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CHARACTERISATION OF AND T CELL RESPONSES TO THE P91A TUMOUR MINUS ANTIGEN

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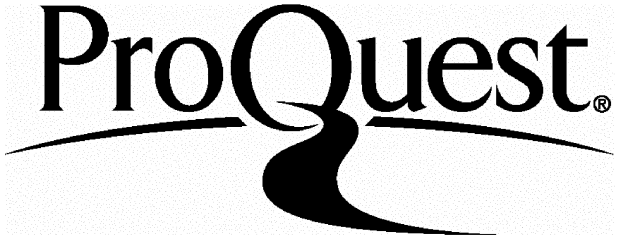

Abstract

The P91A tumour minus (tum^-) antigen is a tumour specific transplantation antigen expressed on the P815 derivative cell line P91. The tumour minus form of the P91A protein has a single amino acid substitution in comparison to the wild-type protein due to a single base change in the P91A tum^- gene. As a result of this amino acid substitution a unique H2-L^d binding peptide is generated and recognised in complex with H2-L^d by CD8+ cytotoxic T cells. P91A transgenic DBA/2 mice have been previously generated. Transgenic and non-transgenic mice differ only in expression of the P91A epitope. P91A peptide specific T cell responses have been examined in P91A transgenic and non-transgenic mice. T cells responsive to low concentrations of P91A peptide are present in non-transgenic mice while high concentrations of peptide are required to elicit equivalent proliferative responses in P91A transgenic mice. These results suggest that high affinity P91A specific T cells may have been deleted in P91A transgenic mice. P91A specific T cell clones have been generated from P91A transgenic and non-transgenic mice and their T cell receptor usage and sequence examined. Where possible this data has been correlated with recognition of the H2-L^d-P91A peptide complex. To further evaluate the interaction between P91A specific T cells and the P91A epitope the exact nature of the naturally processed P91A epitope has been determined and MHC and TCR contact residues defined. The P91A epitope is an octamer peptide which binds to L^d in the absence of consensus binding motif residues. Finally, using a series of P91A based minigenes evidence has been gained which suggests that the P91A epitope can be generated from P91A protein fragments which are targeted to the endoplasmic reticulum.

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1.0 Introduction

1.1 Preface

Innate immune response mechanisms predate the evolution of an adaptive immune system and provide the first line of defence against a world of potential pathogens. Non adaptive responses to pathogenic attack involve a wide variety of effector mechanisms which are characterised by rapid response and general pathogen specificity. Non adaptive immune responses are triggered by receptors which although pathogen specific fail to induce lasting immunity or immunological memory. Although not a cell based mechanism of defence, the activation of complement may represent the most ancient defence mechanism which is operative in vertebrates. Diverse cell types mediate a range of innate recognition and effector mechanisms. For rapid response to pathogenic challenge cell types such as macrophages and neutrophils phagocytose organisms utilising receptors specific for common bacterial components, (CD14/CD18 recognise bacterial lipopolysaccharide, macrophage mannose receptor recognises glycoproteins bearing terminal mannose residues which are expressed on bacteria and yeast). In addition receptors specific for the C3 component of complement and immunoglobulin Fc receptors present on macrophages and neutrophils enable the phagocytosis of opsonised bacteria. Following binding and internalisation pathogens are destroyed and inflammatory mediators released, (e.g. interleukin -1 (IL-1), IL-6, IL-8, IL-12 and tumour necrosis factor-α). Acting in concert these factors increase local capillary dilation, vascular permeability and transmigration of myeloid and lymphoid cells. Innate immune mechanisms eliminate micro-organisms which are encountered on a daily basis and can truly be thought of as the first line of defence.

If innate immune mechanisms fail to eliminate an infectious organism then an adaptive immune response will ensue. While innate immunity relies on the

recognition of conserved proteins, lipids or carbohydrates expressed on pathogens, adaptive immunity has evolved, through the development of unique receptor based strategies, to recognise the myriad of foreign antigens and antigenic peptides expressed on or derived from foreign organisms and differentiate them from components of self, (the mechanism of receptor generation and the nature of the interaction between the T cell receptor and its ligand is discussed in section 1.5 and 1.6).

While the immune system operates throughout the body with specific contributions from non-haematopoietic cells, for example the almost universal expression of MHC class I molecules and the expression of adhesion molecules on vascular endothelium during lymphocyte extravasation, the majority of key cell types differentiate within the haematopoietic system from the common pluripotent bone marrow stem cell. Differentiation to lymphoid (T, B and NK) and myeloid (basophil, eosinophil, monocyte/macrophage and dendritic cells) lineages and the further differentiation and movement of these cells between lymphoid organs, tissue associated lymphoid tissues and the circulation enables effective immune surveillance. T and B lymphocytes comprise the recognition and effector mechanism in an adaptive immune response.

T cells can be distinguished on the basis of co-receptor expression. T cells expressing the CD4 co-receptor recognise peptide, derived primarily from extracellular proteins, in the context of MHC class II molecules while CD8 co-receptor positive T cells recognise peptides, derived from intracellular proteins, in the context of MHC class I molecules, (the generation and binding of peptides to MHC class I molecules is discussed in sections 1.2-1.4). The production of immunoglobulin by cells of the B lineage is the basis of humoral immunity. The specific recognition of antigen by CD4 positive T cells can be considered of central importance both in the direction of an effective humoral immune response and in the maturation of the cytotoxic CD8

positive T cell population involved in the elimination of tumour cells or cells parasitised by intracellular pathogens.

T lymphocyte precursors originate in the foetal liver and bone marrow and migrate to the thymus where a complex series of cell division and differentiation events leads to the emergence of mature T cells. When progenitor cells enter the thymus from the bone marrow they lack most of the cell surface markers characteristic of mature T cells, (e.g. CD3/T cell receptor, CD4, CD8, CD2). Following contact with the thymic micro-environment progenitor cells enter a phase of rapid proliferation and commence rearrangement of the TCRB, TCRG and TCRD loci. Successful rearrangement of a γδ T cell receptor signals the thymocyte to differentiate into a γδ T cell. γδ cells represent a separate lineage of T cells which display a developmentally controlled pattern of TCR segment usage and peripheral distribution. Large populations of γδ T cells bearing identical receptors have been identified in both mouse and man and may be involved in innate immunity to pathogenic organisms. Successful rearrangement of the TCRB locus produces a TCR β chain which pairs with an invariant pre-TCRα chain and signals this population of thymocytes to acquire expression of CD4 and CD8 co-receptor (double positive thymocytes; dp). Rearrangement of the TCRA locus and expression of TCR α chains occurs in dp thymocytes. Double positive thymocytes constitute the largest thymocyte pool (~75%) and along with the $CD4^-CD8^-$ population are found in the thymic cortex. From the pool of double positive thymocytes with functional T cell receptors, negative and positive selection operate to remove autoreactive cells and promote the maturation of thymocytes with receptors of appropriate reactivity to self MHC-peptide, (positive and negative selection of thymocytes is discussed in section 1.8 and Chapter 6). Multiple rearrangements of the TCRA locus allows for the audition of multiple T cell receptors during a period when the thymocyte is available for positive selection and is therefore likely to increase the chances of the latter. Double positive thymocytes may die in the thymic cortex due to the failure to find an appropriate

niche or express a suitable TCR for positive selection, (death by neglect). Those thymocytes which receive a positive selection signal most likely require a prolonged contact period with the thymic micro-environment during which time, non cognate signals may be delivered to the thymocyte. Coincident with positive selection, CD4 or CD8 co-receptor is down regulated, TCR expression is increased and the enzymes required for rearrangement of the TCRA locus are shut down.

Negative selection occurs at both cortical and medullary sites; the factors which determine the anatomical site of negative selection are unclear bur are likely to include the site of cognate antigen expression and the kinetics of the TCR interaction with the MHC-peptide/superantigen complex to which it is reactive. Those thymocytes which receive a positive selection signal migrate to the thymic medulla, and following further interactions with the thymic micro-environment where residual autoreactive cells can be removed, exit into the periphery.

Thymocytes and mature T cells interact with many host cell types. Interaction with cells comprising the thymic micro-environment direct antigen independent aspects of differentiation; a myriad of adhesion molecules (e.g. ICAM-1/2/3, LFA-1, PECAM-1, VCAM-1, L selectin), expressed on antigen presenting cells and endothelium direct antigen independent aspects of cell contact and migration. Co-stimulatory molecule interactions such as CD28-B7-1/B7-2 regulate the activation of T cells while Fas-Fas ligand and CTLA-4-B7-1/B7-2 interactions direct the curtailment of expanded populations of antigen specific T cells. In addition, the principal co-receptor molecules, CD4 and CD8 modulate TCR/MHC-peptide interaction and signalling events in both immature and mature T cells. Many T cell and accessory cell molecules involved in mediating antigen independent interactions are themselves regulated during the differentiation of T cells and immune responses to control lymphocyte migration, extravasation, activation, anergy and death.

The work described in this Thesis deals with the definition of the P91A tum$^-$ epitope and the characterisation of P91A peptide specific T cell responses in P91A transgenic and non-transgenic mice. More specifically the exact nature of the P91A epitope is established and MHC/TCR contact sites determined. T cells are cloned from P91A transgenic and non-transgenic mice and a comparison of TCR usage made. Finally, the intracellular processing of the P91A epitope is examined using a series of truncated/chimeric proteins. The remainder of this introduction will focus on the principal components involved in MHC class I restricted antigen recognition by cytotoxic T cells, namely the T cell receptor, MHC molecule and associated peptide. The interaction of MHC-peptide with the T cell receptor is discussed and models of T cell activation and selection considered.

1.2 The MHC class I processing pathway

Cytotoxic T lymphocytes (CTLs) recognise antigenic peptides in the context of MHC class I molecules. The generation and expression of mature, peptide-loaded class I molecules at the cell surface requires the co-ordination of three essential processes: the degradation of proteins to peptides in the cytosol; the translocation of peptides into the lumen of the endoplasmic reticulum (ER); and the assembly of MHC class I heterodimers and their transport to the cell surface.

1.2.1 Generation of MHC class I binding peptides

The finding that the majority of $CD8^+$ T cells recognise class I associated peptides derived from cytosolic proteins initiated a search for the proteases responsible for peptide generation. Protein degradation in the cytosol is important for the turnover of key regulatory proteins, enzymes, misfolded proteins, normal cellular proteins and in the generation of antigenic peptide fragments (Hershko and Ciechanover, 1992, Rock et al, 1994). Historically the proteasome has been implicated in the generation of class I binding peptides. Townsend and colleagues originally showed that ubiquitination could increase the efficiency of epitope generation (Townsend et al, 1988). Furthermore, γ-interferon inducible components of the proteasome (low molecular weight polypeptides, LMP2 and LMP7) map within the class II region of the MHC locus. Taken together these data suggested that the proteasome may be involved in class I epitope generation. More direct evidence suggesting proteasome involvement in antigen processing is derived from experiments using proteasome inhibitors. Peptide aldehyde inhibitors of the proteasome almost completely inhibit the assembly of the MHC-peptide complex (as assessed by immunoprecipitation) and inhibit MHC class I antigen presentation in a dose dependent manner (Rock et al, 1994). These experiments suggest that not only can the proteasome generate specific peptide ligands but that it is responsible for production the majority of MHC class I binding

peptides. The interpretation of these experiments has been questioned on the basis that the peptide aldehyde inhibitors used can also inhibit lysosomal proteases and calpains. Specific inhibitors of these enzymes however, fail to block the production of antigenic peptides suggesting that they are not primarily involved in class I epitope generation. Additionally, the crystal structure of a prokaryotic 20S proteasome-peptide aldehyde inhibitor complex has revealed binding of the aldehyde inhibitor to all proteolytic active sites within the proteasome (Löwe et al, 1995).

More recently, the streptomyces metabolite lactacystin has been shown to specifically bind to the eukaryotic 20S proteasome and inhibit the generation of the H2-D^b binding influenza nucleoprotein peptide $NP_{366-374}$ from a cytosol located $NP_{327-498}$ protein fragment (Fenteany et al 1995, Cerundulo et al, 1997). Unlike peptide aldehydes, lactacystin appears to be proteasome specific in its action. Taken together, these results demonstrate that proteasome mediated degradation of cytosolic proteins plays an important role in the generation of class I binding peptides.

The proteasome accounts for approximately 1% of soluble cellular protein and is the major neutral proteolytic activity in the cytosol and nucleus (Rivett, 1993). It is a multisubunit complex of approximately 700kDa and has a sedimentation coefficient of 20S (Orlowski et al, 1990, Tanaka, et al, 1992). The quaternary structure of the 20S proteasome as revealed by electron microscopy would appear to have been conserved throughout evolution. It is an elongated cylindrical particle consisting of four stacked rings with a central penetrating channel. The overall dimensions of the complex are 148 Å in length and 113 Å in diameter. The central two rings are each composed of 7 β subunits and the outer rings of 7 α subunits. A single type of α and β subunit is present in the archaebacterium *Thermoplasma acidophilum*, the actinomycete *Rhodococcus sp*. has two types of α and β subunit, yeast proteasomes have 7 types of α and β subunit and mouse and human proteasomes have 7 different α and 10 different β subunits (Tamura et al, 1995, Hilt et al, 1995, Tanahashi et al,

1993, Löwe et al, 1995). It is likely that yeast and mammalian cells will place similar housekeeping demands on the proteasome. The yeast proteasome must therefore be able to fulfil the basic proteolytic requirements of eukaryotic cell function. The development of more sophisticated proteosomes must therefore reflect the ongoing evolution of the cell and sophistication of biological processes. The development of an antigen specific immune system is likely to have been one of the major driving forces in the evolution of the eukaryotic proteasome in jawed vertebrates.

The crystal structure of the 20S proteasome from the archaebacterium *Thermoplasma acidophilum* has been solved at 3.4 Å (Löwe et al, 1995), figure1.1. The central channel of the particle has a maximal diameter of 53 Å and is divided at the junction of adjacent rings into three cavities. Cavities are accessed and connected by four narrow gates formed from α and β hydrophobic loops which protrude into the lumen. Gates are 13 Å in diameter at the particle termini and 22 Å internally. The proteolytic active sites within the proteasome have been defined on the basis of inhibitor interaction. The peptide aldehyde inhibitor acetyl-Leu-Leu-norleucinal (LLnL) binds to all 14 β subunits with the aldehyde function close to the amino terminal threonine residue (Thr^1). Additionally, lactacystin appears to covalently modify Thr^1 of the mammalian proteasome β subunit MB1 and site directed mutagenesis has indicated the importance of Thr^1 and an evolutionary conserved lysine at position 33 (Lys^{33}) in forming the proteolytic active site (Fenteany et al, 1995, Seemüller et al, 1995). Together these data have been interpreted as supporting a novel mechanism of proteolysis in which Thr^1 acts as the nucleophile and proton acceptor in the hydrolysis of peptide bonds. A similar catalytic mechanism is likely to operate in all proteolytically active β subunits. This implication does not conflict with the observation that mammalian proteasomes have multiple cleavage specificities since it is likely that substrate specificity will be dictated by the overall architecture of the β subunit substrate binding pocket (Seemüller et al, 1995). Interestingly the distance between two β subunit N-terminal threonines in the 20S proteasome is 28 Å. Octamer

and nonamer peptides in extended conformation span 30.6 A to 34.5 A (Young et al, 1994). It is therefore possible that concerted dual cleavage of an unfolded polypeptide in extended conformation could result in the generation of peptides of optimal length for transport into the ER and association with class I molecules.

The murine and human proteasome has 10 different β type subunits (7 constitutively expressed, 3 γIFN inducible) and three well characterised proteolytic activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolysing activity) responsible for peptide bond cleavage on the carboxyl side of hydrophobic, basic and acidic residues respectively (Orlowski et al, 1990, Rivett, 1993). In addition to the proteolytic activities described, the existence of up to 5 different proteolytic specificities has been proposed on the basis of inhibitor studies (Orlowski et al, 1993). Although it was thought possible that all β subunits would function in proteolysis, a comparison of amino acid sequence reveals that four of the seven constitutively expressed β subunits lack N-terminal threonine and hence may be catalytically inactive (Seemüller et al, 1995). Those subunits which retain an N-terminal threonine (δ, MB1 and MC14) are more closely related in overall sequence and have γ IFN inducible homologues (LMP2, LMP7 and MECL-1). It is therefore likely that these subunits constitute the catalytic core of the proteasome and confer all enzymatic activity.

1.2.1.1 The involvement of γ interferon inducible subunits in modulating proteasome function

The function of the γIFN inducible β subunits LMP-2 and LMP-7 still remains unclear. Their location within the MHC locus suggests co-evolution with other elements of the immune system but consistent clear evidence of their exact role in immune modulation has not been easy to obtain. *In vitro* studies using fluorogenic tri and tetra-peptides have shown an LMP-2/7 dependent increase in substrate cleavage

after basic and hydrophobic residues and a decrease in cleavage C-terminal of acidic residues (Gaczynska et al, 1993). This pattern of cleavage should generate appropriate C-termini for binding to the majority of MHC class I molecules (Falk et al, 1991, Rammensee et al, 1995). Although LMP-2 and LMP-7 may enhance the production of appropriate class I binding peptides their role in this is not essential. The human B cell lymphoblastoid mutant cell line T2 has a homozygous deletion of the MHC class II region, which encompasses the ATP-binding cassette peptide transporters TAP-1 and TAP-2 and the proteasome subunits LMP-2 and LMP-7 (Salter et al, 1985). T2 cells fail to transport peptides from the cytosol into the lumen of the ER and as a consequence have low level surface MHC class I expression. Transfection of TAP-1 and TAP-2 genes into T2 is sufficient to restore wild-type MHC class I surface expression and sensitise T2 transfectants for CTL mediated kill by two different T cell clones (Arnold et al, 1992, Momburg et al, 1992). These data indicate that LMP-2 and LMP-7 may not be generally required for generating the majority of peptides that are bound by MHC class I molecules.

The role of LMP-2 and LMP-7 has been further investigated in mice with targeted deletions of one or other gene (Van Kaer et al, 1994, Fehling et al, 1994). LMP-2 deficient mice have normal levels of MHC class I surface expression. However, proteasomes from these mice appear to cleave less efficiently after hydrophobic or basic residues suggesting that epitope generation may be impaired. More surprisingly, LMP-2 deficient mice have a 60-80% reduction in the number of CD8+ T cells. This is a difficult observation to explain and leads one to the awkward conclusion that thymic selection may be altered in LMP-2-/- mice. LMP-7-/- mice have a 25-45% decrease in surface MHC class I expression and present a male specific antigen H-Y-D^b epitope less efficiently to anti H-Y-D^b specific TCR transgenic spleen cells (Fehling et al, 1994). The cleavage specificity of LMP-7 -/- proteasomes has been examined and found to be different from that of wild-type proteasomes (Stohwasser et al, 1996). The alteration in specificity is in part due to increased chymotryptic activity

and has been related to increased incorporation of the β sub-unit MB1. This finding is however difficult to reconcile with a phenotype of low surface MHC class I expression since enhanced chymotrypsin-like activity should generate peptides with hydrophobic carboxy termini.

Although the data is confusing, it remains very likely that LMP-2 and LMP-7 modulate proteasome function to the advantage of the immune system. Whether they do so alone or in concert is unclear as is their exact mechanism of action.

The third proteasome β subunit with predicted catalytic activity (MC14 in the mouse, z in humans) has a recently identified γ IFN inducible homologue designated MECL-1 (Groettrup et al, 1996, Nandi and Monaco, 1996, Hisamatsu et al, 1996). The homologous proteasome subunit PUP1 in *S. cerevisiae* appears to preferentially cleave C-terminal of arginine residues (Heinmeyer and Wolf, unpublished). If the same is true of MECL-1 then the generation of at least human class I binding peptides (HLA-A*3101, HLA-A*3302 and HLA-A68.1 all bind peptides with a C-terminal arginine) may be enhanced by its incorporation into the proteasome.

1.2.1.2 The involvement of proteasome activator complexes in antigen presentation

The 20S proteasome can bind at its ends either of two currently described activator complexes; the '19S regulator' and '11S regulator' (or PA28 regulator). The 20S core particle in association with two 19S regulator complexes forms the 26S proteasome and is responsible for the ATP-dependent targeted degradation of ubiquitin-conjugated proteins. The role of ubiquitination and the involvement of the 26S proteasome in antigen processing remains controversial. Ubiquitin tagging was shown to facilitate the presentation of an influenza nucleoprotein epitope (Townsend et al, 1988). However, for only two proteins with intramolecular disulphide bonds (β-

galactosidase and ovalbumin) has a dependence on ubiquitination for class I antigen presentation been established (Grant et al, 1995, Michalek et al, 1996). Interestingly, the 20S proteasome has been shown to be capable of generating peptides from β galactosidase *in vitro* (Dick et al, 1994). However in this study the substrate protein was chemically denatured and reduced prior to processing. The 26S proteasome is involved in the degradation of cellular regulatory and structural proteins and as such must have the capacity to disrupt the conformation of mature proteins in order that they may gain access to the interior of the proteasome. Its involvement in antigen processing is however still unclear.

The PA28 proteasome activator is composed of three subunit types, α and β and γ (Ahn et al, 1995) which form hexa- or heptameric rings, bind at either end of the 20S core particle and modulate the generation of antigenic peptides both *in vitro* and *in vivo* (Gray et al, 1994, Groettrup et al, 1996, Dick et al, 1996). In doing so, the PA28 activator has been shown to enhance 10 fold the presentation of a murine cytomegalovirus pp89 protein-derived epitope (Groettrup et al, 1996). The crystal structure of a PA28 regulator complex composed solely of α subunits has recently been solved at 2.8 Å (Knowlton et al, 1997). In this structure the PA28 regulator is shown to have a 20 Å diameter opening at one end and a 30 Å diameter opening at the presumed proteasome binding end. Based on this crystal structure it is has been proposed that PA28 induces a conformational change in proteasome α subunits which enhances substrate access to the lumen of the proteasome and induces a long range conformational change within the enzymatically active β subunits. In addition to its proposed role in altering proteasome cleavage patterns the PA28 regulator has been suggested to target the proteasome (in a PA28 subunit specific manner) to different intracellular locations (which might include targeting to the ER) and may also bind chaperoned proteins (which may generate unfolded proteins which can gain access to the proteasome central chamber). Both of these mechanisms would tend to increase

the efficiency of generation and delivery of peptides into the class I processing pathway.

It appears that the mammalian proteasome has evolved to have fewer catalytically active constituent β subunits than that of the archaebacterium proteasome. The three remaining active subunits are however unique and have γIFN inducible homologues. In addition, a specific γIFN inducible activator of the proteasome exits and is capable of enhancing the presentation of viral epitopes. The role of the 19S particle (as part of the 26S proteasome) is unclear but it is likely that it is involved either directly or indirectly in unfolding mature conformed proteins (possibly with internal disulphide bonds). Peptide fragments generated by the 26S may be transported into the ER or chaperoned by cytoplasmic heat shock proteins for further processing by the 20S-PA-28 proteasome complex. Denatured and misfolded proteins may be degraded by the 20S proteasome in the absence of any contribution of the 19S subunit. Whether a dynamic equilibrium exists between the binding of the 19S subunit and the PA-28 activator to the 20S core particle is also unclear. Irrespective of the exact contribution to processing made by proteasome activators and subunits, the proteasome generates peptide fragments of approximately 5-15 amino acids in length which must be transported into the ER if they are to escape further degradation in the cytosol and associate with nascent class I heavy chains (Wenzel et al, 1994, Kuckelkorn et al, 1995). The following section examines the mechanism by which peptides generated in the cytosol gain access to the lumen of the ER.

1.2.2 TAP mediated transport of peptides into the endoplasmic reticulum

In order to associate with newly synthesised MHC class I heavy chains, peptide fragments generated by the proteasome must be translocated to luminal side of the ER membrane. This task is performed by the 'transporters associated with antigen processing' (TAP-1 and TAP-2) and is energy dependent. TAP-1 and TAP-2 are

multi-membrane spanning proteins with an ATP-binding cassette and are noncovalently associated as a heterodimer in the ER membrane (Spies et al, 1991, 1992, Kelly et al, 1992). Neither component is individually active and transfection of both is required to correct the MHC class I peptide loading defect in the human mutant cell line T2 (T2 has a homozygous 1 megabase deletion of the MHC class II region which encompasses the TAP-1 and TAP-2 genes), (Arnold et al, 1992, Momburg et al, 1992).

TAP molecules from different species preferentially transport peptides of 8-12 amino acids; longer and shorter peptides are transported less efficiently (Schumacher et al, 1994a and b, Momburg et al, 1994a and b, Androlewicz et al, 1994). This length range is compatible with the requirements for MHC class I binding peptides. This said, 33mer peptides have been eluted from HLA-B27 and TAP dependent translocation of peptides up to 40 amino acids has been demonstrated (Urban et al, 1994, Koopmann et al, 1996).

TAP transporters from different species vary in their ability to transport specific peptides. Human TAP and rat TAP allotype A (TAP^A) are rather indiscriminate regarding the nature of the C-terminal peptide residue, whereas mouse TAP and rat TAP allotype B (TAP^B) preferentially bind and translocate peptides with aromatic or hydrophobic residues (Heemels et al, 1993, Momburg et al, 1994b). Consistent with these transport specificities human MHC class I alleles bind peptides with hydrophobic or basic residues while mouse class I molecules bind only peptides with hydrophobic C-terminal residues (Falk et al, 1991, Rammensee, 1995). The co-evolution of TAP and MHC class I molecules is clearly demonstrated in the rat where the presence of $TAP2^A$ is required for the translocation of MHC class I ($RT1.A^a$) binding peptides which have a C-terminal arginine residue ($RT1.A^a$ prefers to bind peptides with a C-terminal arginine residue), (Heemels et al, 1993, Momburg et al,

1994, Powis et al, 1996). Allelic forms of mouse and human TAP-1/2 do not appear to show variation in peptide specificity (Obst et al, 1995, Schumacher et al, 1994b).

1.2.3 Assembly of the MHC class I-peptide complex

The assembly mechanism of murine and human MHC class I-peptide complexes is shown in figure 1.2. Newly assembled heavy chain-β_2m heterodimers of class I histocompatibility molecules associate with TAP, bind translocated peptides and dissociate from TAP in parallel with their transport from the ER to the Golgi apparatus (Suh et al, 1994, Ortmann et al, 1994, Grandea et al, 1995). ER resident chaperones control the folding of class I heavy chain, its association with β_2m, the interaction of the heavy chain -β_2m heterodimer with TAP and even the rate of intracellular transport of fully assembled class I molecules (Suh et al, 1994, Suh et al, 1996). Calnexin appears to be the major if not the only chaperone involved in class I assembly in the mouse (Suh et al, 1996). Calnexin is a non-glycosylated type-1 membrane protein with a luminal domain homologous to calreticulin. It binds transiently to a diverse array of membrane and soluble proteins shortly after their translocation into the ER and its dissociation is often correlated with polypeptide folding or subunit assembly. Calnexin interacts with the oligosaccharide $Glc_1Man_9GlcNAc_2$ which is attached to nascent polypeptides and additional amino acids within the polypeptide chain (Vassilakos et al, 1996).

Murine MHC class I assembly may proceed as follows. Newly synthesised murine heavy chains (H chains) associate rapidly with calnexin and maintain this interaction during H chain assembly with β_2m (Degen et al, 1992). Calnexin-bound H chain β_2m heterodimers associate with TAP (Suh et al, 1996). The presence of TAP-1 but not TAP-2 is essential for this interaction as is residue 222 of the membrane proximal portion of the H chain α_3 domain (Suh et al 1994, Suh et al, 1996). Following assembly of this large ternary complex, TAP transported peptide associates with the

MHC class I binding groove and class I molecules dissociate from TAP but remain largely associated with calnexin (Suh et al, 1996). Dissociation of calnexin is coincident with transport of fully conformed class I molecules to the Golgi apparatus.

The assembly of human MHC class I-peptide complexes is similar but different to that of the mouse in that assembly and surface expression of conformed class I molecules is unaltered in the absence of calnexin (Scott et al, 1995). Calnexin has been shown to associate with free human class I heavy chains but this would seem not to be essential for assembly (Ortmann et al, 1994). Calreticulin has recently been shown to act as an alternative chaperone to calnexin (Sadasivan et al, 1996). Calreticulin like calnexin is an ER resident protein with lectin like specificity for monoglucosylated N-linked glycans. It associates with H chain-β_2m dimers and is part of the TAP H chain-β_2m ternary complex (Sadasivan et al, 1996). A further component has been implicated in the formation of the H chain-β_2m calreticulin TAP complex. Tapasin is a 48 kDa glycoprotein which immunoprecipitates with TAP, and associates with calreticulin-H chain-β_2m (Ortmann et al, 1994, Sadasivan et al, 1996). It is suggested to form a bridge between calreticulin-H chain-β_2m and TAP. A similar component is likely to exist but has not yet been identified in the mouse.

1.3 On the nature of peptides which bind to MHC class I molecules

Allele specific binding motifs have been established for both murine and human MHC class I and II molecules (see chapter 4.1), (Falk et al, 1991, Hunt et al, 1992, reviewed by Rammensee et al, 1995). Murine class I molecules bind peptides of predominately 8-10 amino acids which have a hydrophobic C-terminal residue. Human class I molecules place a similar length restriction on peptides but can accommodate positively charged C-terminal residues (arginine and lysine) in an allele specific manner (Rammensee et al, 1995). Longer and shorter peptides can associate in non standard conformations with class I molecules (Schulz et al, 1991, Urban et al,

1994, Collins et al, 1994, Bouvier and Wiley, 1994). Elution of peptides from MHC molecules and bulk sequencing of this material has revealed allele specific binding motifs (Falk et al, 1991). Binding motifs reflect peptide C-terminal amino acid preference (which is common to multiple class I alleles) and conservation of amino acid usage which is peptide residue and allele specific. Peptide motifs have been used to successfully predict class I epitopes (Pamer et al, 1992, Cossins et al, 1993). However, as they reflect *per se* the nature of the dominant species of peptide which associates with a specific MHC allele binding motifs cannot be used to predict peptides which bind in non-standard conformations utilising alternative strategies. Scanning of the human papilloma virus E7 protein for H2- D^b and K^b binding motifs identified a K^b binding peptide to which peptide specific T cells could be induced. These T cells however fail to recognise naturally processed E7 peptides, indicating that the natural epitope differs from the predicted peptide. The naturally processed peptide was subsequently identified and shown to have neither a K^b or D^b binding motif (Sadovnikova et al, 1994). This study serves to illustrate that not all peptides which have the potential to bind to class I molecules are necessarily generated *in vivo*. Indeed, the generation of potential class I binding peptides has been shown to be dependent both on epitope location within a protein and on internal epitope residues (Del Val et al, 1991, Eisenlohr et al, 1995, Hahn et al, 1992, Ossendorp et al, 1996).

The examination of bulk peptide sequence has identified general and specific features of MHC peptide binding. An examination of MHC-peptide crystal structure will help identify the structural basis of peptide binding.

1.4 The structural basis for the association of peptides with MHC class I molecules

The crystal structure of the MHC-peptide complex has been determined for both mouse and human class I molecules in association with heterogeneous or single

peptides (Bjorkman et al, 1987a and b, Madden et al, 1991 and 1992, Saper et al, 1991, Fremont et al, 1992, Matsumara et al, 1992, Young et al, 1994, and others). The overall topology and fold structure of the MHC class I H chain-β_2m dimer is conserved between mouse and man (Bjorkman et al, 1987a, Fremont et al, 1992). The MHC class I heavy chain consists of three domains (α1, α2 and α3). The α3 domain and the β_2m subunit have immunoglobulin like folds, interact with each other and lie proximal to the cell membrane, figure 1.3 A and B. The α1 and α2 domains are novel in structure, associate to form a superdomain which binds peptide and are supported in a membrane distal position by the α3 domain and β_2m subunit, figure 1.3 A-C. The α1 and α2 domains are composed of four β strands straddled by a C-terminal α-helical region. The association of α1 and α2 results in an extended eight stranded antiparallel β-sheet which is traversed by two long α-helical segments that run antiparallel to each other and perpendicular to the β-sheet. β strands are connected by non helical loops which are either solvent exposed and extend from below the α helices on either side of the binding groove or are inaccessible to solvent, extend below the floor of the β pleated sheet and interact with the α3 domain or β_2m subunit (Saper et al,1991).

The α helices are comprised of a short vertical helix (H1) followed by a long curved (H2 in α1) or kinked (H2a, H2b in α2) helix. The highest point of each helical region is near the turn at the intersection of helices H1 and H2 (Saper et al, 1991). The α helices therefore conform only in part to the contours of the β pleated sheet being considerably higher towards their N-termini. The orientation of the α1 and α2 helices may have implications for TCR access to peptide (Garcia et al, 1996, Garboczi et al, 1996). The α helices and β pleated sheet form the boundaries of a cleft which is approximately 30 Å in length and binds peptide. The class I peptide binding groove is delimited at the left end (with which the peptide N-terminal interacts), where the α1 and α2 helices come together, by the Trp167 ring contacting Tyr59 and at the right end by Tyr84 contacting the inner face of H1 α2 (Saper et al, 1991).

1.4.1 The interaction of peptide with the MHC class I binding groove

A pocket in an MHC binding groove is a structural unit with an individual affinity to a corresponding peptide side chain or residue. Certain pockets have a well shaped structure, (recess, indentation or chemical composition) that is specific for a single peptide side chain, others do not and in certain circumstances the boundaries between pockets can be ambiguous (Matsumara et al, 1992). Six pockets (A, B, C, D, E and F) have been identified (on the basis of solvent accessibility to a 1.4 A probe) within the MHC class I peptide binding groove, figure 1.4 (Saper et al, 1991, Fremont et al, 1992 and others). Pockets are located at either the junction of the β-pleated sheet and the α helices (B, C, D, and E) or between the α helices (A and F). Pockets A and F lie at either end of the binding groove and are responsible for binding the N- and C-termini of the peptide respectively. The chemical composition of pockets A and F is conserved between the class I alleles of mouse and man. Conserved residues in pockets A and F (predominately tyrosine residues which can hydrogen bond via their free hydroxyl group) form hydrogen bonds with polar main chain atoms and the peptide terminal NH_2 and COOH groups. Hydrogen bonding networks are stabilised in both pockets by the inclusion of a water molecule (Madden et al, 1991, Fremont et al, 1992, Bouvier and Wiley, 1994). Hydrogen bonding between the N- and C-termini and conserved MHC residues in the A and F pockets has been shown to contribute 22^0C to the thermal stability of an HLA-A2 MHC-peptide complex (total thermal stability of complex 65^0C), a contribution which is greater than that of the combined effect of the peptide anchor positions (Bouvier and Wiley, 1994). Although much of the thermal stability of the MHC-peptide complex is derived from conserved N- and C- terminal interactions a decamer peptide has been shown to bind to HLA-A2.1 with the C-terminus extending out of the binding groove (Collins et al, 1994). In this case however, the thermal stability of the complex is 14.1^0C lower than that of the MHC-nonamer complex due to the disruption and loss of three hydrogen bonds to

the peptide carboxylate oxygen. Whether this complex exists *in vivo* as a decamer extending from the groove is unknown.

A comparison of mouse and human peptide backbone structures reveals limited conformational variation at both N- and C- termini, indicating that most peptides interact similarly at both termini (Fremont et al, 1992, Madden et al, 1993). Bulk sequencing of eluted peptides and N-terminal amino acid substitution studies indicate a lack of preference for a specific N-terminal peptide residue (Falk et al, 1991, Bouvier and Wiley, 1994). Consistent with such a finding, an examination of MHC-peptide crystal structures indicates that N-terminal residue side chains point out of the A pocket (Madden et al, 1993). Hydrophobic residues are preferentially bound in the F pocket of mouse class I molecules (Falk et al, 1991). This preference for hydrophobic residues is explained by the finding that the C-terminal residue side chain points down into the hydrophobic interior of the F pocket (Saper et al, 1991, Fremont et al, 1992 and others). Human class I molecules bind peptides with hydrophobic or basic (arginine or lysine) C-terminal residues. An examination of the amino acid composition of the HLA-A68 F pocket reveals a negatively charged aspartate residue at its base and accounts for the ability of this molecule to bind peptides with positively charged C-terminal residues (arginine or lysine). Overall, these results suggest that MHC class I molecules have evolved to conserve important residues at both ends of the peptide binding groove which form universal pockets for binding the charged termini of short peptides.

Most of the peptides which bind to MHC class I molecules are 8-10 amino acids in length and contain one or two major anchor residues in addition to a conserved C-terminal residue (Rammensee et al, 1995). The position, physical and chemical nature of motif residues determines their ability to interact with the polymorphic pockets (B, C, D and E) of the MHC binding groove. In general, peptide anchor residues bind into

deep electrostatically and sterically complementary pockets and contribute to the overall energy of binding.

Peptide position two is an important anchor residue for the majority of mouse and human class I alleles (Rammensee, et al, 1995). The potential for allele specific diversity within this pocket can be seen by examining the mechanism of binding of HLA-A2 and HLA-B27 binding peptides both of which have a preferred second residue (Leucine for HLA-A2 and Arginine for HLA-B27) which binds within the MHC B pocket. In order to accommodate a positively charged arginine residue the HLA-B27 B pocket has a negatively charged glutamate residue at its base. By contrast, the interior of the HLA-A2 B pocket is entirely hydrophobic and is ideally suited to binding the motif residue leucine.

General cleft architecture also plays an important role in determining the nature of peptides which bind to MHC molecules. The greatest structural differences between H2-D^b and H2-K^b occur in the mid cleft region where β sheet residues Glu9 and Gln97 (H2-D^b) replace Val9 and Val97 (H2-K^b), (Young et al, 1994). These changes create a shallow polar ledge in the centre of the D^b cleft at a point where it is already narrowed compared to K^b. This grouping of structural features determines the anchor residue preference of H2-K^b and H2-D^b (P5-Asn for D^b and P5-Tyr for K^b) in that the larger tyrosine residue can be accommodated by the slightly wider and hydrophobic environment of the K^b cleft and the narrower more polar cleft of the D^b molecule makes a good set of contacts (including three hydrogen bonds to Gln97) with the asparagine residue (Young et al, 1994).

Together, specific N- and C-terminal interactions combined with allele specific interactions and general cleft architecture determine the orientation and conformation of peptides bound to MHC class I molecules. As a composite structure the MHC-peptide complex represents the ligand which is recognised by the T cell receptor.

Cytotoxic T cells recognise antigens as small antigenic peptide fragments bound to MHC class I molecules on the surface of antigen presenting cells. The recognition of the MHC class I-peptide complex is accomplished by a heterodimeric T cell receptor (TCR) composed of disulphide linked α and β chains. The following sections will examine the genomic organisation of the TCR α and β loci and the mechanisms responsible for generating T cell receptor diversity.

1.5 The Genomic organisation of the TCR loci

T cell receptor α and β genes are situated in distinct loci on chromosomes 14 (TCRA) and 6 (TCRB) in the mouse and 14 (TCRA) and 7 (TCRB) in humans (orphan TCRB V segments have also been mapped to chromosome 9 (Kranz et al, 1985, Lee et al, 1984, Caccia et al, 1984, Collins et al, 1984, 1985, Cornelis et al, 1993). The TCRB locus in the mouse consists of approximately 23 variable (V), 2 diversity (D), 12 junction (J) and 2 constant (C) functional gene segments, arranged over 800kb (Arden et al, 1995a). The TCRA locus consists of approximately 87 functional V region gene segments arranged, as 20 related subfamilies, over 370kb, 50 J segments and a single C segment arranged over 95kb (Arden et al, 1995a, Jouvin-Marche et al, 1990, Koop et al, 1992). Mouse TCRA V segment sub-families contain members which share over 80% similarity at the DNA level and have been generated through successive rounds of gene duplication (Jouvin-Marche et al, 1990). Accordingly, inter sub-family similarity is below 65% (Arden et al, 1995a). Mouse TCRB V segments generally exist as single member sub-families with below 55% sequence similarity at the DNA level. TCRB V8 and TCRB V5 subfamilies are exceptional, having arisen by two successive rounds of tandem gene duplication and have three TCRB V segments (the third TCRB V5 segment is a pseudogene), (Chou et al, 1987). In contrast to mouse TCRB V sub-families which tend to contain mainly single gene segments mouse TCRA V sub-families generally have multiple members. If anything the opposite is true of human TCRB V and TCRA V sub-families (only two of 32 TCRA V sub-

families have more than two members whereas 7 of 26 TCRB V sub-families have 3 to 9 members), (Arden et al, 1995b). This human/mouse inter-species variation might suggest that T cell receptors in mice and humans have evolved under slightly different selective pressures. An examination of inter sub-family diversity within and between species however reveals that members of murine and human TCR V sub-families are more closely related to each other than they are to other sub-family members of their own species (Clark et al, 1995). This indicates that mouse and human TCR V segments have co-evolved under similar selection pressures from a pool of common ancestral V segments (in which TCR families may have already been established) and that the differences in genomic structure of the α and β loci represent species specific variation on a common theme.

