction was comprised of 1 µg of the relevant oligonucleotide, 10 µCi [γ-^{32}P]ATP (10 mCi/ml; 3000 Ci/mmol), 0.1 volumes of 10x T4 Polynucleotide kinase buffer and 10 U of T4 Polynucleotide kinase, was incubated at 37°C for 10 min followed by heating to 70°C for 10 min to inactivate the kinase enzyme.

Each filter was prehybridised at 55°C in ExpressHyb hybridisation solution (Clontech) for 1-2 h. Filters were then hybridised by the addition of 1µl of either the [^{32}P]β-actin or [^{32}P]tyrosinase cDNA probe for 4-5 h. The filters were subesquently washed twice at 65°C with 2x SSC, 1% (w/v) SDS for 30 min, and at least twice with 1x SSC, 1% (w/v) SDS for 15 min. Further washes

were performed, if necessary, until there was an appreciable reduction in the radioactivity detected with a Geiger counter in the discarded washes, which represents the unbound probe.

Subsequently, filters were subjected to autoradiography for 1-12 h at -70°C and the number of positive colonies counted and expressed as a percentage of the total number of colonies originally transferred to the filter.

The frequency of *E. coli* colonies infected with phage containing β-actin cDNA was found to be 0.03%. The frequency of *E. coli* colonies infected with phage containing tyrosinase cDNA was found to be 0.2%.

6.3.3 Patients

Sera from 10 vitiligo patients (3 male, 7 female; mean age: 52 yr; age range: 23-77 yr) were used in this study (Table 6.1). None of these patients had a related autoimmune disorder or family history of autoimmune disease. All of these vitiligo sera contained antibodies shown to have reactivity with protein bands from Skmel23 melanoma cell line extracts at either 100, 70 or 50 kDa in immunoblotting experiments performed by Dr. E. Helen Kemp (Division of Clinical Sciences (North), University of Sheffield, unpublished data).

6.3.4 Isolation and biotinylation of IgG

IgG was isolated from the sera of patients by protein G Sepharose 4 Fast Flow affinity column chromatography (Amersham Pharmacia Biotech). Eluted IgG fractions were concentrated using an Amicon Concentrator (Amicon Inc., Beverley, MA, U.S.A.). The concentrated IgG was filter-sterilised with a Millex® Filter Unit (Millipore Corp.) and the final concentration measured by photometry at 280 nm. Biotinylation of the IgG was performed using EZ-Link™ Sulfo-NHS-LC-LC-Biotin (Pierce, Rockford, IL, U.S.A.), according to the manufacturer's protocol. Briefly, a 1mg/ml solution of Sulfo-NHS-LC-LC-Biotin was made up immediately prior to setting up the reaction, which comprised 2 mg of IgG sample in 1 ml PBS and 75 µl of the Sulfo-NHS-LC-LC-Biotin solution. The reaction was mixed gently and allowed to proceed for 2 h on ice, after which

Table 6.1: Details of the vitiligo patients used in this study.

Patient	Gender	Age at sample date	Age at onset	Disease duration	Size of immuno-reactive protein/s in Skmel23 cell extract blots[1]	Vitiligo sub-type
1	Male	68 yr	58 yr	10 yr	100 kDa[2]	Symmetrical and peri-orificial
2	Female	43 yr	31 yr	12 yr	70 kDa	Symmetrical
3	Male	77 yr	70 yr	7 yr	50 + 70 kDa	Symmetrical and peri-orificial
4	Female	54 yr	21 yr	33 yr	50 + 100 kDa	Symmetrical and peri-orificial
5	Female	52 yr	46 yr	6 yr	50 + 70 kDa	Symmetrical
6	Female	40 yr	31 yr	9 yr	50 + 70 kDa	Symmetrical and peri-orificial
7	Female	70 yr	70 yr	<1 yr	70 kDa	Symmetrical
8	Female	58 yr	54 yr	4 yr	50 kDa	Segmental
9	Male	23 yr	22 yr	1 yr	70 kDa	Peri-orificial
10	Female	37 yr	10yr	27yr	70 kDa	Symmetrical and segmental

[1]Unpublished data from western blotting experiments performed by Dr E. Helen Kemp (Division of Clinical Sciences (North), University of Sheffield).

[2]kDa, kilodalton.

time unreacted biotin was removed by dialysis against fresh PBS. All IgG samples were stored at 4°C until required.

6.3.5 Paramagnetic bead preparation

Dynabeads® M-280 Streptavidin (Dynal Biotech, Oslo, Norway) were used to immobilise biotinylated IgG during the isolation of phage displaying antibody-binding peptides. For each experiment, a 20-µl (10 mg/ml) sample of beads was washed extensively with 500-µl aliquots of PBS containing Tween 20 at 0.05% (v/v). A Dynal Magnetic Particle Concentrator (Dynal Biotech) was used to separate the beads after each wash and the beads were finally resuspended in 235 µl of sterile water.

6.3.6 Isolation of phage displaying IgG-binding peptides (biopanning)

A 15-µl aliquot of biotinylated IgG was incubated with 235 µl of prepared Dynabeads® and incubated at 4°C for 30 min on a rotating platform to permit antibody-bead binding. The antibody sample used was a pool from ten vitiligo patients with each biotinylated IgG at a concentration of 2 mg/ml. To block any non-specific phage binding to the beads later in the procedure, 300 µl of 2% (w/v) dried milk in PBS containing 10% (v/v) sterile glycerol was added to the bead-IgG suspension and incubation at 4°C continued for 1 h. The bead-IgG complexes were separated from the blocking buffer using a Dynal Magnetic Particle Concentrator, washed twice and finally resuspended in 150 µl of PBS/0.05% (v/v) Tween 20 before the addition of a 100-µl sample of phage-display library containing 1 x 10^{10} cfu. The suspension was then incubated overnight at 4°C to allow interaction of the antibody-bead complexes with peptides displayed on the surface of the phage particles.

The bead-IgG complexes were washed extensively with PBS/0.05% (v/v) Tween 20 to remove any unbound phage. Bound phage were eluted from the bead-IgG complexes with 150 µl of 100 mM HCl (adjusted to pH 2.2 with solid glycine), and the beads then magnetically separated from the supernatant that contained the phage particles. Neutralisation of the supernatant was accomplished by the addition of 9 µl of 2 M Tris-HCl (pH 7.6). The phage

suspension was subsequently used to infect 2 ml of exponentially growing *E. coli* XL1-Blue for 15 min at room temperature. Aliquots of the infected cells were then plated onto LB agar containing 50 µg/ml ampicillin and 10 µg/ml tetracycline to allow the recovery of pJuFo phagemids with cDNA inserts.

To generate phage for a further round of selection, the infected *E. coli* XL1-Blue culture was made up to 10 ml with fresh LB medium and incubated at 37°C with shaking for 1 h before superinfection with 1 x 10^{12} pfu of helper phage VCSM13. Following incubation at room temperature for 30 min, the cells were transferred to 100 ml of LB containing 50 µg/ml ampicillin, 10 µg/ml tetracycline and 10 µg/ml kanamycin. The culture was then incubated with shaking overnight at 37°C. Phage were prepared from the infected culture and titred as described above and stored at –20°C. This first round library enriched in phage displaying IgG-binding peptides was used in a second round of selective enrichment as detailed above. In all, three rounds of biopanning were undertaken.

6.3.7 Analysis of recombinant pJuFo phagemid by PCR amplification

Individual colonies, isolated from the third round of biopanning by plating out the infected *E. coli* XL1-Blue, were grown and pJuFo phagemid prepared using a Wizard Minipreps DNA Purification System (Section 2.7.1) . Phagemid DNA (50 ng samples) was subjected to 36 cycles of PCR amplification as described previously (Section 2.13) with primers 1192 5'CCGCTGGATTGTTATTACTCGC TG3' and 1500 5'TGCAAGGCGATTAAGTTGGGTAAC3' (Life Technologies Ltd.), which flank the *Xba*I-*Kpn*I cloning sites in pJuFo, using the following conditions: 94°C, 1 min; 55°C, 1 min; 72°C, 2 min; and 72°C for 10 min for termination. An aliquot of the PCR amplification products were analysed by electrophoresis in 0.8% (w/v) agarose gels (Section 2.9) to confirm the presence of a cDNA insert and the remaining reaction directly purified according to a Wizard PCR Preps DNA Purification System (Section 2.14) prior to DNA sequencing.

6.3.8 Sequencing and sequence analysis

Sequencing of PCR amplification products was performed according to a Thermo Sequenase Cycle Sequencing Kit (USB Corp.) with [γ-^{32}P]ATP (ICN Pharmaceuticals Ltd.) and primer 5'-CCGAAATCGCGAA CCTGCTG-3' (Life Technologies Ltd.) which lies upstream of the *Xba*I cloning site in pJuFo. Briefly, 20 pmol of the primer was 5'-end labelled using 30 µCi [γ-^{32}P]ATP (10 mCi/ml; 3000 Ci/mmol) as previously described (Section 6.3.2.2). One microlitre of the radiolabelled primer was then used in a cycle sequencing reaction, which consisted of 4 µl PCR amplified template, 2 µl reaction buffer (30 mM Tris HCl, pH 9.5; 7 mM $MgCl_2$), 8 U of Thermo Sequenase polymerase and dH_2O to 17.5 µl. The reaction was divided between four tubes containing 4 µl of either ddGTP (150 µM each dATP, dCTP, 7-deaza-dGTP, dTTP, 1.5 µM ddGTP), ddATP (as ddGTP, except 1.5 µM ddGTP replaced with ddATP), ddTTP or ddCTP termination mix, mixed and overlaid with 10 µl mineral oil. The reaction tubes were then placed in a thermal cycler and subjected to 40 cycles using the following conditions: 95°C, 30 sec; 55°C, 30 sec; 72°C, 1 min 30 sec. When cycling was complete, 4 µl of stop solution (95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF) to each of the termination reactions. Samples were stored frozen until use.

Sequenced DNA was subjected to electrophoresis in 6% (w/v) acrylamide/7M urea gels with glycerol tolerant gel buffer (USB Corp.). Samples were heated to 75°C immediately prior to loading on to gel, and 2-3 µl of the sample termination reaction was loaded on each gel lane. Gels were run for 3-4 h at 2000 V, dried on 3MM chromatography paper and were subjected to autoradiography for 12 h at -70°C. The cDNA sequences were compared with international databases (Genbank) using the BLAST network service of the National Centre for Biotechnology Information (NCBI, National Institutes of Health; Bethesda, MD, U.S.A.).

6.4 Results

6.4.1 Construction of the melanoma cDNA phage-display library

A melanoma cDNA library was initially constructed in a λ-phage expression vector, λTriplEX2. The primary size of independent clones in the original λ-phage library was estimated to be 1 x 10^6 and displayed >99% recombinance assessed by blue/white screening. Following library amplification, a titre of 1 x 10^{10} pfu/ml was achieved. The λ-phage library was then converted into a phagemid pTriplEX2 library, prior to mass excision and subcloning of cDNA inserts into the phage-display vector pJuFo.

The primary size of the pJuFo melanoma cDNA library was 1 x 10^6 independent clones. The titre was assessed by infecting *E. coli* XL1-Blue with the pJuFo library and then plating out the bacteria onto LB agar containing ampicillin and tetracycline. In order to determine the frequency of recombinant phagemid in the pJuFo library, ten individual cfus were picked and grown and the pJuFo phagemid isolated from each culture. Analysis of the phagemids by PCR amplification with primers 1192 and 1500, indicated that 9/10 (90%) of phagemids in the pJuFo library contained a cDNA insert (Figure 6.7). Following amplification with helper phage, a stock phage-display library with a titre of 1 x 10^{11} cfu/ml was produced. The frequency of recombinant phagemid in the phage-display library was determined by picking and growing forty individual cfu and isolating the pJuFo phagemid from each culture. Analysis of the phagemids by PCR amplification with primers 1192 and 1500, indicated that 36/40 (90%) of pJuFo phagemids in the phage-display library contained an inserted cDNA fragment. Figure 6.8 demonstrates that the cloned fragments ranged in size from 800 to ~2,500 bp in length, with the majority of phagemids containing an insert of 1500 bp. Hybridisation experiments revealed that the frequency of β-actin and tyrosinase inserts in the library was 0.03% and 0.2%, respectively, indicating that the library was relatively complex. Although tyrosinase cDNA had a comparatively high abundance, this was as expected in a library derived from deeply pigmented cells. This phage-display library was used for the selective enrichment of IgG-binding proteins from vitiligo patients.