1.5.1 V(D)J recombination and the potential to generate diversity

As a result of evolutionary pressure (the need to express a broad TCR repertoire) the immune system has evolved a mechanism to generate complete TCR genes from multiple gene fragments (V, (D), J and C segments). A functional TCR gene is formed when a single V segment recombines with a D and/or J segment upstream of a C segment. The recombination system (multiple enzymes which recognise, cut, process and ligate DNA) recognises conserved sequence elements, termed recombination signal sequences (RSS), that adjoin the V, D, and J segments (Tonegawa et al, 1983). RSS are composed of two relatively conserved sequences (a heptamer and a nonamer) separated by a spacer (which can be 12 or 23 bases in length) and lie immediately upstream or downstream of segments capable of somatic recombination (Tonegawa et al, 1983, Hesse et al, 1989). RSS with a 23 base spacer may only recombine with those which possess a 12 base spacer. TCR V segments have a 23 base RSS immediately 3' of the coding region and consequently recombine with down stream D or J segments which have a 5' RSS with a 12 base spacer. TCRB D segments also have a 3' RSS with a 23 base spacer which can recombine to a down

stream J segment possessing a 3' RSS with a 12 base spacer. No somatic recombination occurs between the J segment and the TCR constant segment.

The mechanism of recombination is incompletely understood (reviewed by Bogue and Roth, 1996). However certain components are more clearly defined than others. DNA cleavage occurs in two steps, both of which are dependent on the presence of the recombinase activating gene products RAG-1 and RAG-2 (McBlane et al, 1995). First, a single nick is introduced precisely at the border between the RSS and the coding sequence. Second, the free 3' end of the coding segment attacks the 5' end of the coding sequence complementary strand generating a hairpin structure. In doing so, RAG-1 and RAG-2 excise the fragment of intervening DNA which contains both the 3' and 5' RSS and generate two hairpin loops which contain the nucleotides of the coding elements involved in recombination. The DNA ends of TCR coding segments exist only transiently as closed ended hairpin loops before they are subsequently opened by the action of an as yet unidentified nuclease. Opening of a hairpin loop at its terminus generates a blunt ended product which can be ligated to the neighbouring coding sequence involved in the recombination event. The introduction of a nick near to the terminus generates a short palindromic single stranded extension which can be subsequently filled in and incorporated into the rearranged TCR gene as novel junctional sequence. This type of junctional addition is termed template-dependent palindromic (P) and is a mechanism for generating additional sequence diversity within V(D)J junctions. Additional junctional diversity can be introduce during the recombination event by the addition or loss of variable numbers of nucleotides. Terminal deoxynucleotide transferase (TdT) has been identified as the enzyme responsible for template independent addition of (N) nucleotides. Consequently in mice with targeted deletions of the TdT gene (TdT-/- mice) less than 3% of TCR junctional sequences contain template-independent insertions (Gilfillan et al, 1993, Komori et al, 1993).

The generation of junctional diversity is critical to the production of a broad T cell repertoire. Initial predictions that the T cell receptor would largely conform in tertiary structure to that of an immunoglobulin Fab fragment (Davis and Bjorkman, 1988, Chothia et al, 1988, Claverie et al, 1989) have been largely confirmed by the crystallisation of a TCR α domain, β chain, two TCR αβ heterodimers and a single chain TCR Fv fragment in combination with a clonotype specific immunoglobulin Fab fragment (Fields et al, 1995, Bentley et al, 1995, Garcia et al, 1996, Garboczi et al, 1996, Housset et al, 1997). Regions of TCR sequence hypervariability are now known to encode solvent exposed protein loops which interact with the MHC-peptide complex (Chothia et al, 1988, Jores et al, 1990, Garboczi et al, 1996). Furthermore, as initially predicted the third hypervariable loop (CDR3) which is encoded by V(D)J junctional sequence interacts largely with peptide bound in the antigen binding groove (Garcia et al, 1996, Garboczi et al, 1996). Thus V(D)J recombination enables peak diversity to be generated in the region of the TCR which is primarily involved in recognising antigenic peptides.

1.6 Interaction of the TCR with the MHC-peptide complex

The crystal structure of the T cell receptor in complex with MHC-peptide has recently been solved and largely confirms previous predictions that the TCR would conform in tertiary structure to an immunoglobulin Fab fragment (Garboczi et al, 1996, Garcia et al, 1996, Housset et al, 1997, Chothia et al, 1988, Davis and Bjorkman, 1988, Claverie et al, 1989). As is the case for immunoglobulin variable domains the membrane distal domains of the TCR (Vα and Vβ) possess solvent exposed hypervariable loops (termed CDR1-3 after complementarity determining regions initially described for immunoglobulins) which in the case of the TCR are involved in the recognition of the MHC α helices and associated peptide. CDR1 and CDR2 are integral parts of the Vα and Vβ domain while CDR3 is encoded by V(D)J junctional sequence (Chothia et al, 1988, Jores et al, 1990). As a consequence of this, CDR1 and

CDR2 exhibit limited V region specific variation while CDR3 exhibits greater variability which is largely independent of V segment usage. A fourth solvent exposed loop is present in both Vα and Vβ domains. This loop is hypervariable in TCR β chains and is suggested to interact with endogenous and exogenous superantigen (Choi et al, 1990). The crystal structure of a Vβ8.2 chain in combination with staphylococcus enterotoxins C2 and C3 has recently been solved and confirms the involvement of this loop (in addition to β chain CDR2 and to a lesser extent CDR1) in binding exogenous superantigen (Fields et al, 1996). Although this fourth loop may orientate towards CDR1 and CDR2 and has been postulated to interact with the MHC helices and/or peptide (Bentley et al, 1995) an examination of the 2C/dEV8/H2-K^b crystal structure indicates that neither the α or β loop is close enough to the MHC-peptide surface to make any significant interatomic contacts (Garcia et al, 1996). This said, either loop may affect MHC-peptide recognition by making contacts with CDR1 or CDR2 and altering their interaction with the MHC α helices and/or peptide. Furthermore, in the TCR(A6)/Tax/HLA-A2 complex a single residue of loop 4 (α68K), contacts the MHC molecule forming hydrogen bonds to the α2 helix at T163 and E166 (Garboczi et al 1996). The involvement of the fourth hypervariable loop in contacting the MHC-peptide complex would therefore seem not to be extensive but in certain cases possible.

The crystal structure of the TCR(A6)/Tax/HLA-A2 complex has been solved at 2.6 A and reveals a diagonal orientation of the TCR on the MHC class I molecule, figure 1.5 A-D (Garboczi et al, 1996). The TCR is oriented similarly in the 2C/dEV8/H2-K^b complex, figure 1.5 A-C (Garcia et al, 1996). It has been proposed that the diagonal orientation of the TCR on the MHC-peptide complex is a feature general to all TCR/MHC-peptide complexes and results from conserved structural features of both the TCR and the MHC molecule (Garboczi et al, 1996). The contact surface of the TCR is composed of solvent exposed hypervariable loops (CDR1-3) and is relatively flat except for a deep central pocket which is formed where CDR3α and β converge

(Garboczi et al, 1996, Garcia et al, 1996). The nature of this surface does not permit probing of the antigen binding cleft in search of polymorphic peptide residues. Consequently, the TCR must engage a broad area of the MHC-peptide surface in order to specifically interact with peptide TCR contact residues. The crystal structure of a single chain TCR Fv fragment in combination with a clonotype specific antibody has recently been solved and reveals a slightly different CDR3 conformation in that CDR3 α and CDR3 β are closely apposed and a central cleft is not formed. Whether this conformation results from immunoglobulin Fab fragment induced alterations in CDR3 positioning is unclear. If, as is likely, the conformation of the TCR CDR3 region is non artifactual then interaction of this TCR with MHC-peptide ligand would be predicted to involve significant CDR3 conformational changes.

The accessibility of the TCR to MHC-peptide is further complicated when one considers that MHC associated peptides are buried deep in the antigen binding cleft. In the case of MHC class I associated peptides, the octamer and nonamer peptides VSV-8 and SEV-9 in complex with H2-K^b have only 17% and 25% of their respective surfaces accessible to solvent (Fremont et al, 1992). Solvent inaccessibility is primarily due to the mechanism of peptide binding in which both the N- and C-peptide termini and anchor residues are tethered deep in the antigen binding groove. Nevertheless, peptides which are anchored at their termini have the ability to bulge out of the MHC binding cleft in their central region (Fremont et al, 1992, Madden et al, 1991). The bulging of peptide out of the MHC binding groove increases the solvent accessibility of central peptide residues and is essential if longer peptides are to be accommodated within the confines of a closed ended groove. The presence of a more centrally located anchor residue does not necessarily preclude peptide bulging. Indeed the greater solvent accessibility of the SEV-9 nonamer over the VSV-8 octamer is primarily due to a bulge in the centre of SEV-9 just prior to the P6 anchor residue. Central peptide flexibility is further revealed in a structural comparison of five HLA-A2-peptide complexes (Madden et al, 1993). Peptide termini and P2 anchor

side chains are bound similarly in all five cases. In contrast, peptide main and side chains adopt strikingly different conformations in the central part of the peptide binding groove, where they are most accessible to solvent and the T cell receptor.

Although peptide residues may bulge out of the antigen binding cleft they are still positioned well below the apices of the MHC $\alpha 1$ and $\alpha 2$ helices, figure 1.3 (Saper et al, 1991). In order for the fairly flat surface of the TCR to fit at a low enough point on the MHC surface to contact a significant length of exposed peptide, a diagonal orientation (between the apices of $\alpha 1$ and $\alpha 2$ helices) is required, figure 1.5 and 1.6. Translation of the TCR along the peptide binding site or rotation from this orientation is likely to break the shape complementarity. MHC mutation analysis and peptide immunisation of TCR α or β chain transgenic mice further support such an orientation (Sun et al, 1995, Sant'Angelo et al, 1996).

Structural constraints favour a single orientation of the TCR on the MHC-peptide complex. Conserved Vα or Vβ usage in the recognition of a defined peptide has also been suggested to support a single orientation of the TCR (Sant'Angelo et al, 1996). This is weak evidence since it is not unlikely that specific structural features of an MHC-peptide complex (peptide residues or peptide induced conformational changes in the MHC molecule) may be preferentially recognised by CDR1(α or β) or CDR2(α or β). If this happens then V region conservation will be driven by MHC-peptide recognition and not necessarily by an inherent recognition mechanism.

Stronger evidence suggesting a single orientation of the TCR is derived from the finding that certain individual V regions are preferentially expressed in CD4+ or CD8+ peripheral T cell subsets (Jameson et al, 1990, Jameson et al, 1991, Pircher et al, 1992). CD8+/CD4+ subset specific V segment usage suggests that V regions interact preferentially with MHC class I/II or tend to preferentially associate with either CD8 or CD4 co-receptor. In either case a single orientation of the TCR on the

MHC molecule is implied. Closely related members of the Vα3 family undergo selection on different MHC classes (Vα3.1 and Vα3.2 are preferentially expressed on CD4+ and CD8+ peripheral T cells respectively), (Sim et al, 1996). Vα3.1 and Vα3.2 differ by only 4 amino acids of which two are located in CDR1 and CDR2. Reciprocal substitution of one or other of these residues is sufficient to reverse the pattern of Vα3 expression in CD4+ and CD8+ T cells (Sim et al, 1996). These data suggest that residues in CDR1/2 of Vα3.1 and 3.2 dictate specificity for MHC class and imply a single orientation of the TCR on MHC-peptide complex.

An examination of TCR/MHC-peptide crystal structures indicates that the initial model which predicted CDR1/2 to interact with the MHC helices and CDR3 with MHC bound peptide is too rigid (Davis and Bjorkman, 1988, Chothia et al, 1988, Claverie et al, 1989). In the case of the A6/Tax/HLA-A2 complex variable loops CDR1 and 3 of both Vα and Vβ contact the Tax peptide whereas CDR1-3 of Vα and CDR3β contact the HLA-A2 α helices. Furthermore, CDR3β extends across the binding site contacting the α1 helix, peptide residues (Y5, P6, V7 and Y8) and multiple residues on the α2 helix. In contrast, CDR2β fails to make any contacts with the MHC-peptide complex (Garboczi et al, 1996). Although similarly oriented, the hypervariable loops of mouse TCR 2C are utilised differently to contact H2-K^b-dEV8 (Garcia et al, 1996). CDR2β covers most of the carboxyl end the MHC α1 helix and has some contact with C-terminal peptide residues whereas CDR1β covers the N-terminal end of the α2 helix. In both mouse and human TCR/MHC-peptide crystal structures CDR3α and β converge over the centre of the peptide to form a deep cavity which accommodates a major TCR contact residue(P4-K for dEV8 and P5-Y for Tax). This is best observed in the A6/Tax/HLA-A2 crystal structure in which P5-tyrosine is buried in a deep hydrophobic pocket formed from CDR3 α and β. P5-Y is further anchored within the TCR pocket by a hydrogen bond which extends from its hydroxyl group to serine 31 of CDR1α (Garboczi et al, 1996). Whether a central CDR3 pocket is formed or induced to form (following MHC-peptide association) in

other TCR is unknown. However, bearing in mind that central peptide residues often bulge out of the binding groove a pocket in such a location would be ideally situated to interact with solvent exposed peptide side chains.

1.7 The relationship between altered peptide ligands and T cell activation

It has recently been established that T cells can interact productively with suboptimal ligands. A suboptimal ligand (partial agonist) for the purposes of this discussion is an MHC-peptide complex which interacts with a TCR and results in an altered biological response in comparison to that of the optimal MHC-peptide ligand (agonist). Flexibility in TCR recognition of its ligand was initially established in a report describing a CD4+ Th2 clone that responded to an MHC-peptide ligand by proliferating and producing interleukin (IL4) but responded to a single amino acid variant of the peptide by secreting IL4 in the absence of proliferation (Evavold and Allen, 1991). This was followed by a report which showed that proliferation of a CD4+ T cell clone could be inhibited by variants of an agonist peptide which was altered at specific TCR contact residues (De Magistris et al, 1992). Further studies have shown that the recognition of suboptimal ligands is a general characteristic of CD4+ and CD8+ T cells which may result in partial activation, induction of anergy or complete antagonism of the biological response to agonist peptide (Jameson et al, 1993, Sloan-Lancaster et al, 1993 and 1994). Taken together, these findings clearly show that a T cell can respond to an MHC-peptide ligand in a variable manner and that the TCR is not merely an on/off switch.

The finding that T cells respond differently to full agonists and altered peptide ligands suggests that intracellular signalling events induced by these interactions are dissimilar. This was confirmed in an examination of membrane proximal signalling events which revealed that the pattern CD3ζ chain phosphorylation was altered in

response to TCR activation with agonist or partial agonist peptides (Sloan-Lancaster et al, 1994, Madrenas et al, 1995). Two differentially phosphorylated forms of CD3ζ are generated following TCR interaction with a full agonist ligand (p21 and p23 phospho-ζ chains). Following TCR interaction with a partial agonist or antagonist peptide there is a relative or complete absence of p23 phospho-ζ. Following, phosphorylation of CD3ζ in response to agonist peptide the syk family kinase ZAP-70 binds to phosphorylated ζ chains and is itself subsequently activated through phosphorylation. Partial agonist and antagonist peptides fail to induce ZAP-70 phosphorylation although (inactive) ZAP-70 may be found stably associated with p21phospho-ζ chains (Sloan-Lancaster et al, 1994, Madrenas et al, 1995). These experiments show that altered peptide ligands deliver a qualitatively different signal to the cell and not just less of the same signal which is generated by agonist interaction with the TCR.

More recently the activity of altered peptide ligands has been correlated with CD3ζ chain phosphorylation, T cell acid release, intracellular calcium flux and proliferation (Rabinowitz et al, 1996). Calcium flux and acid release are complementary assays which measure T cell activation within minutes of receptor ligation (Rabinowitz et al, 1996). In these experiments, antagonist peptides generally failed to induce acid release or sustained calcium flux whereas agonist peptides induced the full spectrum of responses including proliferation. Furthermore, peptides were identified which could trigger early responses (partial calcium flux and acid release) but failed to sustain an increased intracellular calcium concentration or induce proliferation. These results suggest a hierarchy of T cell activation steps which can be differentially activated in response to altered peptide ligands. The causal relationship between ζ chain phosphorylation, acid release and calcium flux has not been established. However it has been argued that acid release and calcium flux are independent responses triggered by different signalling pathways (Rabinowitz et al, 1996). This remains to be established.

Two basic models have been proposed to account for differential signalling by agonist peptides and altered peptide ligands (reviewed by Madrenas and Germain, 1996). The first model relates to the kinetics of the interaction between the TCR and its MHC-peptide ligand and suggests that the association and/or dissociation constants of the TCR in combination with an MHC-altered peptide ligand complex are significantly different from those of the same TCR in combination with an MHC-agonist peptide complex. In support of this model, the affinities of three different antagonist peptide-H2-E^k complexes have been shown to be ~ 10-50 times lower than that of the wild-type agonist peptide-E^k complex for TCR (Lyons et al, 1996). Furthermore, the lower affinity of T cell receptors for MHC-altered peptide ligands has been shown to be due to increased TCR off-rates (increased rate of TCR dissociation), (Matsui et al, 1994, Lyons et al, 1996). These results suggest that the biological effects of antagonists and partial agonists may be largely based on kinetic parameters. In support of this notion, positive selection of CD8+ T cells has been shown to enhanced by altered peptide ligands which promote lower affinity TCR interactions and increased TCR off-rates (Alam et al, 1996).

A second model of agonist/altered peptide ligand T cell activation contends that agonist and altered peptide ligands induce different conformational changes in the TCR which are able to induce unique signals (Janeway, 1995). At present no direct evidence exists for such a model. Although it is certainly possible that TCR conformation may be subtly changed in response to an interaction with an altered peptide ligand one must weigh this up against the available evidence which suggests that the T cell receptor Vα domain and β chains have a very similar conformation when crystallised alone or as a heterodimer in combination with MHC-peptide (Garcia et al, 1996, Garboczi et al, 1996). Possible flexibility in the Vα hinge region is suggested by the disordered appearance of the Cα domain in the murine 2C αβ heterodimer. However, Cα has a rigid structure in the A6/HLA-A2-Tax complex

indicating that flexibility may be a feature of this area only in uncomplexed soluble TCR αβ heterodimers. Indeed, no overall conformational change has been observed in Fab fragments even when their V domains rotate up to 16° as a result of induced fit in the antigen combining site (Stanfield et al, 1993). Furthermore, a comparison of the crystal structures of TCRs in complex with MHC-peptide complexes suggests that TCRs will be able to interact with the MHC-peptide complexes utilising their CDR3 domains to a greater or lesser extent depending on the exact nature of the ligand. In this respect, the interaction of the TCR with its ligand will therefore differ from that of other receptor-ligand interactions in that the exact nature of the interatomic contacts will differ for each TCR in complex with MHC-peptide. It seems unlikely that this diversity in recognition will promote a uniform conformational change in the overall structure of the TCR. Furthermore, bacterial superantigens, MHC-peptide complexes and TCR clonotype specific antibodies all differ in the way they bind TCR V domains yet are all capable of efficiently activating T cells (Fields et al, 1996, Garboczi et al, 1996, Housset et al, 1997).

Although it is unlikely that a conformational change is responsible for directing a signal through the CD3 complex, it is possible that lesser conformational alterations and/or enhanced rigidity of the overall αβ heterodimer (on ligation of MHC-peptide complex) promote either co-receptor association or TCR dimerisation. Evidence which supports dimerisation is mainly derived from physical and crystallographic studies (Brown et al, 1993, Fields et al, 1995, Reich et al, 1997). MHC class II molecules have been shown to crystallise as a dimer of dimers, suggesting that dimerisation may also occur in vivo (Brown et al, 1993). Likewise mouse TCR α variable domains dimerise on crystallisation (Fields at al, 1995). More recently, MHC-peptide ligand specific oligomerisation of T cell receptor molecules has been shown to occur in solution and is hypothesised to be enhanced *in vivo* in the presence of CD4 or CD8 co-receptor molecules (Reich et al, 1997). Additionally, using a soluble TCR and MHC-agonist peptide complexes bound to surface plasmon

resonance chips, Gascoigne and others have demonstrated significantly decreased TCR off rates at 37° compared to 26° (N. Gascoigne, personal communication [BSI]). Based on the exact characteristics of the binding kinetics this data has been interpreted to suggest that a conformational change occurs in the TCR following MHC-peptide association at 37° which promotes TCR dimerisation. Based on a model of TCR dimerisation or oligomerisation agonist and antagonist peptides could be differentiated on the basis of their ability to induce the formation of stable aggregated TCR clusters. This however does not resolve the issue of whether altered peptide ligands operate on the basis of an induced TCR conformational change (which promotes oligomerisation of TCR) or kinetic parameters (in which rapid dissociation of the TCR/MHC-peptide complex prevents oligomerisation).

Unfortunately a projection of a TCR$\alpha\beta$ dimer of dimers (based on the conformation of the Vα homodimer) onto the human MHC class II dimer of dimers reveals a difference in complementarity such that both T cell receptors may not interact with MHC bound peptide simultaneously (Fields et al, 1996). In addition, murine and human TCR/MHC-peptide complexes crystallise as single units (Garboczi et al, 1996, Garcia et al, 1996). These data call into question the above observations and suggest that oligomerisation may be an *in vitro* effect.

It has recently been established that a single MHC-peptide complex expressed on the surface of an antigen presenting cell (APC) can engage and serially trigger up to 200 T cell receptors (Valitutti et al, 1995). In addition, different T cell responses can be elicited by different levels of T cell receptor occupancy (Valitutti et al, 1996). This suggests that different T cell responses can be triggered by different doses of pure agonist peptide in a manner not dissimilar to that initially shown for partial agonist peptides. How might serial triggering control differential signalling? A distinctive feature of this model is that an appropriate off rate is required to enable a single MHC-peptide complex to engage and trigger multiple T cell receptors within a given

time frame. Optimal TCR triggering will therefore occur when the half life of the TCR/MHC-peptide complex matches the time required to generate a complete activation signal within the cell. Consequently, TCRs which engage MHC-peptide for suboptimal periods may fail to generate complete activation signals. Interestingly, MHC-peptide complexes which are capable of binding TCRs with a very high affinity may also fail to signal effectively due to an inability to dissociate and trigger multiple T cell receptors (this phenomenon may explain the behaviour of high affinity anti-CD3 antibodies which trigger T cells inefficiently), (Viola et al, 1996). It is possible that such an effect would be overcome in the presence of excess MHC-peptide since concomitant engagement of multiple T cell receptors may tend to generate a complete T cell activation signal. However, such an argument assumes that all MHC-peptide and TCR molecules are available to interact on the cell surface. This may not be the case. It is probable that serial triggering occurs within a limited area of T cell-APC contact and will therefore be dependent on the supply of new TCRs and signal transduction components to replace those removed and utilised following TCR/MHC-peptide interaction. If this is the case then even high levels of high affinity MHC-peptide complexes would be incapable of activating sufficient TCRs due to decreased off rates and lack of exposure to T cell receptors outside the T cell-APC contact site. The dependence on sustained serial triggering to initiate and maintain T cell activation suggests that thymocytes may be selected on the basis of critical kinetic parameters which promote optimal triggering of T cell receptors in the periphery.

1.8 The involvement of peptides in thymocyte positive and negative selection

T cell receptor transgenic mice have been used extensively to examine the nature of positive and negative selection and most of what we now know of these processes derives from their use (Kisielow et al, 1988a and b, Teh et al, 1989, Sha et al, 1988a and b, 1990, Berg et al, 1989a and b, Pircher et al, 1989, Mamalaki et al, 1992 and

1993). However, TCR transgenic mice do have their drawbacks in that they only provide a method to spot check selection of a specific T cell receptor. This said, a large number of different TCR transgenics now exist and generalisations concerning positive and negative selection have emerged.

The generation of TAP-/- and β_2m-/- mice has allowed the specific *in vitro* peptide requirements for positive and negative selection to be investigated in foetal thymic organ culture (FTOC). Using the TAP-/- system it has been shown that peptide contributes to the specificity of positive selection (simple stabilisation of class I expression is insufficient to promote positive selection of CD8+ T cells in all cases) and that high and low concentrations of the same peptide can induce negative or positive selection respectively (Ashton-Rickardt et al, 1993, 1994a and b). The concept that it is the avidity of interaction between developing thymocyte and thymic antigen presenting cell which differentiates positive from negative selection is further supported by experiments in β_2m-/-mice (Sebzda et al, 1994). In this model system high and low concentrations of an agonist peptide from lymphocytic choriomeningitis virus glycoprotein (LCMV-gp 33-44) which binds to D^b mediate negative and positive selection of LCMV-gp 33-44 specific TCR transgenic thymocytes respectively in FTOC. An alternative set of data using the β_2m-/- mice has been interpreted to support a model of selection whereby TCR antagonist and agonist peptides promote positive and negative selection respectively (Hogquist et al, 1994 and 1995). In these experiments however, the exclusivity of agonist and antagonist peptides in terms of mediating negative or positive selection was not complete in that antagonist peptides could mediate negative selection in β_2m+/- FTOC. This suggests that quantity as well as quality of response is important in dictating positive and negative selection. Interestingly, in these experiments a strong agonist peptide failed to induce positive selection at any concentration tested. It is possible that for strong agonist peptides a very small peptide concentration window exists which can mediate positive selection. Concentrations marginally above this might mediate negative

selection and concentrations slightly lower might go unnoticed by the developing thymocyte. Antagonist peptides for CD8+ T cells were defined as those peptides which have comparable binding to an MHC molecule as a native agonist peptide but which are capable of inhibiting lysis of agonist peptide pre-pulsed target cells (Jameson et al, 1993). On the basis of the data of Hogquist and colleagues it was proposed that MHC-antagonist peptide complexes engage the TCR in such a way as to promote positive selection of developing thymocytes. Furthermore, these data suggest that positive selection may be mediated *in vivo* by sets of TCR specific antagonist peptides.

The data of Hogquist and colleagues are not irreconcilable with the previous data of Ashton-Rickardt and Sebzda but rather fit into a model of T cell activation and selection in which the kinetics of the TCR/MHC-peptide interaction dictate the ultimate outcome (partial T cell response, positive or negative selection). The interaction of a T cell receptor with its MHC-peptide ligand can be broken down into two phases, association and dissociation (for further discussion see Chapter 6). Each phase has its own rate and the net effect determines the affinity of the interaction. It has recently been demonstrated by two different groups that the affinity of interaction between specified TCRs and sets of antagonist peptides is up to 50 times lower than that of the interactions with agonist peptide (Alam et al, 1996, Lyons et al, 1996). Furthermore, this decrease in affinity is largely due to an increased rate in dissociation of the TCR/MHC-peptide complex and correlates with the ability of antagonist peptides to positively select in foetal thymic organ culture. (Alam et al, 1996, Lyons et al, 1996). A relationship between increased dissociation rate and weak T cell stimulatory response has been previously observed (Matsui et al, 1994). Antagonist peptides would therefore seem to promote low affinity interactions between TCRs and MHC/antagonist ligands. Although this is undoubtedly part of the explanation which underlies positive and negative selection, it cannot be the whole story since low concentrations of agonist peptides are known to be able to positively select

thymocytes in FTOC (Ashton-Rickardt et al, 1994, Sebzda et al, 1994). These two issues can be reconciled if one considers positive and negative selection as being dependent on the avidity of the interaction between the developing thymocyte and the thymic stromal cell rather than the affinity of the TCR for the MHC-peptide complex. If this is the case then a small amount of a high affinity agonist peptide/MHC complex or a large amount of a low affinity antagonist peptide/MHC complex should create an inter-cellular interaction of similar avidity. Although TCR based avidity may correlate with selection the contribution that the TCR/MHC-peptide interaction makes to the overall avidity of the intercellular interaction is unknown. TCR and MHC molecules are membrane proximal structures and require the establishment of intimate membrane contact to interact. It is therefore probable that the strength of adhesion will be crucial in permitting encounters between TCRs and MHC-peptide complexes. The strength of adhesion is also enhanced by TCR signalling (Dustin and Springer, 1989). This might permit continued and strengthened association between the T cell and the APC following an initial encounter. Of further relevance to T cell activation and selection is the finding that multiple T cell receptors are triggered by only a few MHC-peptide complexes (Valitutti et al, 1995, 1996, 1997). Such a mechanism of TCR interaction suggests that TCR/MHC-peptide complexes must dissociate in order for an MHC-peptide complex to engage another TCR. It has been estimated that up to 200 TCRs may be triggered by a single MHC-peptide complex (Valitutti et al, 1995). This mechanism of signalling once again focuses attention on the critical importance of the kinetics of the TCR/MHC-peptide interaction in terms of optimal association and dissociation of the TCR. The involvement of peptides in positive and negative selection is further discussed in Chapter 6.

Figure 1.1: The crystal structure of the 20S proteasome from the Archaeon *Thermoplasma acidophilum* at 3.4 Å resolution. (A) Cα drawing of two α (red) and two β subunits (blue) showing the major contacts betwen the subunits. (B) Top view of the 20S proteasome showing the Cα atoms only. (C) Side view of the 20S proteasome. The overall dimensions are 148 Å in length, 113 Å maximum diameter and 75 Å minimum diameter. (D) View of the proteasome cut open along the longitudinal seven fold axis of symmetry; the three compartments and the gates are clearly visible. Dimensions: entrance to the channel: 13 Å diameter, bottlenecks in centre: 22 Å in diameter, left and right cavity: 50 Å diameter and 40 Å length, central cavity: 53 Å diameter and 38 Å length. Taken from Löwe et al, (1995).

Figure 1.1

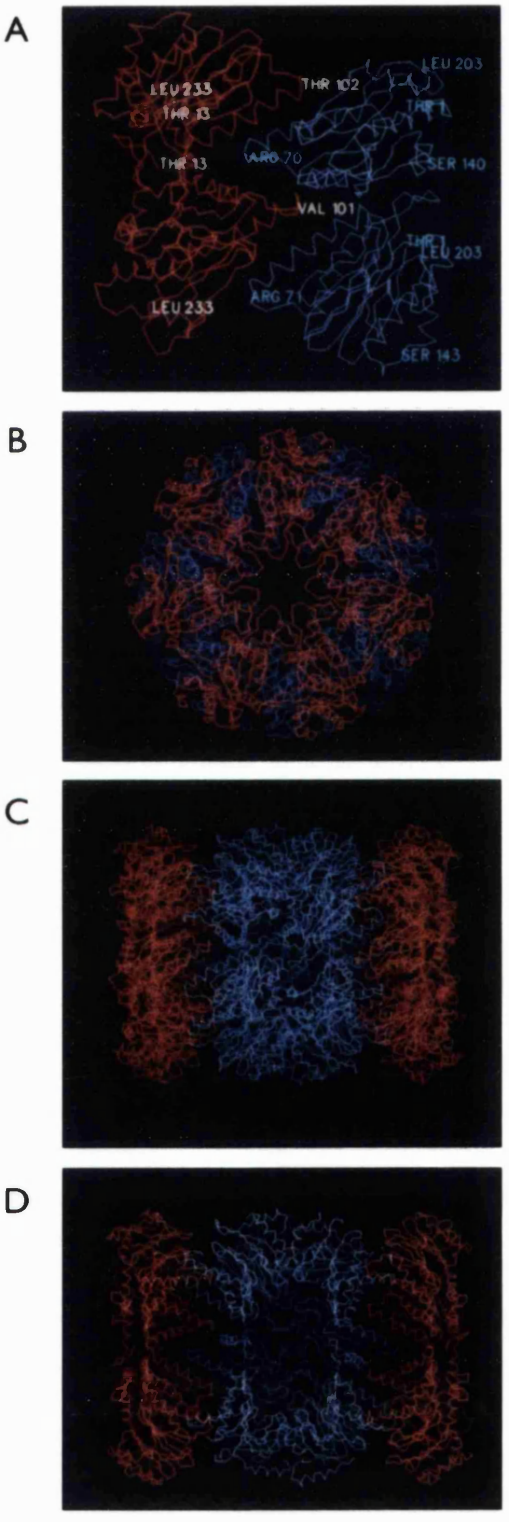

A
LEU 203
LEU 233
THR 102
THR 13
THR 13
ARG 70
SER 140
VAL 101
LEU 203
LEU 233
ARG 71
SER 143
B
C
D

Figure 1.2: Assembly of the MHC class I-peptide complex. The mechanism of MHC-peptide assembly is shown as it is thought to occur in humans. In mice the role of calreticulin is unclear and it may be that calnexin remains associated with class I heavy chain for a longer period, even after dissociation of MHC-peptide from TAP. Taken from Sadasivan et al, (1996).

Figure 1.2

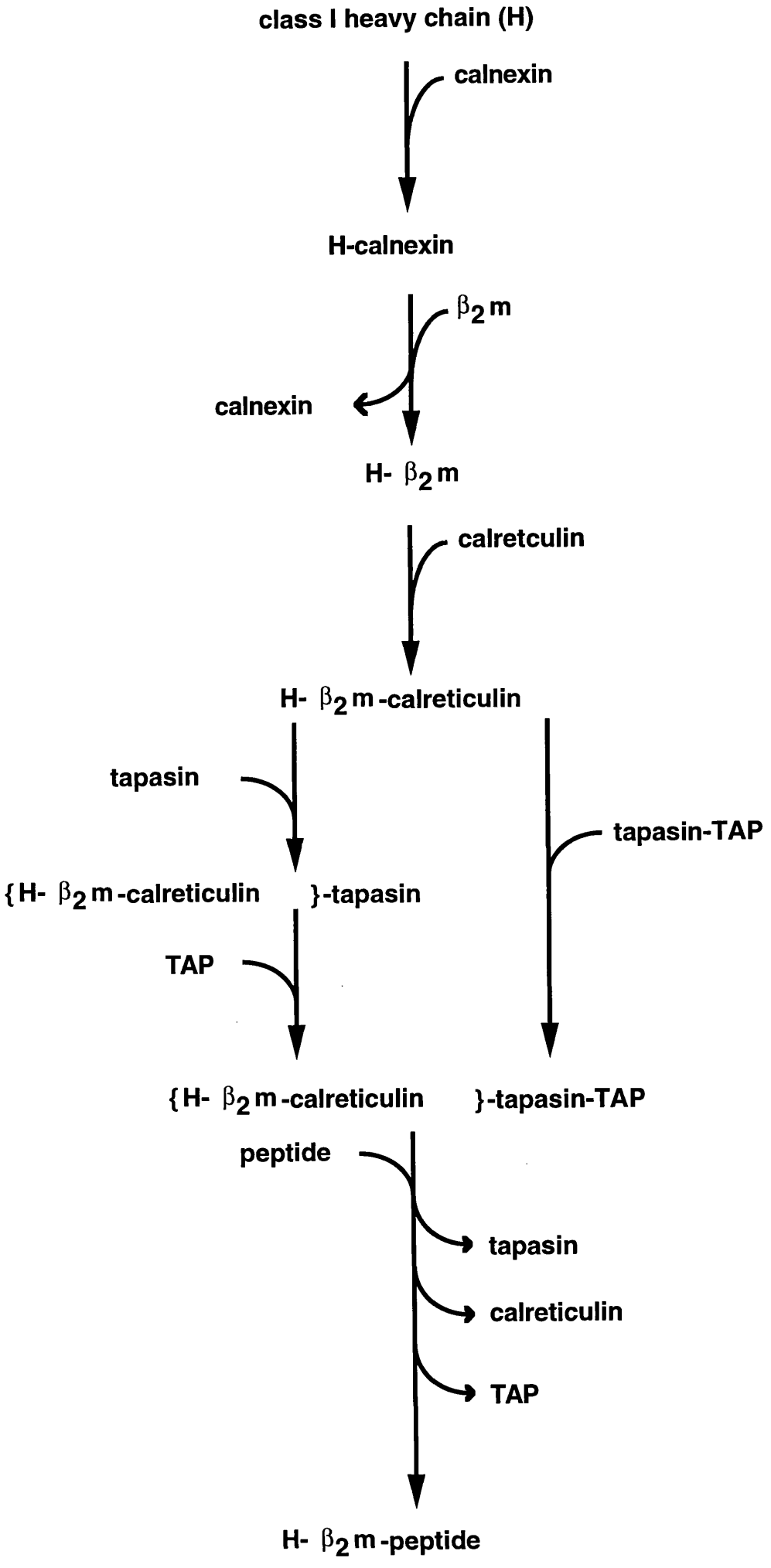

class I heavy chain (H)
calnexin
H-calnexin
β2m
calnexin
H- β2m
calretculin
H- β2m -calreticulin
tapasin
tapasin-TAP
{H- β2m -calreticulin }-tapasin
TAP
{H- β2m -calreticulin }-tapasin-TAP
peptide
tapasin
calreticulin
TAP
H- β2m -peptide

Figure 1.3: Diagramatic representation of an MHC class I molecule based on the refined crystal structure of HLA-A2. (A) Ribbon drawing of the HLA-A2 Cα backbone showing the four domains with the polymorphic α1 and α2 domains at the top and the immunoglobulin like α3 domain and β_2m at the bottom. (B) Ribbon drawing of HLA-A2 viewed perpendicular to the pseudo-dyad axis of the α1α2 domains (blue, helices; red, β-strands). * apices of α1 and α2 helices. (C) α1 and α2 domains viewed from above revealing the peptide binding cleft. Based on figures taken from Saper et al, (1991) and Bjorkman et al, (1987a).

Figure 1.3

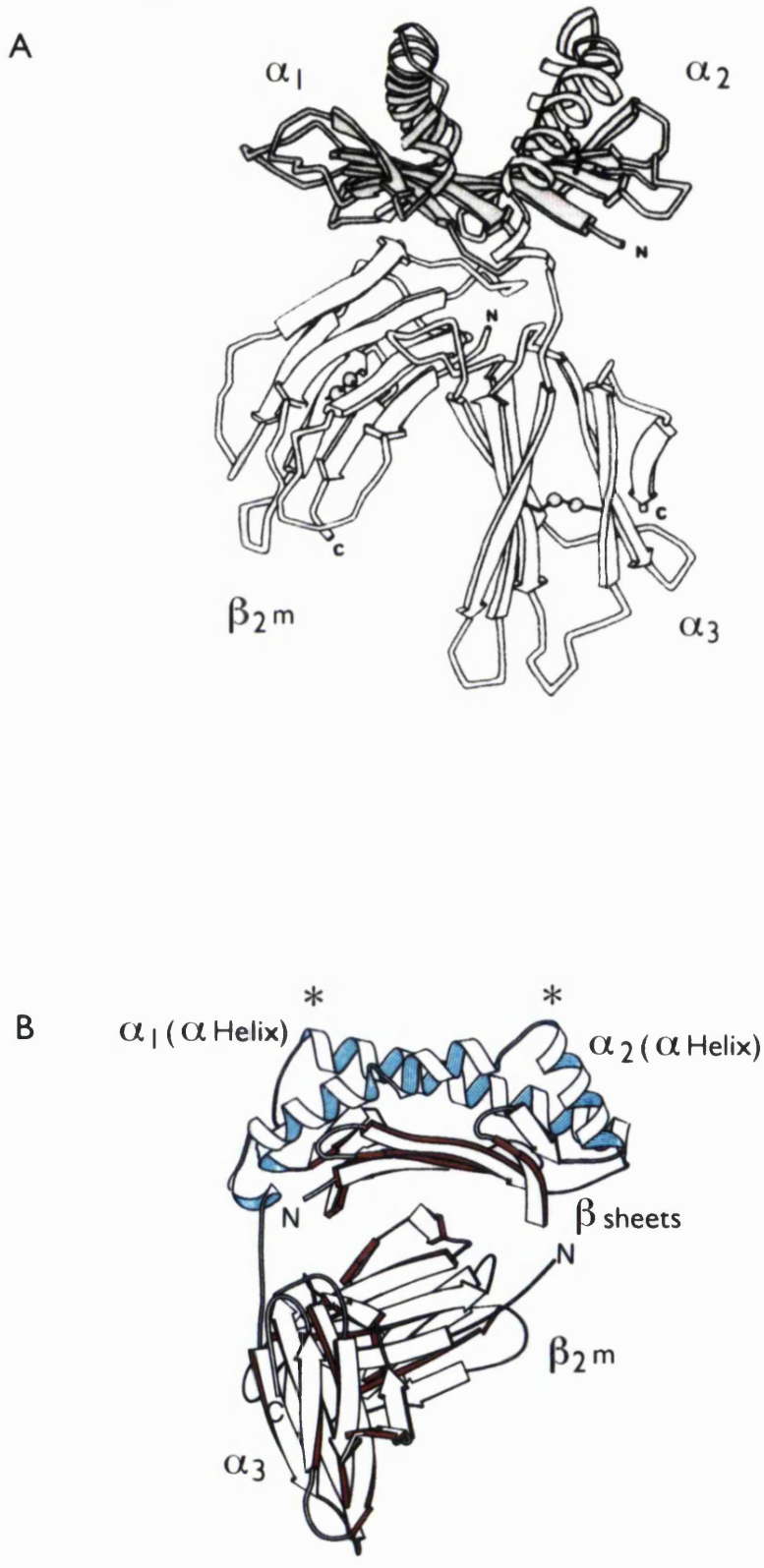


Figure 1.3

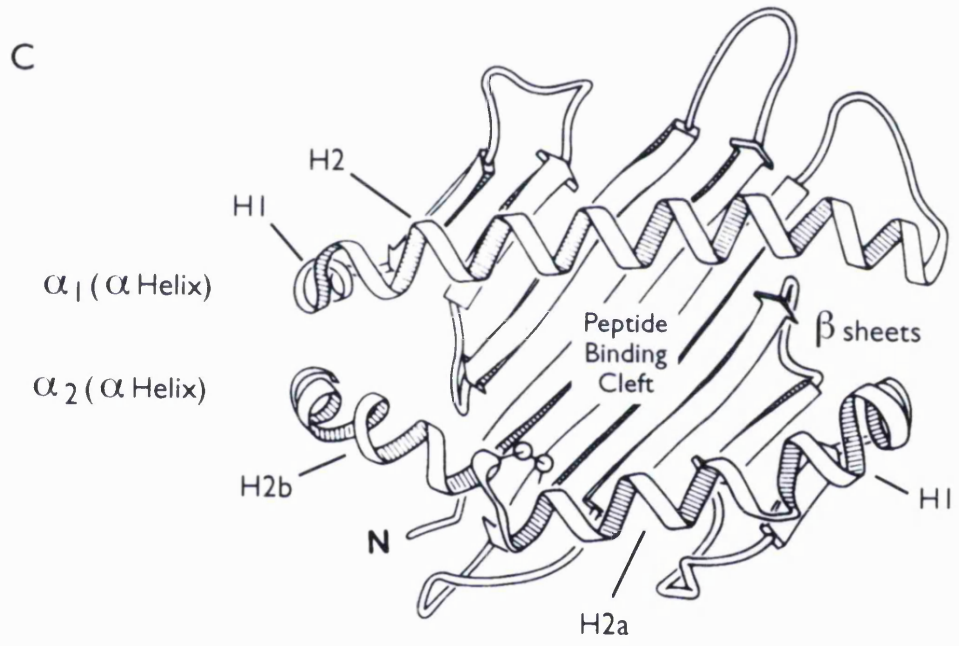

C
H2
H1
α1 (α Helix)
α2 (α Helix)
Peptide
Binding
Cleft
β sheets
H2b
N
H1
H2a

Figure 1.4: Ball and stick representation of the HLA-A2 binding cleft reveals pockets which are involved in peptide binding. Solvent accessible atoms forming the molecular surface of pockets A to F are colour coded by pocket. Only residues having some accessible surface to the pockets are shown. The H1α1 helix is at the top left corner. Taken from Saper et al (1991).

Figure 1.4

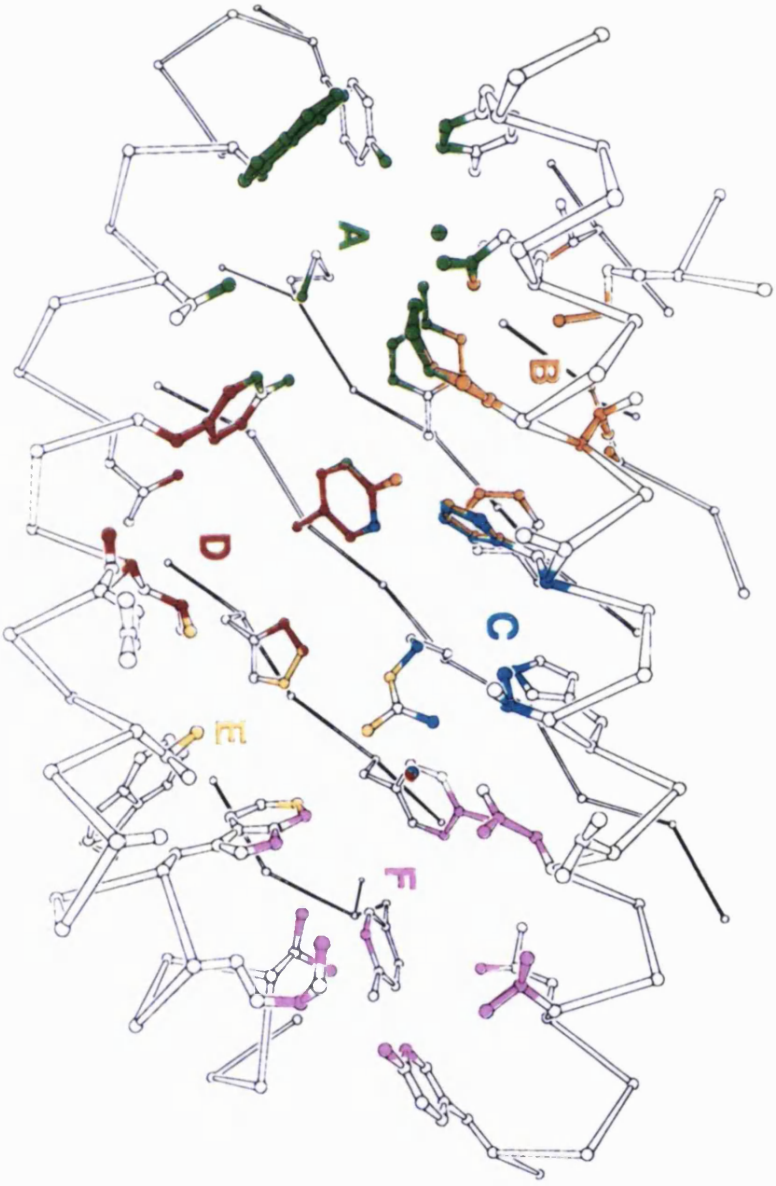
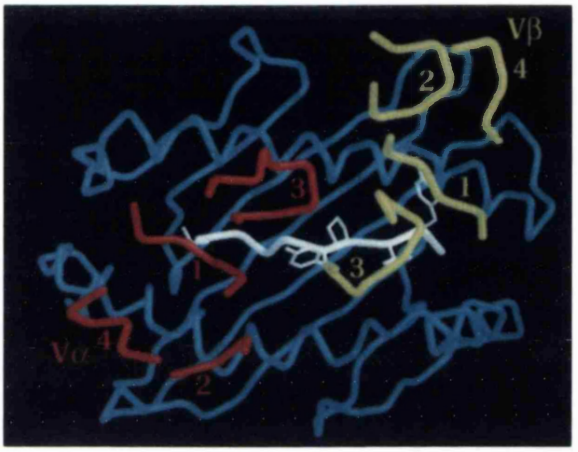


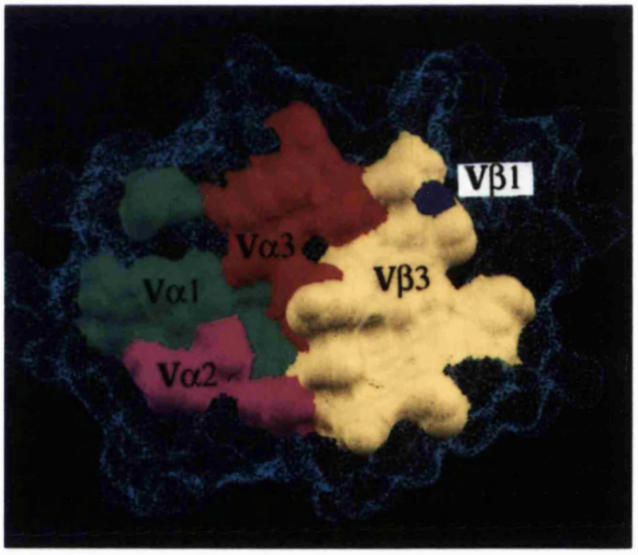
Figure 1.5: Orientation of the human αβTCR (A6) on the HLA-A2/Tax peptide complex.(A) Variable loops of TCR Vα (red) and Vβ (yellow), Tax peptide (white), and HLA-A2 (blue). Peptide side chains contacted by the TCR are shown in white. (B) Interaction of the A6 TCR with Tax peptide and HLA-A2. Solvent accessible surface of the MHC-peptide complex contacted by TCR complementarity determining regions (colour coded). (C) Schematic representation of TCRαβ CDR loops (numbered) shown diagonally orientated between the apices of the MHC class I α1 and α2 helices. (D) View of the A6 TCR as seen looking up from the MHC binding groove reveals a deep central pocket which accommodates the Y5 residue of the Tax peptide. Taken from Garboczi et al, (1996).

Figure 1.5

A



B



C

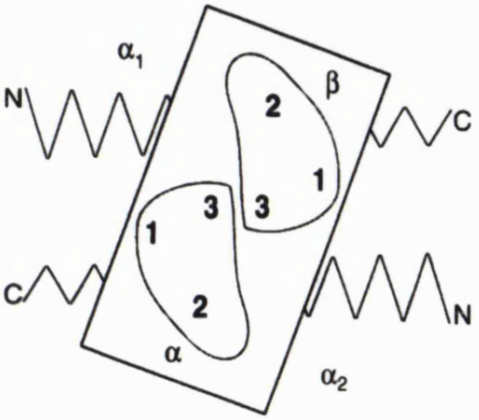


Figure 1.5

D

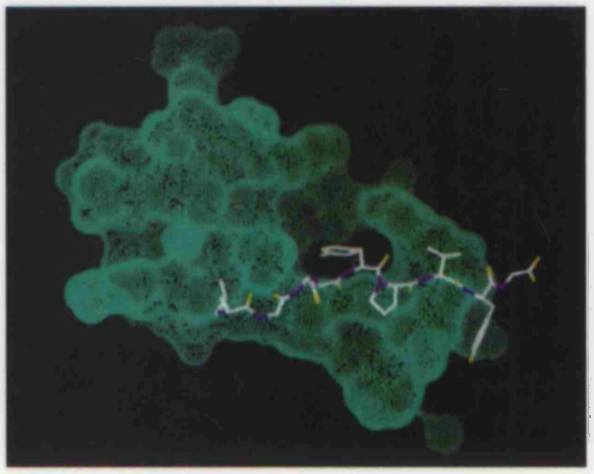
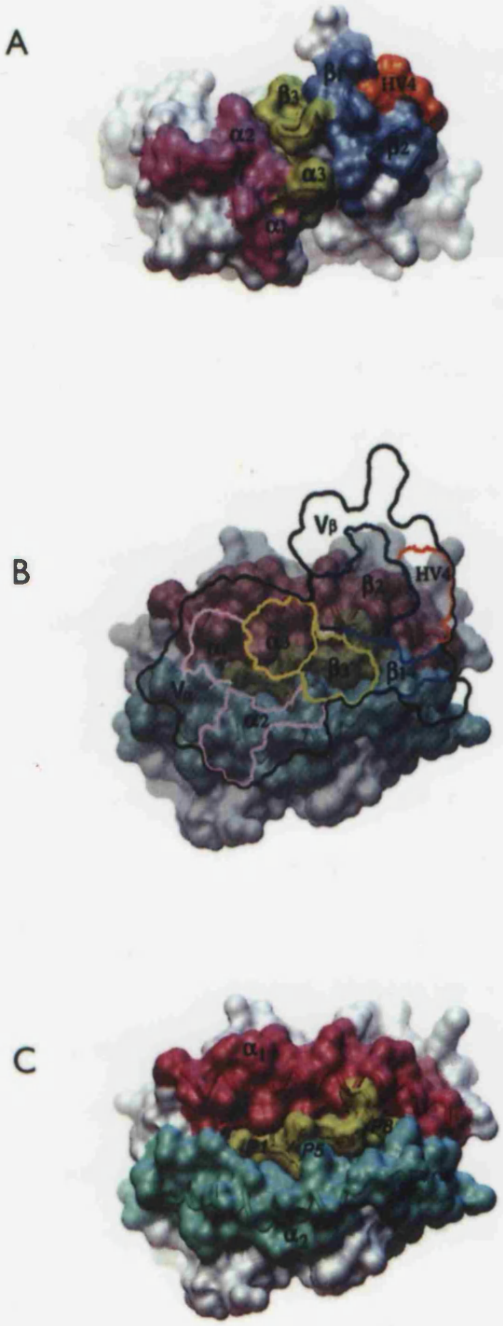


Figure 1.6: Interaction of the mouse 2C TCR with the H2-K^b-dEV8 peptide complex. (A) Molecular surface of the 2C binding site as viewed from above the complementarity determining regions (CDRs). The surface and loop trace of the Vα and Vβ CDRs, α1 and α2 are magenta; CDRs β1 and β2 are blue; Vα and Vβ CDR3s are yellow; and Vβ HV4, orange. A central cleft between CDR3α and CDR3β can be observed. (B) Footprint of the 2C binding site molecular surface on the H2-K^b-peptide molecular surface. The outline and footprint of the TCR molecule was obtained by slicing through the TCR surface in the vicinity of the combining site and projecting that surface onto the MHC-peptide complex. The view is looking directly onto the MHC-peptide complex as in (C) through the 2C TCR complex. The TCR molecular surface is outlined in black and borders delineating CDR regions (α1-3, β1-3 and HV4) are indicated in colour. (C) Molecular surface of the H2-K^b-OVA model used to help determine the position of the H2-K^b-dEV8 in 2C-H2-K^b-dEV8 crystals. Taken from Garcia et al, (1996).

Figure 1.6



2.0 T cell responses to the P91A antigen in P91A transgenic and non-transgenic mice

2.1 Introduction

The P91A tum$^-$ antigen is a novel tumour specific transplantation antigen (TSTA) expressed on the P91 variant of the tumour cell line P815. P815 is mastocytoma cell line initially derived following tumour induction in DBA/2 mice (Dunn et al, 1957) and is both weakly antigenic and highly malignant. A particularly malignant clone P1 has been isolated from the parental P815 line (Uyttenhove, 1980) and following *in vitro* chemical mutagenesis, a panel of 11 clones with decreased tumorigenicity obtained. As a group, these clones form either no or only occasional tumours when injected into DBA/2 mice (even when injected at doses exceeding 1000 times the tumour inducing dose of P1 cells) and have been designated, on the basis of this phenotype, as 'tumour minus' (tum$^-$). The P91 cell line is one such tum$^-$ variant.

Tum$^-$ variants stimulate variant specific protective immunity which can be transferred in the CD8+ T cell population to naive mice and is characterised by T cell mediated tum$^-$ variant specific cytolysis (Boon et al, 1980; Maryanski et al, 1982a and b; Boon, 1989). Together, these experiments suggest that tum$^-$ variants express novel epitopes which are not shared with the parental cell line. This has subsequently been shown to be the case (see below). Having said this, following rejection of a tum$^-$ variant a state of partial immunity can exist to the parental cell line P1. This cross protective immunity is directed at shared antigens found on both the variant and P1 and indicates that although P1 may be only weakly antigenic it can nevertheless be rejected following expansion of a cross protective effector population (Uyttenhove et al, 1980).

Four major TSTAs (A, B, C and D) have been identified on P1 and its derivative cell lines (Uyttenhove, 1983). Antigens A and B are encoded within the same gene while Antigens C and D show no such linkage. All four antigens are recognised by cytotoxic T cells and participate in the rejection response. Antigen loss variants which lack either a shared or tum$^-$ specific TSTA form aggressive tumours (Maryanski et al, 1983a and b). A delicate balance therefore exists between the successful rejection and uncontrolled proliferation of P1 and its tum$^-$ variants.

The P91 cell line expresses two tum$^-$ antigens; P91A and P91B (Maryanski et al, 1983a and b). The P91A gene has been successfully cloned and is comprised of 12 exons extending over approximately 14kb encoding a protein of 529 amino acids (Lurquin et al, 1989). Two cysteines (205 and 478) and two potential N linked glycosylation sites (120 and 483) are present. A comparison of the wild type P91A sequence with that of the tum$^-$ variant reveals a single base change (G to A) in exon four which results in an arginine to histidine substitution in the tum$^-$ version. Peptides synthesised to encompass the altered amino acid sensitise P1 cells for P91A specific kill and a 13mer peptide, P91A (12-24) (ISTQN**H**RALDLVA), was identified as the optimal peptide for induction of cytolysis (Lurquin et al, 1989). MHC transfection and antibody blocking studies subsequently identified H-2L^d as the restricting molecule and a synthetic peptide with wild type sequence (ISTQN**R**RALDLVA) was shown to neither induce cytolysis or compete with the P91A tum$^-$ peptide for MHC binding. These data indicate that an arginine to histidine substitution in the P91A 12-24 peptide results in the generation of a new L^d binding peptide.

P91A is now known to be a non-ATP binding sub-unit of the 19S proteasome regulator (DeMartino et al, 1994) and homologous genes have been identified in both *Drosophila melanogaster* and in the budding yeast Saccharomyces *cerevisiae* (Pentz and Wright, 1991; Kawamura et al, 1996). The P91A homologue SUN2 in yeast functions as a suppresser of the 26S proteasome component NIN1 which in turn is

involved in inhibiting G1/S and G2/M cell cycle progression. Although the function of P91A in mammalian cells is still unknown it is possible that it may perform a similar role to SUN2 in yeast and therefore participate in the regulation of the cell cycle.

We were interested in developing a mouse model system to examine rejection responses and T cell selection to a defined MHC class I epitope. To this end, P91A transgenic mice were generated in collaboration with Dr A. Mellor at the National Institute for Medical Research, Mill Hill. Fertilised DBA/2 oocytes were injected with a 17kb genomic fragment of the P91A(tum$^-$) gene, known to include 3.3kb of 5' and 0.2kb of 3' untranslated sequence. The P91A transgene will therefore operate under the control of its own promoter. A single founder animal was obtained and used to establish a heterozygous transgenic colony. Attempts to breed to homozygosity were unsuccessful. In general the P91A transgenic mice proved a difficult line to maintain. The reason for this is unclear. However non-transgenic DBA/2 mice housed in the same facility also showed low fecundity and high neonatal mortality.

P91A transgenic mice possess 8-10 copies of the transgene integrated as a head to tail concatamer and two copies of the wild type gene (Antoniou et al, 1996). The P91A protein derived from the transgene contains the amino acid histidine as opposed to wild type arginine and thus encodes the P91A tum$^-$ epitope. The functional significance of this amino acid substitution is unknown. Experiments underway in yeast will help to establish whether the tum$^-$ version of P91A is functionally competent. In addition P91A transgenic spleen cells have been shown to express the P91A epitope as assessed by proliferation of the epitope specific T cell clone P91.6 (Antoniou et al, 1996). Together these data and further analysis of P91A gene expression in transgenic mice suggest that the P91A epitope is functionally operative in P91A transgenic mice. The work in this thesis concerns the use of P91A transgenic and non-transgenic mice to study T cell responses to the P91A antigen in a controlled

model system in which the only variable is the expression or lack of expression of the P91A epitope.

2.2 Results and discussion

2.2.1 P91A peptide specific T cell responses in P91A transgenic and non-transgenic mice

2.2.1.1 Expression of the P91A wild type gene in thymic sub populations

Previous experiments in this lab have demonstrated the widespread and potentially ubiquitous expression of P91A mRNA in transgenic mouse tissues. Furthermore the expression of the P91A transgene has been shown to be identical to that of the wild type gene (Antoniou et al, 1995).

The thymic expression of P91A is of specific interest since it is in this location that selection of T cells occurs. Recent work in collaboration with Dr. N. Moore (Dept of Anatomy, University of Birmingham), has demonstrated the expression of P91A wild type mRNA by rtPCR in three thymic stromal sub-populations: CD45 depleted thymic stromal cells, MHC class II expressing thymic epithelial cells and thymic dendritic cells, Figure 2.1A. In addition, P91A message is detected in all thymocyte sub-populations examined, Figure 2.1B. Insufficient numbers of mice have been available to isolate equivalent stromal sub-populations from transgenic thymii. However, since the P91A transgene is of genomic origin and has 3.3kb of 5' untranslated sequence it is presumed that expression will be identical to the wild type gene. Identical expression in all other tissues would tend to support this hypothesis.

2.2.1.2 Expression of the P91A epitope within the thymus

Although isolation of transgenic thymic sub-populations has not been possible the expression of the P91A epitope within the thymus has been demonstrated by the proliferation of the P91A specific T cell clone P91.6 to transgenic thymocytes, Figure 2.2. This assay primarily reflects the expression of epitope on thymocytes, since these cells account for the majority of thymic derived cells. It is however likely that stromal elements and dendritic cells express the P91A epitope since both are known to express P91A wild type message. In any event, evidence from H2-K^b transgenic mice, in which expression of the transgene is limited to cells of the T cell lineage indicates that negative selection of anti K^b specific TCR transgenic thymocytes occurs unimpeded (Schulz and Mellor, 1996). This suggests that thymocyte / thymocyte interaction is sufficient to promote negative selection of a class I restricted TCR and that if class I epitope is expressed within the thymus then at least negative selection of reactive thymocytes will proceed unimpeded.

2.2.1.3 The generation of P91A specific T cell lines from P91A transgenic and non-transgenic mice

The peripheral T cell repertoire largely reflects the outcome of thymic selection. By examining peripheral P91A reactive T cells it may be possible to establish whether P91A transgenic mice negatively select P91A reactive T cells (for further discussion of this approach to examining selection in P91A transgenic mice see Chapter 6.1).

The use of TCR transgenic mice has circumvented the need to search within the peripheral T cell pool for evidence of negative or positive selection (Kisielow et al, 1988a, 1988b, Teh et al, 1989, Sha et al, 1988a, 1988b, 1990, Berg et al, 1989a, 1989b, Pircher et al, 1989, Mamalaki et al, 1992 and 1993). Using TCR transgenic mice a single clonal population can be followed with relative ease through the stages

of selection and out into the periphery. In this way the interaction of a defined TCR with its MHC-peptide ligand can be examined. The present work attempts to look at the interaction of an unmanipulated thymocyte/T cell population with a defined MHC-peptide complex. This approach requires one by necessity to examine the peripheral T cell pool for evidence of selection. This can only be achieved by comparing characteristics of peptide specific T cell lines and clones derived from transgenic and non-transgenic mice.

In vivo peptide immunisation has been used successfully to generate class I epitope specific CTL (Zhou et al, 1992; Kast et al, 1991). In the present study various strategies were employed in an attempt to immunise mice with P91A synthetic peptides and generate CTL *in vivo*. Unfortunately all were unsuccessful. This is in keeping with other laboratories experience using the P91A peptide as immunogen (T. Boon, personal communication). It was therefore necessary to adopt a primary *in vitro* strategy to generate transgenic and non-transgenic P91A specific T cell lines and clones.

Well characterised methodologies exist to generate primary *in vitro* T cell lines (Alexander-Miller et al, 1991; Stauss et al, 1992). All methods use a high concentration of peptide for initial *in vitro* culture with step wise reduction in peptide concentration upon restimulation. The method of Alexander-Miller (1991) was used to derive P91A specific T cell lines from P91A transgenic and non-transgenic mice (see Chapter 7). Following this method, recombinant IL2 (rIL2) or supernatant harvested from Conconavalin A stimulated rat spleen cells (ConA-sup) is added at the time of the third *in vitro* stimulation. The specificity of cultured cells is established following each restimulation by assessing P91A specific cytolysis. Using this type of approach T cell lines were generated from P91A transgenic and non-transgenic mice, Figure 2.3 A-D. Peptide specificity was noted after the second stimulation for non-transgenic lines 1 and 3, Figure 2.3 A and B. A longer period of *in vitro* culture was

however required before peptide specific kill could be detected in the transgenic lines 4 and 14, Figure 2.3 C and D. Transgenic line 14 failed to show any specificity for peptide after three weeks of *in vitro* culture, Figure 2.3 D. On examination this line was found to contain a large number of cells proliferating in response to rIL2 and only a small proportion of cells with a characteristic *in vitro* morphology of CD8+ cells. The low number of CD8+ T cells present in line 14 was confirmed by flow cytometry. Line 14 was FAC sorted on the basis of CD8 expression and re-expanded *in vitro* . By week seven line 14 displayed good peptide specific kill. These experiments show that P91A specific T cells exist in P91A transgenic and non-transgenic mice.

2.2.1.4 Isolation of T cell clones from P91A transgenic and non-transgenic mice

In order to examine more closely the nature of the T cells involved in P91A peptide recognition T cell clones were derived from existing transgenic and non-transgenic T cell lines. T cell clones were obtained by limiting dilution and weekly restimulation with P91A peptide. In all cases ConA-sup was found to be more reliable than rIL2 for clone derivation. Four panels of T cell clones were obtained. The peptide specificity for each panel of clones is shown in Figure 2.4 A-D.

Proliferation assays were used to screen all T cell clones. This assay permits the screening of clones at different P91A peptide concentrations. Three peptide concentrations were used: 6000nM, 600nM and 60nM. A single peptide concentration was thought to be insufficient for screening purposes since very high and very low peptide concentrations may produce close to background proliferations. The low dose through lack of stimulation through the TCR and the high dose through hyperstimulation leading to programmed cell death of the responding cells.

As shown in Figure 2.4 C and D, clones from transgenic mice respond to high concentrations of peptide and only weakly or not at all to low peptide concentrations. The reciprocal pattern of stimulation is seen for clones derived from non-transgenic mice, Figure 2.4 A and B. Certain non-transgenic clones, for instance 3R and 3U, show no significant peptide induced proliferation above background. However, clones such as these can be inhibited from proliferating at higher peptide concentrations indicating that they possess a TCR capable of interacting with the P91A epitope.

2.2.1.5 Peptide responsiveness of T cell lines and clones

In the initial analysis of T cell lines, specific cytolysis of P91A peptide coated target cells was assessed. In this type of assay P1 target cells are incubated with a fixed concentration of peptide, effector cells are added and specific kill measured. In initial experiments the concentration of peptide used was 50μM. The results of the T cell clone screening experiments indicate that transgenic and non-transgenic T cell clones can respond to lower peptide concentrations. In order to examine the peptide response curves of transgenic and non-transgenic T cell lines proliferation assays were set up using serial ten fold dilutions of the P91A 12-24 peptide. As shown in Figure 2.5 A and B, the peptide response curves of transgenic and non-transgenic lines are significantly different with transgenic line 4 requiring 100-1000 fold more peptide than non-transgenic line 3 to elicit half maximal proliferation.

The proliferation of transgenic line 4 and non-transgenic line 3 was also assessed in the absence or at two different concentrations of rIL2. In this set of experiments line 4 and line 3 proliferated in the absence of additional rIL2. This is not to imply that cytokines were not required for proliferation but rather that a sufficient level of cytokines was produced by the responder cells or irradiated syngeneic feeder spleen cells which were present throughout the assay. The addition of increasing concentrations of rIL2 has two effects. First, a large increase in background

proliferation in the absence of peptide. Second, a stepwise shift in the peptide dose response curve to the left. Although rIL2 affects the peptide responsiveness of the transgenic and non-transgenic lines at equivalent rIL2 concentrations transgenic line 4 and non-transgenic line 3 maintain their differential responsiveness to peptide.

The peptide responsiveness of selected T cell clones derived from non-transgenic T cell line 3 and transgenic T cell line 4 is shown in Figure 2.6. In all cases non-transgenic clones are more peptide responsive than transgenic clones. Overall the differential sensitivity was approximately 3 logs; half maximal proliferation at 5 nM for non-transgenic clones and half maximal proliferation at 2500 nM for transgenic clones. This difference in responsiveness to peptide may result from the deletion of high avidity/affinity P91A reactive T cells in P91A transgenic mice (for further discussion see Chapter 6.1). The role of peripheral tolerance mechanisms in the induction and maintenance of tolerance to the P91A epitope in transgenic mice is as yet unknown. However, the finding that P91A peptide specific cells exist in the peripheral T cell pool of transgenic mice with a threshold for activation approximately three logs higher than that of corresponding P91A reactive cells in non-transgenic mice supports the concept that T cells are selected in the thymus on the basis of avidity/affinity for MHC-peptide. If this is the case then P91A reactive T cells from transgenic mice should express either a low level of a high affinity P91A specific TCR or a normal level (comparable to the level expressed on non-transgenic P91A specific T cells) of a low affinity TCR. However the avidity of a cellular interaction is the composite attractive force between two cells and is the outcome of multiple receptor/ligand interactions. Since the major co-receptor present on cytotoxic T cells is the CD8 molecule it is possible that the differential response of transgenic and non-transgenic T cells to peptide reflects the level of this molecule on the cell surface. Furthermore, differences in the level of TCR expression between transgenic and non-transgenic P91A specific T cells may account for differential peptide sensitivity *in vitro*.

2.2.1.6 The level of CD3 and CD8 expressed on transgenic and non-transgenic T cell clones is approximately equal

It is suggested here that transgenic and non-transgenic clones differ in the quality of the TCRs which they express: transgenic clones expressing TCR which interact weakly with the P91A epitope, non-transgenic clones expressing TCRs which interact strongly. In other words, the P91A epitope is acting as a full agonist for non-transgenic clones and as a weak agonist for transgenic clones. It is however clear that T cell activation is dependent on the avidity of the interaction between T cell and APC and that this interaction is dependent on both TCR and co-receptor/adhesion molecule interactions (Springer et al, 1987; Dustin and Springer, 1989; Rodrigues et al, 1992).

The level of CD8, CD4 and TCR on thymocytes has previously been shown to be critical for positive or negative selection and decreasing the expression of any one of these on thymocytes in the appropriate transgenic system can be sufficient to alter the threshold for negative selection (Robey, 1992a,1992b; Fung-Leung et al, 1993; Homer et al, 1993; Teh et al, 1989). Furthermore, experiments using chimeric CD4 and CD8 molecules have shown that these effects can be independent of co-receptor associated kinase activity in that overexpression of CD4 or CD8 molecules which are unable to associate with p56Lck were able to rescue the development of CD4+ or CD8+ thymocytes in CD4 or CD8 null mice respectively (Killeen et al, 1993, Chan et al, 1993). These experiments support the idea that co-receptor engagement increases the avidity of interaction between T cell and APC and can alter the outcome of selection on this basis.

In various TCR transgenic models down regulation of TCR and/or low levels of CD8 expression have been associated with the induction and maintenance of self tolerance (Shönrich et al, 1991; Kisielow et al, 1988a and b). This implies that the overall

threshold for peripheral T cell activation may be altered by decreasing the level of expression of TCR and/or costimulatory molecule (Robey et al, 1992b; Kisielow et al, 1988a and b). Furthermore, epitope specific CD8+ T cell clones have been identified with a spectrum of CD8 dependency that correlates inversely with the epitope density required for T cell activation or kill (Alexander-Miller et al, 1991). These data suggest that the activation state of a T cell in the periphery may be modulated by the level of expression of TCR and/or co-receptor.

The T cell clones isolated in the present study display differential sensitivity to peptide. In order to assess whether this reflects a difference in the level of CD8 or TCR expression, transgenic and non-transgenic T cell clones were stained with anti CD3 or CD8 monoclonal antibodies and analysed by flow cytometry, figure 2.7. Five transgenic clones (4A, 4B, 4D, 4E and 4G) were compared with five non-transgenic clones (3A, 3B, 3C, 3D and 3E). Their is no significant difference between the expression of CD3 on transgenic and non-transgenic clones ($t = 0.086$, $df = 8$, $p < 0.05$). Likewise, CD8 expression is equivalent on transgenic and non-transgenic T cells ($t = 1.33$, $df = 8$, $p < 0.05$). It is therefore unlikely that differences in TCR or CD8 expression account for the differential sensitivity to peptide of transgenic and non-transgenic clones.

2.2.1.7 T cell clones derived from P91A transgenic mice are not activated by the level of P91A epitope expressed on transgenic tissue

The isolation of weak avidity P91A reactive T cells from transgenic mice may mean that potentially autoreactive T cells exist in transgenic animals. Such cells may be rendered tolerant *in vivo* but be capable of *in vitro* activation. If this were the case then P91A specific T cell clones derived from transgenic mice might be able to recognise the level of P91A epitope expressed on transgenic tissue. This possibility

was addressed by examining the ability of P91A transgenic spleen cells to activate transgenic and non-transgenic clones. As can be seen in figure 2.8, T cell clones from transgenic mice fail to proliferate in response to P91A transgenic spleen cells whereas non-transgenic clones show an epitope specific proliferation. It is therefore likely that the level of epitope expressed on transgenic spleen cells is below that required for the activation of low avidity T cells derived from transgenic mice.

2.2.1.8 P91A transgenic mice can generate high avidity T cells to a third party L^d binding peptide

The P91A transgene copy number as estimated by southern blot analysis is 8 - 10 copies (Antoniou et al, 1996). It is therefore possible that an abundance of P91A tum^- message and protein exists in transgenic cells. The tum^- form of the P91A protein may therefore out compete the wild type form for incorporation into the 26S proteasome. The potential functional significance of this is unclear, but the fact that the proteasome is involved in the generation of class I binding peptides makes it a possibility that P91A transgenic mice are defective in generating class I binding peptides and may therefore have dysfunctional T cell selection. Furthermore, unknown integration site specific effects may have a disruptive effect on immune function in P91A transgenic mice.

In order to show that the generation of low avidity T cells is an epitope specific effect in P91A transgenic mice T cells lines were generated which recognise the alternative L^d binding peptide, MCMV $pp89_{168\text{-}176}$ (Reddehase et al, 1989). P91A and MCMV peptide specific lines were derived from a transgenic mouse and show peptide specific kill, figure 2.9 A and B. A proliferation assay using serial titrations of the MCMV peptide reveals a response to peptide which is very similar to that of high avidity P91A specific non-transgenic lines and clones, figure 2.9 C. P91A transgenic mice can therefore generate MCMV $pp89_{168\text{-}176}$ specific T cells which have

comparable peptide responsiveness to P91A specific T cells derived from non-transgenic animals.

2.2.1.9 Conclusions

P91A specific T cells exist in P91A transgenic and non-transgenic mice. This said, P91A reactive T cells derived from non-transgenic and transgenic mice differ in their responsiveness to the P91A peptide in that non-transgenic T cells can be activated by low concentrations of peptide while high concentrations of peptide are required to activate transgenic T cells. This differential sensitivity is not explained by differences in CD8 or TCR expression since the level of TCR and CD8 is equivalent on transgenic and non-transgenic clones. These data are interpreted as being supportive of an avidity/affinity model of negative selection in which high avidity P91A specific thymocytes are deleted in P91A transgenic mice. Furthermore, P91A reactive T cells which escape deletion in P91A transgenic mice cannot be activated by the level of the P91A epitope expressed on peripheral P91A transgenic tissues. These results are further discussed in conjunction with later results on TCR usage and fine specificity of clones in Chapter 6.

2.2.2 Induction of peripheral tolerance to the P91A tum$^-$ antigen in non-transgenic mice

Having made an attempt to address the issue of central tolerance to the P91A tum$^-$ antigen in transgenic mice an examination of *in vivo* responses to the P91A tum$^-$ antigen was conducted in non-transgenic mice. The expression of the P91A tum$^-$ antigen is critical for the successful rejection of the P91 tumour line as indicated by the failure of DBA/2 mice to reject P91A antigen loss variants (Uyttenhove, 1983). Interestingly, rejection appears to occur in the absence of an obvious CD4+ T helper response. Additionally, P815 has no constitutive MHC class II expression and

attempts to induce MHC class II expression on P815 using γ-interferon have been unsuccessful (T.Boon, personal communication). Although reprocessing and presentation of tumour antigens by host antigen presenting cells (macrophages or dendritic cells) may occur, the possibility exists that a CTL response may be mounted against the P91A epitope in the absence of T cell help. If this occurs then it is also possible that P91A transgenic DBA/2 skin may be rejected by non-transgenic DBA/2 mice. In other words the P91A epitope may be sufficient to constitute a minor histocompatibility antigen rejection barrier.

Using the P91A transgenic mouse model it has become evident that in itself, the P91A epitope does not constitute a complete minor histocompatibility antigen. This is evidenced by the acceptance of female P91A transgenic DBA/2 skin by non-transgenic female DBA/2 mice (Antoniou et al, 1996). It has been further shown that this lack of rejection results from the absence of a suitable MHC class II helper determinant expressed in female transgenic donor skin. Since female DBA/2 mice fail to reject male DBA/2 skin an attempt was made to constitute a complete minor histocompatibility antigen by combining the P91A epitope with the DBA/2 H-Y determinant (DBA/2 mice are an H-Y non responder strain as assessed by the failure of male DBA/2 mice to reject female DBA/2 skin). When this was done, it became clear that female non-transgenic mice could reject male transgenic skin and that the combination of the P91A epitope with a component of the H-Y antigen was sufficient to elicit graft rejection (Antoniou et al, 1996). The fact that H-Y can facilitate the rejection of P91A+ H-Y+ skin indicates that H-Y reactive cells are present in DBA/2 mice. Furthermore, mice treated with an anti CD4 depleting antibody failed to reject P91A+ H-Y+ skin indicating that the H-Y component is likely to be a MHC class II helper determinant (Antoniou et al, 1996). In all experiments prior i/p immunisation with P91A+ H-Y+ spleen cells was required before any rejection of P91A+ H-Y+ skin was observed. The protocol for induction of a rejection response is shown in figure 2.10 A.

The P91A model of skin graft rejection can be interpreted as displaying features of the two signal hypothesis of B cell activation (Bretscher and Cohn, 1970). This hypothesis states that a B cell must receive two consecutive signals in order to become activated: signal one via the antigen receptor and signal two from a second cell specific for a component of the same antigen. In this model the second signal is derived from antigen specific T helper cells and can largely be substituted by IL2. The two signal model of B cell activation and more specifically the requirement for CD4 T lymphocyte help has been applied to the activation of naive CD8+ T cells (Stuhler and Walden, 1993; Guerder and Matzinger, 1989 and 1992; Rees et al, 1990; Vandervegt and Johnson, 1993).

In the P91A model of skin graft rejection help is derived from H-Y specific CD4+ T cells and the effector cells are presumed to be the CD8+ P91A reactive T cells. This interpretation assumes that the CD4+ H-Y reactive cells do not directly participate in the rejection response but exert their effect through CD8+ effector cells. This is likely to be the situation in DBA/2 mice which are known H-Y non responders. However, it has recently been demonstrated that CD8 knock out mice are capable of rejecting allogeneic skin demonstrating that in certain circumstances rejection can be mediated by CD4+ T cells (Krieger et al, 1996; Dalloul et al, 1996a). Furthermore a recent study has demonstrated that adoptive transfer of naive or sensitised $CD4^+$ cells from CD8 knockout mice into nude mice, which have been grafted with skin from mice deficient in MHC class I or class II respectively can reconstitute rejection (Dalloul et al, 1996b). In this case, although MHC class I expressing allografts were rejected, $CD4^+$ cells did not display allo-antigen specific cytotoxic activity, though they did proliferate *in vitro* in response to allo-antigen . Although CD4+ cells would seem capable of eliciting a rejection response independent of CD8+ cells the converse does not appear to be true of CD8+ cells in CD4 knockout mice (Krieger et al, 1996). Together, these results suggest a critical involvement of CD4+ T cells in skin graft rejection.

P91A+H-Y+ skin graft rejection appears to conform to a model in which CD4+ and CD8+ T cells collaborate to effect rejection and as such are consistent with a two signal model of T cell activation in which CD4+ H-Y specific T cells are required in the activation of P91A specific CD8+ T cells. A further prediction of the two signal hypothesis is that a state of T cell tolerance may be induced if signal one is encountered in the absence of signal two (Guerder and Matzinger, 1989, 1992; Rees et al, 1990). The characteristics of P91A+H-Y+ skin graft rejection allow the two signal model of peripheral T cell tolerance to be tested.

2.2.2.1 *In vivo* demonstration of peripheral tolerance to the P91A tum$^-$ epitope

In order to test out the two signal hypothesis of peripheral T cell tolerance female DBA/2 mice were given an initial i/p injection of female transgenic or male non-transgenic spleen cells (representing signal I or signal II). After two weeks all mice were given a second injection of male transgenic spleen cells (representing combined signals I and II). A further two weeks later all mice were grafted with male transgenic skin and subsequently examined for signs of acute or chronic rejection. The experimental protocol is outlined in figure 2.10B.

Prior immunisation with either the class I CTL epitope P91A or the class II helper epitope H-Y results in long term survival of P91A+ H-Y+ skin with a median survival time of over 100 days (MST > 100 days), table 2.1 A and B. By contrast, mice which did not receive an initial injection of P91A+ or H-Y+ cells reject P91A+ H-Y+ skin with a MST of 48 days, table 2.1 C. This result supports and extends the initial predictions of the two signal model of peripheral T cell tolerance and indicates that presentation of the MHC class II epitope H-Y to the immune system in the absence of the class I epitope P91A induces long term tolerance to grafted P91A+ H-Y+ skin.