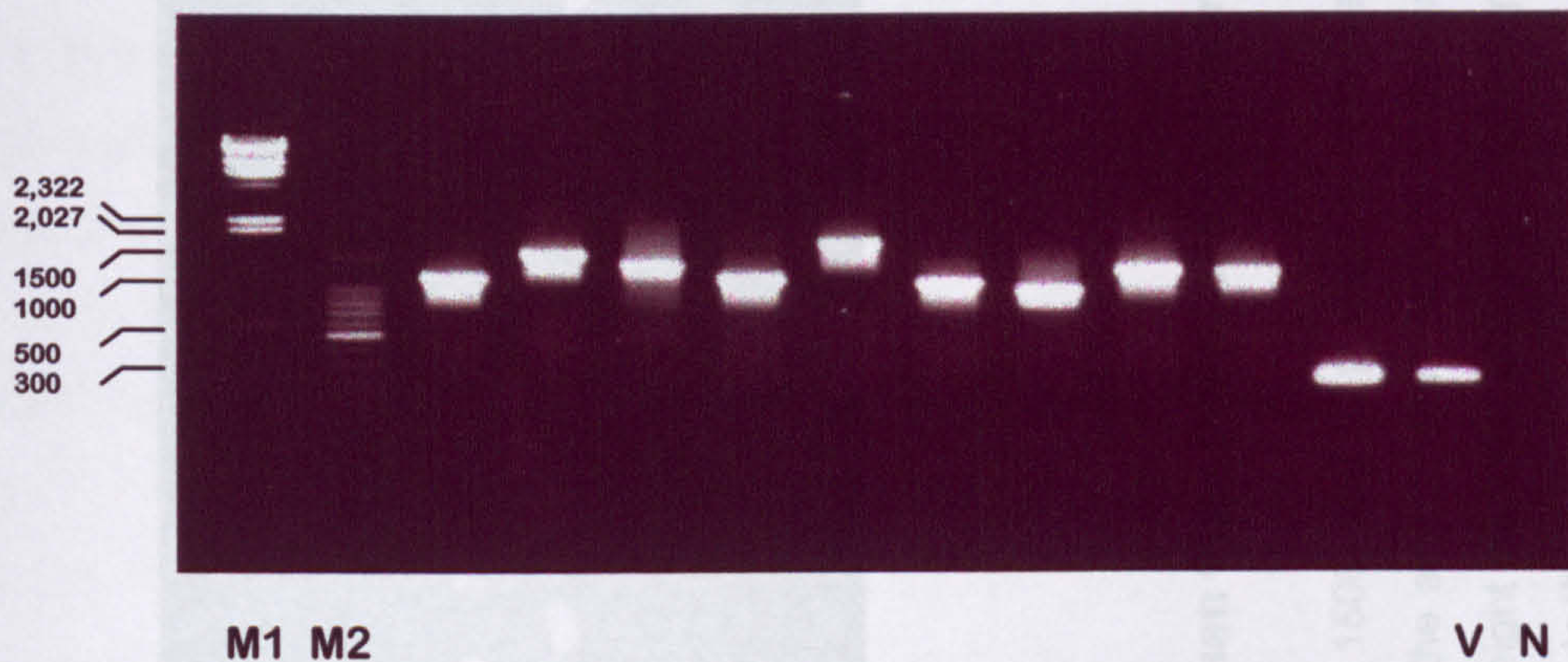


Figure 6.7: PCR amplification of the inserts of 10 clones randomly chosen from the primary pJuFo melanoma library.

PCR amplification of pJuFo phagemid clones was carried out using primers 1192 and 1500, and 10-µl aliquots of the resulting products were subjected to agarose gel electrophoresis in a 0.8% agarose gel. Lanes are included containing the amplification product of the empty vector pJuFo (V; 308 base pairs), a negative control reaction with no DNA template (N), and both Lambda *Hind*III DNA molecular weight markers (M1) and 100 base pair molecular weight markers (M2) (Promega). The sizes (base pairs) of the relevant molecular weight markers are shown to the left of the figure.

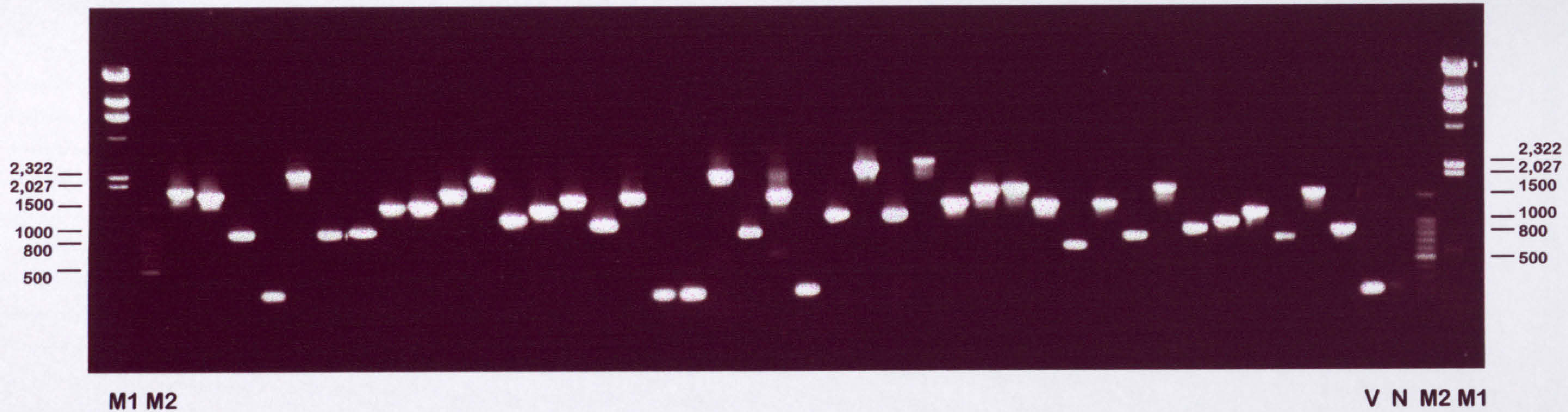


Figure 6.8: PCR amplification of the inserts of 40 clones randomly chosen from the pJuFo melanoma phage-display library.

PCR amplification of pJuFo phagemid clones was carried out using primers 1192 and 1500, and 10-µl aliquots of the resulting products were subjected to agarose gel electrophoresis in a 0.8% agarose gel. Lanes are included containing the amplification product of the empty vector pJuFo (V), a negative control reaction with no DNA template (N), and both Lambda *Hind*III DNA molecular weight markers (M1) and 100 base pair molecular weight markers (M2) (Promega). The sizes (base pairs) of the relevant molecular weight markers are shown to the left and right of the figure.

6.4.2 Enrichment of phage displaying IgG-binding peptides

The melanoma cDNA phage-display library was subjected to three rounds of biopanning against a pool of biotinylated IgG from ten vitiligo patients who had no other autoimmune disease. Three rounds of enrichments were performed and twenty-four individual cfus were picked and grown from each round, and the pJuFo phagemid isolated from each culture. Analysis of the phagemids by PCR amplification with primers 1192 and 1500 is shown in Figure 6.9 and Table 6.2. A divergence on a particular cDNA insert size was not observed and the range of inserts sizes remained similar during biopanning. The percentage of recombinant phage decreased between the unselected and enriched libraries, which might have been due to a selective growth advantage of phage particles bearing empty phagemid and therefore not required to secrete a fusion protein.

Forty individual ampicillin/tetracycline resistant bacterial colonies from the third round of enrichment were picked and grown for sequence analysis. Phagemid DNA was isolated from each culture and analysed by PCR amplification with 1192 and 1500 primers. The PCR amplification products from thirty recombinant phagemid were purified and sequenced in order to aid identification of the cDNAs by BLAST searches of international databases (Genbank). Of the 30 clones sequenced, 28/30 (93%) showed homology with DNA sequences present in the databases. Of these homologies, 24/28 (86%) corresponded to known genes and the remaining 4/28 (14%) to unidentified human cDNA. Approximately one quarter of the clones with correspondence to known genes encoded proteins that had not yet been assigned an identity or function. The results of the sequence analysis are summarised in Table 6.3.

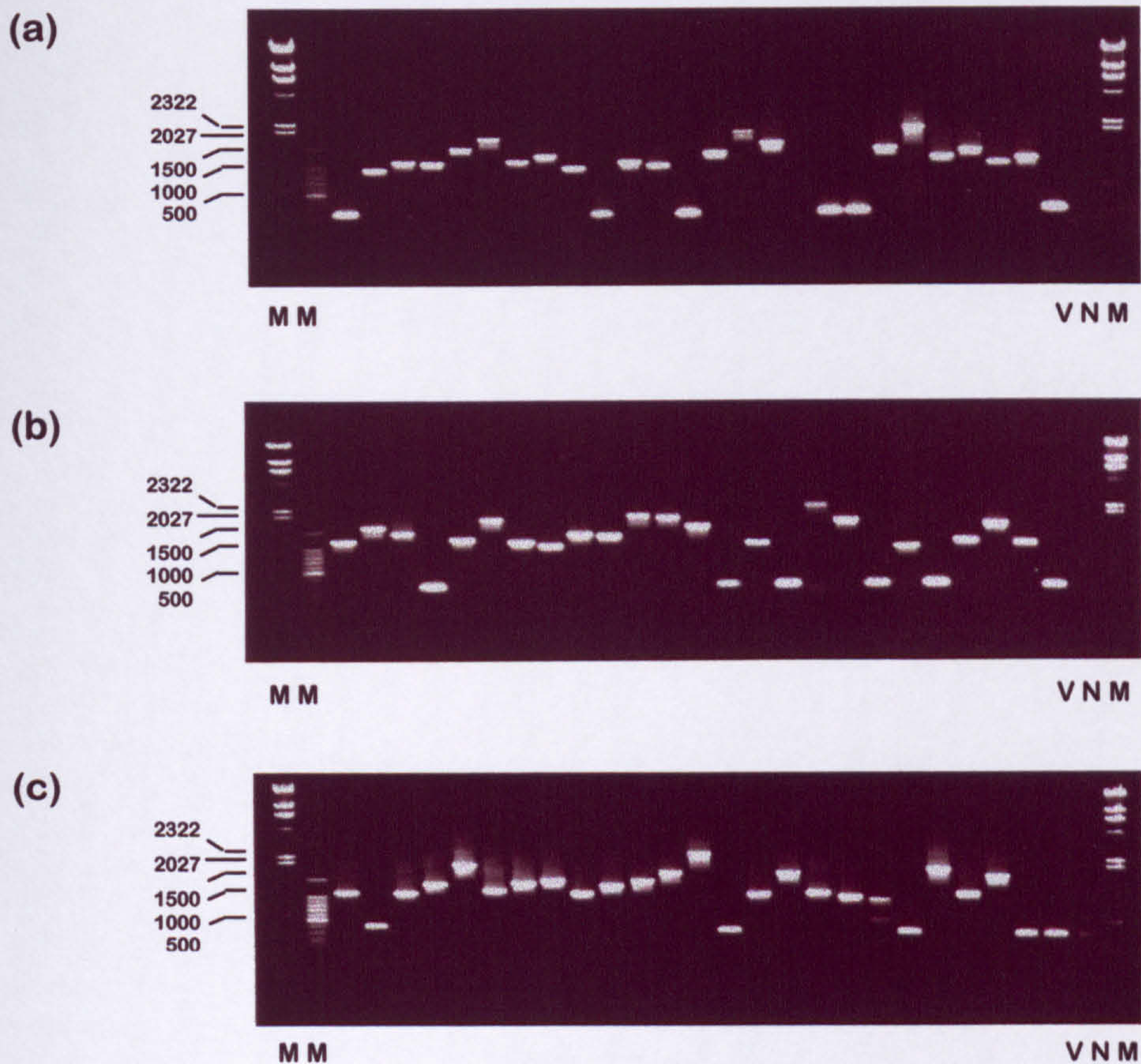


Figure 6.9: PCR amplification of phagemid cDNA inserts from three libraries selected by biopanning using vitiligo patient IgG: Round 1 (a), Round 2 (b) and Round 3 (c).