H-Y specific CD4+ T cells appear to be rendered tolerant by an initial exposure to H-Y in the absence of P91A. This suggests that H-Y specific CD4+ T cells are regulated in their response by the simultaneous recognition of the P91A epitope by CD8+ T cells. CD8+ T cells have previously been implicated in the regulation of *in vivo* and *in vitro* immune responses and are capable of secreting IL2 and γ- interferon (Inaba et al, 1987; Gill, 1993; Kemeny et al, 1994; Stuhler and Walden, 1993).

A further possibility which could explain non responsiveness to P91A H-Y after initial exposure to H-Y is the development of a post activation refractory period in H-Y responsive T cells. This seems unlikely since 14 days is the standard period between P91A+ H-Y+ spleen cell immunisation and P91A+ H-Y+ skin grafting. A protocol which is known to induce graft rejection, table 2.1 C.

Non professional antigen presenting cells can tolerise naive T cells, (Fuchs and Matzinger, 1992). Mixed spleen cells, which contain only ~1% professional APCs, are used as the immunising population in the present experiment. It is therefore highly likely that the majority of H-Y responsive CD4+ T cells will encounter antigen on non professional APCs and become tolerised. The presence of multiple non professional APCs may tend to decrease the probability of H-Y reactive CD4+ T cells interacting with antigen on a professional APC. This in turn may have the same effect as decreasing the precursor frequency of H-Y reactive cells, since interactions with non professional APCs will anergise the responding cell. It is possible that only a very small percentage of H-Y specific CD4+ T cells become fully activated upon initial exposure to H-Y+ male spleen cells and that a situation of near mass tolerance is induced. Such an outcome may possibly be avoided in the presence of P91A specific CD8+ T cells. It is now known that CD8+ T cells may become activated in the absence of specific T cell help if there is CD28/B7 co-receptor engagement and may even be activated in the absence of CD28/B7 interaction if there is persistence of antigen (Harding et al, 1992; Kündig et al, 1996). It is possible that P91A specific

CD8+ T cells gather in conjunction with H-Y specific CD4+ T cells round non professional APCs and via the secretion of cytokines or induction of costimulatory molecules on the antigen presenting cell support the activation or prevent the tolerisation of the CD4+ cells.

2.2.2.2 *In vitro* demonstration of tolerance to the P91A tum$^-$ epitope in non-transgenic mice

Having established a sensitive *in vitro* technique for generating P91A specific T cells this method was subsequently used to examine P91A specific responses in tolerised, (table 2.1, groups A and B), non tolerised (table 2.1 group C) and naive DBA/2 mice. As in previous experiments, successive rounds of *in vitro* peptide stimulation were used to expand P91A specific T cells.

As can be seen in figure 2.11A mice which have received an initial injection of female transgenic spleen cells (table 2.1 group A), fail to show any peptide specific kill following the first or second round of *in vitro* stimulation and display only weak peptide specific kill following the third stimulation. Mice which did not receive an initial injection of P91A or H-Y+ spleen cells and subsequently rejected male transgenic skin (table 2.1 group C), exhibit strong peptide specific kill following only a single *in vitro* stimulation which increases in strength over subsequent restimulations, figure 2.11 B. Naive DBA/2 mice show no peptide specific kill following initial *in vitro* culture but exhibit P91A specific kill following the second and third rounds of *in vitro* stimulation, figure 2.11 C. This experiment supports the view that P91A reactive T cells are rendered tolerant following initial exposure to the P91A epitope. The low level of P91A specific cytolysis observed after three rounds of *in vitro* stimulation in tolerised animals, figure 2.11 A, most likely reflects the expansion of low avidity P91A reactive T cells. Alternatively small numbers of P91A specific T cells may fail to be tolerised and are subsequently expanded over the

extended three week culture period. The introduction of cytokines may encourage such a process, although in these experiments ConA-sup was not added until the time of the third *in vitro* stimulation. The issue of whether the *in vitro* addition of cytokines could reverse the state of tolerance induced *in vivo* was addressed in experiments in which rIL2 or ConA-sup was added to primary cultures from tolerised, non tolerised and naive animals. Unfortunately these experiments were repeatedly unsuccessful with the addition of cytokines resulting in *in vitro* cell death and poor T cell yields.

The state of tolerance induced by an initial i/p injection of male spleen cells (table 2.1 group B) is also characterised by *in vitro* non-responsiveness to P91A, figure 2.12 A. Furthermore, tolerance following an initial injection of P91A+ female spleen cells is confirmed, figure 12 B and effector cells from mice which have rejected P91A+ H-Y+ skin and naive DBA/2 mice show P91A peptide specific kill, figure 12 C and D. In addition, third party responses can be elicited from animals which have received an initial injection of P91A+ or H-Y+ spleen cells, indicating that tolerance is epitope specific, figure 12 E.

2.2.2.3 The relative efficiencies of priming and tolerance induction

To gain insight into the relative efficiencies of priming and tolerising and to exclude the possibility of clonal exhaustion as the mechanism of tolerance to P91A a titration experiment was performed in which the pre-immunising injection consisted of varying percentages of male and female transgenic spleen cells, table 2.2. This experiment indicates that clonal exhaustion cannot be the mechanism of tolerance since pre-immunising with P91A+ H-Y+ spleen cells promotes accelerated graft rejection (MST = 24 days). Furthermore, priming and tolerance induction appear to be of similar efficiency, the outcome reflecting the cellular composition of the pre-immunising injection.

This is a surprising result since one might predict that priming might be easier to achieve than tolerance. This stems from the idea that all cells must be tolerant for tolerance to exist but only some cells need to become activated to prime. The finding that 10% P91A+ H-Y+ cells fail to prime in the presence of 90% P91A+ cells is at odds with this hypothesis and suggests that the rejection/tolerance is directly related to the composition of the immunising population. The fact that when equal numbers of P91A+ and P91A+ H-Y+ cells are injected half the mice reject their grafts while half retain them supports such a view. It is also possible that a form of infectious tolerance is operating to prevent naive T cells from becoming activated. Infectious transplantation tolerance has been previously demonstrated and shown to be an efficient way of tolerising naive T cells (Qin et al, 1993). The kinetics of such a process if it is operating in this model system are unknown. However, lesser numbers of tolerant cells may be capable of inducing tolerance in a large number of naive T cells.

2.2.2.4 Conclusions

Signal one (the P91A epitope) in the absence of signal two (a class II epitope derived from H-Y) leads to long term tolerance of P91A specific T cells. Furthermore, initial exposure of H-Y specific T cells to H-Y in the absence of P91A results in altered activation or tolerisation of H-Y specific cells such that they are incapable of providing help for P91A specific T cells. In the absence of help, P91A specific T cells encountering P91A+ H-Y+ cells fail to become activated and may themselves be rendered tolerant. The mechanism of tolerance is not clonal exhaustion and activation and tolerance induction appear to be of similar efficiency.

In all experiments performed, live spleen cells were injected. It is therefore likely that a state of cellular chimerism exists in all animals. It is unknown whether the persistence of P91A+ or H-Y+ spleen cells is required for the maintenance of long

term tolerance. Furthermore, recent thymic emigrants may interact with P91A+ H-Y+ spleen cells. The outcome of this type of interaction appears to be functional tolerance as indicated by long term graft survival. This may reflect a lack of a specific T cell help which exists for lone P91A specific T cells exiting the thymus. The chance of a P91A specific T cell interacting with P91A and finding itself in the immediate vicinity of a CD4+ H-Y reactive competent helper T cell may well be very low. Additionally, engrafted professional APCs are unlikely to remain as part of the chimeric population with the remaining population of T and B cells more likely to induce tolerance than activation in potential responder T cells. Alternatively, recent thymic emigrants may be a special class of T cells more prone to tolerisation than activation. Such a mechanism if it exists might be of benefit in maintaining tolerance to antigens which are not expressed in the thymus but are found in the periphery.

Cellular chimerism has previously been suggested to account for tolerance to Qa-1 (Guerder and Matzinger, 1992; Vandervegt and Johnson, 1993). In order to assess the involvement of cellular chimerism in maintaining long term tolerance to P91A the present experiments need to be repeated using irradiated or mitomycin-C treated spleen cells for injection. Although it is difficult to examine the state of cellular chimerism that exists within the animals used in these experiments, since neither P91A or H-Y can be detected by antibody, it would however be possible to analyse general aspects of cellular chimerism using a discriminatory PCR approach.

Figure 2.1: RT PCR analysis of thymic stromal sub sets and thymocyte sub populations. RT PCR was performed on stromal and thymocyte sub populations isolated from day 16 foetal thymic organ cultures. P91A specific primers (5' WTG [AAGATCAGCACTCAGAATCG] and 3' PE8 [GACTAATAGGCTGACGCTGT]) amplify a 618bp product which encompasses P91A exons 4, 5, 6, 7 and 8. (A) 1: $CD45^-$ stroma, 2: MHC class II^+ thymic epithelial cells, 3: MHC class II^+ thymic dendritic cells. (B) 1: $CD4^-CD8^-$ thymocytes, 2: $CD4^+CD8^+$ TCR^{low} thymocytes, 3: $CD4^+CD8^+$ TCR^{high} thymocytes, 4: $CD4^+$ and $CD8^+$ thymocytes.

Figure 2.1


A
1 2 3
738 bp
615 bp
492 bp
369 bp
123 bp
B
1 2 3 4
738 bp
615 bp
492 bp
369 bp
123 bp

Figure 2.2

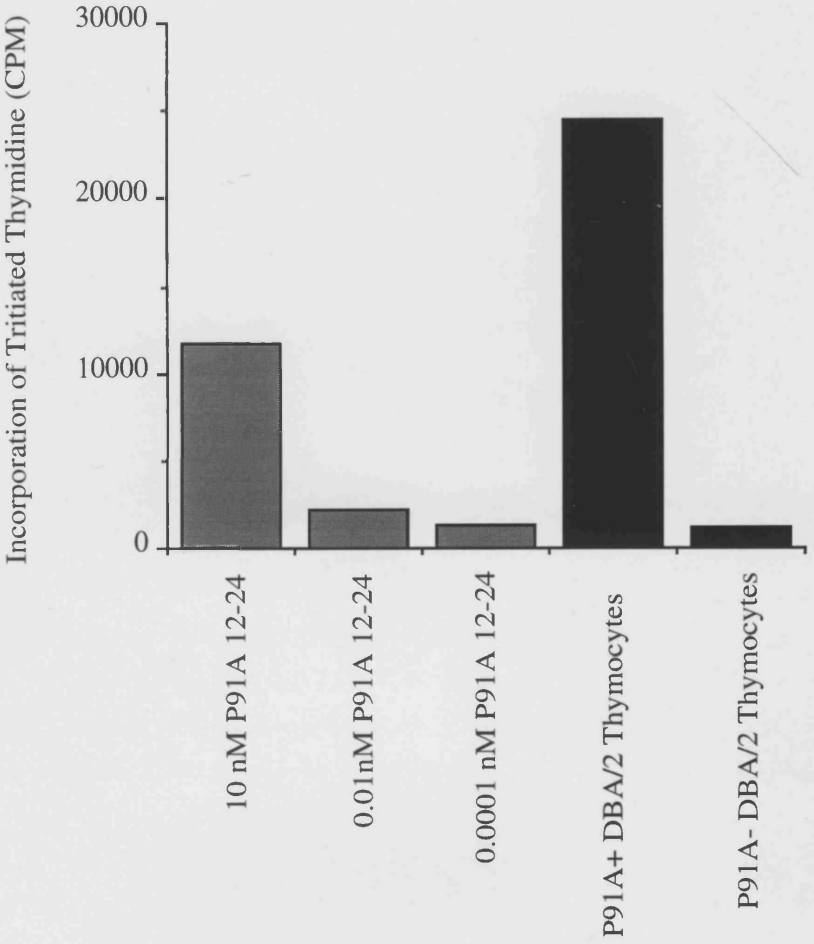


Figure 2.2: Expression of the P91A epitope within transgenic thymus. Proliferation of the P91A specific T cell clone P91.6 was assessed in presence of P91A 12-24 peptide (Lurquin et al, 1989) and 5 X 10^5 irradiated syngeneic feeder spleen cells ▩ , or in the presence of 5 X 10^5 irradiated P91A transgenic or non transgenic thymocytes ■.

Figure 2.3 : *In vitro* generation of P91A specific T cell lines from P91A transgenic and non-transgenic mice. T cell lines were generated from non-transgenic mice 1 and 3 (2.3A and 2.3B) and transgenic mice 4 and 14, (2.3C and 2.3D). P91A specific kill was assessed following stimulation on days 4, 9, 16 and 23, (i), (ii), (iii) and (iv) respectively. In the case of transgenic line 14 no kill was apparent following three *in vitro* restimulations. This line was FAC sorted on the basis of CD8 expression and a small population of CD8+ cells expanded *in vitro*. Line 14 was re-assayed on day 54 following a 7th *in vitro* restimulation, (2.3D(iv)). Percent specific lysis of P1 cells was measued in the presence —□— or absence —◆— of 50μM P91A 12-24 peptide.

Figure 2.3 A

Non transgenic line 1

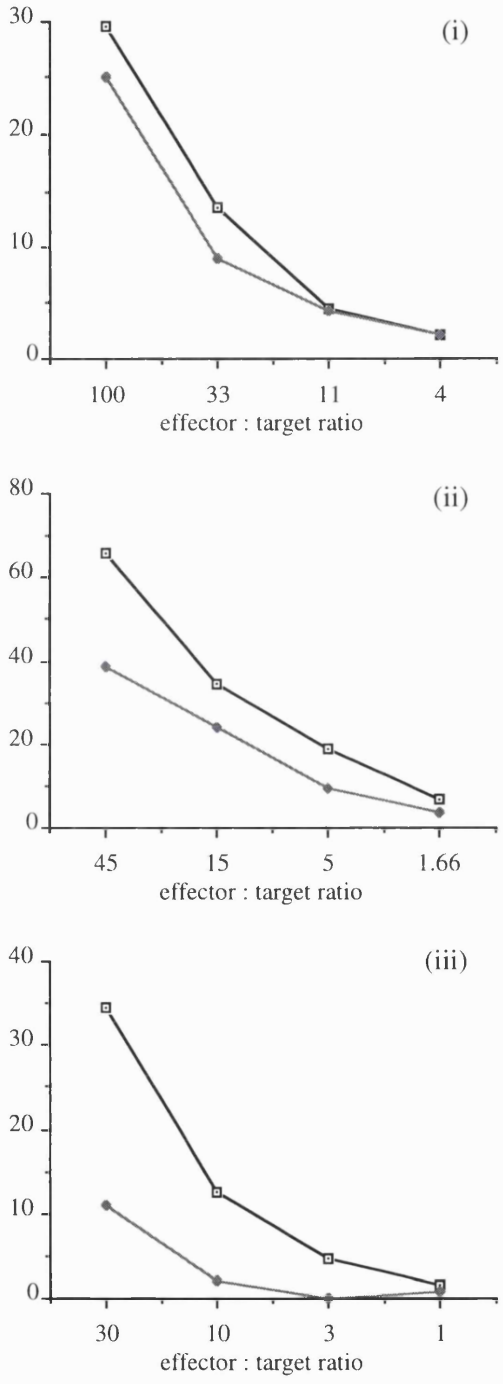


Figure 2.3 B

Non transgenic line 3

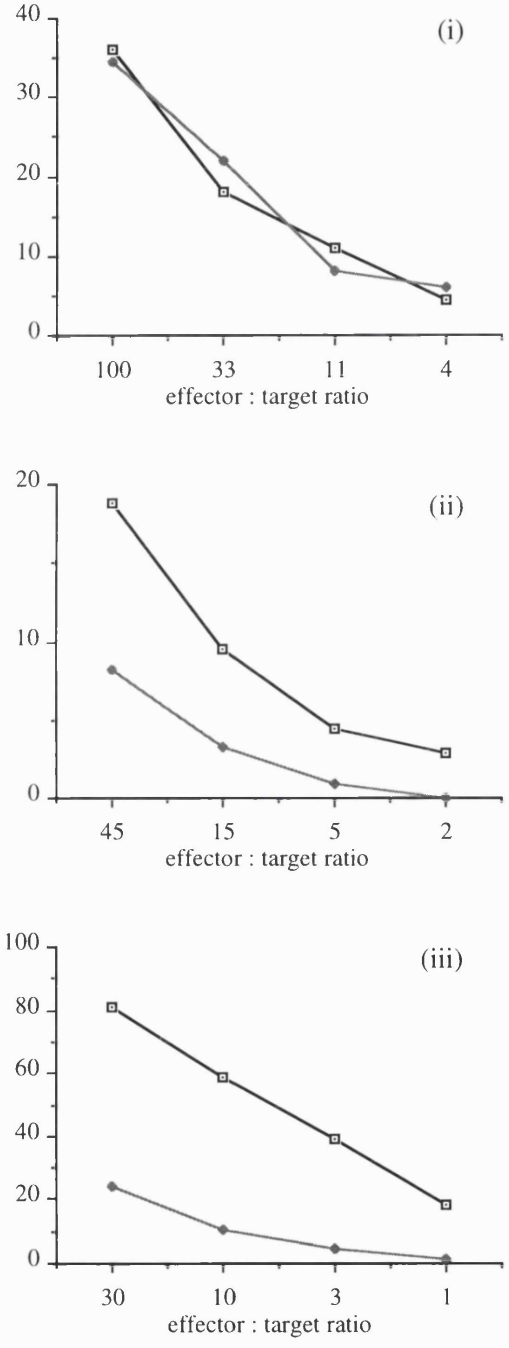


Figure 2.3 C

P91A Transgenic line 4

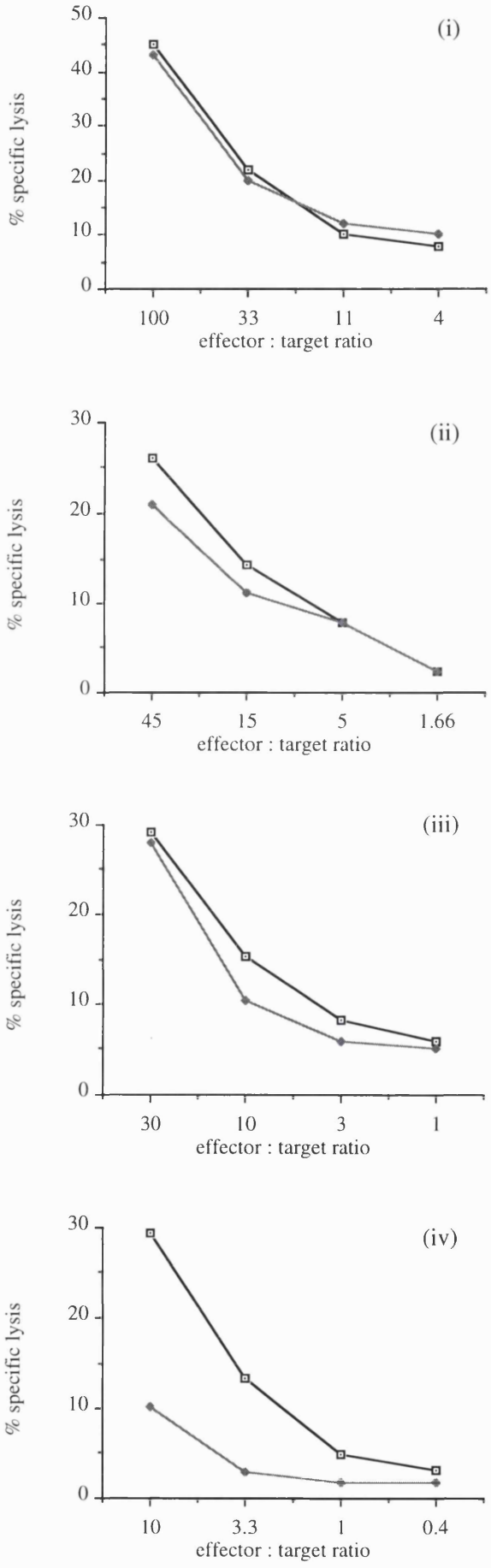


Figure 2.3 D P91A transgenic line 14

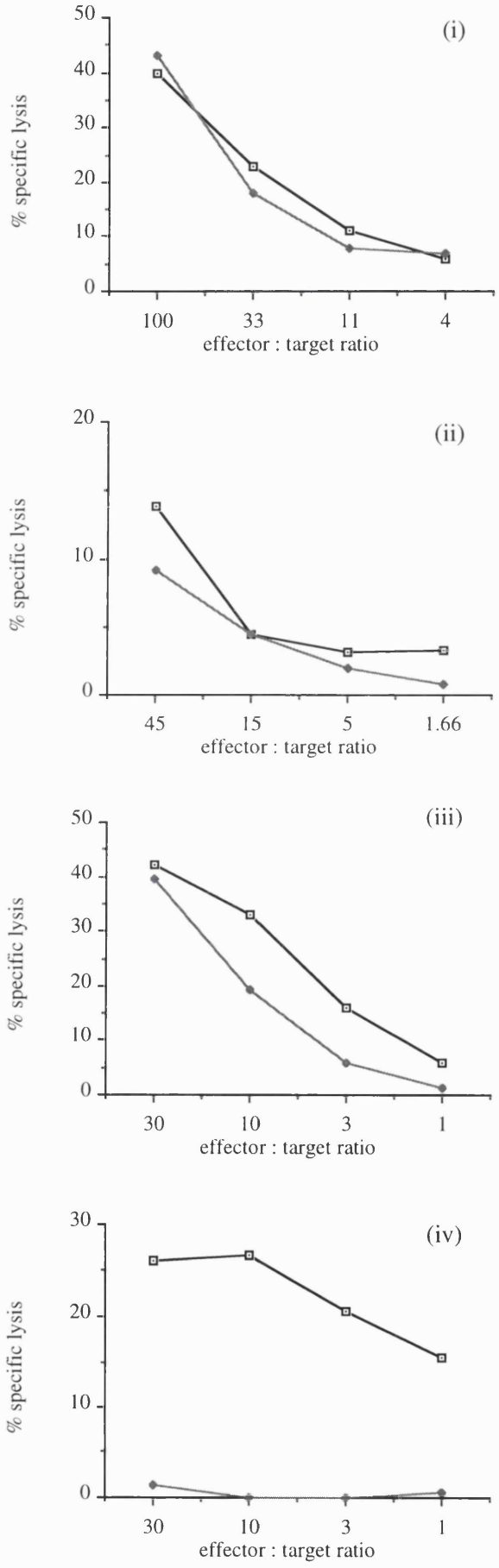


Figure 2.4 : The derivation of T cell clones from transgenic and non-transgenic mice. T cell clones were derived by limiting dilution and weekly restimulation with P91A peptide and 2% rat ConA sup. The proliferative responses of clones derived from non-transgenic lines 1 and 3, (A and B) and transgenic lines 4 and 14, (C and D) are shown. Proliferation is assessed at three concentrations of peptide in the presence of 2% rat ConA sup.

Figure 2.4

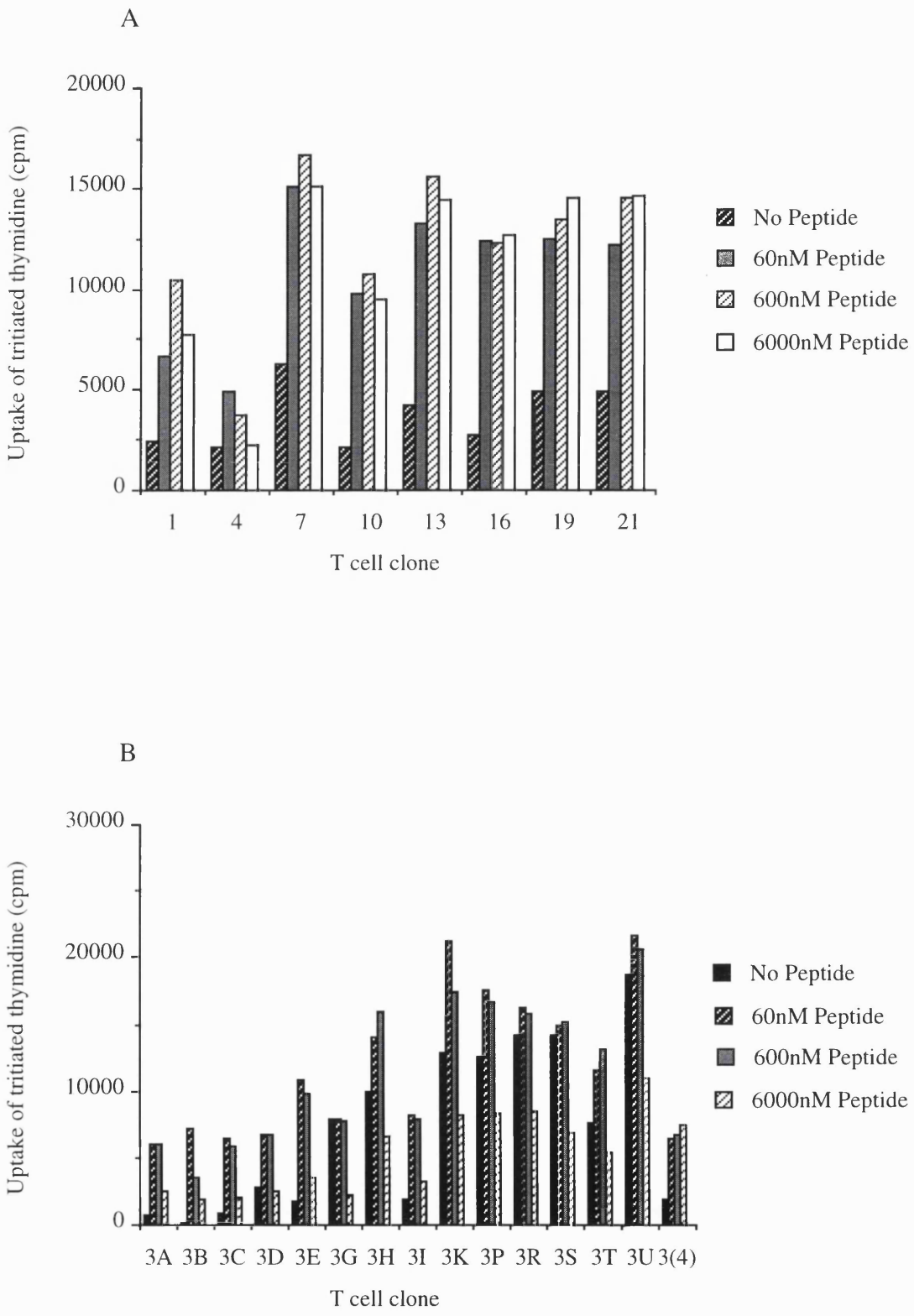
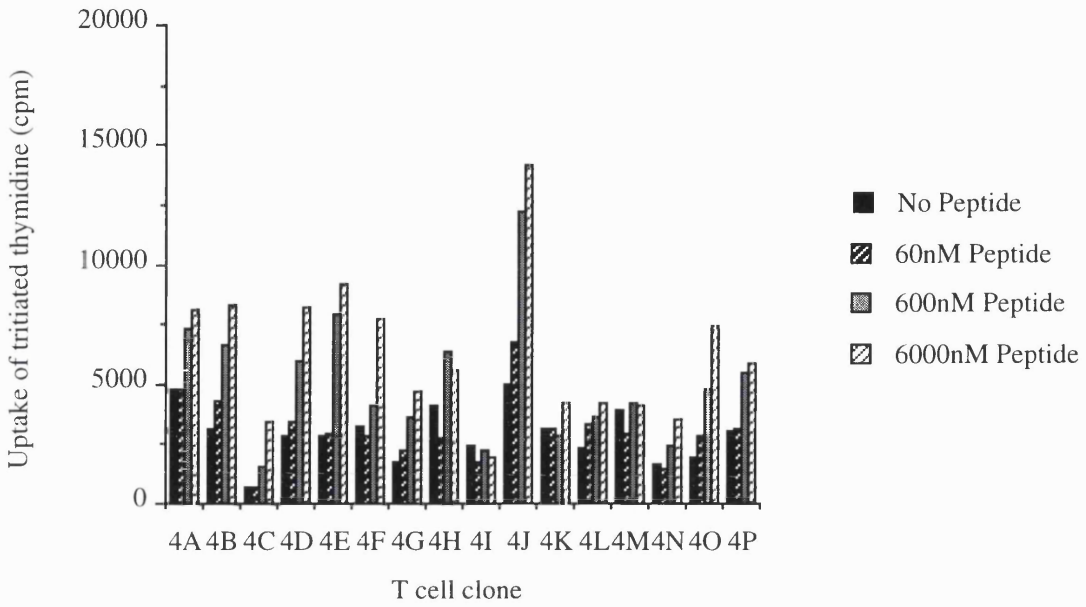

A
20000
15000
10000
5000
0
Uptake of tritiated thymidine (cpm)
1
4
7
10
13
16
19
21
T cell clone
No Peptide
60nM Peptide
600nM Peptide
6000nM Peptide
B
30000
20000
10000
0
Uptake of tritiated thymidine (cpm)
3A 3B 3C 3D 3E 3G 3H 3I 3K 3P 3R 3S 3T 3U 3(4)
T cell clone
No Peptide
60nM Peptide
600nM Peptide
6000nM Peptide

Figure 2.4

C


20000
15000
10000
5000
0
Uptake of tritiated thymidine (cpm)
4A 4B 4C 4D 4E 4F 4G 4H 4I 4J 4K 4L 4M 4N 4O 4P
T cell clone
No Peptide
60nM Peptide
600nM Peptide
6000nM Peptide

D

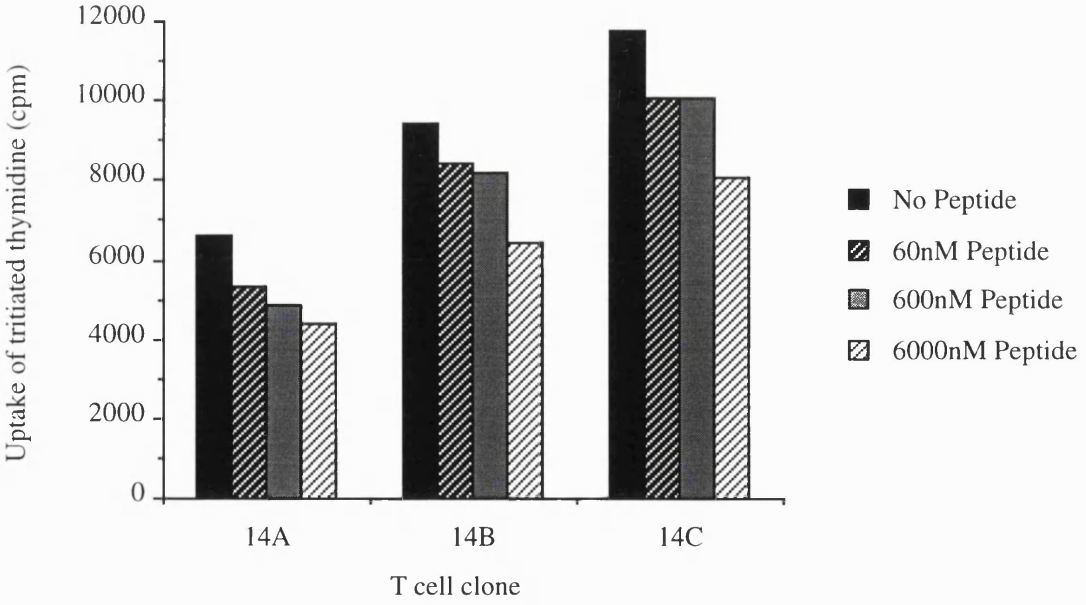

12000
10000
8000
6000
4000
2000
0
Uptake of tritiated thymidine (cpm)
14A
14B
14C
T cell clone
No Peptide
60nM Peptide
600nM Peptide
6000nM Peptide

Figure 2.5 : Peptide responsiveness of transgenic and non-transgenic T cell lines.
The proliferation to P91A peptide of (A) non-transgenic line 3 and (B) transgenic line 4 was assessed (I), in absence of rIL2, (II) in the presence of 5iu/ml rIL2 or (III) in the presence of 20iu/ml rIL2. Incorporation of tritiated thymidine was measured in the presence —⊡— or absence * of P91A 12-24 peptide.

Figure 2.5

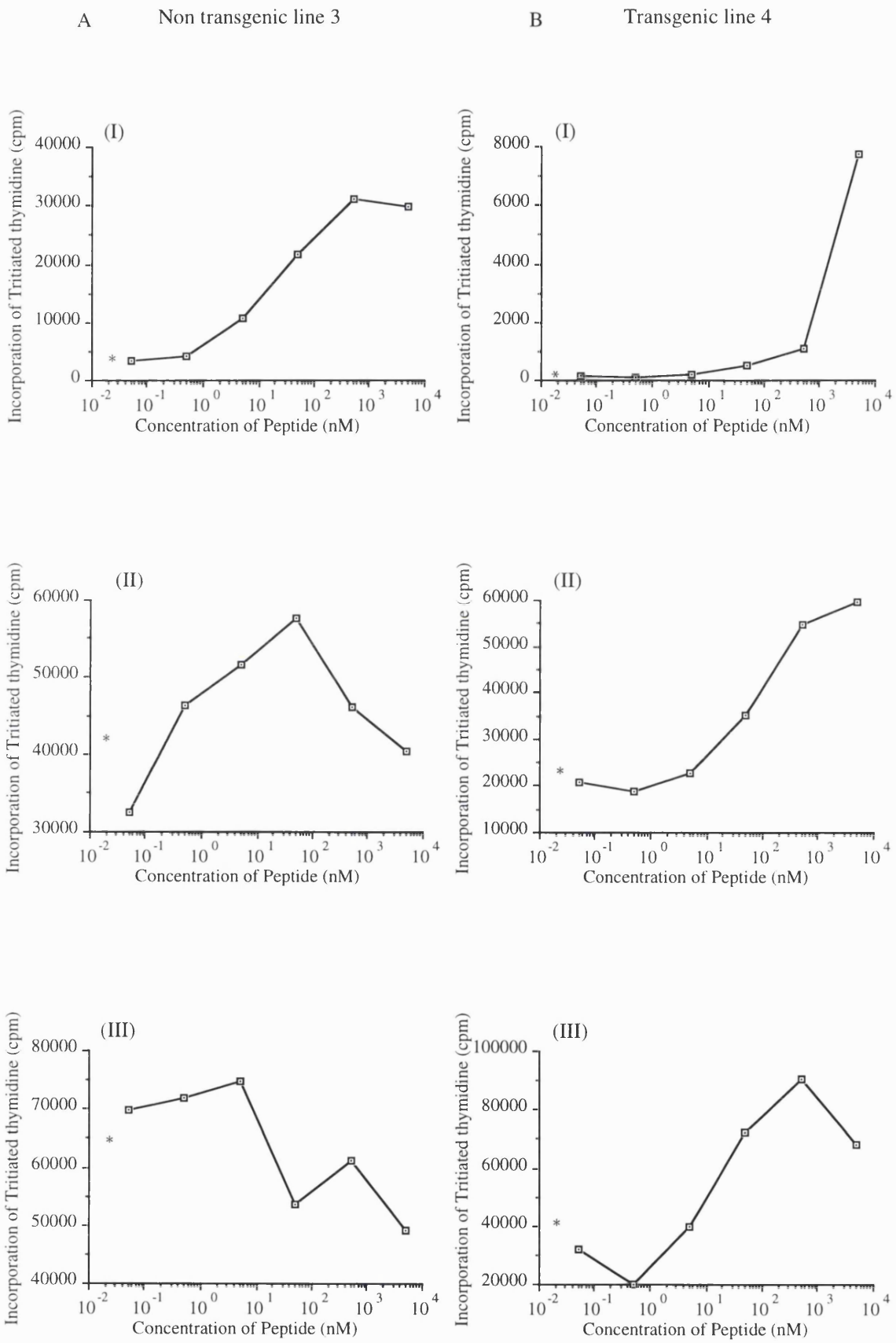

A
Non transgenic line 3
B
Transgenic line 4
(I)
(II)
(III)
Incorporation of Tritiated thymidine (cpm)
Concentration of Peptide (nM)
40000
30000
20000
10000
0
8000
6000
4000
2000
60000
50000
40000
30000
20000
10000
80000
70000
60000
50000
40000
100000
80000
60000
40000
20000
*
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4}

Figure 2.6 : Peptide responsiveness of transgenic and non-transgenic T cell clones. The proliferation of non-transgenic T cell clones (3A, 3B, 3C, 3D and 3E) and transgenic T cell clones (4A, 4B, 4D, 4E and 4G) in the presence —⊡— or absence * of P91A 12-24 peptide.

Figure 2.6

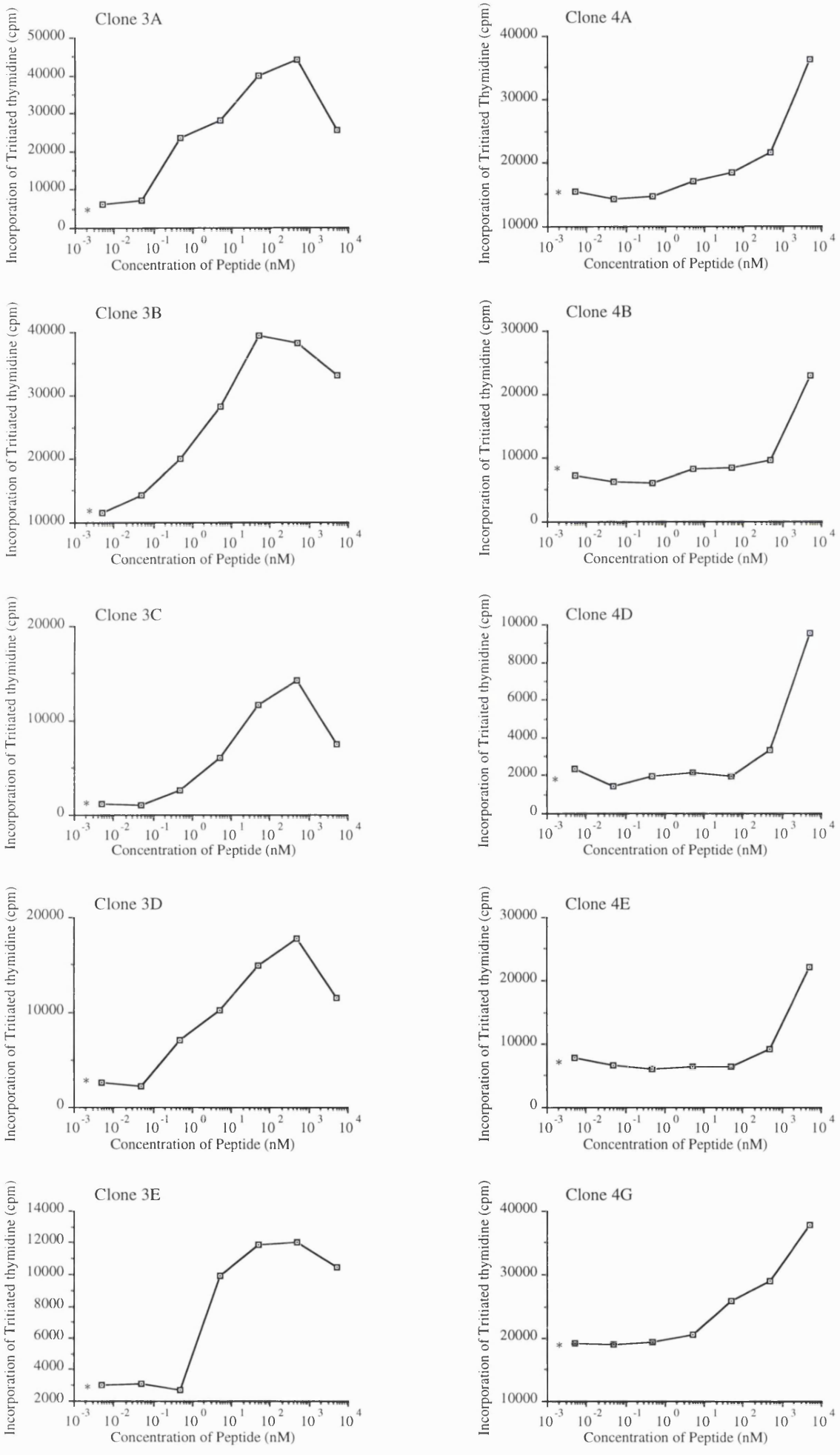

Clone 3A
Clone 4A
Clone 3B
Clone 4B
Clone 3C
Clone 4D
Clone 3D
Clone 4E
Clone 3E
Clone 4G
Incorporation of Tritiated thymidine (cpm)
Incorporation of Tritiated Thymidine (cpm)
Concentration of Peptide (nM)

Figure 2.7: The levels of expression of TCR and CD8 are equivalent on transgenic and non-transgenic clones. Transgenic and non-transgenic clones were stained with (A) an anti CD3 monoclonal antibody (KT3), (Tomonari et al, 1988) or (B) an anti-CD8 monoclonal antibody (KT15), (Tomonari et al, 1990). Detection in both cases was with FITC conjugated goat anti-mouse Fc. Background fluorescence was measured post staining with FITC conjugated goat anti-mouse Fc.