Phagemid DNA was prepared from twenty-four individual ampicillin and tetracycline resistant library colonies from each round of selection and subjected to PCR amplification using primers 1192 and 1500. Ten-microlitre aliquots of the resulting products were subjected to agarose gel electrophoresis in a 0.8% agarose gel. Lanes are included which contain the amplification product of the empty vector pJuFo (V), a negative control amplification with no DNA template (N), and Lambda *Hind*III DNA and 100 base pair molecular weight markers (M) (Promega). The sizes (base pairs) of the relevant molecular weight markers are shown to the left of the figure.

Table 6.2: Analysis of phagemid cDNA inserts from three libraries selected by biopanning using vitiligo patient IgG.

	No. of phage screened	No. of phage eluted after biopanning	% phage eluted after biopanning	No. of phage analysed by PCR	% recombinant phage determined by PCR	Avergae size of cDNA insert (bp)[1]	Range of sizes of cDNA inserts (bp)
Unselected	–	–	–	40	36/40 (90%)	1500	800--~2500
Round 1	1.30×10^{11}	7150	5.5×10^{-6}	24	19/24 (79%)	1000-1500	1000-2300
Round 2	3.33×10^{10}	1200	3.6×10^{-6}	24	19/24 (79%)	1000-1500	1000--~2500
Round 3	2.25×10^{10}	100	4.4×10^{-7}	24	20/24 (83%)	1000-1500	800--~2500

[1]bp, base pairs.

Table 6.3: cDNAs isolated from selective enrichment of the melanoma cDNA phage-display library with vitiligo IgG.

Protein encoded by cDNA	No. of library clones	Genbank accession number	Size of insert carrying cDNA (bp)[1]	Previously identified as an autoantigen	Reference
Ubiquitin-conjugating enzyme	2/30	BC000468	900	No	
Integrin beta-4 binding protein	2/30	XM009691	1000-1500	No	
Osteopontin	2/30	XM011125	1000-1500	Type 1 diabetes mellitus. Rheumatoid arthritis.	Fierabracci *et al.* 1999. Sakata *et al.* 2001.
Translation elongation factor 1 alpha	1/30	BC006102	1500-2000	Systemic lupus erythematosus.	Frampton *et al.* 2000.
Heat shock protein 90	1/30	D87666	800	Systemic lupus erythematosus. Melanoma with MAH[2].	Conroy *et al.* 1994. Kiniwa *et al.* 2001.
Alpha-enolase	1/30	BC004458	1500-2000	Systemic lupus erythematosus	Pratesi *et al.* 2000.
Gamma-enolase	1/30	BC002745	1000-1500	No	
GTP-binding protein SAR1	1/30	BC003658	1500-2000	No	
Melanin-concentrating hormone receptor 1	1/30	U71092	1500-2000	No	
Ribosomal protein L9	1/30	NM000661	800	No	
Ribosomal protein L24	1/30	AF212226	1500	No	
Translation initiation factor- 2	1/30	BC002513	1500-2000	Melanoma with MAH	Kiniwa *et al.* 2001.
Signal sequence receptor	1/30	XM044177	1500	No	
PTD 017	2/30	AAH05373	1000	No	
PTD 010	1/30	XM043589	1000-1500	No	
CGI-34	1/30	AF132968	1500	No	
CGI-107	1/30	NM016045	1500	No	
CGI-115	1/30	BC020641	1500-1000	No	
My 028	1/30	AF061731	1000	No	
Un-named protein	1/30	XM041928	1500	-	
Unidentified	1/30	XM031540	1500	-	
Unidentified	1/30	AL136941	800	-	
Unidentified	1/30	AC002044	800	-	
Unidentified	1/30	AC006992	1000	-	
No significant matches	1/30	-	1500-2000	-	
No significant matches	1/30	-	1000-1500	-	

[1] bp, base pairs. [2] MAH, melanoma-associated hypopigmentation.

6.5 Discussion

The combination of affinity selection and biological amplification employed in phage-display technology makes it a particularly useful technique for the isolation of novel antigens. Large cDNA libraries, containing up to 10^{11} independent clones, can be screened in a single experiment and IgG-binding peptides identified, even if their expression in the original library was rare, through repeated rounds of enrichment. Furthermore, displayed proteins are thought to assume native structure (Skerra & Pluckthun 1988) and are screened in liquid-phase in contrast to conventional immunoscreening, which requires adsorption of the proteins to nitrocellulose membranes. Indeed, a phage-display strategy has been recently applied to the discovery of antigen in chronic human inflammatory CNS disease (Burgoon *et al.* 2001). Additionally, phage-display based on the pJuFo system has been successfully employed in this laboratory for the isolation of autoantigens in SLE (Kemp *et al.*, unpublished data, Division of Clinical Sciences (North), University of Sheffield) and polymyositis (Herd *et al.*, unpublished data, Division of Clinical Sciences (North), University of Sheffield).

Several putative antigens were enriched from the melanoma library by their ability to bind IgG from vitiligo patients (Table 6.3). A number of these proteins had been characterised, or were structurally related to proteins that had been characterised, as autoantigens in other disorders. Two distinct isoforms of the glycolytic enzyme enolase were enriched in the third round of selection. Alpha-enolase is ubiquitously expressed in both cytoplasm and cell membrane (Moscato *et al.* 2000; Gitlits *et al.* 2001) and has been described as an autoantigen in SLE (Pratesi *et al.* 2000). Gamma-enolase has not previously been reported as an autoantigen and its expression is thought to be limited primarily to neurons (Gitlits *et al.* 2001). However, since melanocytes are originally derived from the neural crest and are capable of neuronal behaviour, such as the synthesis of neurochemicals (Iyengar 1989), its presence in the library is perhaps not unsurprising. Heat-shock protein 90 was also enriched by vitiligo IgG and has been identified as an antigen in SLE (Conroy *et al.* 1994). Furthermore, the heat shock proteins have been implicated in the initiation of autoimmune disorders (Elson & Thompson 1994) and the presence of

antibodies to this highly conserved protein in patients with vitiligo may represent a marker of autoimmunity. Additionally, vitiligo IgG enriched osteopontin, a calcium-binding glycoprotein recently described as an autoantigen in type 1 diabetes mellitus (Fierabracci *et al.* 1999) and rheumatoid arthritis (Sakata *et al.* 2001). A further candidate antigen was the GTP-binding protein SAR1 and antibodies to a melanocyte-expressed GTP-binding protein rab38 have been previously demonstrated in melanoma patients (Jager *et al.* 2000). Interestingly, a recent study detected antibodies to two of the vitiligo putative antigens reported here, heat shock protein 90 and translation initiation factor 2, by screening of a λ-phage cDNA library also derived from Skmel23 cells with sera from a melanoma patient with melanoma-associated hypopigmentation (Kiniwa *et al.* 2001).

In addition, three putative antigens with an association to epidermal and melanocyte function were isolated. Firstly, the ubiquitin-conjugating enzyme huBC9 which has been demonstrated to mediate degradation of the microphthalmia-associated transcription factor (MITF), a protein which is known to regulate transcription of genes involved in melanogenesis (Xu *et al.* 2000). Secondly, a binding protein was enriched that is specific to integrin beta-4, an adhesion molecule shown to be vital for attachment of the epithelial cells to the basal layer of the epidermis (van der Neut *et al.* 1996). Finally, the G protein-coupled receptor for melanin-concentrating hormone, which may play a role in the regulation of melanocyte behaviour and, indeed, was recently shown to have an inhibitory affect on alpha melanocyte-stimulating hormone (α-MSH)-induced melanogenesis (Hoogdijn *et al.* 2001). The melanin-concentrating hormone receptor was selected for further study of its immunoreactivity with vitiligo antibodies because of its possible regulatory function in skin pigmentation.

The diversity of clones still apparent in the cDNA library after three rounds of biopanning suggests that further rounds of selection might be necessary to demonstrate dominant enrichment of a specific IgG-binding protein. Additionally, the numbers of phage displaying IgG-binding proteins eluted from each subsequent round of biopanning decreased (Table 6.2). The poor elution could be attributed to a rare abundance of antigens in the original library and to the use of a relatively dilute pool of patient IgG, in which individual titres of autoantibodies may be low. Nevertheless, a profile of sequences was

revealed which includes proteins with previously demonstrated IgG-binding properties and association with autoimmune disease, and a candidate autoantigen with a proposed specific function in melanocytes.

Although these results are encouraging, phage-display does have limitations in its application to antigen discovery. Surface-protein expression may be prevented by a number of factors, therefore reducing the proportion of the cDNA library inserts represented by the phage particles. The displayed proteins must be efficiently secreted through the inner membrane of E. coli, fold into their correct three-dimensional structures in the periplasmic space and maintain their correct configuration on the surface of the phage particle after exposure to an oxidising environment (Sambrook & Russell 2001). Furthermore, proteins that naturally associate with membranes may not be able to pass into periplasm, and proteins containing charged residues might not be secreted (Wilson & Finlay 1998). An additional problem is that the computer analysis of the enriched proteins can only be as comprehensive as the available sequence databases. The complexity of the library is also limited by the quality of the cDNA inserts, which may not possess an open reading frame, and by its source messenger RNA. It would have been preferable here to use a melanocyte, rather than melanoma, cell line in order to better represent the proteins expressed by melanocytes in vitiligo.

In summary, the use of phage-display appears to be applicable to the isolation of peptides which are bound by antibodies from vitiligo patients. However, further experiments are needed to confirm the reactivity of the putative autoantigens to individual vitiligo sera. This can be achieved by testing antibody binding to [^{35}S]-labelled antigen produced in the TnT® system (Section 4.3.1.4) in radioimmunoassay. Due to its reported effects on melanocyte behaviour, the melanin-concentrating hormone receptor was singled out for further analysis in the subsequent chapter.

7 An investigation of autoantibody responses to the melanin-concentrating hormone receptor 1 in vitiligo patients

7.1 Introduction

7.1.1 The melanin-concentrating hormone

Melanin-concentrating hormone (MCH) is a cyclic peptide originally identified in the pituitary gland of teleost fish by its ability to induce aggregation of pigment granules thereby lightening the appearance of the scales (Kawauchi *et al.* 1983). The physiological roles of MCH in mammals have yet to be fully elucidated but since it is highly expressed in regulatory centres of the brain, particularly the hypothalamus, and throughout the central and peripheral nervous systems, it is thought to act as a neuromodulator or neurotransmitter mediating a broad range of behavioural responses (Skofitsch *et al.* 1985; Bittencourt *et al.* 1992; Mouri *et al.* 1993; Knigge *et al.* 1996). Most functional studies of MCH have concentrated on its influence on feeding (Qu *et al.* 1996), a role which is highlighted by the lean phenotype created by knocking-out the function of the receptor in transgenic mice (Shimada *et al.* 1998) and by the obese phenotype of mice engineered to over-express MCH (Ludwig *et al.* 2001). The action of MCH on the regulation of pigmentation in higher vertebrates, including humans, is currently unknown. A recent study demonstrated expression of MCH throughout human epidermis and showed a partial inhibition of α-melanocyte-stimulating hormone (α-MSH)-induced melanogenesis by MCH in human melanocytes (Hoogdijn *et al.* 2001). Some of the characterised physiological functions of MCH, with reference to the antagonistic effects of MCH on α-MSH, are shown in Table 7.1. Recently, several studies have identified a somatostatin-like receptor SLC-1 (Kolakowski *et al.* 1996) as the MCH-receptor, which was consequently renamed MCHR1 (Bachner *et al.* 1999; Chambers *et al.* 1999; Saito *et al.* 1999; Shimomura *et al.* 1999).

Table 7.1: Physiological functions influenced by MCH[1] and α-MSH[2].

Location	Ligand	Receptor	Function	Reference
Fish melanophore	Salmon α-MSH	Not characterised	Pigment dispersion.	Kawauchi *et al.* 1983.
	Salmon MCH	Not characterised	Pigment aggregation.	
Human melanocyte	Human α-MSH	MC1R[3]	Increases $[Ca^{2+}]_i$[6]. Melanogenesis.	Hoogdijn *et al.* 2001.
	Human MCH	MCHR1[4]	Decreases α-MSH stimulated $[Ca^{2+}]_i$. Partially inhibits α-MSH induced melanogenesis.	
Mouse melanoma cells	Rat α-MSH	MC1R	Melanogenesis.	Ludwig *et al.* 1998.
	Rat MCH	?	No apparent effect on melanogenesis.	
Mammalian brain	Rat α-MSH	MC4R[5]	Inhibits feeding.	Ludwig *et al.* 1998.
	Rat MCH	MCHR1	Increases feeding.	

Adapted from Saito *et al.* (2000).

The large brackets indicate a functionally antagonistic relationship between MCH and α-MSH.

[1] MCH, melanin-concentrating hormone. [2] α-MSH, α-melanocyte-stimulating hormone.
[3] MC1R, melanocortin 1 receptor. [4] MCHR1, melanin-concentrating hormone receptor 1.
[5] MC4R, melanocortin 4 receptor. [6] $[Ca^{2+}]_i$, intracellular calcium.

7.1.2 Melanin-concentrating hormone receptor 1 structure and expression

The melanin-concentrating hormone receptor 1 (MCHR1) is a G protein-coupled receptor (Bachner *et al.* 1999; Chambers *et al.* 1999; Saito *et al.* 1999; Shimomura *et al.* 1999) that specifically binds MCH. The G protein-coupled receptors are classified in a superfamily of membrane proteins, with a distinguishing arrangement of seven transmembrane domains, encompassing hundreds of receptors for a diverse range of chemical messengers including hormones and neurotransmitters (Iismaa & Shine 1992; Eckard & Beck-Sickinger 2000). Whilst the precise structure of the MCHR1 protein remains to be determined, its primary amino acid sequence is most similar to that of the somatostatin and opioid receptor subfamilies (An *et al.* 2001). Characteristically, G protein-coupled receptors require a guanine triphosphate (GTP)-binding protein, or G protein, in order to signal through a number of intracellular pathways (Hawes *et al.* 2000). The MCHR1 is capable of coupling to multiple types of G protein, which can lead variously to the activation of the MAP kinase signalling pathway, to an increase in the level of intracellular calcium ($[Ca^{2+}]_i$) and to a decrease in cyclic adenosine monophosphate (cAMP) when bound by MCH (Hawes *et al.* 2000).

The expression of MCHR1 has been detected in numerous regions of the brain (Kolakowski *et al.* 1996; Bachner *et al.* 1999; Chambers *et al.* 1999; Saito *et al.* 1999; Hervieu *et al.* 2000; Kokkotou *et al.* 2001) with relatively high expression in the hypothalamus. Moderate expression has also been noted in the eye, skeletal muscle and tongue of the rat, although the function of the receptor at these locations is unknown (Saito *et al.* 1999; Saito *et al.* 2000). The receptor is expressed by both human melanocytes (Hoogdijn *et al.* 2001) and keratinocytes (Burgaud *et al.* 1997) and by human (Hoogdijn *et al.* 2001; Saito *et al.* 2001) and mouse melanoma cell lines (Drozdz *et al.* 1995; Hoogdijn *et al.* 2001).

7.2 Aim

MCHR1 cDNA was recovered following the third round of biopanning against a melanoma cDNA phage-display library with a pool of vitiligo IgG from ten patients (Chapter 6). The aim of the present study was to confirm immunoreactivity against the receptor and establish its frequency in sera from patients with vitiligo using radiobinding assays with [^{35}S]-labelled MCHR1.

7.3 Materials and methods

7.3.1 Patients

Sera from 55 vitiligo patients (22 male, 33 female; mean age: 48 yr; age range: 14-77 yr) were used in this study. Of these patients, 41 had no other autoimmune disorder and no family history of autoimmune disease and 14 had at least one other autoimmune disorder: autoimmune thyroid disease 9; alopecia areata, 3; systemic lupus erythematosus, 1; scleroderma, 1. Sera from 28 healthy individuals (10 male, 18 female; age range: 21-59 yr; mean age: 34 yr) were used as controls. As a further two sets of controls, 20 sera from patients (5 male, 15 female; mean age: 44 yr; age range: 22-84 yr) with Graves' disease (GD) and 16 sera from patients (7 male, 9 female; mean age: 48 yr; age range: 26-77 yr) with Addison's disease (AD) were tested.

7.3.2 Specific antisera

Rabbit polyclonal anti-MCHR1 antiserum MCHR11-S was purchased from Alpha Diagnostic International (San Antonio, TX, U.S.A). Other rabbit polyclonal antisera against melanocyte-specific proteins that were used as controls included: anti-tyrosinase (αPEP7; Tsukamoto *et al.* 1992); anti-tyrosinase-related protein-2 (αPEP8; Tsukamoto *et al.* 1992); anti-Pmel17 (AZN-LAM; Schreurs *et al.* 1997).

7.3.3 Cloning of human MCHR1 cDNA by RT-PCR

Total melanocyte RNA (1 µg) was used to prepare cDNA in a 30-µl reaction containing 10 mM DTT, 1 mM dNTPs (Promega), 250 ng random primers (Promega), 600 U M-MLV Reverse Transcriptase (RT; Promega) and 1X M-MLV RT buffer (Promega). The cDNA was subjected to 36 cycles of PCR amplification (Section 2.13) with MCHR1-specific primers 5'TTGAATTC GCCGCC**ATG**TTGTGTCCTTCCAAG3' and 5'AATCTAGACGC**CTA**TCAGGTG CCTTTGCTTC3' using the following conditions: 94°C, 1 min; 55°C, 1 min; 72°C, 2 min; and 72°C for 10 min for termination. The ATG translation initiation codon in the forward primer and the TAG translation termination codon in the reverse primer are shown in bold-type face. Restriction sites for *Eco*RI and *Xba*I were incorporated into the forward and reverse primers (underlined in the text), respectively, in order to allow subcloning of the PCR amplification product into pcDNA3 and subsequent expression of the MCHR1 cDNA from the T7 promoter in the vector.

The 1300 bp PCR amplification product was purified using a Wizard PCR Preps DNA Purification System (Section 2.14), restricted with *Eco*RI and *Xba*I (Section 2.8), ligated into *Eco*RI-*Xba*I-restricted pcDNA3 (Section 2.11) and then used to transform *E. coli* JM109 (Section 2.12). Plasmid DNA was purified from individual transformants using a Wizard Minipreps DNA Purification System (Section 2.7.1), restricted with *Eco*RI and *Xba*I and analysed by electrophoresis in a 0.8% (w/v) agarose gel (Section 2.9). One recombinant plasmid was sequenced according to a T7 Sequenase® Version 2.0 DNA sequencing kit (Amersham), as previously described (Section 2.15), using T7 and SP6 primers with [α-^{35}S]dATP, and according to a Thermo Sequenase Cycle sequencing kit (USB Corp.; Section 6.3.8) using the primers listed in Table 7.2 with [γ-^{32}P]ATP, to verify that no sequence errors had been introduced. The recombinant plasmid, pcMCHR1, was purified with a Qiagen Plasmid Maxi Kit (Qiagen Ltd.) (Section 2.7.2).

Table 7.2: Oligonucleotide primers used to sequence the MCHR1

Primers[1]	Primer sequences
501	5'AGGATTCCAGATGAACGG3'
631	5'AGCATCTCCTACATCCAA3'
851	5'TGTGGCACTTTGGGGAGA3'
911	5'TCACCAGCACCTACATCC3'
1091	5'CAGGAGGTGCAGTGGGCT3'
1141	5'CTCTACTGGTTCACCCTG3'
1321	5'TCCTTTGTGTGCTGGGCA3'
1348	5'GTGCTACAGCTGACCCCA3'
1441	5'TGCCTCAACCCCTTTGTG3'

[1] The numbers correspond to the first base pair of the target annealing sequence in the MCHR1.

7.3.4 *In vitro*-coupled transcription and translation

In vitro transcription-translation of pcMCHR1 was performed using a TnT® T7 Coupled Reticulocyte Lysate System with translation-grade [^{35}S]methionine as detailed elsewhere (Section 4.3.1.4). The percentage incorporation of [^{35}S]methionine was determined by TCA precipitation according to the manufacturer's protocol. Glycosylation of MCHR1 was achieved by the inclusion of 4 µl of canine pancreatic microsomal membranes (Promega) in the standard 50 µl TnT® T7 Coupled Reticulocyte Lysate reaction mixture. SDS-PAGE of *in vitro* translated MCHR1 was carried out in 12% (w/v) polyacrylamide gels as described previously (Section 2.17). Gels were fixed in 10% (v/v) glacial acetic acid/25% (v/v) isopropanol and then soaked in Amplify scintillant before drying at 60°C for 2 h onto 3MM chromatography paper. Autoradiography was carried out at –70°C using Fuji RX x-ray film. Pre-stained SDS-PAGE standards (Low Range) were from Bio-Rad Laboratories Ltd.

7.3.5 Radiobinding assays

For each assay, an aliquot of the *in vitro* translation reaction mixture (equivalent to approximately 100,000 cpm of TCA precipitable material) was suspended in 50 µl of immunoprecipitation buffer. Serum was then added to a final dilution of 1:20. Specific rabbit polyclonal antisera (MCHR11-S; αPEP7; αPEP8; AZN-LAM) were used at a final dilution of 1:100. For dilution experiments, three vitiligo sera positive for MCHR1 antibodies and a pool of six healthy control sera were used in the radiobinding assay at final dilutions of 1:20, 1:50, 1:100, 1:200, 1:500; 1:1000 and 1:2000. Radiobinding assays were carried out as previously described (Section 4.3.1.5).

Antibody levels were expressed as a relative index. An Ab index for each serum tested in the immunoprecipitation assay was calculated 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. The upper level of normal for the assay was calculated using the mean Ab index + 3 SD of the population of 28 healthy individuals. Any serum with an Ab index above the upper level of

normal was designated as positive for antibody reactivity. For dilution experiments, Ab indices were calculated for each serum tested as: cpm immunoprecipitated by tested serum at each dilution/the cpm immunoprecipitated by a pool of six healthy control sera at each dilution.

For analysis of immunoprecipitated proteins by SDS-PAGE and autoradiography, the protein G Sepharose-antibody complexes were resuspended in 50 μl of SDS sample buffer, heated at 100°C, centrifuged and the supernatant recovered for electrophoresis in a 12% (w/v) polyacrylamide gel which was processed as detailed above.

7.3.6 Statistical analysis

The frequency of specific antibodies was compared between patient groups and controls using Fisher's exact test for 2 x 2 contingency tables. P values < 0.05 (two-tailed) were regarded as significant.