Figure 2.7 A

T cell clone	Mean Fluorescence (Background)	Mean Fluorescence (anti-CD3)	Increase in Fluorescence	Mean Increase in Fluorescence
3A	6.63	16.84	10.21	
3B	4.17	12.42	8.25	
3C	6.02	13.69	7.67	7.79
3D	3.54	11.13	7.59	
3E	5.85	11.11	5.26	
4A	2.58	8.35	5.77	
4B	4.74	12.84	8.1	
4D	2.94	15.5	12.56	7.66
4E	1.87	7.22	5.35	
4G	3.4	9.94	6.54	

Figure 2.7 B

T cell clone	Mean Fluorescence (Background)	Mean Fluorescence (anti-CD8)	Increase in Fluorescence	Mean Increase in Fluorescence
3A	2.53	51.13	48.6	
3B	6.48	64.23	57.75	
3C	2.37	73.5	71.13	55.81
3D	6.54	52.69	46.15	
3E	1.99	57.43	55.44	
4A	9.13	71.57	62.44	
4B	2.51	81.77	79.26	
4D	2.31	53.34	51.03	64.21
4E	2.43	68.53	66.1	
4G	6.48	68.72	62.24	

Figure 2.8

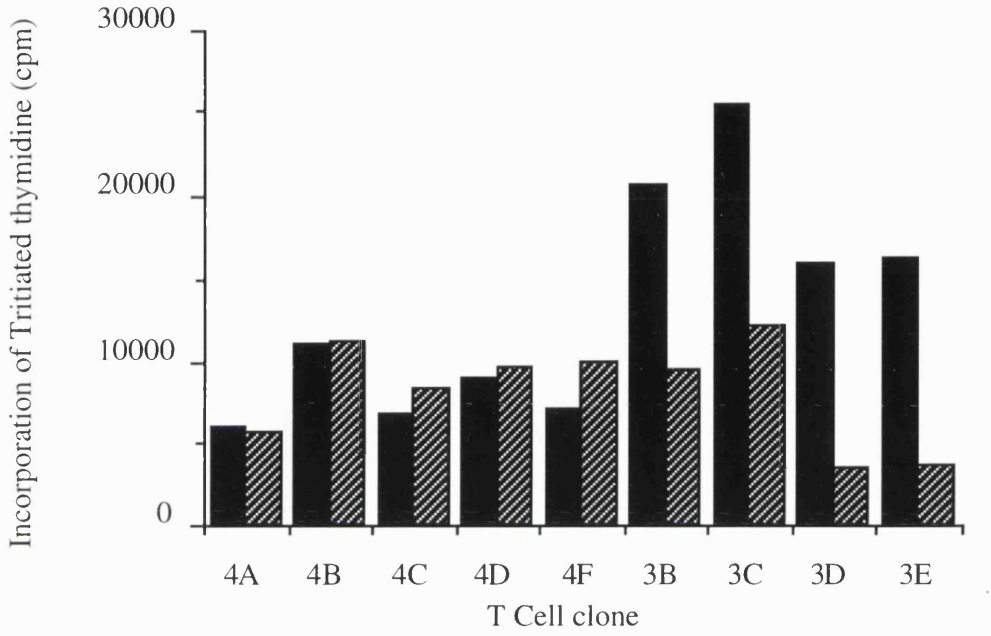


Figure 2.8 : T cell clones from P91A transgenic mice fail to proliferate in the presence of P91A transgenic spleen cells. T cell clones were plated at 1 X 10^4 cells / well onto 5 X 10^5 irradiated transgenic ■ or non transgenic ▨ spleen cells in the presence of 2% ConA sup. Incorporation of tritiated thymidine was measured at 72 hours following a 12 hour pulse period. Non transgenic T cell clones 3B, 3C, 3D and 3E are compared with transgenic clones 4A, 4B, 4C, 4D and 4F.

Figure 2.9 : The proliferative response of T cells derived from a P91A transgenic mouse to an alternative L^d binding peptide. CTL lines specific for either the P91A 12-24 —◆— or MCMV $pp89_{168-176}$ —□— peptide were derived from a transgenic mouse as described previously. Peptide specific kill of P1 targets was measured in the presence of 50μM MCMV peptide (A) or P91A 12-24 peptide (B). The proliferative response of the MCMV specific line to peptide was measured (C). * background proliferation in the absence of peptide.

Figure 2.9

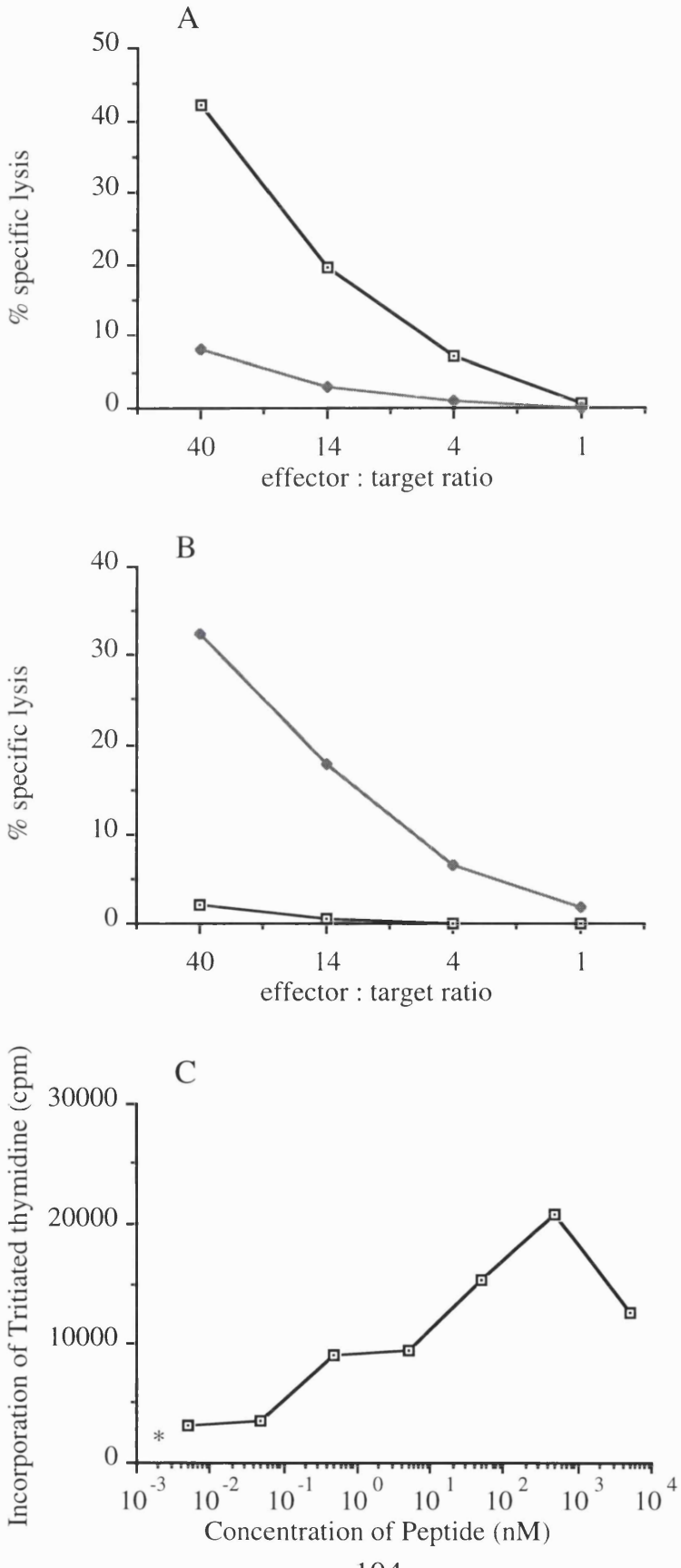
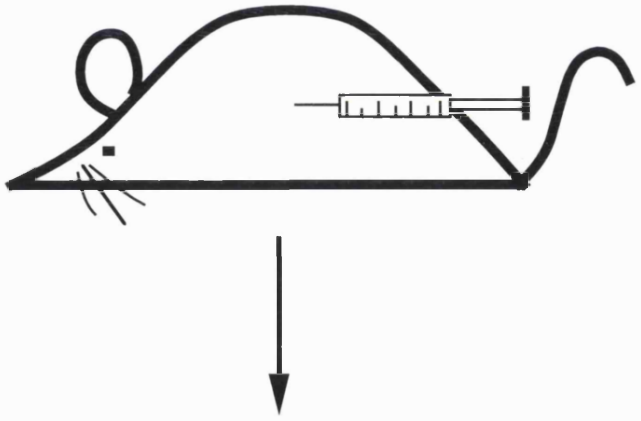

A
50
40
30
20
10
0
% specific lysis
40
14
4
1
effector : target ratio
B
40
30
20
10
0
% specific lysis
40
14
4
1
effector : target ratio
C
30000
20000
10000
0
Incorporation of Tritiated thymidine (cpm)
*
10 -3
10 -2
10 -1
10 0
10 1
10 2
10 3
10 4
Concentration of Peptide (nM)

Figure 2.10 A

Protocol for inducing rejection of male transgenic skin
by non transgenic female DBA/2 mice

Day 0: Immunise i/p with 1 X 10^7 P91A+H-Y+ spleen cells



Day 14: Graft with P91A+H-Y+ tail skin

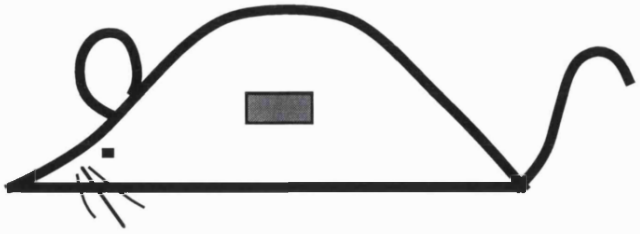
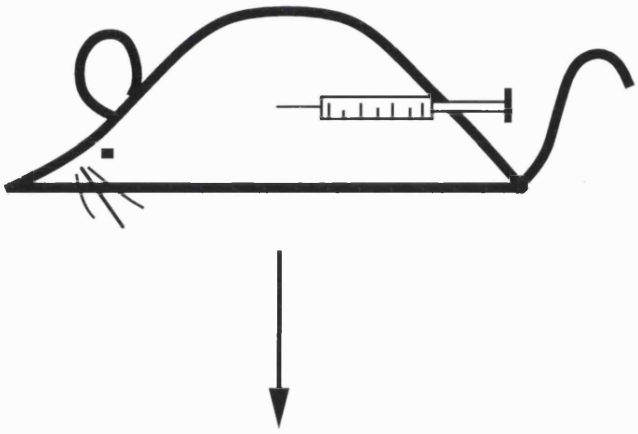


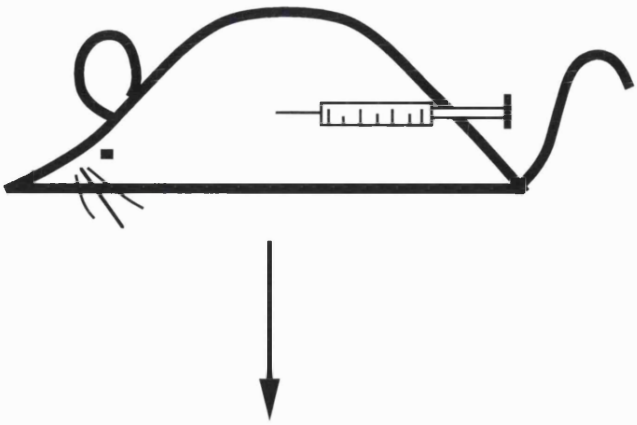
Figure 2.10 B

Protocol for inducing tolerance to male transgenic skin in DBA/2 mice

Day 0: Pre-immunise i/p with 1 X 10^7 P91A+ female or P91A- male spleen cells



Day 14: Immunise i/p with 1 X 10^7 P91A+H-Y+ spleen cells



Day 28: Graft with P91A+H-Y+ tail skin

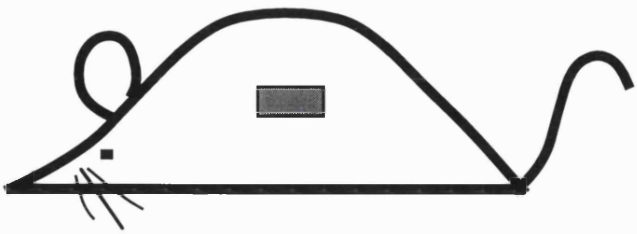


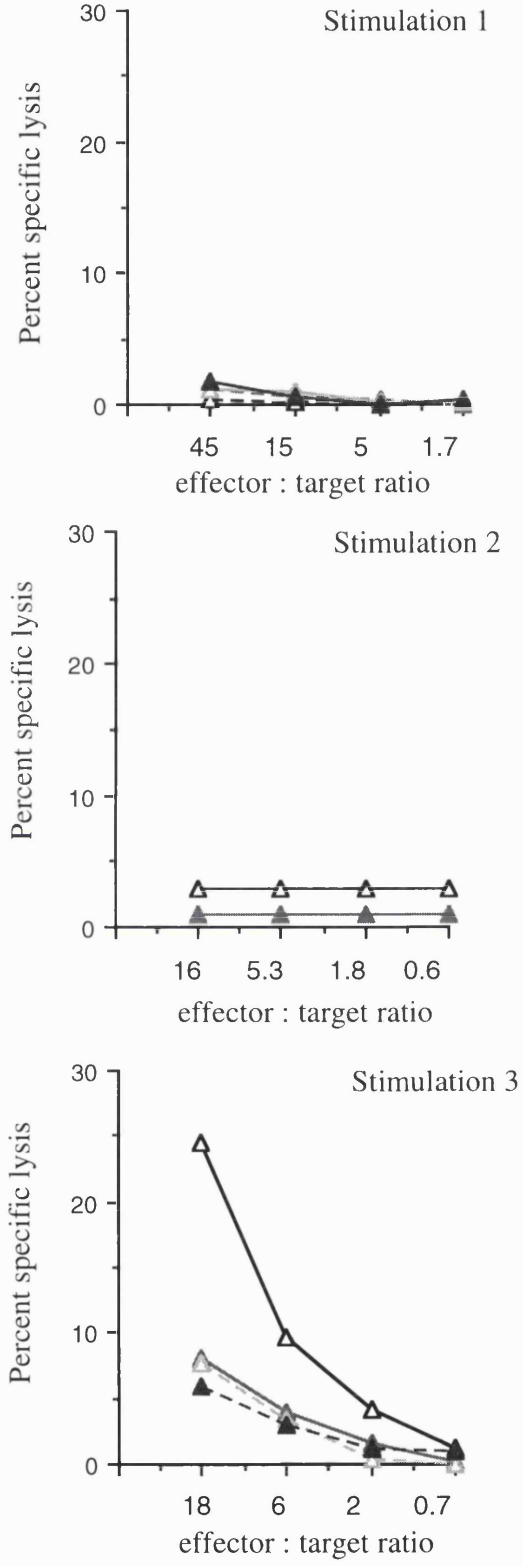
Table 2.1

Group	Pre-immunistion		Test immunisation		Skin challenge		MST (days)	Rejection Number (days)
	P91A	H-Y	P91A	H-Y	P91A	H-Y		
A	+	-	+	+	+	+	>100	1/13 (d68)
B	-	+	+	+	+	+	>100	1/10 (d60)
C	-	-	+	+	+	+	48	12/12 (d18, d28, d38, 2x d41, 2x d48, d52, d60, d72, d75, d102

Table 2.1 Peripheral tolerance induction in DBA/2 mice following pre-immunisation with P91A+ or H-Y+ DBA/2 spleen cells. Mice were immunised and grafted as shown in figure 2.10 B. Median survival time (MST) is the time when 50% of grafts are rejected.

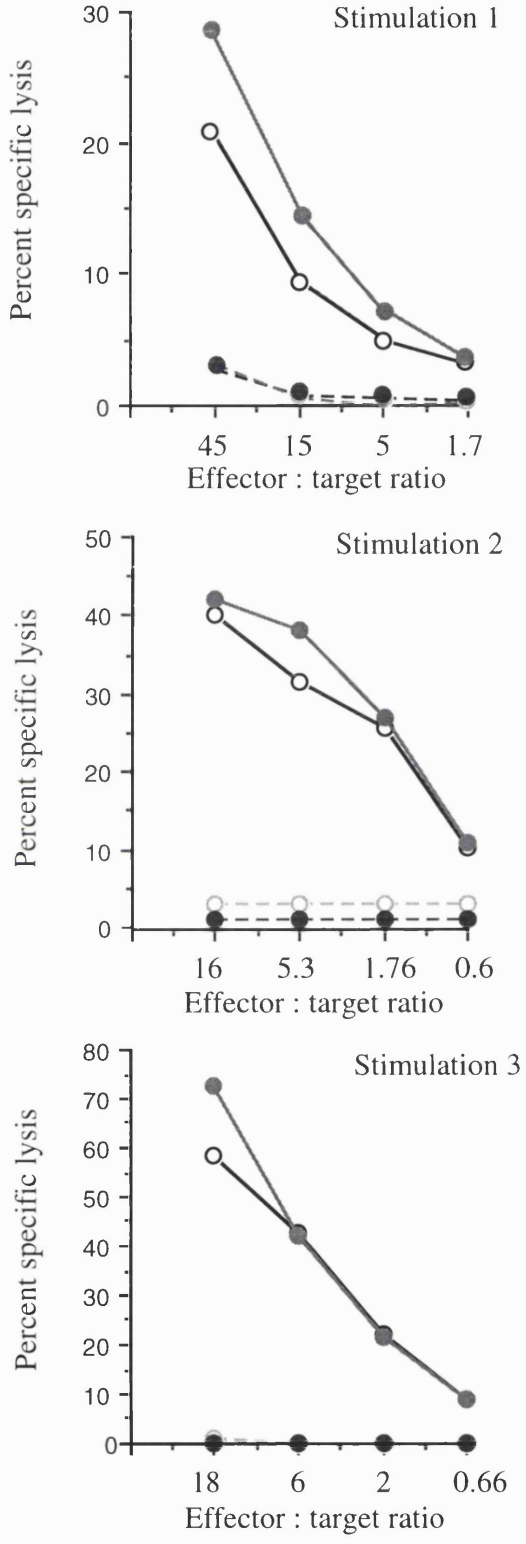
Figure 2.11 : *In vitro* demonstration of tolerance to P91A. Spleen cells from (A) P91A pre-immunised mice which have accepted P91A+H-Y+ skin (table 2.1 group A), (B) mice which have received no pre-immunisation and have rejected P91A+ H-Y+ skin, (table 2.1 group C) and (C) naive DBA/2 mice were cultured *in vitro* to generate P91A peptide specific T cells. Percent specific lysis was measured four days after the first, second or third restimulation. P1 cells in the presence (solid lines) or absence (dashed lines) of 50μM P91A 12-24 peptide acted as targets in all cases.

Figure 2.11 A



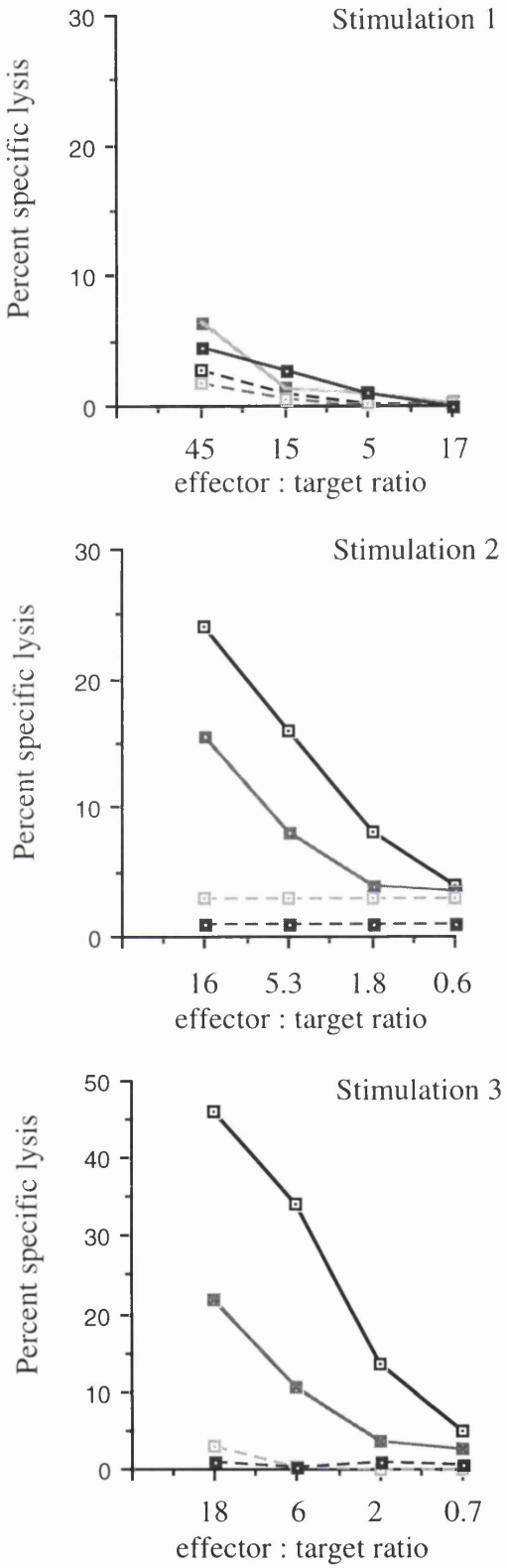
Effector cells from two DBA/2 mice which have been preimmunised with P91A+ spleen cells (table 2.1 group A) assayed in the presence (solid lines) or absence (dashed lines) of 50μM P91A 12-24 peptide. Mouse one: open symbols, mouse two: solid symbols.

Figure 2.11 B



Effector cells from two mice which have rejected P91A+H-Y+ donor skin, (table 2.1group C) assayed in the presence (solid lines) or absence (dashed lines) of P91A 12-24 peptide. Mouse one: open symbols, mouse two: solid symbols.

Figure 2.11 C



Effector cells from two naive DBA/2 mice assayed in the presence (solid lines) or absence (dashed lines) of P91A 12-24 peptide. Mouse one: open symbols, mouse two: solid symbols.

Figure 2.12 : *In vitro* demonstration of tolerance to P91A induced by either pre-immunising with P91A+ or H-Y+ spleen cells. Effector spleen cells (A -D) were cultured for 5 days in the presence of 50μM P91A 12-24 peptide after which time specific lysis of P1 targets was measured in the presence (solid lines) or absence (dashed lines) of 50μM P91A 12-24 peptide. Effector spleen cells from tolerant mice (A and B) show good allospecific kill of H-2^b targets (E).

(A) Effector cells from two DBA/2 mice which have been preimmunised with male DBA/2 spleen cells (table 2.1 group B).

(B) Effector cells from two DBA/2 mice which have been preimmunised with female P91A transgenic spleen cells (table 2.1 group A)

(C) Effector cells from two DBA/2 mice which have received no preimmunising injection and have rejected male P91A transgenic skin.

(D) Effector cells from two naive DBA/2 mice

(E) Spleen cells from H-Y or P91A tolerised mice (A) and (B) were cultured with irradiated C56Bl/10 (H-2^b) spleen cells for five days after which allospecific kill of EL4 target cells was measured. Open triangles: effector cells from an H-Y preimmunised mouse. Closed squares: effector cells from a P91A preimmunised mouse.

Figure 2.12

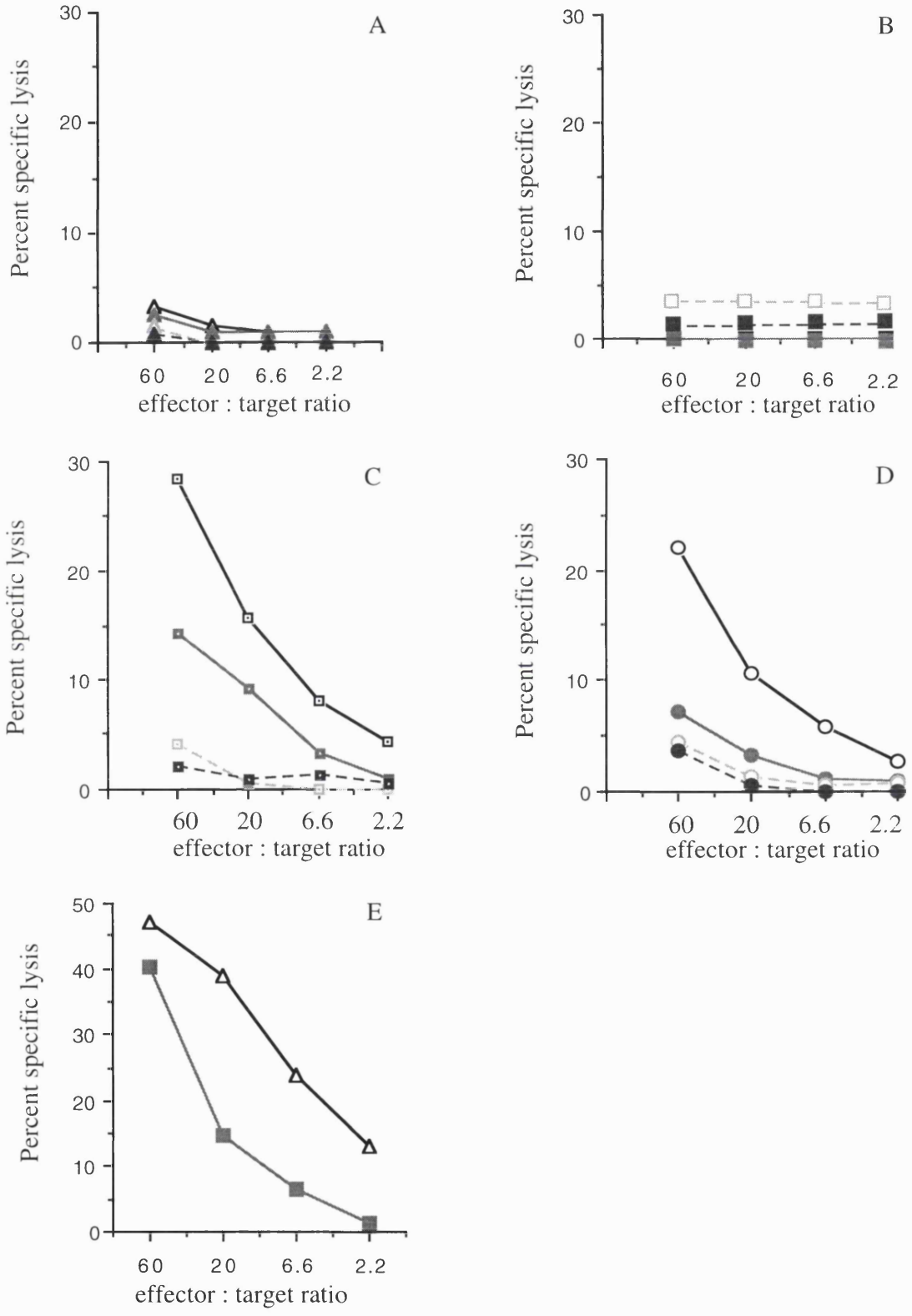

A
B
C
D
E
Percent specific lysis
effector : target ratio
60
20
6.6
2.2
0
10
20
30
40
50

Table 2.2

Group	Pre-immunistion		Test immunisation		Skin Challenge		MST (days)	Rejection Number (days)
	% P91A	% P91A/ H-Y	P91A	H-Y	P91A	H-Y		
1	100	-	+	+	+	+	>100	0/7
2	90	10	+	+	+	+	>100	0/4
3	50	50	+	+	+	+	53	3/6 (28, 28, 53)
4	10	90	+	+	+	+	24	4/4 (24, 24, 31, 45)
5	-	100	+	+	+	+	24	4/4 (21, 24, 35, 53)

Table 2.2 The relative efficiencies of priming and tolerance induction. DBA/2 recipient mice were pre-immunised i.p. with a titrated mix (10^7 total) of tolerising P91A+ and immunising P91A+H-Y+ spleen cells. All recipients were skin grafted 14 days after the test immunisation.

3.0 Analysis of T cell receptors expressed on P91A transgenic and non-transgenic clones

3.1 Introduction

Several studies have attempted to correlate specific TCR gene product usage with the recognition of specific MHC or MHC-peptide complexes. Studies involving both class I and class II restricted T cells have reported a correlation between particular TCR Vα or Vβ gene segment usage and MHC/antigen recognition (Fink et al, 1986, Winoto et al, 1986, Urban et al, 1988, Johnson et al, 1989, Acha-Orbea et al, 1988, Moss et al, 1991, Boitel et al, 1992, Cose et al, 1995). Furthermore, in peptide specific responses conserved CDR3 junctional sequences and the localisation of charged residues within this region have been correlated with peptide recognition (Engel et al, 1988, Sorger et al, 1990, Danska et al, 1990, Lai et al, 1990, Wither et al, 1991, Jorgenson et al, 1992, Bowness et al, 1993, Kelly et al, 1993, Sun et al, 1995, Sant'Angelo et al, 1996, Turner et al, 1996). Analysis of TCR and CDR3 sequences using anchored or inverse PCR techniques has been used to examine T cell usage and CDR3 characteristics in both healthy individuals and disease states where the nature of the antigenic stimulation is unknown (Moss et al, 1993, Moss et al, 1995, Wang et al, 1995a, Hall and Lanchbury, 1995, Bowman et al, 1997). Conservation in the usage of both Vα and Vβ chains has been observed in certain peptide specific responses. Analysis of H2-D^b-restricted CTL specific for a lymphocytic choriomeningitis virus glycoprotein peptide (LCMV-$GP_{275-289}$) reveals preferential usage of Vα4/Vβ10 but junctional comparisons were not made in this study (Aebischer et al, 1990). In addition, conserved Vα (Vα 12.1, 14.1, 22) and Vβ (Vβ7) usage has been shown in the response of three unrelated donors to the HLA-B27 restricted influenza A virus nucleoprotein epitope $NP_{383-391}$ (Bowness et al, 1993). Although the CDR3 region is intimately involved in MHC-peptide recognition there are examples where V region usage rather than CDR3 sequence correlates more

closely with MHC-peptide recognition. Dominant Vβ13 usage by K^d-restricted clones specific for a *Plasmodium Berghei* nonapeptide has been reported (Casanova et al, 1991). However in this study, analysis of α and β junctions as well as Vα chains reveals heterogeneity in the panel of clones. This suggests that residues encoded within the TCRB V region can in certain circumstances direct peptide specific responses in the face of CDR3 diversity and fits a model of MHC-peptide recognition in which rigid categorisation of CDR (1, 2 and 3) function is less critical.

A study examining the T cell receptor usage of P91A specific T cell clones was published during the course of this work (Solheim et al, 1993a). T cell clones were generated *in vitro* using high concentrations of peptide and varied greatly in their CD8 dependency as defined by the ability of anti-CD8 monoclonal antibody to block lysis of peptide pulsed target cells. Moreover, the CD8 dependency of each clone correlated with the determinant density required to elicit specific kill, in that CD8-independent clones required much higher concentrations of peptide for equivalent lysis. TCR analysis of P91A specific clones indicated a dominant usage of Vβ8 by 12 of 17 clones. In addition there was an apparent restriction in Vα and Jα usage although this did not correlate with CD8 dependency. It was further speculated that a negatively charged residue (Asp or Glu) found within CDR3α might interact with the positively charged residues (His or Arg) located within the P91A epitope. Taken together, these data suggest the dominant usage of a specific Vβ, limited J segment usage and conserved CDR3α junctional sequence in the recognition of the P91A epitope.

Large panels of T cell clones have been generated from P91A transgenic and non-transgenic mice. Whether these clones derive from independent progenitor cells or represent sister clones is unknown. The following section investigates the TCR usage of P91A specific T cell clones derived from transgenic and non-transgenic mice. TCR gene segment usage and V(D)J junctional sequences are compared between low and

high affinity clones and similarities and differences correlated with peptide responsiveness.

3.2 Results and discussion

3.2.1 T cell receptors expressed on P91A peptide specific T cell clones

3.2.1.1 The genomic rearrangement of T cell receptors in P91A transgenic and non-transgenic T cell clones

When T cells are cloned from an expanded population of cells it is unfortunately probable that the resultant clones will be composed of a mixture of sister clones derived from the same progenitor cell and clones derived from independent peptide specific T cells. Sister clones will bear identical T cell receptors and have the same genomic rearrangement of V(D) and J segments while peptide specific clones which have originated from independent T cells will have different patterns of V(D)J recombination and express unique T cell receptors. This difference is however not immediately apparent since sister and unrelated T cell clones may show similar or identical proliferative responses to peptide.

In an attempt to define the heteroclonality of T cell clones derived from transgenic and non-transgenic mice genomic recombination within the TCRBJ2 locus was examined. During the process of VDJ recombination intervening V, D and J segments are deleted. This enables specific V, D and J segments to become directly apposed at the genomic level. Using a TCRB J2 probe (Palacios and Samaridis, 1991) which hybridises 3' of the last TCRB J2 segment and a Hind III digest of genomic DNA it has been possible to examine genomic recombination to TCRB J2 segments in P91A specific T cell clones, figure 3.1. This method has been previously used to examine genomic recombination within in the TCRB, D, and G loci of extrathymic T cells in

nude mice (Palacios and Samaridis, 1991). In the present study only rearrangement to the TCRB J2 cluster was examined.

The rearrangement of a specific V segment to the TCRB D2/J2 cluster will automatically result in the excision of all intervening DNA. This will include all 3' V segments, the TCRB D1/J1 cluster and the TCRB C1 segment. Furthermore, rearrangement of TCRB D2 to a TCRB J2 segment will result in the excision of intervening TCRB J2 segments. There is no rearrangement to TCRB C2 the intervening sequence being spliced out of the resultant TCR messenger RNA. TCRB rearrangement can be attempted on both copies of chromosome six. If this occurs then following digestion of genomic DNA with the appropriate restriction enzyme (HindIII in this case) two bands of equal intensity, which differ in size from that of the unrearranged locus will be produced. A single rearrangement will produce a band which varies in size depending on which V/J2 segment has been used and will be of equal intensity to the band derived from digestion of the unrearranged TCRB locus. Failure to rearrange a V segment to either copy of the TCRB J2 locus will result in a double intensity band of predicted unrearranged size (in this case 5.2kb).

Only those clones which show good specificity for the P91A peptide were examined. As can be seen in figure 3.2 A and B, all panels of T cell clones contain multiple sister clones. T cell clones derived from non-transgenic line 3 fall into two categories, both of which appear to have rearranged both TCR loci to the TCRB D2/J2 cluster. Clones derived from non-transgenic line 1 also fall into two unique categories which once again appear to have rearranged both TCRB loci to the TCRB D2/J2 cluster. Unfortunately transgenic line 14 failed to yield any peptide specific T cells and was therefore not examined further. Transgenic line 4 produced two types of T cell clone the majority of which have a single rearrangement to the TCRB D2/J2 cluster; a 5.2kb band of equal intensity to the rearranged band is present in these clones and indicates that no recombination to TCRB D2/J2 has occurred on the sister chromosome. Clone 4A is the only clone derived from line 4 which has a different pattern of

rearrangement. In this case clone 4A also appears to have rearranged both TCR loci to the TCRB D2/J2 cluster. It should be noted that even in those clones which have rearranged both TCRB loci to the D2/J2 cluster a weak band representing the unrearranged TCRB J2 cluster is present. This band is derived from irradiated mouse feeder spleen cells which are required for growth of the T cell clones. Feeder spleen cell DNA will not produce bands of polymorphic size even though multiple rearrangements will be present within this population since recovery of sufficient DNA to produce a band of defined size is unlikely to be possible. Having said this, every T cell which has not rearranged a V segment to the TCRB J2 locus on one chromosome will produce a fragment of DNA of defined non rearranged length (in this case 5.2kb). The number of such T cells will be large and recovery of DNA sufficient even from a dying population of feeder cells to produce a weak band representing the unrearranged locus.

In total, six different types of T cell clone have been isolated: four from two non-transgenic mice and two from a transgenic mouse. This is a disappointing result since it was hoped that a large scale analysis of T cell receptor usage would be possible in transgenic and non-transgenic mice. The fact that so many clones derive from the same progenitor must reflect the strategy which was used to derive the lines and clone the cells. It is likely that *in vitro* priming is a less effective method of priming T cells than that which occurs *in vivo*. If this is the case then the initial activation of progenitor resting T cells may have been suboptimal. Furthermore, following activation those cells which proliferate better *in vitro* will have a competitive advantage over their neighbours. This effect will be exaggerated over time and may result in the culture becoming dominated by a clonal or oligoclonal population. This is undoubtedly what occurred in this case. If cloning had been attempted at an earlier stage then it is probable that the diversity of T cell clones generated would have been greater. In the present work good quality lines were first derived from which T cells were cloned. It was hoped that such lines would still contain a largely mixed

population of peptide specific T cells. With hindsight it would have been better to clone earlier. Having said this, it was not uncommon for *in vitro* priming to be unsuccessful and cloning from early cultures would therefore have often been unsuccessful.

3.2.1.2 The further examination of T cell receptor usage by transgenic and non-transgenic T cell clones

In order to further examine the nature of the T cell receptors used by transgenic and non-transgenic T cell clones two complementary rtPCR approaches were used. The first approach utilises a panel of TCRA V specific sense oligonucleotides and a TCRA C antisense primer to screen cDNA from selected T cell clones, figure 3.3 A. This panel of primers was initially used to examine the T cell receptor α and β chain usage of cytotoxic T cells specific for a the *Plasmodium berghei* nonapeptide (PbCs 252-260) in association with H2-K^d (Casanova et al, 1991). The second approach utilises sets of TCRA V or TCRB V degenerate primers in conjunction with a nested set of antisense TCRA C or TCRB C primers, figure 3.3 B (Candeias et al, 1991). The use of a set of sense primers which are degenerate for TCR V recognition permits priming of all possible TCR V segments. This mixture of primers would result in non specific priming and lack of specificity if the number of PCR cycles was high and a single TCR C region antisense primer was used. By restricting the number of PCR cycles to 26 for each amplification and by using a nested set of constant region antisense primers specificity for TCR chains is introduced into this method. Both methods generate PCR products which can be sub-cloned and sequenced.

Initial attempts to define the TCR usage of clones was thwarted by contamination of T cell clone RNA with RNA derived from feeder spleen cells. Although not a problem for genomic analysis (at the sensitivity of the assay contaminant DNA will not be picked up by southern analysis) small amounts of feeder cell derived

messenger RNA will be amplified in either PCR approach and result in false positive results. Attempts to separate live T cells from irradiated feeder cells were unsuccessful and T cell clones failed to grow in the absence of a feeder population. In an attempt to overcome this problem athymic BALB/c (nu/nu) mice were used as a source of feeder cells. These mice are grossly deficient in peripheral T cells. Total RNA was prepared from T cell cultures and used to make cDNA for use in either of the two rtPCR techniques.

Using the panel of TCRA V specific primers transgenic clone 4A was shown to express two α transcripts: TCRA V2 and TCRA V8, figure 3.4. Due to continued problems using the panel of TCRA V specific primers the second PCR method was used to amplify the α and β transcripts from all other clones. Unlike the first method the second approach gives no direct information on TCR chain usage. All PCR products were sub-cloned into the TA vector (Invitrogen) and manually sequenced. The junctional sequences and V, D and J usage of all clones are shown in figure 3.5 A and B. V segment usage was confirmed by sequencing up to 150 bases 5' of the V(D)J junction. The junctional sequences of the TCRA and TCRB transcripts present in the P91A specific T cell clone P91.6 are included.

Five high affinity non-transgenic (3B, 3E, 16, 19 and P91.6) and two low affinity transgenic clones (4A and 4G) are included in this analysis. These clones were previously shown to have rearranged different T cell receptors and are therefore of independent origin, figure 3.2. Five of the seven clones use TCRB V8 segments, with V β 8.3 being used by four clones (4A, 3E, 16 and 19) and V β 8.2 by the tum$^-$ specific clone P91.6. TCRB V8 usage is not restricted to non-transgenic clones which show a high level of peptide responsiveness since transgenic clone 4A which proliferates only weakly to the P91A peptide is Vβ 8.3 positive. Non-transgenic clone 3B and transgenic clone 4G have rearranged TCRB V10 S1 and TCRB V1 respectively. Both of these Vβ segments have less than 50% similarity with Vβ 8.2 or

8.3 segments in a pairwise comparison (Arden et al, 1995b). Interestingly, analysis of the genomic sequence of TCRB V10 S1 identifies an in frame stop codon two bases from the 3' end of the open reading frame. In the cloned transcript this has been removed through exonuclease activity at the time of V(D)J recombination. It would be interesting to examine whether the presence of this in frame stop codon affects TCRB V10 S1 usage in peripheral T cells.

These results suggest a dominant role for Vβ8 positive T cells in the recognition of the P91A/L^d complex. Previous studies have demonstrated a dominant role for the usage of Vβ 8 positive T cells in alloresponses to L^d and in the L^d restricted response to MCMV (Goss et al, 1993; Rodewald et al, 1989). Furthermore, the alloreactive repertoire of CTL recognising the self peptide p2Ca, which is derived from the mitochondrial enzyme α-ketoglutarate and is restricted to L^d, is biased towards Vβ8 usage (Tjoa and Kranz, 1994a, 1994b). Taken together, these results indicate that Vβ8 may be more concerned with the recognition of the L^d molecule than with the peptide presented by it.

All the TCRB transcripts which were cloned derive from productive rearrangements of the TCRB locus. Three clones have rearranged to the TCRB J1/C1 (4A, 16, P91.6) and four to TCRB J2/C2 (4G, 3B, 3E and 19). Five clones use TCRB D1 and two clones use TCRB D2. In clones 4A, 3E, 16 and 19 the D segment has been reduced in size by exonuclease activity to only two or three bases. The presence of non germline encoded N/P nucleotide additions either before or after the D segment is shown in figure 3.5 B. The J segment usage of the clones is relatively conserved. Four clones use TCRB J2 S2, two clones TCRB J1 S1 and P91.6 uses TCRB J1 S6. TCRB J2 S6 and TCRB J1 S1 usage does not correlate with the origin of the clone since examples of both exist in transgenic and non-transgenic clones. In general the J segment used is derived from the same cluster as the D segment. However, this is not the case for

clones 4G and 3B in which the TCRB D1 segment is rearranged to a downstream TCRB J2 segment. This type of standard rearrangement is not uncommon.

Transgenic clone 4A and non-transgenic clone 16 have both rearranged to the TCRB D1/J1 cluster. This result is at odds with the previous genomic analysis of the TCRB J2 region which suggests that both clones have attempted to rearrange to TCRB D2/J2 on both copies of chromosome 6, Figure 3.2. Such a pattern of rearrangement would automatically remove the TCRB D1/J1/C1 region from the genome. A possible explanation for this result is that a rearrangement to the TCRB D2/J2 cluster exists on one copy of chromosome six, a second rearrangement to TCRB D1/J1 exists on the sister chromosome and a further D2-J2 rearrangement exists within the TCRB C2 cluster on this chromosome. This would give the appearance of two TCRB V-D2-J2 recombination events at the TCRB D2/J2 cluster while in fact only a single V-D-J and a D-J rearrangement had occurred.

TCRA V usage is also relatively conserved within the clones analysed. Three clones utilise a TCRA V3 segment (3B, 3E and 19), two clones use the TCRA V4 S4 segment (4G and 16) and the remaining two clones use TCRA V8 S13 (4A) and TCRA V18 $S^{P91.6}$ (P91.6). Clone 4A has rearranged both TCRA loci resulting in the presence of a second transcript for TCRA V2 S5. Only TCRA V8 S13 is rearranged in frame. The finding that clone 4A has two α transcripts and is the only clone for which the panel of TCRA V specific primers was used suggests that it is possible that other clones might also have a second α or β transcript which is not detected by the second PCR method. Failure to detect a second transcript might result from the preferential priming and amplification of TCR messenger RNA by degenerate primers. Such an effect would be compounded if the TCR message for a second α or β chain was present in low abundance. In the present study while multiple clones were sequenced a poor yield of a second PCR product could still go undetected.

TCR V3 segments are used by three of the five non-transgenic clones (3B, 3E and 19). Clones 3B and 3E use TCRA V3 S7 and TCRA J41 and have identical junctional sequences, while clone 19 uses TCRA V3 S5 in conjunction with the TCRA J7 sequence. Interestingly the H2-L^d alloreactive T cell clone 2C which recognises L^d in conjunction with the octameric α-ketoglutarate peptide (LSPFPFDL) utilises a TCRA V3 segment (TCRA V3 S1). This clone also uses a TCRB V8 segment (TCRB V8 S2) as do clones 3E and 19. TCRs from clones 3E and 19 may therefore have a similar structure to the 2C TCR.

Transgenic clone 4G and non-transgenic clone 16 both utilise TCRA V4 S4. A previous study of this type has shown a high affinity P91A specific T cell clone to express TCRA V4 S4 in combination with TCRB V8 S2 (Solheim et al, 1993a). In the present study, high affinity clone 16 utilises TCRB V8 S3 and low affinity clone 4G uses TCRB V1 S1. It is therefore possible that TCRV4 S4 is favoured for recognition of the P91A/L^d complex but that this potential for recognition is only revealed when it is expressed in combination with a TCRB V8 segment. In support of this idea, the usage of T cells expressing TCRB V8 segments is known to be favoured in L^d restricted responses.

No specific TCRA J segment is used by non-transgenic clones and although three such clones utilise TCRA J41 this junction region is also found in transgenic clone 4A. The P91A tum^- specific clone P91.6 uses a novel TCRA V segment which has been designated TCRA V18 $S^{P91.6}$. Two previous members of the TCRA V18 family have been cloned, TCRA V18 S1 and S2 (Pircher et al,1987 and Couez et al, 1991). It is at present unclear whether TCRA V18 $S^{P91.6}$ is an allele of a previously cloned TCRA V18 segment. However attempts to PCR clone the complete TCRA V18 $S^{P91.6}$ segment using primers based on the published sequences have not been successful.

It is difficult to establish any definitive traits in V(D)J usage for transgenic and non-transgenic clones. Taken as a group and ignoring the transgenic non-transgenic distinction TCRA V and TCRB V usage is consistent with other studies examining L^d restricted allospecific and peptide specific responses (Solheim et al, 1993a, Louie at al, 1996). In the present study clone 4A utilises a member of a TCRA V sub-family and a TCRB V sub-family which have previously been shown to be favoured for L^d allo and peptide specific responses (Solheim et al, 1993a, Louie at al, 1996). Since clone 4A is a low affinity clone for the P91A-L^d complex this might suggest that CDR3 interactions are primarily responsible for this effect. This idea is explored further in Chapter 4.2.8.

Clone 4G uses a preferred TCRA V segment (TCRA V4 S4); utilised by a previously isolated P91A specific clone and an L^d alloreactive clone (Solheim et al, 1993a, Louie et al, 1996). In the case of the P91A specific clone (7H5, Solheim et al, 1993a) a TCRB V8 S2 chain was paired with the TCRA V4 S4 chain. This resulted in a high affinity P91A reactive clone. Clone 4G uses a TCRB V1 segment in combination with TCRA V4 S4 and is a low affinity clone. It is tempting to speculate that poor reactivity of this clone might stem from the usage of a TCRB segment which can interact only poorly with the L^d molecule. An examination of the CDR2 of TCRB V1 reveals a sequence which is grossly different from that of TCRB V8 segments and might suggest a reason for a poor interaction with L^d. Such an interpretation however may not hold up under further scrutiny in that TCRB V10 S1 (expressed in high affinity clone 3B) has a very similar CDR2 sequence to that of TCRB V1 (low affinity clone 4G).

As a group, transgenic and non-transgenic clones use TCRA V segments which have been shown to be used by other L^d specific clones (TCRA V8, TCRA V4 and TCRA V3), (Solheim et al, 1993a, Louie et al, 1996, Saito et al, 1984a and b). The finding of similar α chain usage in P91A specific clones is not so surprising. However, the

finding that this conservation of usage extends to multiple alloreactive clones and the alloreactive T cell clone 2C suggests that specific α chains may be important in determining reactivity to L^d. If anything, the α chain usage of P91.6 represents the exception to the rule in that an α chain (TCRA V $S18^{P91.6}$) that has not previously been associated with recognition of L^d is used. This clone was raised against the P91A tum^- antigen following tumour rejection and recognises the low level of P91A epitope expressed on the P91 tum^- cell line. It is possible that the high level of peptide which was used to generate P91A specific T cell clones *in vitro* has a detrimental effect on the isolation of this type of clone.

Similar to other studies, transgenic and non-transgenic clones appear to preferentially use TCRB V8 segments. In the single case where a P91A specific non-transgenic clone utilises an alternative TCRB V segment (clone 3B) a segment is used (TCRB V10 S1) which has been shown to be preferred in allorecognition of L^d (Louie et al, 1996). In the latter study, four out of eight clones utilised a TCRB V10 segment while only one used a TCRB V8 segment. Interestingly the clone which utilised TCRB V8 S3 was less cross reactive than other clones which had been isolated; interacting strongly with only L^d (Killion et al, 1995). Taken together the data presented here are in accordance with previously published data showing limited diversity of both α and β chain usage in L^d peptide and alloreactive T cell clones.

3.2.1.3 Analysis of the complementarity determining regions of T cell receptors from P91A transgenic and non-transgenic T cell clones

The predicted amino acid sequences of the complementarity determining regions (CDRs) of P91A transgenic and non-transgenic T cell receptor α and β chains is shown in figure 3.6 A and B. The complementarity determining regions of the TCR α and β chains of the T cell clone 2C are included for comparison. 2C utilises TCRB V8 S2 and TCRA V3 S1 and recognises the H2-K^b/dEV8 peptide complex

(syngeneic) and the H2-L^d/p2Ca complex (allogeneic). The three dimensional structure of the 2C TCR has recently been solved at 2.5 angstroms and crystallisation of a complex of 2C and H2- K^b/dEV8 has allowed the orientation of the TCR on the MHC molecule to be visualised for the first time (Garcia et al, 1996). 2C recognises the H2- L^d/p2Ca complex with a relatively high affinity ($K_d \sim 0.5 \times 10^{-6}M$) compared to H2- K^b/dEV8 ($K_d \sim 0.5 \times 10^{-5}M$) and utilises a TCR α and β chain which are known to be favoured for L^d recognition (Solheim et al, 1993a, Louie et al, 1996). A comparison between the CDRs of P91A transgenic and non-transgenic TCRs with the crystal structures of known TCR α and β chains may help to identify common features which are important in the recognition of L^d.

As shown figure 3.6B, CDR1β length is identical in transgenic and non-transgenic clones and is also the same as that found in the β chain of the 2C TCR. Furthermore, Gln^{25} and His^{29} are common residues in all clones. The presence of Gln^{25} is a feature of many but not all TCRB V regions. In cases where Gln^{25} is absent proline is often found in its place. Interestingly, an examination of alloreactive (Louie et al, 1996), P91A specific (Solheim et al, 1993a) and P91A transgenic and non-transgenic CDR1β regions reveals conserved usage of Gln^{25} across the multiple TCRB V regions used (TCRB V1, TCRB V7, TCRB V6, TCRB V10, TCRB V8 S1-3). The crystal structures of TCRB V8 S2 when crystallised in isolation (Bentley et al, 1995) or as part of the 2C TCR (Garcia et al, 1996) indicate that the amide group of Gln^{25} stabilises the CDR1 loop by forming four hydrogen bonds to the main chain atoms of Asn^{27} and His^{29}. The sequences of many TCRB V segments are similar to that of the isolated clones (Arden et al, 1995b) suggesting that hydrogen bonding between residues 25 and 29 may maintain a relatively conserved CDR1 conformation for the majority of TCR β chains. In the very few cases where residue 25 is hydrophobic in nature (TCRBV15 S1 and S2 and TCRB V18 S1), then residue 29 is also hydrophobic. This suggests that hydrophobic interactions may operate to maintain a similar CDR1 conformation in the absence of the more usual hydrogen bonding

strategy. Transgenic clone 4G has a hydrophobic amino acid (Leu) at position 27 and is therefore incapable of hydrogen bonding to Gln^{25}. This may affect the overall conformation of the CDR1 loop and result in poorer recognition of the H2-L^d molecule. This possibility however seems unlikely since high affinity clone 3B also has leucine at this position.

The length of CDR2β is constant for all clones isolated and is also identical to that of the β chain of 2C. The CDR2 loop of clone 2C (TCRB V8 S2) has an extended hairpin conformation which is stabilised by hydrogen bonding between the side chain of Ser^{49} and the main chain atoms of the loop (Garcia et al, 1996). The loop is further stabilised by two hydrogen bonds which extend from the amine group of Arg^{69} to Ser^{49} and the main chain at residue 53. All clones with the exception of 4G and 3B utilise TCRB V8 segments and will therefore adopt the conformation of the CDR2 loop of Vβ8.2. The CDR2 loop of clone 3B may have a slightly different conformation since it possesses neither Ser^{49} or Arg^{69} but has Tyr^{49} and Ser^{69} at these positions. Having said this, it is likely that the hydroxyl group of Tyr^{49} will be capable of forming hydrogen bonds to main chain atoms within the CDR2 loop and possible that Ser^{69} will form hydrogen bonds to Tyr^{49} in a similar way to that in which Arg^{69} hydrogen bonds to Ser^{49}. Transgenic clone 4G also has a tyrosine at position 49 but in this case has a cysteine at position 69. It is again possible that the overall conformation of the CDR2 loop will be similar to that of Vβ8.2 since internal hydrogen bonding to main-chain atoms may maintain loop structure. However, the ability of cysteine at position 69 to form hydrogen bonds to the loop cannot be predicted and will depend upon the proximity of the two residues within the tertiary structure. With this in mind it is possible that the very long side arm of Arg^{69} which is present in all TCRB V8 segments facilitates hydrogen bonding to residues within the CDR2β loop.

The length of CDR1α varies from eight to eleven amino acids in transgenic and non-transgenic clones and is nine amino acids in the majority of cases. This variation in length covers the spectrum of possible lengths for CDR1α sequences (Arden et al, 1995b). Loop length does not correlate with affinity since both longer (eleven residues) and shorter (nine residues) loop lengths are represented in high and low affinity P91A specific T cell clones. Furthermore, the T cell clone P91.6 which utilises TCRB V18 $S^{P91.6}$ has the shortest CDR1α sequence (eight amino acids). Although CDR1α length may vary, the overall conformation of the loop is likely to be maintained by a similar set of interactions. In contrast to CDR1β loop conformation the structure of the CDR1α loop is maintained by predominantly hydrophobic interactions between Tyr^{24} and Leu^{32}. These residues are conserved in all transgenic and non-transgenic α chains and are generally conserved in both mouse and human α chains (Fields et al, 1995). Loop conformation is further stabilised by hydrophobic interactions between Leu^{32} and a hydrophobic residue at position 66 (Leu, Phe or the phenol ring of Tyr). The presence of a residue which can have hydrophobic properties at position 30 also contributes to the stabilisation of the CDR1α loop (Fields et al, 1995). This again is a constant feature of the transgenic and non-transgenic α chains which have been cloned. It is therefore predicted that the conformation of the CDR1α loop of transgenic and non-transgenic clones will be essentially similar and furthermore, that most TCR α CDR1 regions will conform to this structure.

In the present set of clones CDR2α displays only limited variation in length being either eight or nine amino acids. This length range is similar to that of many TCRA V segments. Having said this, CDR2α may vary in length by up to five amino acids (Arden et al, 1995b) and is more variable in primary amino acid sequence than CDR1α. It is therefore not surprising that the crystal structures of the Vα 4.2 homodimer and the Vα 3.1 chain of the 2C TCR reveal different conformations for the CDR2α loop (Garcia et al, 1996, Fields et al, 1995). The CDR2α loop of clone 2C

is stabilised by a hydrogen bond from Tyr^{49} to the main-chain carbonyl of residue 52. Hydrophobic interactions between the phenol ring of Tyr^{49} and Phe^{66} and Phe^{73} help to further stabilise the loop. This pattern of hydrogen bonding and hydrophobic interactions generates a conformation in which the top of the CDR2 loop bulges away from the centre of the antigen combining site. This conformation of the CDR2α loop may exist for clones 4A, 3B, 3E and 19. Clones 4G, 16 and P91.6 may have a different CDR2α conformation since all have a hydrophobic residue at position 49 (Val or Iso) which cannot form a hydrogen bond to the main-chain carbonyl of residue 52. In the case of these TCR α chains alternative hydrogen bonding is likely to maintain loop conformation. This has been demonstrated in the case of Vα 4.2 (Fields et al, 1995). Although the CDR2α of clones 4G, 16 and P91.6 may differ from 4A, 3B, 3E and 19 the presence of a hydrophobic residue at position 49 (Val or Iso) should maintain hydrophobic interactions to the phenol ring of Tyr^{66} and Phe^{73} (4G and 16) or Leu^{66} in the case of P91.6. These interactions may help to maintain the general stability of the CDR2α loop in the presence of individual TCRA V specific differences. Although clones 4G and 16 and the 1934.4 TCR (Fields et al, 1995) utilise TCRA V4 segments there would appear to be significant sequence variation in the CDR2 region between sub-members of this family. Clones 4G and 16 both use TCRA V4 S4 and have hydrophobic amino acids at positions 49, 50 and 51. In this respect they differ from Vα 4.2 (1934.4 TCR) which has hydrophilic/charged residues at positions 50 and 51. How residues 49, 50 and 51 might interact to generate the fine structure of the CDR2α loop of clones 4G and 16 is a matter of speculation.

For clone P91.6, hydrogen bonding between Arg^{50}, Ser^{51} and Arg^{54} may help to maintain the conformation of the TCRA V18 $S^{P91.6}$ CDR2 loop. This pattern of hydrogen bonding is very similar to that found in the CDR2α region of the Vα 4.2 homodimer (Fields et al, 1995). Unlike the CDR2α of the 2C α chain which orientates away from the antigen binding cleft the CDR2α of Vα 4.2 is directed towards the peptide binding groove where it could possibly interact with the N-

terminal half of MHC bound peptide. It is therefore possible that P91.6 utilises residues within CDR2α to interact with the P91A peptide. The predicted orientation of CDR2α in other clones (4A, 3B, 3E and 19) would seem to be incompatible with such a method of MHC-peptide recognition.

The structure of the CDR3 loop of both transgenic and non-transgenic α and β chains is difficult to predict and cannot easily be inferred from pre-existing crystal structures. This is a result of the greater sequence variability of the CDR3 loop and reflects the dominant function of this region in recognising MHC bound peptide. Further interpretation of CDR3 sequence and structure will follow in section 4.2.8.

3.2.2 The making of a low affinity P91A specific T cell receptor transgenic mouse

Experiments involving the addition of defined peptides to *in vitro* cultured foetal thymii from TCR transgenic TAP deficient or β2m deficient mice do not examine positive and negative selection as it occurs on endogenous MHC-peptide complexes (Chapter 1.8 and 6.3). The P91A transgenic mouse provides a system where this could possibly be examined. P91A transgenic and non-transgenic mice differ in the expression of a single H2-L^d binding peptide. It is possible that this difference results in a subtle alteration in the spectrum of T cells which are positively selected. This in turn might account for the presence of a distinct population of T cells in P91A transgenic mice which are selected on the basis of an interaction with the P91A epitope. Furthermore, such T cells may be reactive in the periphery for peptides which are related in primary sequence to the P91A epitope. In an attempt to examine whether T cells of this type exist in P91A transgenic mice TCR and MHC contact residues of the P91A peptide were defined, (Chapter 4.2.6). This information was to be used to construct a panel of altered peptide ligands which were to be used to generate T cell lines from transgenic and non-transgenic mice in the hope of

establishing whether the P91A epitope was capable of positively selecting T cells which were unique to the P91A transgenic mouse. Unfortunately, the difficulties involved in generating peptide specific lines and clones both *in vivo* and *in vitro* made this approach unfeasible.

Having said this, P91A specific T cell clones have been derived *in vitro* from transgenic and non-transgenic mice and been shown to differ in their sensitivity to peptide (Chapter 2.2.1.5). In order to investigate the selection of low affinity P91A specific T cells more thoroughly an attempt was made to generate a TCR transgenic mouse bearing a T cell receptor from a low affinity T cell clone. It was thought possible that low affinity clones could have been positively selected on the basis of an interaction with the transgene encoded P91A-L^d complex. If this were the case then selection of such cells could be investigated by crossing low affinity P91A specific TCR transgenic mice with P91A transgenic mice. The selection of T cells bearing a low affinity P91A specific TCR could be investigated further in FTOC using a panel of altered peptide ligands based on the P91A peptide. Such experiments would give an indication of the peptide requirements for positive and negative selection of low affinity P91A reactive thymocytes.

3.2.2.1 Obtaining full length TCR α and β transcripts from a low affinity P91A specific T cell clone

In order to follow the fate of thymocytes in TCR transgenic mice good quality antibodies are required which are capable of recognising both the α and the β chain of the T cell receptor. Low affinity clone 4A uses the TCRA V8 S13 segment and TCRB V8 S3. Expression of both of these chains can be followed with pre-existing well characterised antibodies (F23.1 recognises Vβ 8.3 and B21.14 recognises Vα 8). Initial attempts to PCR clone the TCR chains of clone 4A were unsuccessful with PCR products having one or more PCR errors. In order to circumvent the problem of

PCR artifact a cDNA library was constructed from clone 4A and screened for the presence of TCR α and β chains. α and β chain PCR products which had been previously shown to have occasional PCR errors were TA cloned and following insert excision and purification used to screen the library.

The cDNA library was plated out at 2.5 X 10^5 colonies/large plate. Four plates were screened in duplicate using a mixture of α and β PCR products to probe. Multiple positive colonies were present on all four filters and on the filter duplicates. A representative primary screen filter is shown in figure 3.7 A. Fifteen positive colonies were picked from this filter and rescreened individually with either the β or α probe, figure 3.7 B and C. This secondary screening identified five α and six β colonies. Primary and secondary screening of other filters identified a further five α and three β colonies. Colonies were picked, expanded in L broth containing ampicillin and plasmid DNA extracted. Colony insert lengths were assessed after digestion of miniprep DNA with EcoR1, figure 3.8 A and B. The digestion of miniprep DNA from β positive colonies has not proceeded to completion. Furthermore, digestion of plasmid DNA from β positive colonies with EcoR1 appears to have generated more than one insert band of lower molecular weight indicating the presence of multiple internal EcoR1 sites within certain β transcripts.

The 4A T cell clone produces two α transcripts, TCRA V2 S5 and TCRA V8 S13. Although the Vα8 probe (TCRA V8 S13 PCR product) was used to screen the cDNA library it is still likely that colonies containing Vα2 will be detected since the Vα8 probe includes the α constant domain. Miniprep DNA from α containing colonies was subjected to PCR analysis using either a Vα2 or Vα8 specific 5' primer and colonies 2, 3, 4, 5, 6, 9 and 10 shown to contain Vα8 transcripts, figure 3.8 C and D. Vα2 transcripts were not detected in any colony examined. This and further analysis of the library confirmed that the out of frame Vα2 transcript was present in very low abundance. This is line with recent findings which show that T cell receptor mini-

gene mRNA which contains stop codons is down regulated in the nucleus (Li et al, 1997). The mechanism of down regulation is unknown but seems to involve some form of sequence proof reading.

Four colonies, designated on the basis of their miniprep numbers as, α9 and α10 and β4 and β6 were sequenced to establish the exact nature of their 5' and 3' ends, figure 3.9 A, B and C. Inserts β4 and β6 are full length TCRB V8 S3 transcripts. The β4 transcript has only two bases of 5' untranslated sequence compared to β6 which has 12 bases 5' of the start codon. Intron one has not been spliced out of either the β4 or β6 transcript and contains an EcoR1 restriction site. Both β4 and β6 transcripts have a complete 3' untranslated region and poly (A) tail. α9 and α10 are transcripts of TCRA V8 S13. The α10 transcript has a long 5' untranslated sequence and has successfully spliced out intron one, figure 3.9 B. This is in contrast to the α9 insert which derives from a partial cDNA terminating within intron one, figure 3.9 B. The 3' end of α9 has a complete 3' untranslated region terminating in a poly (A) tail. This is in contrast to the 3' end of α10 which appears to have lost its poly (A) tail and terminates 11 bases upstream of the poly (A) tail of the α9 transcript. One must assume that the α10 transcript possessed a poly (A) tail at one point since the library was constructed from oligo-dT primed cDNA.

On the basis of this sequence analysis the full length TCRA V8 S13 and TCRB V8 S3 transcripts α10 and β6, were selected for sub-cloning into the transgene expression vector VA-hCD2 (Zhumabekov et al, 1995).

3.2.2.2 Sub-cloning TCR α and β transcripts into the transgene expression vector VA-hCD2

A locus control region (LCR) has been identified within the 3' flanking region of the human CD2 (hCD2) gene (Greaves et al, 1989). This LCR contains a strong T cell

specific enhancer and additional regulatory elements which are responsible for maintaining an active chromatin structure (Lake et al, 1990). A human CD2 minigene which contains the natural hCD2 gene with all but the first intron deleted, together with 5 kb of 5' promoter sequence and 5.5 kb of the 3' LCR can direct T cell specific expression of integrated TCR α and β cDNA, murine CD8α cDNA and other genes in transgenic mice (Mamalaki et al, 1993, Zal et al, 1994, Robey et al, 1991). Transgene expression is independent of integration site specific effects and is transgene copy number dependent (Greaves et al, 1989). Herein lies part of the problem with the initial hCD2 minigene cassette. In order to achieve detectable levels of expression more than ten copies of the transgene were frequently required. Furthermore, to achieve expression levels equivalent to endogenous TCR gene expression it was often necessary to generate mice carrying 50-100 copies of the transgene (Zhumabekov et al, 1995).

The vector used in the present work is derived from the initial hCD2 cassette. It includes 5 kb of promoter sequence and 5.5 kb of the 3' LCR. In the new cassette transgene cDNA is inserted into the second exon of the hCD2 minigene, leaving the promoter and the first exon intact. The start codon and other potential start codons located upstream of the reporter gene (transgene) have been mutated and an artificial poly-linker containing EcoR1, SnaB1 and Sma1 restriction sites has been engineered into the vector at the end of the first intron (Zhumabekov et al, 1995). All other hCD2 coding sequence has been removed and a 500bp fragment from the 3' untranslated region retained which contains two hCD2 poly-adenylation signals (Lang et al, 1988). The hCD2 minigene resides in the Bluescript SK($^-$) plasmid and the entire VA-hCD2 vector is 14.2 kb in size, figure 3.10.

Several TCR transgenic lines have been generated using this vector (Moskophidis and Kioussis, unpublished and Zhumabekov and Kioussis, unpublished) and transgenic mice carrying as few as 2-4 copies of the transgene (α and β chains) been shown to

express transgenic TCR on the surface of peripheral T cells at levels similar to or higher than that of endogenous TCRs. This contrasts with the situation using the old hCD2 cassette in which no expression was detected in any of six transgenic lines carrying 5-15 copies of TCR α and β TCR cDNA (Moskophidis and Kioussis, unpublished and Zhumabekov and Kioussis, unpublished). The VA-hCD2 vector therefore represents a highly efficient mechanism for expressing transgenes in an integration site independent, copy number dependent T cell specific manner.

The VA-hCD2 expression vector has three neighbouring restriction sites into which insert DNA can be sub-cloned; EcoR1, SnaB1 and Sma1, figure 3.10. Digestion of the vector with EcoR1 produces cohesive ends while digestion with SnaB1 or Sma1 both produce blunt ended linearised vector. The cDNA library from clone 4A was initially constructed such that each cDNA molecule was flanked with BstX-1/EcoR1 adapters. This enabled cDNA to be cloned into the library vector (pcDNAII) using the rare cutting enzyme BstX-1. pcDNAII has a multi-restriction enzyme poly-linker with the BstX-1 site towards the centre. Insert cDNA can therefore be retrieved from the library following digestion with either EcoR1, BstX-1 or any of a series of restriction enzymes which cut within the poly-linker either side of the BstX-1 site.

The TCRB V8 S3 transcript (β6) has two internal EcoR1 sites: one within intron one and a second immediately 5' of the stop codon. It was initially hoped to clone TCR α and β cDNA into VA-hCD2 using the restriction enzyme EcoR1. However, digestion with EcoR1 results in the production of multiple fragments, figures 3.8 B and 3.13 C. EcoR1 is the only enzyme which generates cohesive ends and is suitable for use in sub-cloning into the VA-hCD2 vector. Since ligations involving cohesive ends are theoretically easier to achieve than those involving blunt ends a partial digest strategy was adopted to try and obtain a full length TCRB V8 S3 transcript. Unfortunately generation of the full length β6 transcript was very poor (even when the restriction

enzyme concentration was limiting) so much so that it was thought unlikely to be able to recover a sufficient amount of full length insert for successful sub-cloning.
As an alternative approach neighbouring restriction sites Xba1 and BamH1 (3' and 5' of the BstX-1 site) within the pcDNAII poly-linker were used to excise the β6 transcript. Both of these enzymes produce 5' overhangs which were subsequently blunt ended using the Klenow fragment of DNA polymerase I. The α10 transcript was excised from the library vector pcDNAII using EcoR1. The α10 and β6 transcripts were gel purified, sub-cloned into VA-hCD2 which had been linearised with either EcoR1 or Sma1 respectively and transformed into *Epicurian coli* (Stratagene).

Twenty antibiotic resistant VA-hCD2-α10 and twenty-eight VA-hCD2-β6 colonies were picked and plasmid DNA prepared. An EcoR1 digest of VA-hCD2-α10 and VA-hCD2-β6 DNA is shown in figure 3.11 A and B. Miniprep digest VA-hCD2-α10 number 11 displays the correct digest pattern of large vector band (14.2 kb) and correct size insert band. The majority of the other VA-hCD2-α10 miniprep digests seem to indicate that the VA-hCD2 vector has rearranged itself within the host bacteria. Miniprep digests VA-hCD2-β6 numbers 2, 3, 4, 19 and 20 all appear to have correct sized insert bands. The banding pattern of all other VA-hCD2-β6 miniprep digests once again suggests that rearrangement of the vector has occurred within the host bacteria (in the case of β6 ligation four attempts at ligation and transformation of bacteria were required and over 160 colonies screened before the correct digest pattern was observed). Since this work was completed other people in this laboratory have experienced similar problems with the VA-hCD2 vector.

Since the sub-cloning of β6 and α10 cDNA into the VA-hCD2 vector is non-directional potential positive colonies must have the orientation of the ligated insert checked. This was achieved by using a discriminatory PCR method, figure 3.12. As shown in figure 3.13 A the TCRA V8 S13 transcript (α10) has been ligated into the

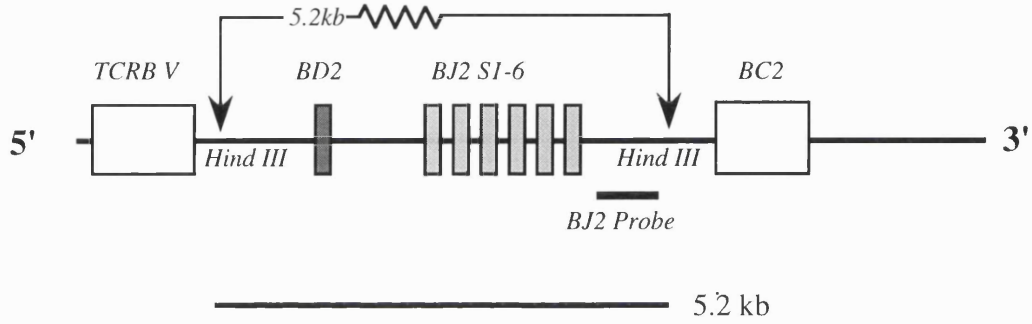
VA-hCD2 vector in the correct orientation in VA-hCD2-α10 colony 11(VA-hCD2-$\alpha 10^{11}$). Furthermore, the TCRB V8 S3 transcript (β6) has been ligated into the VA-hCD2 vector in the correct orientation in VA-hCD2 β6 colonies 2 and 20 and is present in VA-hCD2 β6 colonies 3, 4 and 19 in the wrong orientation, figure 3.13 B (I and II).

Colonies VA-hCD2-$\alpha 10^{11}$ and VA-hCD2-$\beta 6^{20}$ were selected and plasmid DNA prepared. A check EcoR1 digest was performed and revealed the correct banding patterns for both α and β transcripts, figure 3.13 C. Finally, the Bluescript SK(-) vector was excised and the α and β transgenes gel purified. Injection of (CBA x B6) F2 eggs was performed by Dr. Colin Hetherington at the Biomedical Services Transgene Unit, John Radcliffe Hospital, Oxford. To date a single founder transgenic for both the α and β chain and a single founder transgenic for only the α chain have been obtained. Unfortunately, FACS analysis of thymocytes derived from transgene expressing offspring using TCR α and β chain specific monoclonal antibodies reveals no surface expression of either chain. Further injections are ongoing.

Figure 3.1: Examination of genomic rearrangement within the TCRB locus using a Southern blot technique: pictorial representation. (A) following digestion of genomic DNA with the restriction enzyme Hind III and detection with the TCRBJ2 probe, (Palacios and Samaridis, 1991) a 5.2kb band is obtained from an unrearranged TCRB locus. (B) Rearrangement within the TCRB locus results in the apposition of specific V, D and J segments. This recombination results in novel digestion products the size of which will vary depending on the TCRB V segment which has undergone recombination.

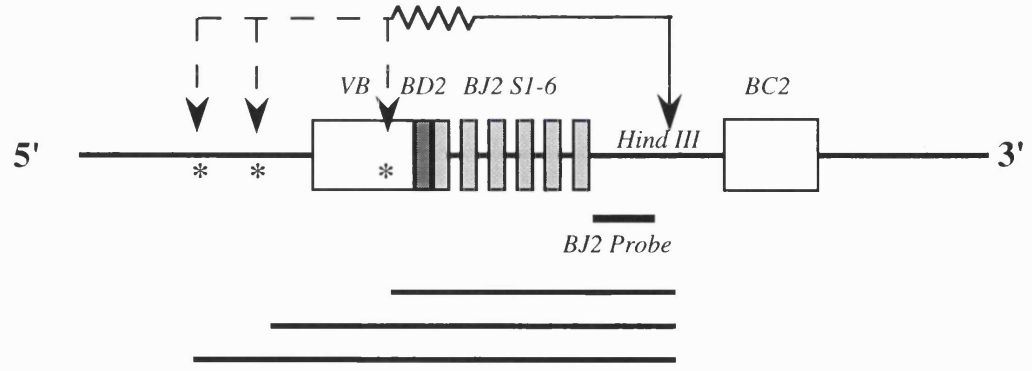
Figure 3.1

(A) Genomic rearrangement of the TCRB locus prior to VDJ recombination



Digestion with Hind III and Southern analysis using the BJ2 specific probe produces a 5.2kb band

(B) Genomic rearrangement of the TCRB locus post VDJ recombination



Digestion with Hind III and Southern analysis using the BJ2 specific probe produces a band of novel size

* Potential Hind III Sites

Figure 3.2: Genomic recombination within the TCRB locus of high and low affinity T cell clones. (A) Multiple sister clones are present in all panels of T cell clones; Southern analysis of T cell clones from transgenic line 4 (lanes 1, 2, 3, 4 and 5)and non-transgenic line 3 (lanes 6, 7 and 8) illustrate this point. (B) Lane 1; digestion of genomic BALB/c X DBA/2 tail DNA with Hind III and detection with the TCRBJ2 probe produces a single 5.2kb band. Lane 2; low affinity clone 4A appears to have rearranged both TCRB loci to the D2/J2 cluster. Lane 3; low affinity transgenic clones 4B, 4C, 4D, 4E, 4F, 4G, 4J, 4O and 4P have a single rearrangment to TCRB D2/J2. Lane 4; High affinity non-transgenic clones 3A, 3B, 3C, 3D, 3H, 3T and 3(4) have rearranged both TCRB loci to D2/J2. Lane 5; high affinity non-transgenic clones 3E and 3I have rearranged both TCRB loci to D2/J2. Lane 6; high affinity non-transgenic clones 1, 4, 10 and 19 have two rearrangements to D2/J2. Lane 7; high affinity clones 7, 13, 16 and 21 have two rearrangements to D2/J2.

Figure 3.2

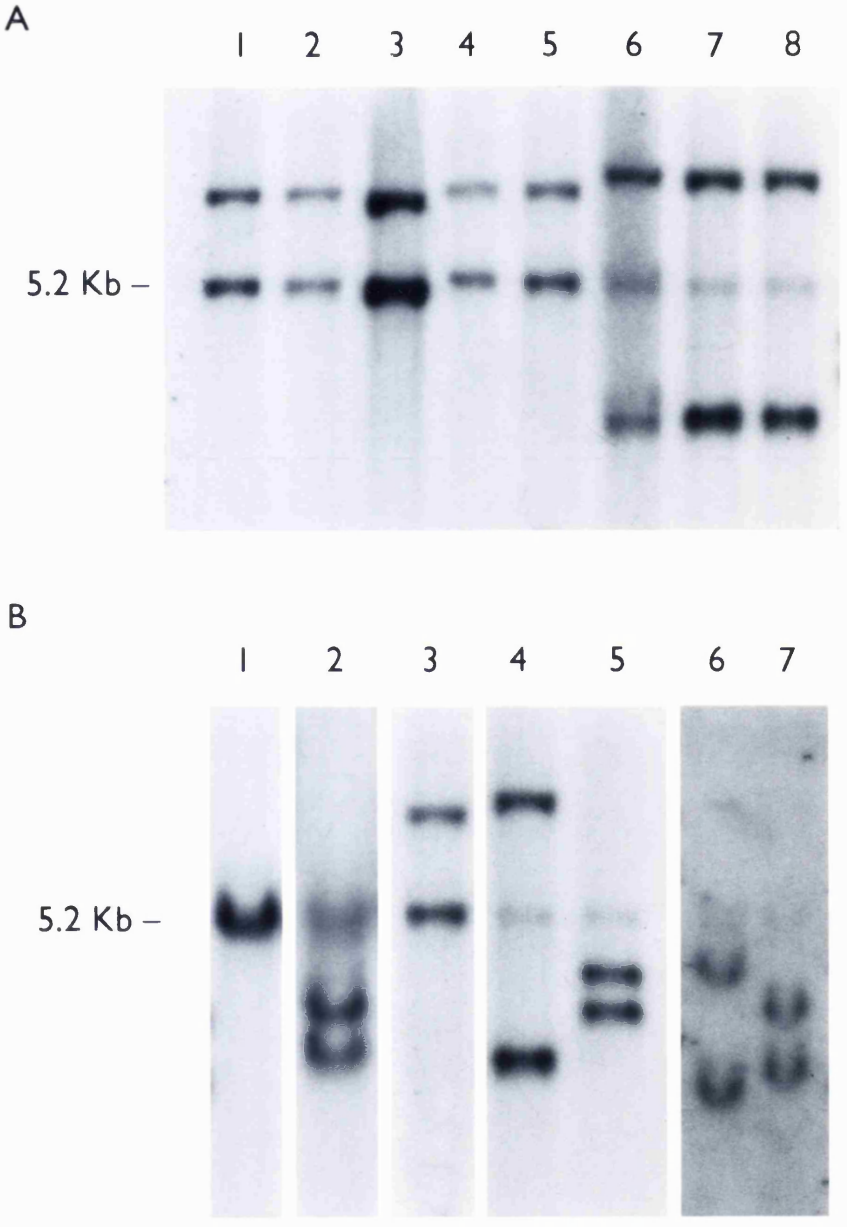


Figure 3.3: Strategies used to clone and sequence TCRA and TCRB V(D)J junctional regions in transgenic and non-transgenic T cell clones. Those clones which displayed specificity for the P91A epitope and had unique rearrangements of the TCRB locus (clones 4A, 4G, 3B, 3E, 16 and 19) were investigated further to establish V segment usage and junctional sequence. Two PCR based strategies were used. (A) The first approach utilised a panel of TCRA V specific 5' primers (see Appendix 1), (Casanova et al, 1991) and an antisense TCR constant region primer to amplify random hexamer primed cDNA from transgenic and non-transgenic clones. This method gives immediate information on V segment usage. For further information on junctional sequences PCR products were sub-cloned into the TA cloning vector (Invitrogen) and sequenced. (B) A second PCR based approach was used to clone and sequence TCRA and TCRB transcripts, (Candeias et al, 1991). cDNA was prepared from T cell clones as for method (A) and amplified by two (α chain) or three (β chain) rounds of PCR as indicated. For the β chain, the first amplification was performed with an equimolar mixture of three degenerate V region primers at the 5' end (NK121, NK122 and NK123) and a constant region 3' primer MQ284; the second amplification was performed with the same set of V region primers and the MS175 3' primer. For the α chain, the first amplification was performed with an equimolar mixture of three degenerate V region primers at the 5' end (NW36, NW37 and NW38) and a constant region 3' primer (NJ108); the second and third amplifications were with the same set of 5' degenerate primers and NJ109 or NJ110 at the 3' end respectively. All products were TA cloned and sequenced using OE6 (TCRA) or OE7 (TCRB) or T7 or Sp6 primers located within the TA cloning vector. All primer sequences are shown in Appendix 1.