7.4 Results

7.4.1 *In vitro* transcription-translation of MCHR1

Plasmid pcMCHR1 was transcribed-translated *in vitro* in a TnT® T7 Coupled Reticulocyte Lysate System. The quality of the *in vitro* translated radiolabelled receptor was evaluated by SDS-PAGE and autoradiography. This revealed a protein product with an estimated molecular weight of 45 kDa (Figure 7.1), a size that is in close agreement with the molecular weight of 39 kDa predicted from the amino acid sequence of the protein (Kolakowski *et al.* 1996). On the addition of canine pancreatic microsomal membranes to the *in vitro* translation reaction, a protein band with an increased molecular weight of 50 kDa was visible after SDS-PAGE and autoradiography (Figure 7.1). This was assumed to be glycosylated MCHR1.

7.4.2 Immunoreactivity of MCHR1

The immunoreactivity of the *in vitro* translated radiolabelled MCHR1 was analysed in radiobinding assays using rabbit polyclonal antisera. MCHR1 was immunoprecipitated by MCHR1-specific antiserum MCHR11-S but not by antisera αPEP7, αPEP8 or AZN-LAM (Figure 7.2), thereby demonstrating the immunoreactivity of labelled MCHR1 to the receptor-specific antiserum.

7.4.3 Detection of antibodies against MCHR1 in sera from patients with vitiligo

Sera from 55 vitiligo patients, 28 healthy controls, 16 AD patients and 20 GD patients were tested for immunoreactivity to MCHR1 in radiobinding assays. For each serum, an Ab index was assigned, this being the mean antibody index of at least two experiments (Figure 7.3). The mean Ab indices ± SD of the control, GD, AD, vitiligo groups were: 0.95 ± 0.16, 0.88 ± 0.19, 0.92 ± 0.26, 1.07 ± 0.62, respectively. The upper level of normal for the radiobinding assay (mean Ab index + 3 SD of 28 healthy controls) was estimated as an Ab index of 1.48

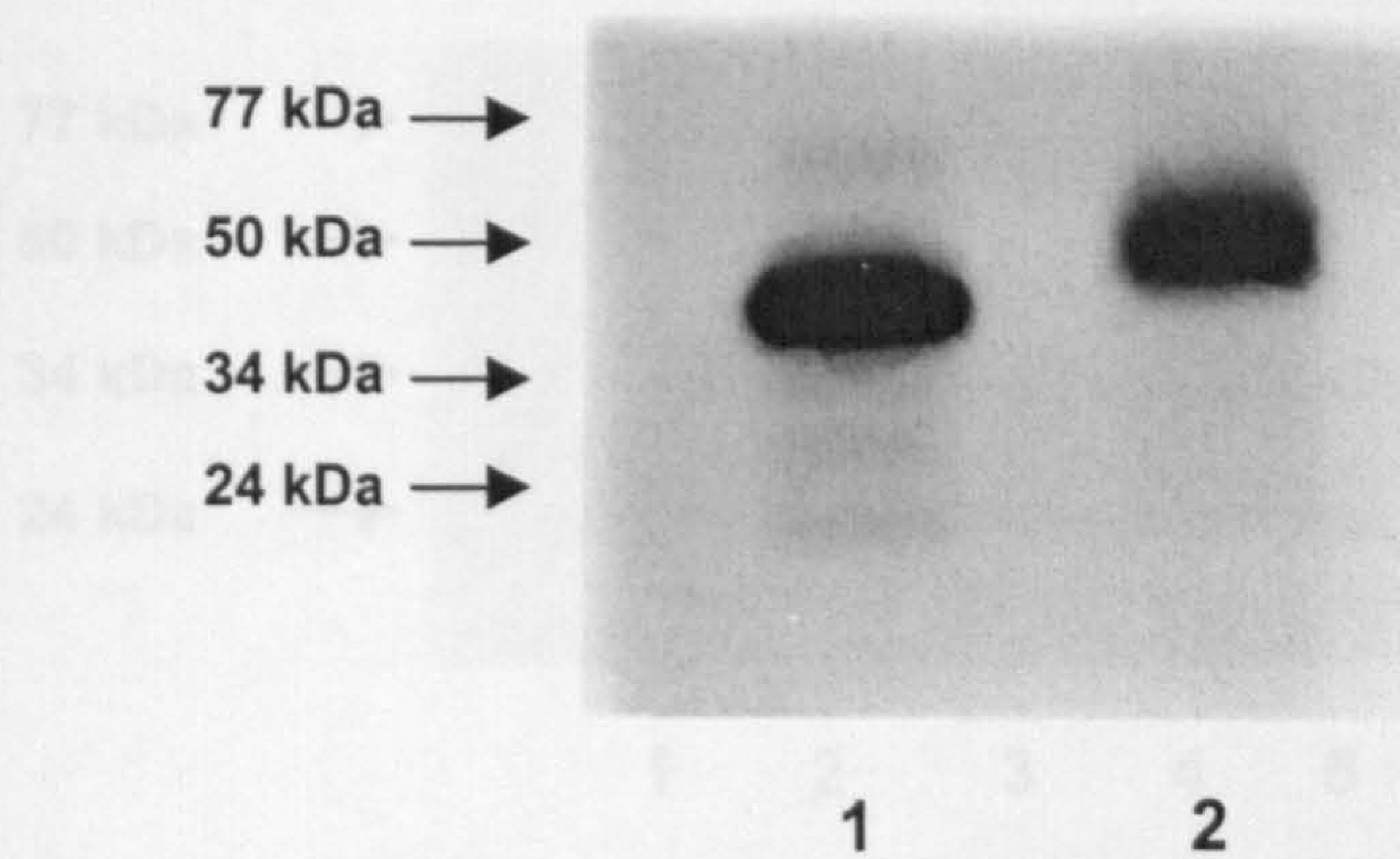


Figure 7.1: SDS-PAGE and autoradiography of *in vitro* translated MCHR1.

MCHR1 was translated in vitro in a TnT® T7 Coupled Reticulocyte Lysate System. To allow glycosylation MCHR1, canine pancreatic microsomal membranes were added to the transcription-translation reaction. Samples of the *in vitro* translation reaction were mixed with SDS sample buffer and electrophoresed in a 12% SDS-polyacrylamide gel followed by autoradiography. *In vitro* translated MCHR1 (lane 1); *in vitro* translated and glycosylated MCHR1 (lane 2).

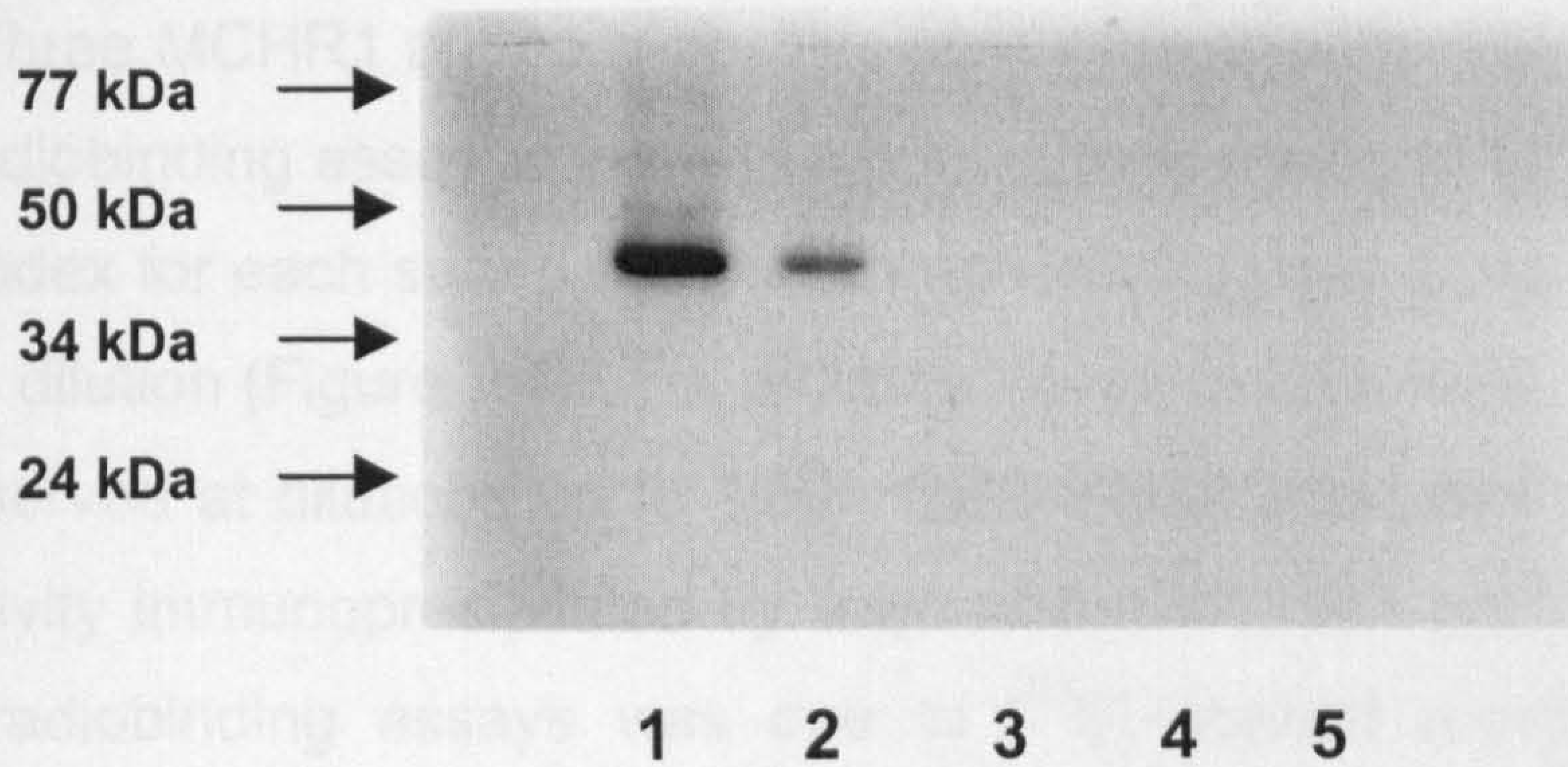


Figure 7.2: SDS-PAGE and autoradiography of *in vitro* translated and immunoprecipitated MCHR1.

MCHR1 was produced *in vitro* in a TnT® T7 Coupled Reticulocyte Lysate System and then used in radiobinding assays with either antisera MCHR11-S, αPEP7, αPEP8 or AZN-LAM. Immunoprecipitated protein was electrophoresed in a 12% polyacrylamide gel followed by autoradiography. *In vitro* translated MCHR1 (lane 1); *in vitro* translated MCHR1 immunoprcipitated with MCHR11-S (lane 2); *in vitro* translated MCHR1 immunoprcipitated with αPEP7 (lane 3); *in vitro* translated MCHR1 immunoprcipitated with αPEP8 (lane 4); *in vitro* translated MCHR1 immunoprcipitated with AZN-LAM (lane 5).

(Figure 7.3). Any serum with an Ab index above this level was considered positive for MCHR1 antibodies. On this basis, none of the healthy individuals was positive for MCHR1 antibodies. Sera from 20 patients with GD and 16 with AD were all negative for antibodies to the receptor. Of the vitiligo patient sera examined, 9/55 (16.4%) were considered positive for MCHR1 antibodies. The frequency of MCHR1 antibodies in the vitiligo patient group was significantly greater than normal (P = 0.025). The use of glycosylated MCHR1 in the radiobinding assay did not alter the Ab index of any of the vitiligo or healthy control sera tested.

Three MCHR1 antibody-positive sera were analysed at different dilutions in the radiobinding assay along with a pool of sera obtained for healthy subjects. An Ab index for each serum sample at each dilution was plotted as a function of 1/serum dilution (Figure 7.4). For all three vitiligo patient sera, saturated binding was observed at dilutions up to 1:50. SDS-PAGE was used to check that the radioactivity immunoprecipitated by each of the MCHR1 antibody-positive sera in the radiobinding assays was due to [^{35}S]-labelled receptor. Figure 7.5 indicates that the positive sera immunoprecipitated a protein band of the correct size when compared with *in vitro* translated MCHR1.

7.4.4 Analysis of MCHR1 antibody-positive vitiligo patients

The details of the MCHR1 antibody-positive and antibody-negative patients vitiligo patients are compared in Table 7.3. No association was evident between the presence of MCHR1 antibodies and either the age of the patient at time of serum sampling, the age of the patient at onset of disease, the duration of the disease or the gender of the patients. In addition, the clinical sub-type of vitiligo did not appear to be related to the occurrence of receptor antibodies and three patients who had active vitiligo were all negative for antibodies against MCHR1. Furthermore, the presence of an autoimmune disorder did not correlate with MCHR1 antibody reactivity. Among the MCHR1 antibody-positive vitiligo patients, only 1/9 (11%) was positive for antibodies to tyrosinase, TRP-1, TRP-2 and Pmel17, and none was positive for antibodies to SOX10, although not all sera were tested for each antibody (Table 7.4).

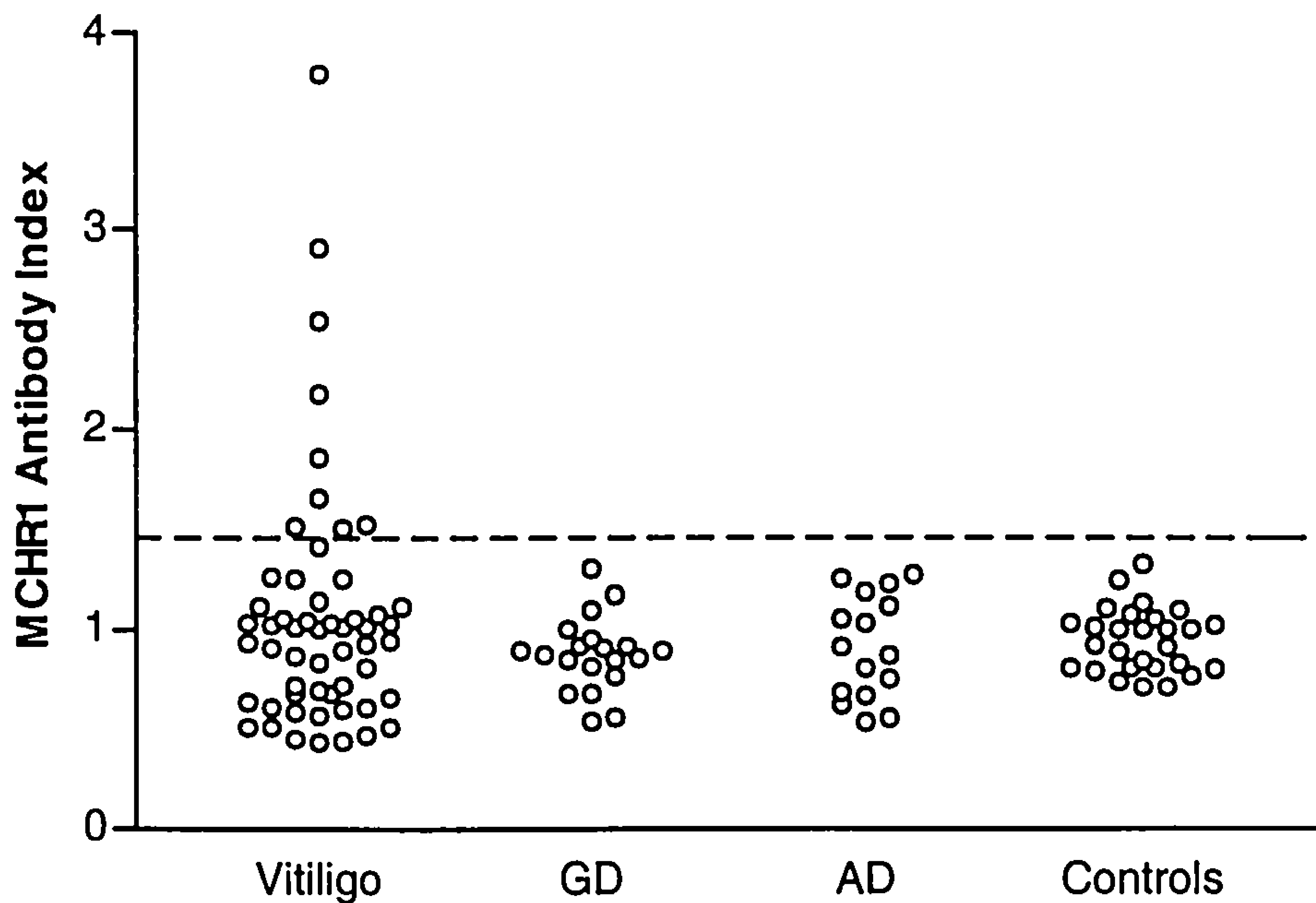


Figure 7.3: Radiobinding assays with patient and control sera.

Vitiligo sera (n = 55), Addison's disease (AD) patient sera (n = 16), Graves' disease (GD) patient sera (n = 20) and healthy control sera (n = 28) were analysed in radiobinding assays. An Ab index was calculated for each serum sample tested as: cpm immunoprecipitated by serum sample/mean cpm immunoprecipitated by 28 healthy controls. The Ab index shown for each serum is the mean of at least two experiments. The dotted line shows the upper level of normal of 1.48 (mean Ab index + 3 SD of 28 healthy controls) for the radiobinding assay.

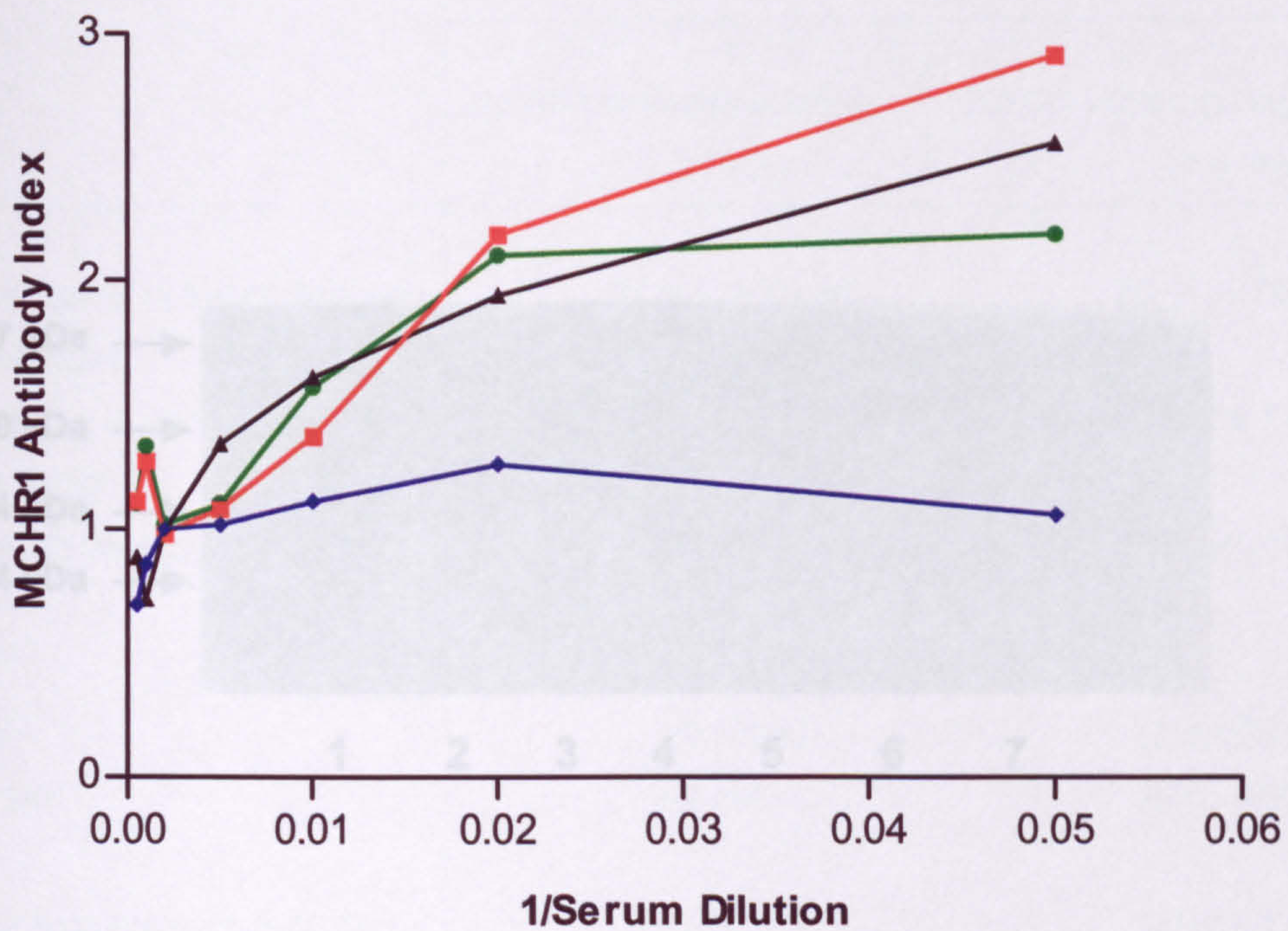


Figure 7.4: Dilution experiments.

Three MCHR1 antibody-positive sera and a pool of six healthy control sera were analysed at different dilutions in the radiobinding assays. The Ab index for each serum at each different dilution was calculated as: cpm immunoprecipitated by serum sample/mean cpm immunoprecipitated by healthy control pool. The Ab index of each positive serum and the pool of healthy control sera at each dilution is plotted as a function of 1/serum dilution. MCHR1 antibody positive serum 1 (■); MCHR1 antibody positive serum 2 (▲); MCHR1 antibody positive serum 3 (●); healthy control sera (◆).

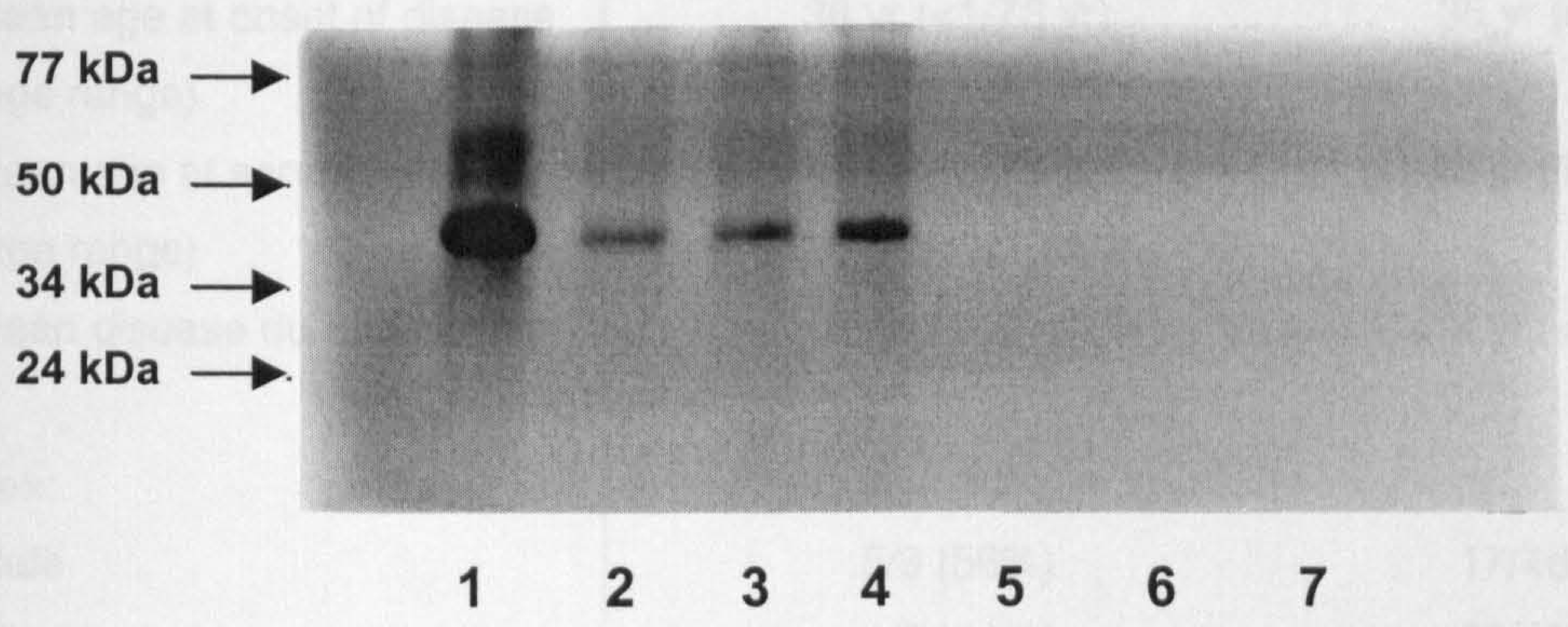


Figure 7.5: SDS-PAGE and autoradiography of MCHR1 immunoprecipitated with either MCHR1 antibody-positive vitiligo or healthy control sera.