Figure3.3 A

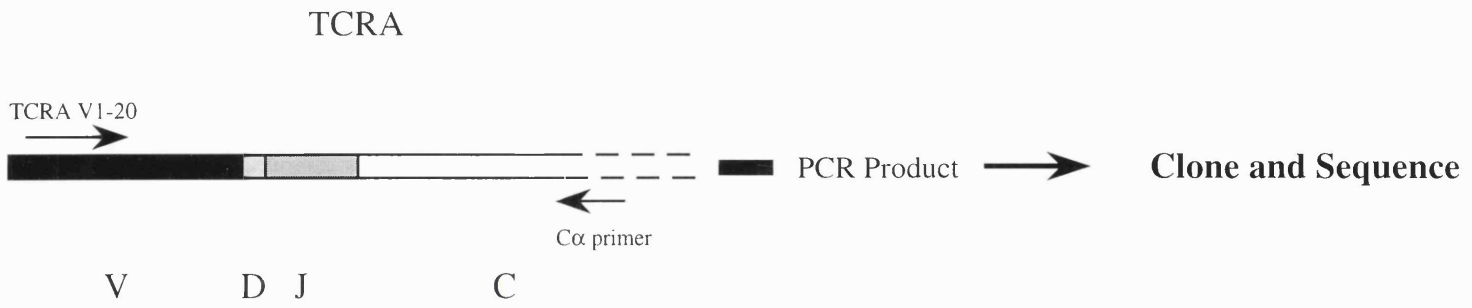


Figure 3.3 B

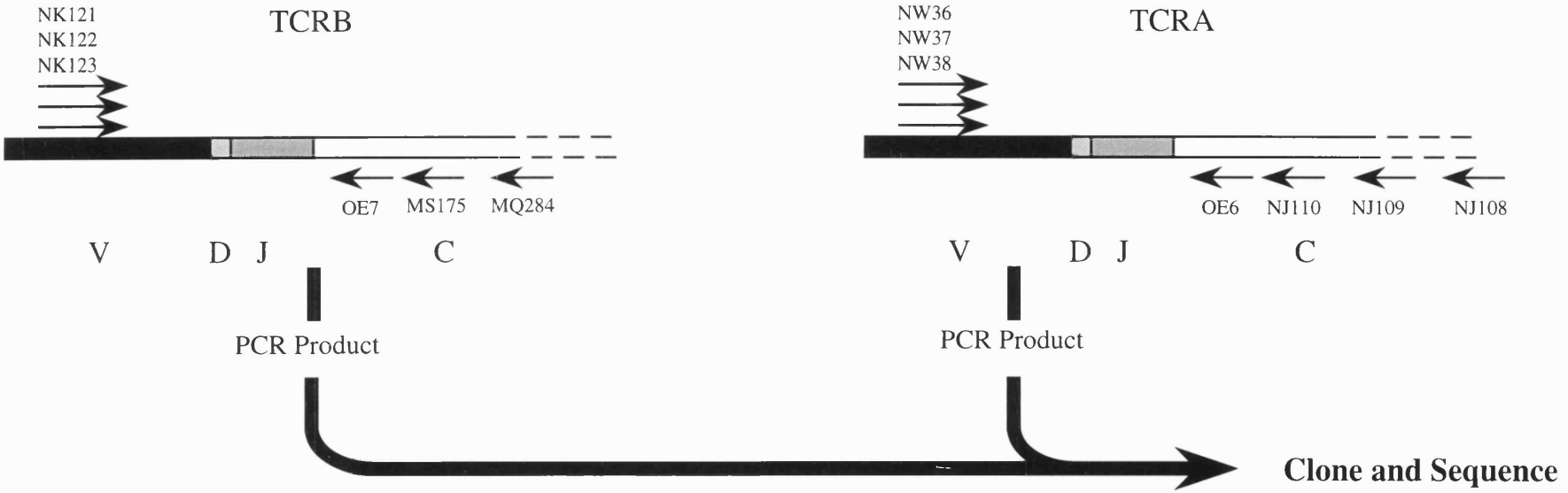


Figure: 3.4

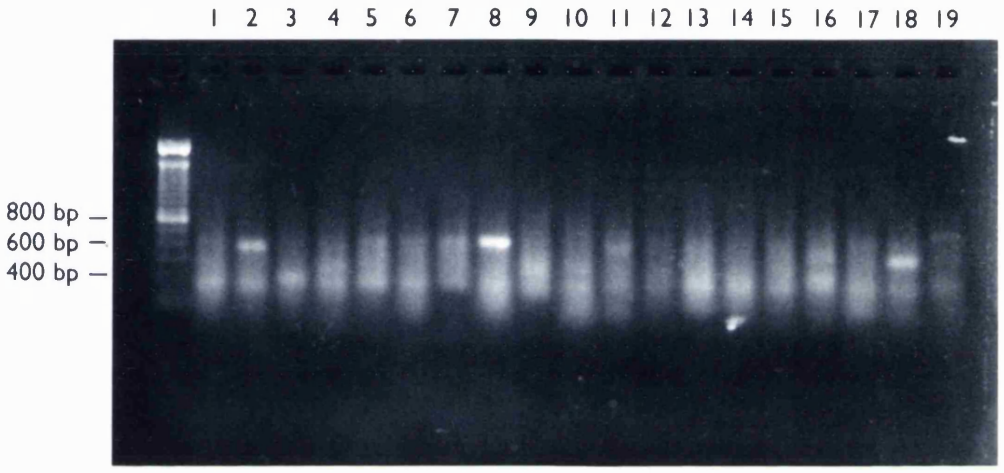


Figure 3.4: TCR analysis of transgenic clone 4A using a panel TCRA V specific primers. cDNA was prepared from clone 4A and amplified using a panel of TCRA V specific primers (Casanova et al, 1991) and a Cα 3' primer (5' GCGAATTCCAGAC CTCAACTGGACCACAG 3') which hybridises at the 3' end of the α constant region. Correct size PCR products are obtained with TCRA V2 (547bp) and TCRA V8 (597bp) specific primers. Lane 1: TCRA V1, Lane 2: TCRA V2, Lane 3: TCRA V3, Lane 4: TCRA V4, Lane 5: TCRA V5, Lane 6: TCRA V6, Lane 7: TCRA V7, Lane 8: TCRA V8, Lane 9: TCRA V9, Lane 10: TCRA V10, Lane 11: TCRA V11, Lane 12: TCRA V12, Lane 13: TCRA V13, Lane 14: Vα34S 281, Lane 15: TCRA V16, Lane 17: TCRA V18, Lane 18: TCRA V19, Lane 19: TCRA V20.

Figure 3.5: Junctional sequences of low and high affinity P91A specific T cell clones from transgenic and non-transgenic mice. (A) TCRA junctional sequences, (B) TCRB junctional sequences. Clone 4A has rearranged two α transcripts; TCRA V8 S13 and TCRA V2 S5. TCRA V2 S5 is out of frame. All other transcripts result from in frame rearrangements of the TCRA and TCRB loci.

Figure 3.5 A

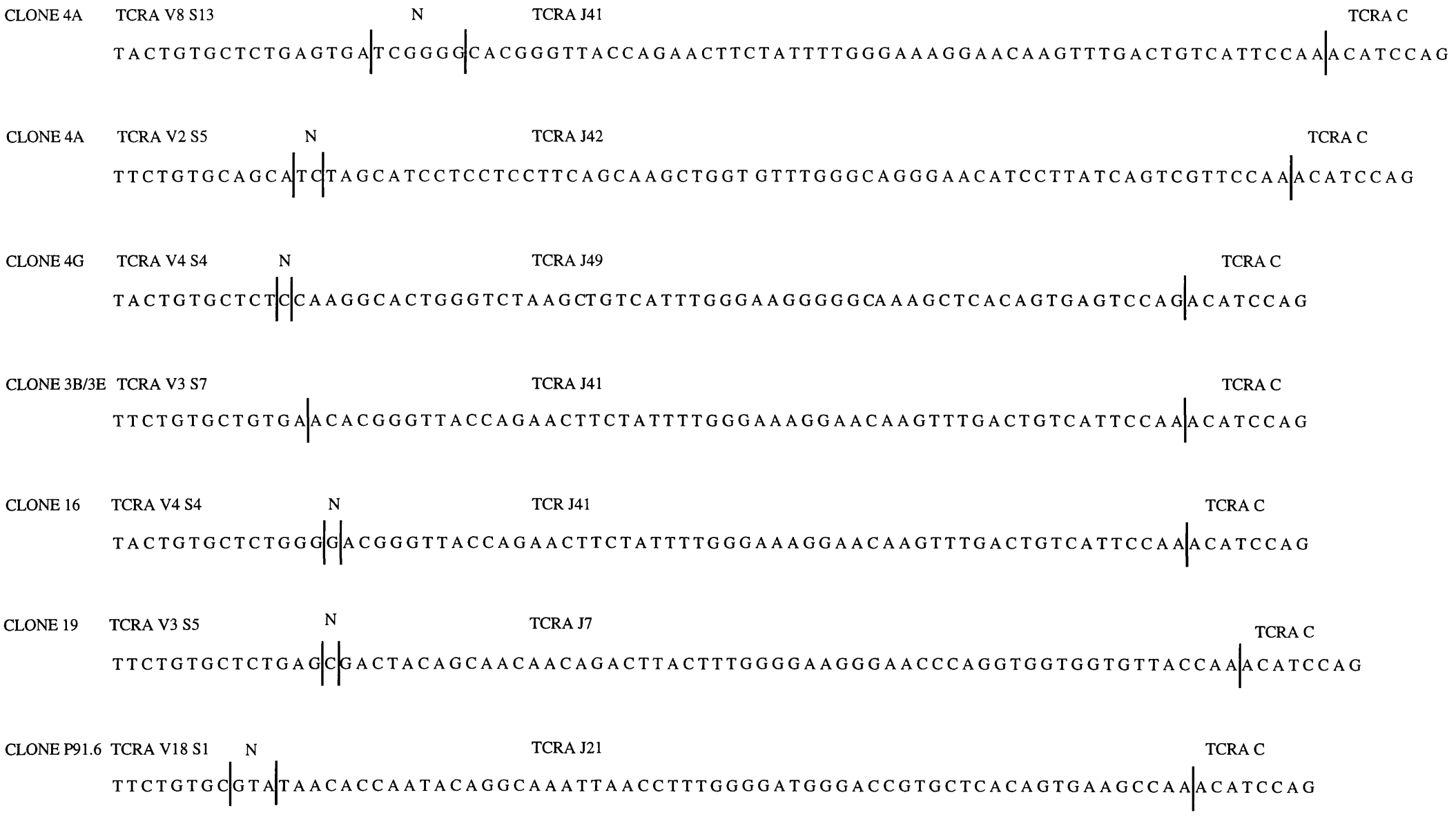

CLONE 4A
TCRA V8 S13
N
TCRA J41
TCRA C
TACTGTGCTCTGAGTGA|TCGGGG|CACGGGTTACCAGAACTTCTATTTTGGGAAAGGAACAAGTTTGACTGTCATTCCAA|ACATCCAG
CLONE 4A
TCRA V2 S5
N
TCRA J42
TCRA C
TTCTGTGCAGCA|TC|TAGCATCCTCCTCCTTCAGCAAGCTGGT GTTTGGGCAGGGAACATCCTTATCAGTCGTTCCAA|ACATCCAG
CLONE 4G
TCRA V4 S4
N
TCRA J49
TCRA C
TACTGTGCTCT|C|C|AAGGCACTGGGTCTAAGCTGTCATTTGGGAAGGGGGCAAAGCTCACAGTGAGTCCAG|ACATCCAG
CLONE 3B/3E
TCRA V3 S7
TCRA J41
TCRA C
TTCTGTGCTGTGA|ACACGGGTTACCAGAACTTCTATTTTGGGAAAGGAACAAGTTTGACTGTCATTCCAA|ACATCCAG
CLONE 16
TCRA V4 S4
N
TCR J41
TCRA C
TACTGTGCTCTGGG|G|ACGGGTTACCAGAACTTCTATTTTGGGAAAGGAACAAGTTTGACTGTCATTCCAA|ACATCCAG
CLONE 19
TCRA V3 S5
N
TCRA J7
TCRA C
TTCTGTGCTCTGAG|C|GACTACAGCAACAACAGACTTACTTTGGGGAAGGGAACCCAGGTGGTGGTGTTACCAA|ACATCCAG
CLONE P91.6
TCRA V18 S1
N
TCRA J21
TCRA C
TTCTGTGC|GTA|TAACACCAATACAGGCAAATTAACCTTTGGGGATGGGACCGTGCTCACAGTGAAGCCAA|ACATCCAG

Figure 3.5 B

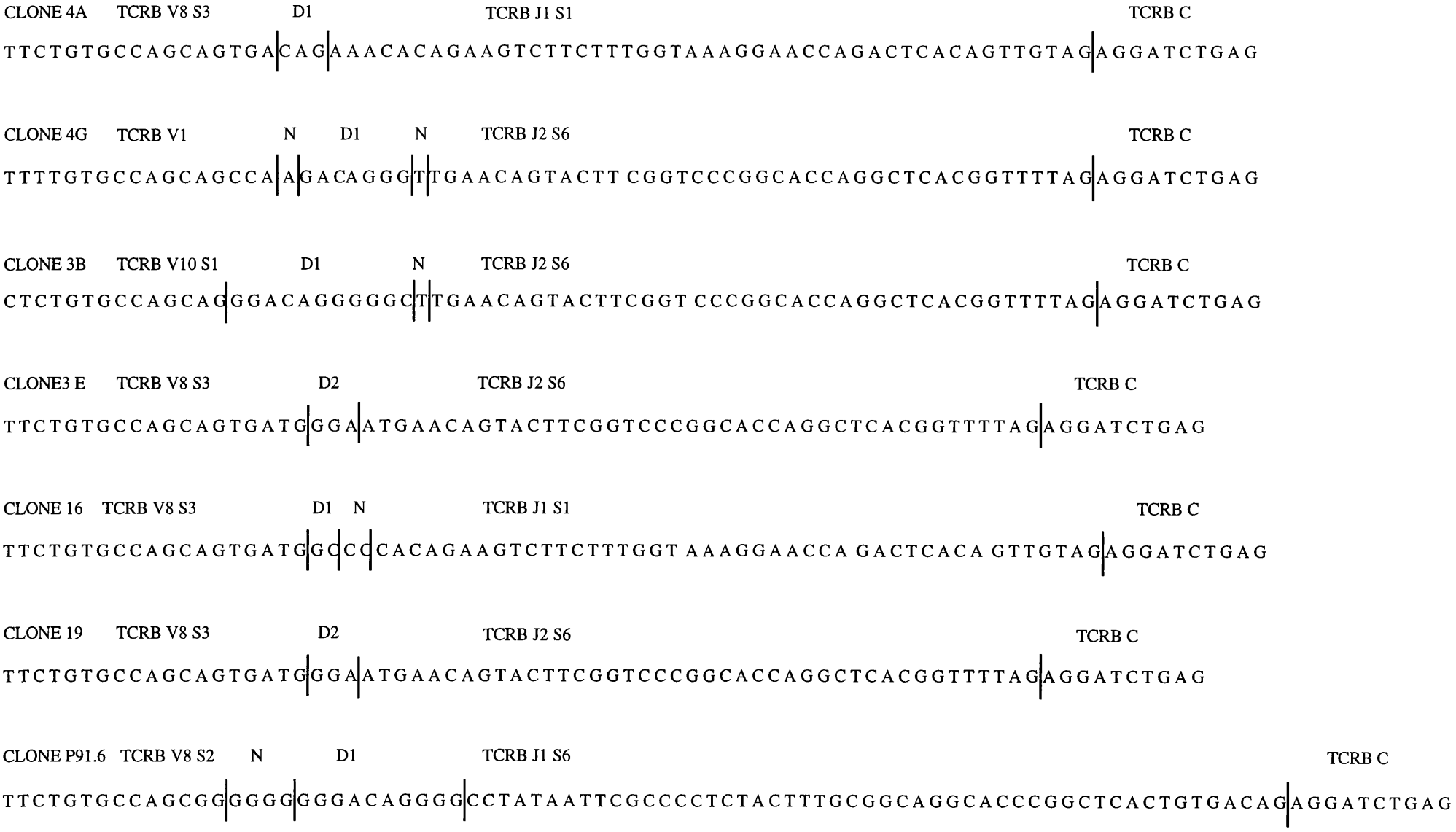

CLONE 4A TCRB V8 S3 D1 TCRB J1 S1 TCRB C
TTCTGTGCCAGCAGTGA|CAG|AAACACAGAAGTCTTCTTTGGTAAAGGAACCAGACTCACAGTTGTAG|AGGATCTGAG
CLONE 4G TCRB V1 N D1 N TCRB J2 S6 TCRB C
TTTTGTGCCAGCAGCCA|A|GACAGGG|T|TGAACAGTACTT CGGTCCCGGCACCAGGCTCACGGTTTTAG|AGGATCTGAG
CLONE 3B TCRB V10 S1 D1 N TCRB J2 S6 TCRB C
CTCTGTGCCAGCAG|GGACAGGGGGC|T|TGAACAGTACTTCGGT CCCGGCACCAGGCTCACGGTTTTAG|AGGATCTGAG
CLONE3 E TCRB V8 S3 D2 TCRB J2 S6 TCRB C
TTCTGTGCCAGCAGTGATG|GGA|ATGAACAGTACTTCGGTCCCGGCACCAGGCTCACGGTTTTAG|AGGATCTGAG
CLONE 16 TCRB V8 S3 D1 N TCRB J1 S1 TCRB C
TTCTGTGCCAGCAGTGATG|GC|C|CACAGAAGTCTTCTTTGGT AAAGGAACCA GACTCACA GTTGTAG|AGGATCTGAG
CLONE 19 TCRB V8 S3 D2 TCRB J2 S6 TCRB C
TTCTGTGCCAGCAGTGATG|GGA|ATGAACAGTACTTCGGTCCCGGCACCAGGCTCACGGTTTTAG|AGGATCTGAG
CLONE P91.6 TCRB V8 S2 N D1 TCRB J1 S6 TCRB C
TTCTGTGCCAGCGG|GGGG|GGGACAGGGG|CCTATAATTCGCCCCTCTACTTTGCGGCAGGCACCCGGCTCACTGTGACAG|AGGATCTGAG

Figure 3.6: Amino acid sequence of TCR complementarity determining regions (CDR) 1, 2 and 3 expressed in transgenic and non-transgenic clones. (A) CDR1, 2 and 3 of T cell receptor α chains, (B) CDR1, 2 and 3 of T cell receptor β chains. The margins of CDR1 and CDR2 have been set using two sets of criteria; firstly, CDR regions are defined on the basis of residues that lie outwith the TCR framework, (Chothia et al, 1988), secondly, based on the crystal structure of the 2C TCR, CDR regions are defined as those residues that are accessible to antigen, (Garcia et al, 1996). Gaps are maintained in the loops corresponding to CDR1 and CDR2 according to the method of Arden and colleagues (1995). In this approach to sequence comparison β strand framework residues were aligned using the Clustal W program and gaps introduced into the CDR1 and CDR2 loops, (Arden et al, 1995). Based on the published crystal structures of a mouse TCR α V domain, β chain and αβ heterodimer, additional residues which are important in maintaining loop conformation are included and shown in plain font, (Fields et al, 1995, Bentley et al, 1995, Garcia et al, 1996). Residues in bold are hypervariable. CDR3 regions are defined according to Moss and Bell, (1995) and have boundries based on the consensus sequence C-A-X-|- - - - - - - | F-G. This method of defining CDR3 allows TCRB and TCRA to be directly compared. The sequences of the 2C TCR α and β CDR1, 2 and 3 regions are included for comparitive purposes.

Figure 3.6 A

		CDR1	CDR2			CDR3
Clone 4A	**TCRA V8 S13**	Y Q T A Y - S - T F L	K S S T D N K - - - R	L	F	C A L S D R G T G Y Q N F Y F G
Clone 4G	**TCRA V4 S4**	Y S T T S I A Y P N L	K V I T A G Q - - - K	Y	F	C A L Q G T G S K L S F G
Clone 3B	**TCRA V3 S7**	Y S S S - - V T P Y L	K Y Y S G D P Y - - V	F	F	C A L N T G Y Q N F Y F G
Clone 3E	**TCRA V3 S7**	Y S S S - - V T P Y L	K Y Y S G D P Y - - V	F	F	C A L N T G Y Q N F Y F G
Clone 16	**TCRA V4 S4**	Y S T T S I A Y P N L	K V I T A G Q - - - K	Y	F	C A L G T G Y Q N F Y F G
Clone 19	**TCRA V3 S5**	Y S Y S - - Q T P Y L	K Y Y S G D P V - - V	F	F	C A L S D Y S N N R L T L G
Clone P91.6	**TCRA V18 S$^{P91.6}$**	Y T T - - - S I T A L	L I R S N E R - - - E	L	S	C A Y N T N T G K L T F G
Clone 2C	**TCRA V3 S1**	Y S Y - - S A T P Y L	K Y Y S G D P V - - V	F	F	C A V S G F A S A L T F G
Position		**24 —— 32**	**48 —— 56**	**66**	**73**	**90**

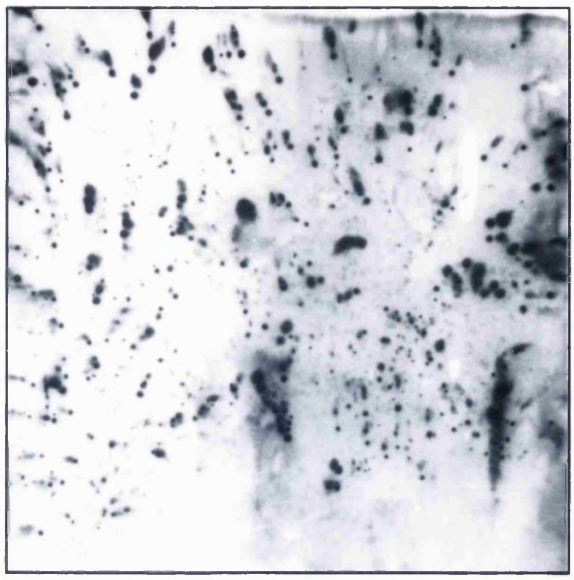
Figure 3.6 B

		CDR1	CDR2		CDR3
Clone 4A	**TCRB V8 S3**	Q T N S H N Y M Y	**Y S Y G A G N L Q I G D V**	R	C A S **S D R N T E V F** F G
Clone 4G	**TCRB V1**	Q **H L G** H N **A** M Y	**L** Y N **L K Q L I R** N E **T** V	C	C A S **S Q D R V E Q Y** F G
Clone 3B	**TCRB V10 S1**	Q **T L G** H **D T** M Y	**S** Y N **N K Q L I V** N E **T** V	S	C A S **R D R G L E Q Y** F G
Clone 3E	**TCRB V8 S3**	Q **T N S** H **N Y** M Y	**Y S Y G A G N L Q I G D V**	R	C A S **S D G N E Q Y** F G
Clone 16	**TCRB V8 S3**	Q **T N S** H **N Y** M Y	**Y S Y G A G N L Q I G D V**	R	C A S **S D G P T E V F** F G
Clone 19	**TCRB V8 S3**	Q **T N S** H **N Y** M Y	**YS Y G A G N L Q I G D V**	R	C A S **S D G N E Q Y** F G
Clone P91.6	**TCRB V8 S2**	Q **T N N** H **N N** M Y	**Y S Y G A G S T E K G D I**	R	C A S **G G G T G A Y N S P L Y** F A
Clone 2C	**TCRB V8 S2**	Q **T N N** H **N N** M Y	**Y S Y G A G S T E K G D I**	R	C A S **G G G G T L Y** F G
Position		**25 —— 33**	**48 —— 60**	**69**	**92**

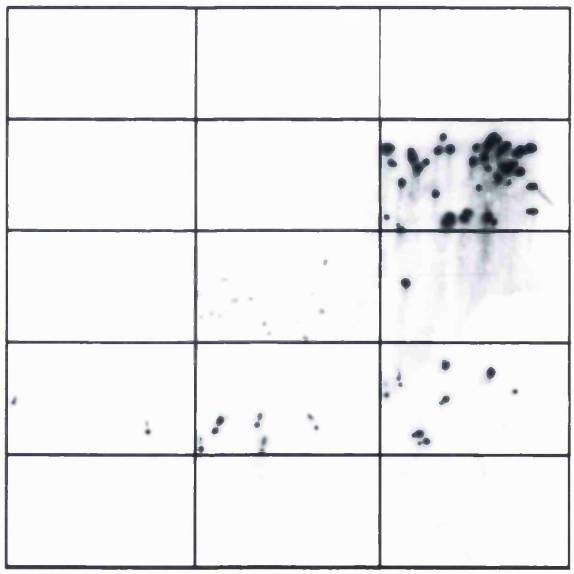
Figure 3.7: Primary and secondary screening of a cDNA library prepared from low affinity transgenic clone 4A. 2 X 10^8 T cells were cultured, snap frozen in liquid nitrogen and a custom cDNA library prepared, (Invitrogen). (A) The library was plated at approximately 2.5 X 10^5 colonies per plate, filters prepared and probed with a mixture TCRA V8 and TCRB V8 specific probes (the probes used to hybridise to the filter were TCR PCR products derived from clone 4A which had previously been shown to contain PCR errors). Multiple positive colonies were detected and fifteen picked for further characterisation. Colonies were expanded for 1 hour in antibiotic containing L broth and replated. Filters were prepared and probed individually with either the TCRB (B) or TCRA (C) specific probe. Six colonies containing a TCRB transcript (B) and five colonies containing a TCRA transcript (C) were detected.

Figure 3.7

A



B



C

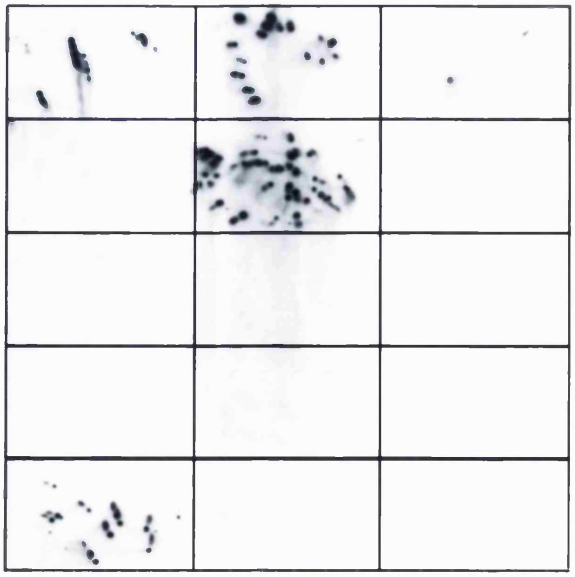


Figure 3.8: Restriction digest and PCR analysis of plasmid DNA extracted from library colonies which are positive for the presence of TCR transcripts. Plasmid DNA was prepared from TCRA or TCRB positive colonies and digested with EcoR 1. (A) Colonies 1-6, 9 and 10 have TCRA inserts in the range 700bp - 1200bp. (B) DNA has not digested to completion. However, colonies 4 and 6 have large TCRB insert bands of approximately 900-1000bp. PCR analysis of TCRA V positive colonies 1-10 using TCRA V2 (C) and TCRA V8 (D) specific 5' primers (Casanova et al, 1991) and the TCRA C 3' primer (5' GCGAATTCCAGACC TCAACTGGACCACAG 3') which hybridises at the 3' end of the TCRA constant region. (D) Colonies 2, 3, 4, 5, 6, 9 and 10 are positive for TCRA V8. (C) No colonies are positive for TCRA V2.

Figure 3.8

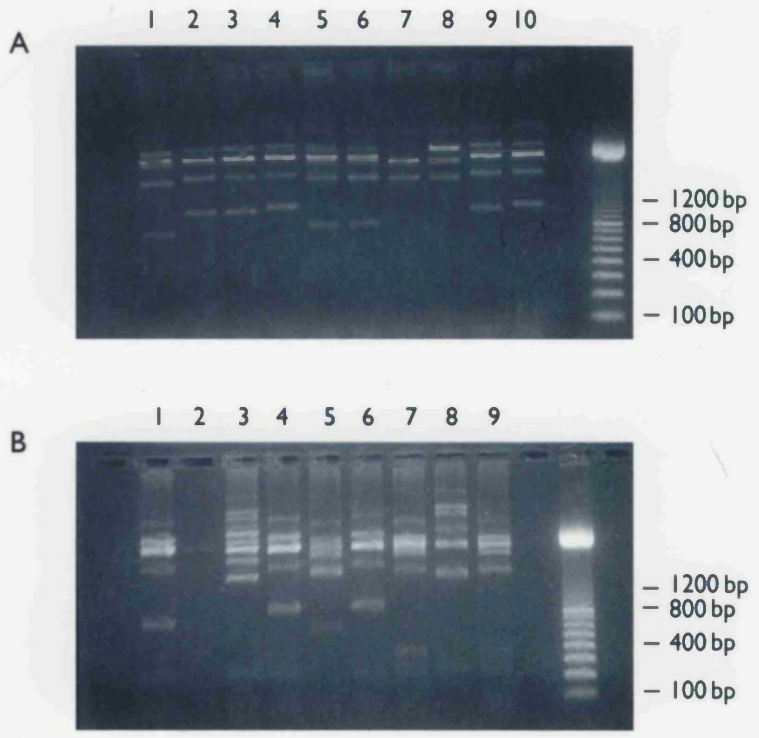

A
1 2 3 4 5 6 7 8 9 10
1200 bp
800 bp
400 bp
100 bp
B
1 2 3 4 5 6 7 8 9
1200 bp
800 bp
400 bp
100 bp
C
1 2 3 4 5 6 7 8 9 10
800 bp
600 bp
D
1 2 3 4 5 6 7 8 9 10
800 bp
600 bp

Figure 3.9: Sequence of the 5' and 3' termini of T cell receptor transcripts TCRB V8 S3 (β4 and β6) and TCRA V8 S13 (α9 and α10). On the basis of plasmid digest (figure 3.8 A and B) colonies with the largest inserts were selected for further sequence analysis to determine the exact nature of 5' and 3' termini. (A) 5' and 3' sequence of TCRB V8 S3 β4 and β6 transcripts. The beginning of the β4 transcript is indicated by an asterisk (*). (B) 5' sequences of TCRA V8 S13 α9 and α10 transcripts. (C) 3' sequences of TCRA V8 S13 α9 and α10 transcripts. EcoR1 restriction sites are underlined and polyadenylation signal sequences are boxed.

Figure 3.9 A

5' Insert Sequences of TCRB V8 S3 Library Colonies β4 and β6

5' Untranslated Region * Exon 1: Signal Sequence

GAATTCCTTGGTCGCGAGATGGGCTCCAGGCTCTTTCTGGTCTTGAGCCTCCTG

Intron

TGT ACAAGTGAGTGCTGGTTAGCCCAAGTGTGCTTCTCTCCCCTGAATTCCCAA

Exon 2

GTCTTTCTGCTCAGATGACATCATCAGGCTTTGTCTTTCTCTATCATAGAACACA

TGGAGGCTGCAGTCA

3' Insert Sequences of TCRB V8 S3 Library Colonies β4 and β6

3' Untranslated Region

Poly (A) Tail

TTACAATCAAACCAATAAACATGTTCTAGGACGGCCTGAAAAAAAAAAAAAAAA

AAAAAAAGAATTC

3' Untranslated Region

Poly (A) Tail

TTACAATCAAACCAATAAACATGTTCTAGGAAAAAAAAAAAAAGAATTC

Figure 3.9 B

5' Sequence of Insert from TCRA V8 S13 Library Colony α9

Intron 1 Exon 2

GAATTCTTTCTTTCTTTCTTTCTTATTTTCTTCCTTATTTTCAGGAAGGAGCAATGGAGAC

TCAGTGACCCAGACAGAAGGCCTGG

5' Sequence of Insert from TCRA V8 S13 Library Colony α10

5' Untranslated Region

GAATTCGAGGAGTGTGAGGAGATCCTGCAGGAGGATTGCCCTGTGAGATTGGTGCACTC

Exon 1: Signal Sequence

AGGGACCAAGTGTCATTTCTTCCATGAACATGCATCCAGTCACCTGCTCAGTTCTTGTGC

Exon 2

TCCTCCTAATGCTCAGAAGGAGCATTGGAGACTCAGTGACCC

Figure 3.9 C

3' Sequence of Insert from TCRA V8 S13 Library Colony α9

3' Untranslated Sequence Poly (A) Tail

CCCACTCAGCTCCCTCACTGCTGCTGACCATTCACAAAAAAAAAAAAAAAAGAATTC

3' Sequence of Insert from TCRA V8 S13 Library Colony α10

3' Untranslated Sequence

CCCACTCAGCTCCCTCACTGCTGCGAATTC

Figure 3.10

Structure of the VA-hCD2 vector

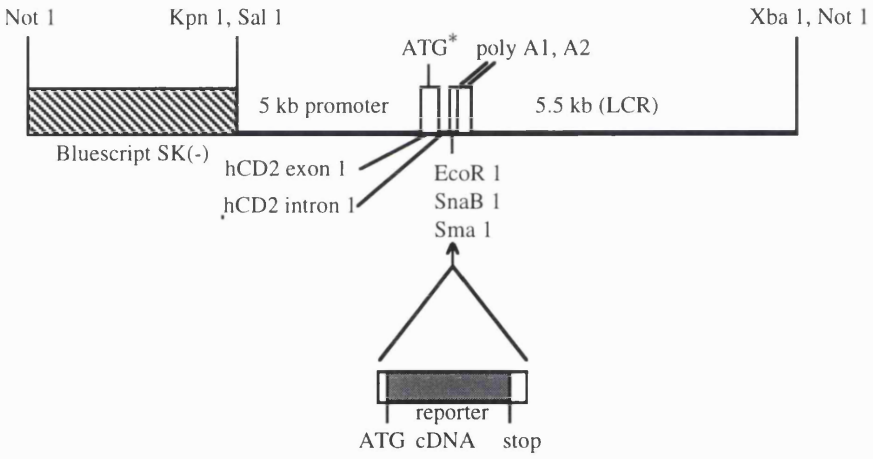


Figure 3.11: Subcloning full length TCRA V8 and TCRB V8 transcripts into the VA hCD2 expression vector. (A) EcoR1 restriction digest analysis of plasmid DNA prepared from 20 VA hCD2/TCRA colonies. Colony 11 has a large full length vector band (14.2kb) and the correct sized TCRA V8 S13 insert band (1109bp). (B) EcoR1 restriction digest analysis of plasmid DNA prepared from 28 VA hCD2/TCRB colonies. Colonies 2, 3, 4, 19 and 20 have the correct banding pattern; vector (14.2kb) and insert bands of 915bp, 228bp and 108bp. The smaller digestion products are poorly visible and are due to internal EcoR1 sites within intron 1 and at the end of the TCRB constant region, see figures 3.9 A and 3.13.

Figure 3.11

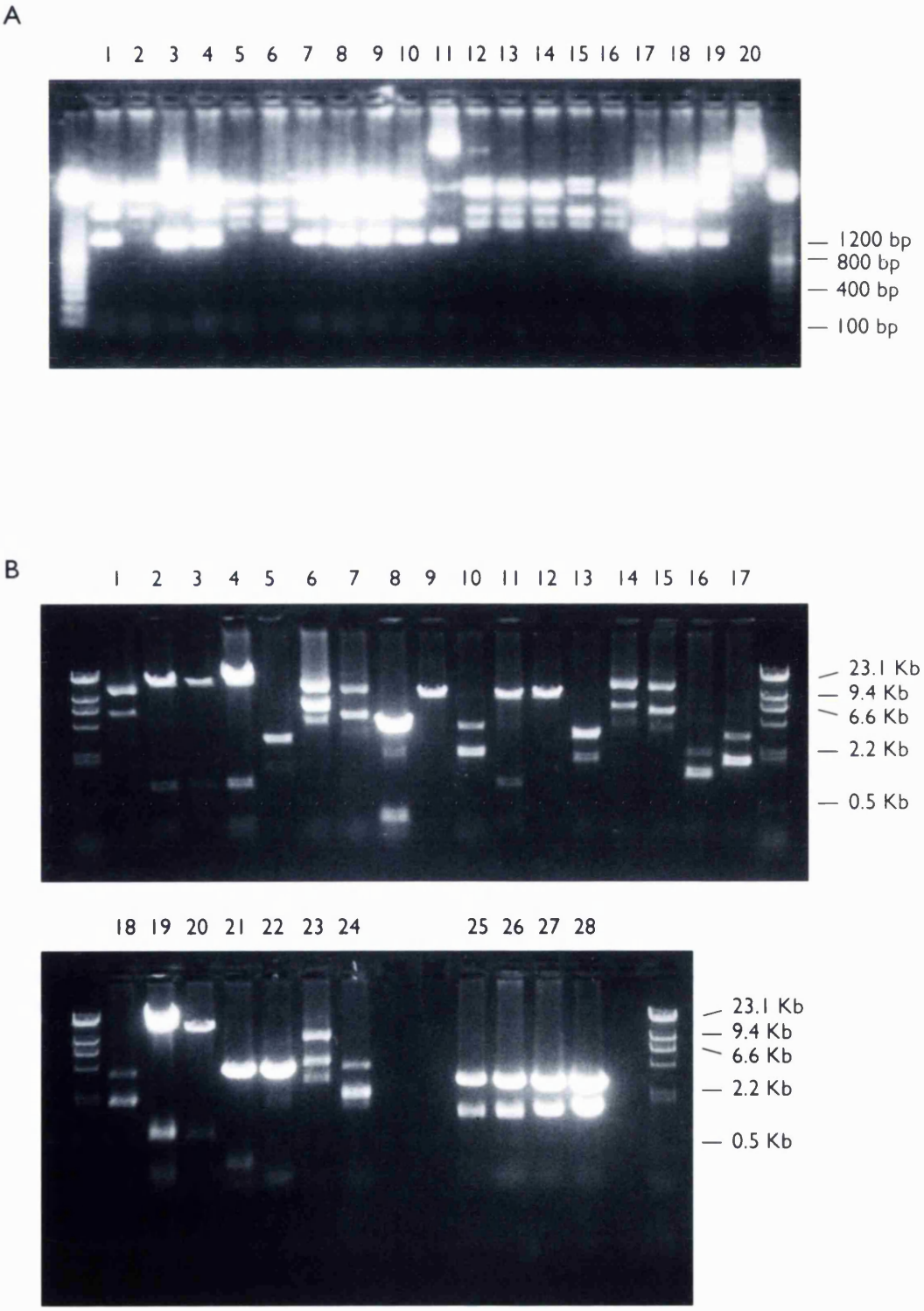
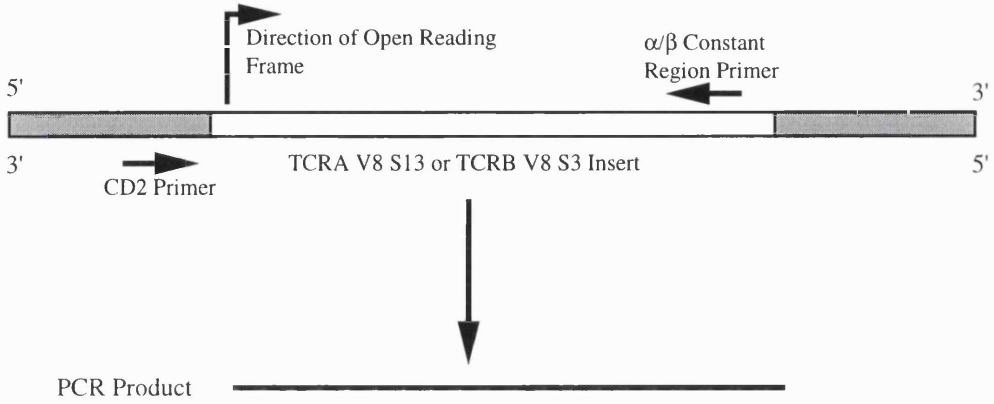
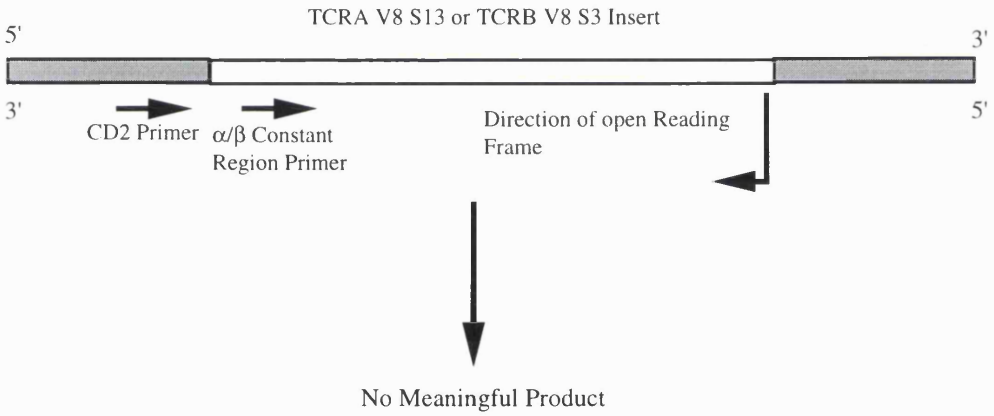