MCHR1 was produced *in vitro* in a TnT® T7 Coupled Reticulocyte Lysate System and then used in radiobinding assays with either vitiligo or healthy control sera. Immunoprecipitated protein was electrophoresed in a 12% polyacrylamide gel followed by autoradiography. *In vitro* translated MCHR1 (lane 1); *in vitro* translated MCHR1 immunoprecipitated with vitiligo MCHR1 antibody-positive sera (lanes 2-4); *in vitro* translated MCHR1 immunoprecipitated with healthy control sera (lanes 5-7).

Table 7.3: Comparison of MCHR1 antibody-positive and antibody-negative vitiligo patients[1].

	Vitiligo patients ($n = 55$)	
	MCHR1 antibody-positive ($n = 9$)	MCHR1 antibody-negative ($n = 46$)
Mean age at onset of disease (age range)	38 yr (<1-73 yr)	35 yr (6-70 yr)
Mean age at serum sample (age range)	47 yr (23-75 yr)	48 yr (14-77 yr)
Mean disease duration (range)	10 yr (1-37 yr)	13 yr (<1-49 yr)
Sex:		
Male	5/9 (56%)	17/46 (37%)
Female	4/9 (44%)	29/46 (63%)
Other autoimmune disorder	2/9 (22%)[2]	12/46 (26%)
Vitiligo sub-type:		
Peri-orificial	1/9 (11%)	1/46 (2%)
Symmetrical	6/9 (67%)	36/46 (78%)
Symmetrical & peri-orificial	1/9 (11%)	4/46 (9%)
Symmetrical & segmental	1/9 (11%)	2/46 (4%)
Segmental	0/9 (0%)	1/46 (2%)
Focal	0/9 (0%)	1/46 (2%)
Universal	0/9 (0%)	1/46 (2%)

[1] The characteristics of the MCHR1 antibody-positive and antibody-negative vitiligo patients were compared using Fisher's exact test. All P values were > 0.05, indicating no significant difference between the two groups.

[2] Autoimmune thyroid disease was diagnosed in both patients.

Table 7.4: Incidence of anti-melanocyte antibodies in MCHR1 antibody-positive patients[1].

	MCHR1 antibody-positive vitiligo patient								
	1	2	3	4	5	6	7	8	9
Incidence of antibodies to:									
Tyrosinase	–	nd	–	–	nd	nd	–	nd	+
TRP-1	–	nd	–	–	–	nd	–	nd	+
TRP-2	–	nd	–	–	–	nd	–	nd	+
Pmel17	–	nd	–	–	–	nd	–	nd	+
SOX10	–	nd	nd	–	–	–	–	–	nd

[1] Negative for antibody reactivity, –; positive for antibody reactivity, +; not determined, nd.

7.5 Discussion

Previously, phage-display technology was employed with a melanoma cDNA library to identify MCHR1 as a novel target of autoantibody reactivity in vitiligo patients (Chapter 6). Radiobinding assays using [^{35}S]-labelled MCHR1 have confirmed the immunoreactivity against the receptor in sera from patients with vitiligo. Of the 55 vitiligo sera analysed, antibodies to the receptor were detected in 16.4% whereas AD, GD and control sera showed no reactivity, indicating a high disease-associated specifity. Two out of the pool of ten vitiligo patients used for biopanning against the library (Chapter 6) were among those patients reactive against the MCHR1, demonstrating that the original enrichment of the receptor from the library was specific. It would also appear that during the cDNA library biopanning experiments the reactivity of MCHR1 antibodies from these patients was not diluted out by the IgGs of the eight unreactive patients, demonstrating the feasibility of using a pool of patients with different specificities.

Glycosylation of MCHR1 did not alter the antibody binding index of any of the vitiligo patients, suggesting that any receptor epitope recognised by the antibody-positive sera was not altered by glycosylating the protein. Additionally, this finding suggests that vitiligo patient sera negative for MCHR1 antibodies do not recognise either glycosylated epitopes or epitopes that might be altered by post-translational processing of the receptor. Similar findings have been reported for the thyroid autoantigens thyroglobulin and thyroid peroxidase, as deglycosylation of these molecules had no effect on autoantibody binding (Kiso *et al.* 1992). It is likely that receptor autoantibodies targeting conformational epitopes were not detected in the radiobinding assay employed here which used recombinant MCHR1. Indeed, the most prevalent antibodies to the thyroid-stimulating hormone receptor (TSHR) in Graves' disease patients only react with native TSHR as they recognise conformation-dependent epitopes (Morgenthaler *et al.* 1999). The frequency of MCHR1 antibodies in vitiligo may, therefore, be higher if native receptor was used to analyse vitiligo sera. This could be accomplished by the expression of MCHR1 in a mammalian cell line, which could then be used in a flow cytometric assay or whole cell immunoprecipitation assay.

Analysis of the MCHR1 antibody-positive vitiligo patients revealed no obvious association between the presence of receptor antibodies and either patient age at the time of serum sampling, patient age at the onset of disease, sex, disease duration or vitiligo sub-type. Furthermore, the occurrence of MCHR1 antibodies did not correlate with the presence of an autoimmune disorder. This finding was in contrast to previous studies in which antibodies to the melanocyte-specific enymes TRP-1, TRP-2 and Pmel17 were only detected in vitiligo patients who had an autoimmune disorder (Kemp *et al.* 1997b; Kemp *et al.* 1998b; Kemp *et al.* 1998c), and antibody reactivity to tyrosinase and SOX10 was identified predominantly in patients with autoimmune disease (Kemp *et al.* 1997a; Hedstrand *et al.* 2001). The presence of MCHR1 antibodies was not related to the occurrence of humoral responses to other melanocyte antigens: in only one individual with tyrosinase, TRP-1, TRP-2 and Pmel17 antibodies was immunoreactivity to the receptor also identified. Two other vitiligo patients tested in this study were positive for antibodies to all the melanogenic autoantigens and one was positive for antibodies to tyrosinase. However, neither of them had antibodies to MCHR1.

The role of the MCH/MCHR1 signalling pathway in the regulation of human melanocyte behaviour has yet to be established, but a recent study suggested that it might function in the regulation of α-MSH induced melanogenesis (Hoogdijn *et al.* 2001). It is possible, therefore, that MCHR1 autoantibodies might adversely affect the functioning of the receptor leading to the disruption of normal pigmentation. Autoimmune disorders that result directly from autoantibody production include Graves' disease and myasthenia gravis and in the former, the G protein-coupled TSHR, present on thyroid follicular cells, is the target of autoantibodies that stimulate TSHR leading to an increase in the synthesis of thyroid hormones and the subsequent clinical symptoms of hyperthyroidism (Meek *et al.* 1964). Future studies will address the issue of whether or not the MCHR1 antibodies reported here can alter the activity of the receptor with respect to pigmentation.

MCHR1 is expressed on the surface of melanocytes. It is of interest, therefore, to note that autoantibodies in vitiligo patients are most commonly directed against antigens on the cell surface (Cui *et al.* 1992; Cui *et al.* 1995b). Previously, only intracellular melanocyte proteins such as tyrosinase have been

reported as autoantigens in vitiligo (Song *et al.* 1994a; Baharav *et al.* 1996; Kemp *et al.* 1997a; Kemp *et al.* 1997b; Kemp *et al.* 1998b; Kemp *et al.* 1998c; Okamoto *et al.* 1998; Hedstrand *et al.* 2001) and this is the first time a surface receptor has been specifically identified as an antigen in this disease. Expression of MCHR1 is not limited to melanocytes and the receptor is found, for example, within the central nervous system (Chambers *et al.* 1999; Saito *et al.* 1999) and in keratinocytes (Burgaud *et al.* 1997). Interestingly, a previous study reported that some of the antibodies in vitiligo patients do target antigens present on other cell types (Cui *et al.* 1992). If indeed MCHR1 antibodies are pathogenic, and this has yet to be determined, the selective destruction of pigment cells, as observed in vitiligo, might result from the relative sensitivity of melanocytes to immune-mediated injury, as compared with, for example, fibroblasts and keratinocytes (Norris *et al.* 1988a). The ability of vitiligo autoantibodies to destroy melanocytes has been previously demonstrated *in vitro* with cultured pigment cells (Norris *et al.* 1988b) and *in vivo* following passive immunisation of mice grafted with human skin (Gilhar *et al.* 1995). Similar studies could be performed on isolated MCHR1-specific antibodies in order to determine their potential pathogenicity.

The G protein-coupled receptor family is thought to consist of one of the largest group of proteins in the human genome, and new receptors and receptor subtypes are rapidly being identified (Evans *et al.* 2001). A second form of the receptor, designated MCHR2, with an approximate 36% overall homology to the MCHR1, was recently characterised (An *et al.* 2001; Mori *et al.* 2001; Sailer *et al.* 2001; Wang *et al.* 2001). In addition, a splice variant of the MCHR2 has been identified, which might modulate activity of the MCHR1 or MCHR2 receptors by heterodimerisation (An *et al.* 2001). It would be of interest, therefore, to determine whether the MCHR1 autoantibodies identified here showed any reactivity to the different forms of the receptor.

In summary, it has been confirmed that the use of phage-display enabled the isolation of a novel autoantigen in vitiligo, MCHR1. The exact role the MCHR1 autoantibodies play in the pathogenesis of vitiligo is, as yet, unknown. The stimulus for their production may stem from a genetic predisposition to autoimmunity, or from a cross-reactive antigen expressed on infecting micro-organisms. Equally, antibodies against the receptor might represent a

secondary phenomenon arising as consequence of injury to the melanocyte via other mechanisms, although they may then act to further exacerbate the condition. Alternatively, it is possible that the MCHR1 autoantibodies play no part in vitiligo aetiology but might, however, indicate the presence of autoreactive anti-MCHR1 T lymphocytes.

8 General discussion

Several hypotheses have been proposed to explain the destruction of cutaneous melanocytes in vitiligo. Persuasive observational and experimental evidence suggests that immune mechanisms play a role in the development of the disease (Section 1.3.6). Characterisation of the autoimmune reactivities in vitiligo may be beneficial in the advancement of more appropriate therapies and diagnostic tools and, perhaps, in determining the aetiology of the disease. Furthermore, similarities between the generation of autoantibodies and autoreactive T cells against melanocyte antigens observed in vitiligo, and the tumour immunity observed in malignant melanoma immunosurveillance, suggest that elucidating the immune response in vitiligo may provide new strategies for the treatment of both diseases.

In the present work, the autoimmune aspects of vitiligo were investigated with particular reference to the identification of melanocyte antigens recognised by autoantibodies in sera from vitiligo patients. In brief, the thesis aimed to examine: (i) possible genetic association of an immune regulatory protein with vitiligo, (ii) B cell reactivity to two previously identified vitiligo autoantigens, (iii) the prevalence of autoantibodies to a melanocyte antigen which forms a significant target for cytotoxic T cells in vitiligo and (iv) the application of a novel technique to the identification of additional disease autoantigens.

Genetic studies of vitiligo have demonstrated the existence of several susceptibility genes, which contribute to an overall predisposition to vitiligo (Majumder *et al.* 1993; Kim *et al.* 1998; Acros *et al.* 1999). Candidate genes include not only those with a role in melanogenesis and other aspects of melanocyte behaviour, but also those whose altered function affects immune regulation. This latter category is the most likely explanation for the fact that polymorphic regions of the CTLA-4 gene are associated with several autoimmune disorders and may represent a generalised marker of autoimmunity. A microsatellite polymorphism of the CTLA-4 gene was, therefore, analysed in a series of vitiligo patients and healthy control subjects (Chapter 3). The study failed to reveal an association of the gene polymorphism with vitiligo, at least in those patients without an associated autoimmune disorder, although the data did suggest that autoimmune endocrinopathy

patients carrying the CTLA-4 disease-associated allele may have a greater risk of developing vitiligo. Consequently, I was unable to identify a subgroup of vitiligo patients in which autoimmune responses may be more likely, for further study in subsequent experiments, since it is possible that immune responses are the primary cause of vitiligo in some patients but are absent or secondary to other pathological mechanisms in others. However, relatively small data sets were used and future analysis of this gene would benefit from larger populations and transmission studies using family groups. Indeed, genetic association was suggested between vitiligo and the CTLA-4 gene in recent case-control (Kristensen *et al.* 2000) and family studies (McCormack *et al.* 2001b) with greater subject numbers.

The greater part of this thesis has concentrated on the identification and characterisation of the melanocyte-specific autoantigens recognised by autoantibodies in sera from vitiligo patients. By defining the targets of the humoral immune response in vitiligo, it was hoped information would be gained as to its relationship to the disease initiation, process and pathogenesis. Moreover, the autoantibodies may serve as markers of an autoantigen against which there may be a pathologically more important cellular immune response.

Molecular analysis of the B cell epitopes on two previously identified vitiligo autoantigens, the melanosomal enzymes tyrosinase and Pmel17, was performed (Chapter 4). Multiple regions of tyrosinase were found to be epitopes for autoantibodies from the vitiligo patients studied, two of which are likely to contain epitopes that are cross-reactive with both TRP-1 and TRP-2. Additionally, at least two epitope domains are present in Pmel17. The finding that neither Pmel17 epitope shares sequence homology with other melanogenic proteins suggests that the IgG response to Pmel17 is distinct from that to the other melanocyte-specific antigens. It has been speculated that the immune response in vitiligo may arise from cross-reactivity between self-proteins and those of infecting micro-organisms (Le Poole *et al.* 1993a; Grimes *et al.* 1996). However, no similarity was apparent between the sequence of the identified epitope regions and that of microbial peptides to lend support to this theory with these particular antigens, with respect to sequenced pathogens. Also, none of the epitopes resided in areas of the proteins with recognised functional activity. However, the methodology employed here is only suitable for the study of linear determinants and further work is needed to fully assess the epitopes recognised

by tyrosinase and Pmel17 autoantibodies exploiting techniques which use native protein and purified monoclonal antibodies. In particular, the application of phage-display (Scott 1992; Williams *et al.* 2001) to future epitope mapping studies would allow expression of antigen in a native arrangement and, therefore, enable detection of conformational epitopes. Moreover, the isolation of monoclonal antibodies from vitiligo patients would allow a more complete analysis of the precise B cell epitope regions, than that obtained from heterogeneous sera.

In addition to the characterisation of known autoantigens, I attempted to identify novel targets of vitiligo patient autoantibodies. The presence of autoantibodies to MelanA in vitiligo sera was examined (Chapter 5) since autoimmune cellular responses to this melanocyte protein have been reported in vitiligo and melanoma patients. No MelanA-antibodies were isolated, suggesting that either MelanA is not a target of the humoral response in vitiligo patients or that any antibody reactivity to the protein is not detectable using the techniques applied. This does not rule out the presence of antibodies that target conformational epitopes on the native protein and future studies could employ mammalian expression systems to produce MelanA protein with native conformation to address this.

A further strategy for identifying vitiligo melanocyte autoantigens was the construction and screening of a melanoma cDNA expression library (Chapter 6). The recently developed pJuFo phage-display system was used to make the cDNA library and to express the proteins encoded therein on the surface of filamentous phage. Phage-display based on the pJuFo cloning system (Crameri & Suter 1993; Crameri *et al.* 1994) has previously been used to identify proteins that bind to human IgE (Crameri & Blaser 1996; Crameri *et al.* 1996; Lindborg *et al.* 1999) and IgG antibodies (Kemp *et al.*, unpublished data; Herd *et al.*, unpublished data). Several possible autoantigens were enriched from the melanoma library by their ability to bind IgG from vitiligo patients, which include proteins formerly characterised as autoantigens in other disorders. In addition, vitiligo-specific putative antigens with an association to epidermal and melanocyte function were isolated.

Most significantly, the library screening led to the discovery of the melanin-concentrating hormone receptor (MCHR1) as a novel target of vitiligo autoantibodies. Immunoreactivity against the receptor in sera from patients with

vitiligo was subsequently confirmed using radiobinding assays with [^{35}S]-labelled MCHR1 (Chapter 7). Antibodies against MCHR1 were found to be highly disease-specific and were detected more frequently than vitiligo-specific autoantibodies, tyrosinase, TRP-1, TRP-2 and Pmel17 described in prior radiobinding assay experiments (Kemp *et al.* 1997a; Kemp *et al.* 1997b; Kemp *et al.* 1998b; Kemp *et al.* 1998c). The specific identification of a surface receptor as an antigen in this disease is in agreement with previous reports that the autoantibodies in vitiligo patients are most commonly directed against cell-surface antigens (Cui *et al.* 1992; Cui *et al.* 1995b). It is of note that the MCHR1 autoantibodies might have additional functional significance to vitiligo if they could disrupt normal pigmentation by adversely affecting the role of the receptor in metabolically active melanocytes.

Work is currently in progress, in collaboration with Dr. Brian Hawes (Schering-Plough Research Institute, New Jersey, U.S.A.) to examine the effects of vitiligo patient IgG containing anti-MCHR1 antibodies on receptor activity. The level of receptor activity will be measured indirectly by calcium flux in a MCHR1-transfected mammalian cell line. Additionally, the ability of these antibodies to fix complement and, therefore, their potential to inflict cellular injury through complement-mediated lysis will be determined by flow cytometric analysis. Complement-fixing ability can be measured by the incubation of anti-MCHR1 IgG with MCHR1-transfected cells, and untransfected control cells, followed by the addition of serum containing all complement factors except C7, thereby preventing cell lysis by the exclusion of a terminal complement component. The level of deposition of C3 on the cells is subsequently measured by flow cytometric analysis and compared between control cells and those expressing the receptor. Attempts to characterise the B cell epitopes on MCHR1 by construction of a MCHR1 random cDNA fragment phage-display library, and by antibody binding to deletions of MCHR1, are also presently being undertaken. It would be useful to establish a mammalian cell line stably transfected with the receptor in our laboratory for future studies of antibody reactivity using immunoblotting, flow cytometric assay or whole cell immunoprecipitation assay. In addition, the incorporation of a histidine tag to the receptor terminal, prior to expression in mammalian cells, would provide a means of preparing sufficient quantities of the pure protein to develop ELISA.

The relationship between the MCHR1-antibodies, and indeed all vitiligo autoantibodies, and the pathology of the vitiligo lesions is yet to be established. The possibility that the MCHR1 autoantibodies play no part in vitiligo aetiology but rather indicate the presence of autoreactive anti-MCHR1 T lymphocytes should be addressed by an investigation into the T cell reactivities to MCHR1. MCHR1 antibodies may nevertheless serve as potentially useful markers of cellular disruption in the epidermis of vitiligo patients, even if they prove to be neither directly pathogenic nor functionally significant in the immune response. Additionally, melanoma patient sera reactivity to MCHR1 could be examined since it may represent a novel target for immunotherapy for this disease.

In conclusion, the work in this thesis has led to the identification of a novel autoantigen in vitiligo in addition to several potential targets. The preliminary characterisation of humoral reactivity to these antigens, especially MCHR1 described in Chapter 7, will be expanded upon in ongoing studies. Although this work may contribute to a greater understanding of the autoimmune aspects of vitiligo, the ways in which these responses fit into the aetiological and pathological disease mechanisms remain uncertain. Whilst vitiligo satisfies most of the defining criteria for autoimmune disease, as shown in Table 8.1, it is unclear to what extent it can be classified as a truly autoimmune disorder. Characterising the specificities of vitiligo autoantibodies will help to resolve the relation of the disease to the first, fourth, and possibly the eighth, criteria (Table 8.1). Additionally, the present work suggests that the detection of expression of MCHR1 antigen in vitiligo lesions may increase the likelihood of providing evidence to fulfil the fourth criterion. However, it is possible that vitiligo represents a spectrum of diseases, which result in the same endpoint, in which autoimmune reactivities are involved to a greater or lesser degree.

Future work is needed to establish whether the immune reactivities in vitiligo are driven by several principal antigens, or whether they are elicited to diverse immunogenic peptides released from melanocytes perhaps injured by other mechanisms. Further characterisation of the putative antigens isolated by the library screening may help to address this question. The construction of a melanocyte cDNA phage-display library, in addition to the existing melanoma library, and refinement of the current screening protocols might allow for identification of further autoantigens. The technique could be expanded to include the use of smaller pools of patient IgG and an initial round of 'negative'

Table 8.1: Summary of defining criteria[1] for autoimmune disease in relation to vitiligo.

Criterion	Comment	Reference
1.Autoantibodies transfer disease	Transfer of IgG from vitiligo patients into nude mice grafted with human skin elicits melanocyte destruction[2].	[2]Gilhar *et al.* 1995.
2.Reproduction of disease in experimental animal models	Depigmentation induced by IgG from vitiligo patients when injected into nude mice with grafts of human skin[2].	[2]Gilhar *et al.* 1995.
3.Genetically determined (spontaneous) animal models	Well-studied Smyth line chickens[3].	[3]Smyth 1989.
4.Identification within lesion of autoantibody or autoreactive T cell	Autoreactive T cells and autoantibodies in vitiligo lesions not so far demonstrated. Circulating, melanocyte-specific T cells[4,5] and autoantibodies[6] have been identified.	[4]Ogg *et al.* 1998. [5]Lang *et al.* 2001. [6]Naughton *et al.* 1983a.
5.Statistical association with particular MHC haplotype	Inconsistent observations but significant association of allele HLA-DR4 reported for several populations[7,8,9].	[7]Venneker *et al.* 1993. [8]Dunston & Halder 1990. [9]Foley *et al.* 1983.
6.Lymphocyte infiltrate in target organ	Infitrating activated T lymphocytes observed at the periphery of vitiligo lesions[10,11].	[10]Al Badri *et al.* 1993b. [11]van den Wijngaard *et al.* 2000.
7.Association with other autoimmune diseases in the same individual or family	Vitiligo is frequently associated with disorders such as autoimmune thyroiditis[12] and Addison's disease[13].	[12]Ochi & DeGroot 1969. [13]Dunlop 1963.
8.Favourable response to immunosuppression	Topical steroids[14], topical cytotoxic drugs[15] and PUVA[16] are reasonably effective treatments.	[14]Kumari 1984. [15]Tsuji & Hamada 1983. [16]Parrish *et al.* 1976.

[1]Rose & Bona 1993. Adapted from Kemp *et al.* 2001b.

biopanning using healthy control IgG to pre-adsorb non-specific IgG-binding proteins. It would also be useful to establish the limits of the technique by performing control experiments with a defined antibody or antisera.

In summary, the work in this thesis has demonstrated the usefulness of applying molecular techniques to the study of vitiligo. In particular, the application of filamentous phage-display has resulted in the discovery of a novel vitiligo autoantigen and is a potentially powerful tool for characterising the immune response in this disease.

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