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1200 bp
800 bp
400 bp
100 bp
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
23.1 Kb
9.4 Kb
6.6 Kb
2.2 Kb
0.5 Kb
18 19 20 21 22 23 24 25 26 27 28
23.1 Kb
9.4 Kb
6.6 Kb
2.2 Kb
0.5 Kb

Figure 3.12: Assessing the orientation of TCRA V8 S13 and TCRB V8 S3 transcripts within the VA-hCD2 vector: diagrammatic representation

(A) Insert Ligated into VA-hCD2 Vector in Correct Orientation



(B) Insert Ligated into VA-hCD2 Vector in Wrong Orientation



(C) Insert Ligated into VA-hCD2 Vector in Wrong Orientation

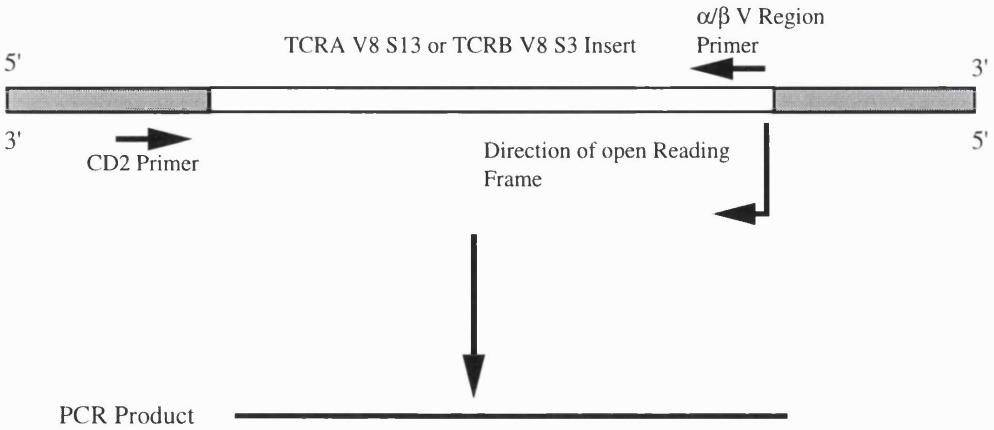
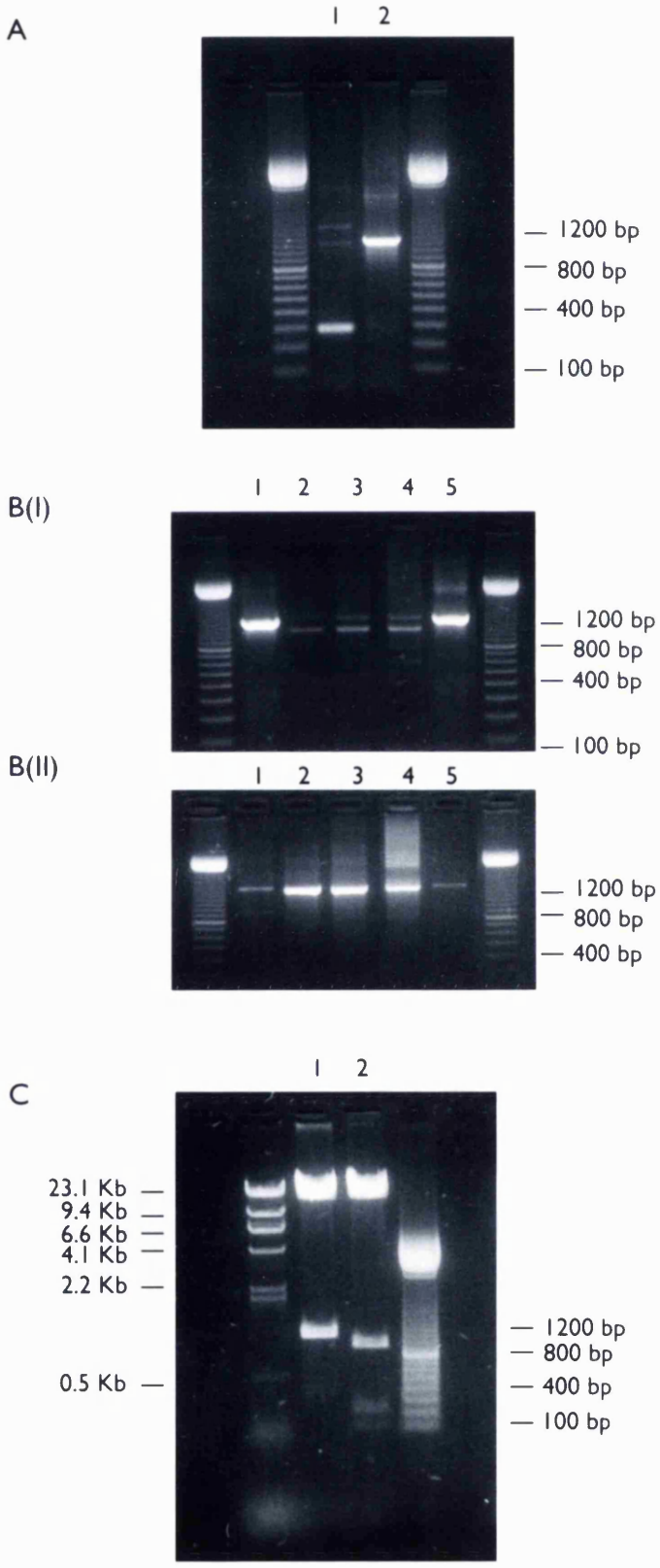


Figure 3.13: Assessing the orientation of TCRA V8 S13 and TCRB V8 S3 transcripts within the VA-hCD2 vector. (A) 1: PCR analysis of VA hCD2/TCRA V8 S13 colony 11 using a 5' primer located within the hCD2 5' untranslated region (5' TGTGGACTC CACCAGTCTCAC 3') and a 3' primer which hybridises at the 5' end of TCRA V8 S13 exon 1 (5' GCGGAATTCTCTTCCCTGAACATGCGTCCTGACACCTGC 3') fails to produce a band of predicted length 1237bp which represents the TCRA V8 S13 transcript ligated into VA-hCD2 in the wrong orientation. (A) 2: PCR analysis of VA hCD2/TCRA V8 S13 colony 11 using a 5' primer located within the hCD2 5' untranslated region (5' TGTGGACTC CACCAGTCTCAC 3') and a 3' primer which hybridises at the end of the TCRA constant region (5' GCGAATTCCAGACCT CAACTGGACCACAG 3') results in a PCR product of predicted length 1119bp and indicates that the TCRA transcript has been ligated into the VA-hCD2 vector in the correct orientation. (B(I) and B(II)), PCR analysis of VA-hCD2/TCRB V8 S3 colonies 2, 3, 4, 19 and 20 are shown in lanes 1, 2, 3, 4 and 5 respectively. B(I) PCR using a 5' primer located within the hCD2 5' untranslated region (5' TGTGGACTC CACCAGTCTCAC 3') and a 3' primer located at the end of the TCR B C1 constant region (5' GCGAATTCCTGACTTCATGAATTTTTTCT 3') results in the production of a 1243bp band if the TCRB transcript has ligated into VA-hCD2 in the correct orientation, (lanes 1 and 5). B(II) PCR using a 5' primer located within the hCD2 5' untranslated region (5' TGTGGACTC CACCAGTCTCAC 3') and a 3' primer located at the start of TCRB V exon 1, (5' GCGGAATTCTGGTCGCGAGATGGGCTCCAG GCTCTT 3') results in the production of a 1461bp product if the TCR B V transcript has ligated into VA-hCD2 in the wrong orientation, (lanes 3, 4 and 5). (C) EcoR1 digest of VA-hCD2-$\alpha 10^{11}$ (lane 1) and VA hCD2 $\beta 6^{20}$ (lane 2).

Figure 3.13


A
1 2
1200 bp
800 bp
400 bp
100 bp
B(I)
1 2 3 4 5
1200 bp
800 bp
400 bp
100 bp
B(II)
1 2 3 4 5
1200 bp
800 bp
400 bp
C
1 2
23.1 Kb
9.4 Kb
6.6 Kb
4.1 Kb
2.2 Kb
0.5 Kb
1200 bp
800 bp
400 bp
100 bp

4.0 Characterisation of the P91A epitope

4.1 Introduction

Crystallisation of MHC class I molecules has revealed the presence of a peptide binding groove containing heterogeneous material (Bjorkman et al, 1987a and b, Garrett et al, 1989, Saper et al, 1991). Structural information derived from the crystallisation of class I molecules containing naturally processed heterogeneous peptides (Silver et al, 1992) or with individual defined peptides (Fremont et al, 1992, Madden et al, 1993) has enabled a general molecular basis for peptide interaction with class I heavy chain to be established. The amino and carboxy termini of peptides form extensive hydrogen bonding networks to conserved residues present at either end of the MHC binding groove (Silver et al, 1992, Fremont et al, 1992, Madden et al, 1993). Polymorphic residues within the binding groove determine allele specific properties of individual molecules and account for the allele specific motifs identified by bulk peptide elution and sequencing (Falk et al, 1991). Pockets in the binding cleft which accommodate residues of particular size and character are of primary importance in the determination of allele specificity (Saper et al, 1991, Madden et al, 1993). Mouse and human allele specific motif residues are usually found at two or three positions and include the carboxy-terminus (Falk et al, 1991, Rammensee et al, 1995). The predominant length of class I associated peptides is 9 amino acids, although peptides of 8 amino acids may be favoured for some alleles. Longer peptides may be accommodated in the binding groove through either a central bulge or by carboxy terminal extension from the groove (Guo et al, 1992, Collins et al, 1994). Furthermore, peptides which fail to reach the amino terminal binding pocket have also been observed to bind, leaving that pocket unoccupied (P. Ghosh and D.C. Wiley, unpublished results quoted in Bouvier and Wiley, 1994). Non-motif positions can also exhibit some residue preference and for peptides which lack characteristic anchor residues, interactions of non-motif positions with the MHC molecule may be

of particular importance. Motifs based on the dominant length of peptides associated with particular MHC molecules can provide invaluable predictive information for epitope identification (Pamer et al, 1992).

The P91A tum$^-$ peptide (Boon et al, 1989, Lurquin et al, 1989) is derived from a mutated form of the S3 subunit of the PA700 regulatory unit (19S unit) of the 26S proteasome (DeMartino et al, 1994) and was originally defined as the 13mer peptide P91A 12-24 [residue number in exon 4 of P91A] (Lurquin et al, 1989). More recent reports have suggested that shorter nonamer peptides P91A 12-20 (Corr et al, 1992) and 14-22 (Alexander-Miller et al, 1994) represent the P91A epitope. Examination of the P91A 12-24 peptide sequence reveals that the P91A epitope does not conform to the defined L^d binding motif (P2-proline, P9-methionine, isoleucine, leucine) and yet is a strong MHC binding peptide (Falk et al, 1991, Corr et al, 1992). The P91A peptide may therefore bind to H2-L^d in a manner distinct from that of other H2-L^d binding peptides and be representative of a more general mode of peptide binding which is utilised by a specific subset of L^d associated peptides.

To model potential interactions between L^d and P91A (and its processing intermediates) and the L^d/P91A complex with T cell receptors of different avidities/affinities it is important to define the exact P91A epitope which is naturally processed and presented. Using MHC stabilisation, T cell stimulation, peptide elution and molecular modelling the P91A epitope is identified as the octamer P91A 15-22 (QNHRALDL). The P91A epitope does not conform to the L^d peptide binding motif and residues which are important for MHC binding and interaction with T cell receptors from transgenic and non-transgenic T cell clones are identified.

4.2 Results

4.2.1 Definition of a P91A optimal binding peptide

H2-L^d is present on the cell surface as either a tri-molecular complex of conformed heavy chain, β_2m and peptide (L^d) or as partially folded heavy chain in the absence of bound peptide (L^dalt), (Smith et al, 1993). Incubation of L^d expressing cell lines with L^d binding peptides results in an increase in conformed L^d molecules, a decrease in L^dalt and an overall increase in surface expression of L^d (Lie et al, 1990 and 1991; Smith et al, 1993). Furthermore, L^dalt can be converted to conformed L^d by the addition of L^d binding peptides to cell lysates, and fails to accumulate on the surface of L^d transfected RMA-S cells (Smith et al, 1993). These findings support the notion that L^dalt molecules accumulate at the cell surface as a result of peptide dissociation from conformed L^d molecules and that the addition of exogenous peptide induces partially folded L^dalt to re-assume a mature L^d conformation. The maintenance of a high concentration of exogenous peptide may help to prevent reversion of L^d to L^dalt. The ability of exogenous peptides to increase surface levels of L^d can be used as a biological assay of peptide binding (Lie et al, 1990, 1991).

The P91A epitope was initially defined as the 13mer P91A 12-24 (ISTQNHRALDLVA), (Lurquin et al, 1989). This peptide is now known to be outside the length range for the majority of MHC class I binding peptides (Rammensee et al, 1995). It is therefore likely that a smaller peptide within P91A 12-24 represents the true P91A epitope.

To define the N-terminus of the P91A peptide a series of N-terminal truncated peptides were synthesised, figure 4.1A, and used in an H2-L^d surface stabilisation assay, figure 4.2A. P91A 12-24, 13-24, 14-24 and 15-24 all bind and increase cell surface expression of L^d. Maximal stabilisation is achieved with P91A peptides 12-24

and 15-24. Peptides shorter than P91A 15-24 fail to bind to L^d. The maximal binding observed with P91A 15-24 combined with the failure of peptides shorter than P91A 15-24 to bind demonstrate the critical importance of position 15 (Q) in the formation of the P91A epitope. If position 15(Q) is a candidate amino terminal residue for the P91A epitope then one must account for the ability of longer peptides to bind in this assay. It is probable that longer peptides which appear to bind to L^d are processed either intracellularly or extracellularly to the P91A 15-24 peptide. A dependence on extracellular processing for *in vitro* generation of MHC class I binding peptides from longer peptides has previously been shown (Kozlowski et al, 1992 and 1993; Sherman et al, 1992).

Using a similar approach to that above, a panel of C-terminal truncated peptides based on P91A 15-24 was synthesised, figure 4.1B, and used to stabilise surface L^d expression, figure 4.2B. P91A 15-24, 15-23, 15-22 and 15-21 all stabilise and increase the surface expression of L^d, figure 4.2B. Stabilisation is best achieved with P91A 15-24 and the 7mer peptide P91A 15-21 with P91A 15-22 and 15-23 both slightly less effective. These data suggest that much of the ability of the P91A epitope to bind to L^d resides in the small 7mer peptide P91A 15-21. However, unlike the situation for the N-terminus a strict requirement for a specific C-terminal residue cannot be determined, with peptides P91A 15-24, 15-23, 15-22 and 15-21 all binding efficiently.

4.2.2 T cell stimulation by N- and C-terminal truncated P91A peptides

Having defined a series of truncated peptides capable of binding to L^d their ability to stimulate the P91A specific T cell clone P91.6 was assessed. P91.6 is the T cell clone originally used to define the P91A tum^- antigen (Maryanski et al, 1983a). Using amino terminal truncated peptides P91A 15-24 is identified as the peptide with highest stimulatory capacity, figure 4.3 A. Peptides P91A 12-24, 13-24 and 14-24 are

all strong stimulators of P91.6, but are at least 10 fold less active than P91A 15-24. P91A 16-24 is approximately 3 logs less active than P91A 15-24. The shorter peptides P91A 17-24 and 18-24 are a further 1-2 logs less active. These data confirm the importance of the N-terminal glutamine in the formation of the P91A epitope.

P91A peptides 12-24, 13-24, 14-24 and 15-24 all stimulate P91.6 in the nanomolar range. It is however unlikely that each of these peptides binds to L^d in its native form and stimulates the T cell clone. This would require P91.6 to recognise four differently sized peptides forming four differently shaped epitopes. The processing of longer peptides, by foetal calf serum (FCS) associated exopeptidases, to generate the P91A 15(Q) amino terminus is likely to account for these results. Processing of the carboxy terminus is also likely to occur and may be required for epitope generation from longer peptides.

C-terminal truncated peptides were also examined for their ability to stimulate P91.6, figure 4.3B. This group of peptides all bind and stabilise L^d surface expression. As shown in figure 4.3B, P91A 15-24 and 15-22 are potent stimulators of P91.6 while P91A 15-23 and 15-21 are both 2-3 logs less active. It is clear from these data that P91A 15-22 and 15-24 are both candidate P91A epitopes. It is however unlikely that both P91A 15-22 and P91A 15-24 bind to L^d in their native form and stimulate P91.6.

To address the possibility that P91A 15-24 is processed by FCS associated exopeptidases to the shorter peptide P91A 15-22 cell surface stabilisation with both peptides was assessed in the absence of serum, figure 4.4. In the absence of FCS, the longer peptide P91A 15-24 fails to stabilise surface expression of L^d, while the shorter peptide P91A 15-22 remains active. In the presence of FCS, P91A 15-24 and P91A 15-22 are both effective in stabilising surface L^d expression. These data suggest that the longer peptide P91A 15-24 must undergo additional processing in the extracellular environment to generate the P91A 15-22 epitope. Paradoxically P91A

15-24 is slightly more active than P91A 15-22 in the presence of FCS. This is most likely due to the dicarboxypeptidase activity of serum angiotensin converting enzyme. This enzyme will tend to generate the biologically active peptide P91A 15-22 from the longer peptide P91A 15-24 and destroy the biological activity of exogenously added P91A 15-22. This feature has been previously demonstrated (Kozlowski et al, 1993). In conclusion, P91A 15-22 (QNHRALDL) is identified as the minimal peptide which retains maximal MHC binding and T cell stimulatory activity.

4.2.3 Interaction of the N-terminus of the P91A epitope with the H2-L^d A pocket

Like many proteins and peptides which possess an amino terminal glutamine, the P91A 15-22 and 15-24 peptides are inherently unstable. This instability results from the propensity of the N-terminal glutamine residue to form the cyclised secondary amino acid pyroglutamic acid, figure 4.5. Much of the energy of interaction between peptides and MHC molecules derives from hydrogen bonds that exist between conserved MHC class I residues in the A and F pockets (figure 1.4) and the peptide terminal NH_2- and COOH- groups. Furthermore, substitution of the terminal NH_2- and COOH- groups with methyl groups has been shown to decrease substantially the thermal stability of the MHC class I/peptide complex (Bouvier and Wiley, 1994). P91A peptides which have a pyroglutamyl N-terminus will not have a free terminal amine group with which to interact with A pocket residues. As a result of this, P91A-pyroglutamyl peptides may bind less efficiently to the H2-L^d molecule. Although peptides were synthesised to possess N-terminal glutamine it is likely that during the course of stabilisation and proliferation assays that pyroglutamate forms of P91A 15-22 and P91A 15-24 are generated. Indeed HPLC analysis of synthesised P91A peptides bearing an N-terminal glutamine residue reveals a substantial proportion of peptide with a pyroglutamyl residue. The biological activity of P91A-pyroglutamyl peptides is unknown.

In order to assess the biological activity of a peptide possessing a pyroglutamyl N-terminal residue, the P91A 15-24 peptide was subjected to acid treatment during synthesis and a substantial quantity of pyroglutamyl-P91A 15-24 purified. As shown in figure 4.6A, P91A 15-24 and pyroglutamyl-P91A 15-24 bind equivalently to L^d. This indicates that the loss of a free terminal amine group and the substantial repositioning of amino terminal side chain atoms is insufficient to destabilise the interaction of pyroglutamyl-P91A 15-24 with the H2-L^d molecule. The ability of pyroglutamyl-P91A 15-24 to bind may be due to an alternative hydrogen bonding strategy in the H2-L^d A pocket. The interaction of the HIV1 octamer peptide (Nef 75-82) with HLA-B*3501 reveals a non standard pattern of hydrogen bonding in the A and F pocket (Smith et al, 1996b). The binding of Nef 75-82 at the N-terminus is maintained by the addition of an extra water molecule in the A pocket which is capable of forming hydrogen bonds to the peptide amino terminus and conserved tyrosine hydroxyl groups in the pocket. A similar strategy may exist in the binding of a pyroglutamyl residue in the A pocket of L^d.

Alternatively, the interaction of peptide anchor residues or the peptide carboxy terminus with the H2-L^d molecule may contribute largely to the overall binding energy of the P91A peptide. Stable MHC class I/peptide complexes have been shown to form in which the amino terminus of the binding peptide fails to reach the class I A pocket (Bouvier and Wiley, 1994). Furthermore, in the present study the 7mer peptide P91A 15-21 binds and stabilises L^d surface expression and yet is physically incapable of binding in both the A and F pockets. These and other results (Bouvier and Wiley, 1994), suggest that the combined interaction of both peptide termini with the A and F pockets may not be required for stable peptide binding. The binding energy of pyroglutamyl-P91A 15-24 may therefore derive from a combination of anchor residue and carboxy terminal interactions within the L^d binding groove. Having said this, in the case of the P91A peptide an amino terminal residue would seem to make a

contribution to the overall binding energy since complete loss of the N-terminal residue in P91A 16-24 leads to greatly diminished binding.

P91A 15-24 and pyroglutamyl-P91A 15-24 were used to stimulate the T cell clone P91.6, figure 4.6B. Pyroglutamyl-P91A 15-24 is approximately ten fold less active than P91A 15-24. This result suggests that the presence of an N-terminal pyroglutamic acid residue induces a conformational change in the T cell epitope recognised by P91.6. Epitope alteration may be as a result of the loss of the glutamine side chain, alteration of the positions of neighbouring peptide residues or repositioning of MHC α helical residues. The first two are likely to occur and the latter has been documented in the binding of the Nef 75-82 to HLA-B*3501 (Smith et al, 1996a).

4.2.4 The P91A 12-20 peptide is not the P91A tum$^-$ epitope

H2-L^d binding peptides are characteristically 9 amino acids in length, have a proline at position 2 and a hydrophobic carboxy terminal residue (Falk et al, 1991 and Corr et al, 1992). The P91A 15-22 peptide is 8 amino acids in length and does not have proline at position 2. One other H2-L^d binding peptide is known which does not conform to the L^d binding motif. The p2Ca peptide (LSPFPFDL) which sensitises H2-L^d expressing cells for allospecific kill by the T cell clone 2C (Udaka et al, 1992) is an octamer peptide which has serine at position 2. Serine is of similar volume and hydrophobicity to proline. During the course of this work a prediction was made that the P91A tum$^-$ epitope would be P91A 12-20 (ISTQNHRAL), (Corr et al, 1992). This prediction was largely based on sequence similarity to the p2Ca peptide and made the assumption that the P2 residue (serine) of p2Ca was a critical anchor residue. In addition, molecular modelling of the P91A 12-20/L^d complex suggested that this peptide would make a favourable set of interactions with the L^d binding groove (Corr et al, 1992).

Although the data presented suggest that the natural P91A epitope is the octamer P91A 15-22 the activity of the P91A 12-20 peptide has not been assessed. P91A 12-20 was synthesised and its ability to bind to L^d and stimulate the T cell clone P91.6 assayed, figure 4.7A and B. P91A 12-20 fails to bind to L^d or stimulate P91.6 indicating that this peptide does not represent the P91A tum^- epitope.

4.2.5 Definition of the P91A tum^- epitope by molecular modelling

Having established a P91A peptide with optimal biological activity a molecular modelling exercise was performed to identify the optimal P91A binding peptide and gain further insight into the mechanism of interaction between the P91A peptide and the H2-L^d molecule. To this end, the binding energies of a series of N-terminal tetra-peptides and C-terminal tri-peptides were calculated, table 4.1. This allows a prediction for the optimal P91A N- and C-termini to be made. These data attribute favourable binding energies to the P91A 15-22 termini (P91A 15-18: -155.474 kcal/mol and P91A 20-22: -12.524 kcal/mol) and therefore support the prediction that the P91A 15-22 peptide is the natural P91A epitope. The binding energy (-135.779 kcal/mol) of the N-terminal tetra-peptide (YPHF) of the MCMV pp89 L^d epitope is included for comparative purposes. The predicted binding energy of the N-terminal tetrapeptide P91A 16-19 is 356.891 kcal/mol. This is the N-terminal tetrapeptide of the P91A 16-24 peptide which has been shown to bind poorly to L^d and stimulate P91.6 weakly, figure 4.2A and 4.3A. An inspection of P91A 16-24 sequence reveals the presence of an arginine residue at peptide position 3. The P91A tum^+ peptide also has arginine at position 3 and is an L^d non binder (Lurquin et al, 1989). Taken together these data suggest that the presence of an arginine residue at position 3 has a de-stabilising effect on peptide binding.

The L^d motif residue P2 proline is predicted to interact with the interior of the L^d B pocket (Corr et al, 1992; Thorpe, 1995). Since the P91A 15-22 peptide does not have

either the L^d binding motif proline or the less common serine at position 2, a comparative modelling exercise was performed to establish the ability of the L^d B pocket to accommodate asparagine at P2 (asparagine is the P2 residue of the P91A 15-22 peptide). The fit of amino acid side chains into pocket B was assessed by searching for areas of contact between peptide amino acid side chain atoms and atoms contributed to the pocket by the L^d heavy chain. Contact was assessed by imaging incursions into the pocket walls by peptide side chain atoms. The interaction of the B pocket with the P91A P2 asparagine or the H2-L^d binding motif residues proline or serine is shown in figure 4.8A-C. The small side chains of proline, serine and asparagine display no steric clash with the internal walls of the B pocket indicating that all residues may be accommodated. Furthermore, the asparagine residue has good complementarity with the B pocket and is predicted to form hydrogen bonds with the hydroxyl group of tyrosine 45 and the carboxyl group of glutamic acid 9.

Having examined the interaction of the B pocket with the P2 residue of P91A 15-22 the interaction of the P3 residues histidine (tum^-) and arginine (tum^+) with the L^d D pocket was explored. The P91A (tum^+) peptide is an L^d non binder and has arginine at position 3 of the P91A 15-22 peptide. P91A 15-22 (tum^-) has histidine at this position. The difference between the biological activities of these two peptides can be explained by examining the nature of the interaction between their P3 residues and the H2-L^d D pocket. The failure of the tum^+ peptide QNRRALDL to bind appears to be due to a steric clash between the long side chain of the P3 arginine and the walls of the highly hydrophobic D pocket, figure 4.8E. By contrast, P3 histidine of the P91A 15-22 tum^- peptide makes a good set of contacts with the D pocket and is complementary in both size and character, figure 4.8D. These data confirm that P3 arginine cannot be accommodated in the L^d D pocket and account for the failure of the P91A tum^+ peptide to bind to L^d.

4.2.6 Definition of MHC and TCR contact residues within the P91A tum$^-$ peptide

L^d stabilisation and T cell stimulation data indicate that P91A 15-22 (QNHRALDL) rather than P91A 15-24 (QNHRALDLVA) is the natural P91A tum$^-$ epitope. To further confirm that residues 23 and 24 do not form part of the native P91A tum$^-$ peptide and to determine residues within the peptide capable of interacting with MHC or TCR a series of amino acid substituted peptides based on P91A 15-24 were synthesised, figure 4.1C. As shown in figure 4.9 A, B, C and Table 4.2, P91A residues 23 and 24 do not appear to be important for either MHC binding or T cell recognition. This supports the notion that these residues are not part of the P91A tum$^-$ epitope and that the activity of P91A 15-24 and its substituted variants is dependent on processing to the shorter peptide P91A 15-22. Further analysis of L^d stabilisation and T cell stimulation by amino acid substituted variants indicates that peptide positions 5, 6, 7 and 8 are important for MHC binding and that positions 1, 3 and 4 strongly influence T cell recognition.

Although position 2 is normally an anchor residue of L^d binding peptides, alanine substitution of this residue appears to have no effect on P91A peptide binding, figure 4.9A, table 4.2. Furthermore, substitution of P2 asparagine for glutamine, alanine or proline does not affect the ability of P91A 15-22 substituted variants to bind to L^d, figure 4.10A. These data suggest that P91A 15-22 binds to L^d in a substantially different way to that of the majority of L^d binding peptides. The major MHC contact residues of the P91A 15-22 peptide reside in the carboxy terminal half of the peptide with the amino terminal part of the peptide seeming to contribute little to the specificity of the interaction. In order to test whether the carboxy terminal half of the P91A 15-22 peptide contains all the necessary information required to confer L^d specificity, a synthetic peptide was synthesised with alanine or glutamine substitutions of the first four residues (AQAAALDL). Position 2 was changed to a

glutamine since this residue is permissive for L^d binding and is hydrophilic in nature. It was hoped that by including a hydrophilic amino acid the overall hydrophobicity of the peptide would be decreased and the solubility of the peptide in water maintained. As shown in figure4.10B, synthetic peptides AQAAALDL and QNHRALDL appear to bind equivalently to L^d. These data suggest that the specificity of the interaction between P91A 15-22 and H2-L^d is conferred by the carboxy terminal half of the peptide and that major alterations in N-terminal sequence can be tolerated without affecting binding. P91A 15-22 therefore binds to L^d using an alternative strategy to that of other L^d binding peptides.

4.2.7 Identification of the naturally processed P91A peptide

To establish whether P91A 15-22 (QNHRALDL) represents the peptide which is naturally processed and presented, low molecular weight material was extracted from P91A transgenic spleen cell lysates and separated by HPLC. Fractions of collected material were used to stimulate the P91.6 T cell clone. In multiple experiments stimulatory activity was present in a single fraction, figure 4.11A. Under identical HPLC conditions the synthetic peptide P91A 15-22 was shown to co-elute with the naturally active peptide, figure 4.11B. This and previous data confirms the identity of the P91A tum^- epitope as the octamer P91A 15-22 (QNHRALDL).

4.2.8 Interaction of the P91A-L^d complex with the low affinity T cell clone 4A

The P91A peptide like other MHC class I binding peptides has residues which are primarily concerned with MHC binding (P5, P6, P7 and P8) and T cell recognition (P1, P3 and P4). In this and other studies the P4 residue arginine has been shown to be an important TCR contact site for P91A specific T cell clones (in all but one clone analysed P4 arginine was found to be critical for T cell recognition), (Alexander-

Miller et al, 1994). Although peptide main chain conformation can be altered by amino acid substitution and therefore alanine substitution of a specific residue may affect the overall conformation of the peptide in the binding groove it remains likely that P4 arginine interacts directly with the TCR. Indeed, molecular modelling of the P91A peptide/H2-L^d complex reveals a solvent exposed arginine side chain directed away from the MHC binding groove (Thorpe, 1995). The nature of this residue is further confirmed by amino acid replacements (Ala, Gln, Glu and Lys) which fail to disrupt peptide binding (Robinson and Lee, 1996). P4 arginine would therefore seem to be play little part in the interaction of the P91A peptide with the MHC molecule but be a critical T cell receptor contact residue for the majority of P91A specific T cell clones.

It has been suggested that P91A transgenic mice delete high affinity P91A reactive T cells and that those cells which escape deletion interact only weakly with the P91A epitope. Since P4 arginine is a TCR contact residue for the majority of P91A specific T cell clones it is probable that P91A reactive T cells are deleted in the thymus at least in part on the basis of their ability to interact with this residue. If this is the case then T cells which escape deletion may do so on their capacity to interact weakly with the L^d-P91A complex as a result of clashing either sterically or electrostatically with the P4 arginine residue. If this is the case, then interaction of the P4 arginine residue with a TCR from a P91A specific transgenic T cell clone would be a de-stabilising force promoting a weak interaction. Arginine is a large positively charged amino acid. The substitution of this bulky, charged residue for a small neutral alanine residue may therefore lead to enhanced interaction between a low affinity transgenic TCR and the L^d-peptide variant complex. In order to examine the nature of transgenic TCR contact residues, low affinity transgenic clone 4A was stimulated with a panel of alanine substituted peptides, figure 4.12. The majority of substitutions resulted in the failure of peptide variants to stimulate the clone. This is to be expected since any alteration which decreases the already weak affinity of the TCR/MHC-peptide interaction or

lowers the MHC-peptide ligand density and so decreases the avidity of the interaction will result in the failure of T cell activation. However, alanine substitution of position 4 arginine results in enhanced stimulation of the low affinity clone 4A (the alanine substituted peptide variant is 10-100 fold more active than native P91A peptide). Since position 4 of the P91A epitope is not involved in MHC binding the increased activity of this peptide is likely to result from enhanced TCR/MHC-peptide interaction and supports the notion that the TCR from clone 4A has a low affinity for the L^d-P91A complex as a result of an unfavourable interaction with P91A P4 arginine.

Two ternary complexes of TCR/MHC-peptide have been crystallised (Garcia et al, 1996 and Garboczi et al, 1996). Both of these crystal structures reveal a diagonal orientation of the TCR on the MHC molecule with CDR3 α and β converging over the centre of the peptide to form a deep central pocket, figures 1.5 and 1.6. This pocket has been shown to accommodate the HTLV-1 Tax peptide TCR contact residue Y5 (Garboczi et al, 1996) and is hypothesised to bind a large side chain from the dEV8 peptide (Garcia et al, 1996). In the present study multiple P91A specific non-transgenic and transgenic TCRs have been cloned. The amino acid sequences of transgenic and non-transgenic CDR3 regions are shown in figure 4.13A and B. Although it may not be reasonable to speculate about CDR3 structure outwith the confines of the overall $\alpha\beta$ TCR structure, if one examines the CDR3α and β sequences of clone 4A the presence of a non germline encoded arginine residue is found in both regions. This feature is not found in any other sequence examined. Arginine is a positively charged amino acid with a long side chain. The presence of two such residues in a central TCR pocket formed by the convergence of CDR3α and β may be incompatible with the accommodation of P91A P4 arginine. This failure to accommodate could be based on both steric and electrostatic incompatibility. The substitution of P4 arginine for alanine may remove this potential for clash and repulsion.

It has been reported that the presence of charged peptide TCR contact residues can correlate with oppositely charged residues in TCR junctional sequences (Jorgensen et al, 1992). A previous analysis of TCRs from P91A specific T cell clones has suggested that negatively charged residues glutamic acid and aspartic acid found at similar positions in CDR3α sequences interact with the positively charged peptide residues P3 histidine and P4 arginine (Solheim et al, 1993b). In the present study no such correlation with CDR3α sequence was shown to exist. This said, negatively charged aspartic acid is present at the same position in four out of five non-transgenic CDR3β regions. Whether this residue interacts with positively charged P3 histidine or P4 arginine is unknown. The aspartic acid found in non-transgenic CDR3β regions is generally germline encoded. The presence of aspartic acid at this position may therefore reflect the preferred usage of TCRB V8 segments in the recognition of H2-L^d. Aspartic acid is the last amino acid encoded by TCRB V8 S3 and in the absence of exonuclease activity will be present at the start of CDR3β. Furthermore, aspartic acid is found at the same position in the CDR3β of low affinity clone 4A and is absent in the CDR3β of tum^- specific clone P91.6.

4.3 Discussion

The P91A epitope has been identified as the octamer P91A 15-22 (QNHRALDL). Previous reports have suggested the identity of the P91A epitope to be the 13mer P91A 12-24 (Lurquin et al, 1989), the 9mer P91A 12-20 (Corr et al, 1992) or the 9mer P91A 14-22 (Alexander-Miller et al, 1994). The two latter reports utilised a single approach (Lurquin et al, 1989) or made an initial assumption regarding epitope length (Alexander-Miller et al, 1994). In this study, the P91A peptide is defined using a variety of complementary approaches all of which indicate the natural epitope to be P91A 15-22.

Using a panel of amino and carboxy terminal truncated peptides, P91A position 15 glutamine [P91A-15(Q)] is identified as critical for MHC binding. Truncated peptides which lack P91A-15(Q) fail to bind to H2-L^d. Since P91A-15(Q) can be substituted with alanine or tyrosine (Alexander-Miller et al, 1994) with no resultant decrease in MHC binding activity, it is likely that the glutamine residue participates in binding through conventional hydrogen bonding of the terminal NH_2- group and the peptide backbone to conserved MHC residues (Silver et al, 1992, Madden et al, 1992, Fremont et al, 1992, Bouvier and Wiley, 1994). Furthermore, position 1 glutamine can be a residue for T cell recognition, implying that the side chain points away from the floor of the binding groove. Molecular modelling also supports such a view of the P1 glutamine residue (Thorpe, 1995).

The C-terminus of the P91A peptide displays a less stringent requirement for a specific terminal residue with the heptamer P91A 15-21 binding as efficiently as the longer peptides P91A 15-22, 15-23 and 15-24. It is probable that the longer peptides which bind to L^d are processed in the presence of serum proteases to shorter peptides which are biologically active (Sherman et al, 1992, Kozlowski et al, 1992, 1993). P91A 15-23 which stabilises L^d poorly may bind directly to the L^d molecule or may be efficiently processed to the heptamer P91A 15-21. Such processing may be the result of abundant serum carboxy dipeptidases (Kozlowski et al, 1993). The 7mer peptide P91A 15-21 is too short to bind in both the A and F pockets. Since there is a strict requirement for P91A-15(Q) at the N-terminus it is likely P91A 15-21 binds in the absence of filling the C-terminal F pocket. This type of binding has previously been described and may be energetically stable (Bouvier and Wiley, 1994). Although both P91A 15-21 and 15-23 bind to L^d neither peptide displays significant T cell stimulatory activity. The heptamer P91A 15-21 must therefore bind in a conformation which does not permit recognition by the T cell clone P91.6. Interestingly, the longer peptide P91A 15-23 also fails to stimulate P91.6. This implies that processing of P91A 15-23 to P91A 15-22 is either inefficient or does not occur and is once again

suggestive of the actions of a dicarboxypeptidase which is capable of generating P91A 15-21 from P91A 15-23. Importantly, independent truncation of both termini has allowed definition of an optimal octamer sequence (P91A 15-22). The use of overlapping peptides of fixed length (L^d predominantly associates with nonamers) would not readily have identified this peptide.

Further support for this interpretation comes from the analysis of amino acid substituted peptides for which it is shown that neither valine (P9) or alanine (P10) are crucial for MHC binding or T cell recognition. To show definitively that the P91A 15-22 peptide represents the naturally processed and presented epitope, HPLC separated peptides eluted from P91A transgenic spleen cells were used to stimulate the tum^- specific T cell clone P91.6. The naturally processed peptide elutes at the same position as the candidate synthetic peptide P91A 15-22.

P91A 15-22 does not have either P2 motif residue (proline or the less common serine) but has an asparagine residue at this position. All other known L^d epitopes have one or other of the motif residues at this position implying a critical role for P2 in MHC binding. However for the 2C peptide (Udaka et al, 1992) substitution of P2 serine with alanine did not have a major effect on MHC binding (Al-Ramadi et al, 1995). The amino acid substitution analysis performed here shows that for the P91A epitope, P2 asparagine is similarly not critical for MHC binding or T cell recognition. Furthermore, molecular models show the asparagine residue to fit comfortably into the B pocket and form two hydrogen bonds with L^d heavy chain residues tyrosine 45 and glutamic acid 9. The molecular basis for the differential binding of the P91A tum+ and tum- peptides was also examined. The P3 arginine residue of the tum+ peptide clashes unfavourably with the end and side walls of the highly hydrophobic D pocket preventing binding. In contrast, the P3 histidine residue of the tum- peptide makes a good set of contacts with the pocket and is complementary in both size and character. Molecular modelling of the P91A peptide thus provides complementary

data supporting P91A 15-22 as the P91A epitope and provides a molecular explanation for the differential binding of the tum- and tum+ forms of the peptide.

Finally, amino acid substitution analysis was used to help define residues within the P91A epitope which influence MHC binding and TCR interaction. This analysis reveals that positions 5, 6, 7 and 8 participate in MHC binding or are sufficiently close to the MHC groove to permit only limited amino acid diversity at these positions. Positions 1, 3 and 4 are critical for T cell recognition. The two T cell clones used in this study recognise different residues of the P91A epitope; positions 1, 3 and 4 are critical for clone 3B with position 4 being of major importance for clone P91.6. The P91A peptide would therefore appear to an example of a peptide where TCR contact residues are located towards the N-terminus and MHC contact residues towards the C-terminus. The finding that the peptide AQAAALDL (which has amino acid substitution of the C-terminal half) binds and stabilises surface L^d expression supports such a notion.

Interestingly, the L^d binding peptide LSPFDFDL identified as a T cell epitope for the L^d specific alloreactive T cell clone 2C is also an octamer (Udaka et al, 1992) and contains serine rather than the major motif residue proline at position 2. Of a number of described L^d binding peptides analysed (Solheim et al, 1993b), P91A and 2C were noted to alter the conformation of L^d as detected by the L^d specific monoclonal antibody, B22/249. This effect was attributed to the absence of proline at P2 and the lack of a proline induced conformational change in the α1 helix in the region of the L^d B pocket. Since 2C and P91A were the only two octamers in the group of six peptides analysed, it is possible that the length of the bound peptide is also a factor in determining the conformation of the B22/249 epitope, indeed the structure of HLA-B*3501 complexed with an octamer peptide reveals a substantial shift in part of the α2 helix (Smith et al, 1996b). Furthermore, in this study the authors also demonstrated that the positions of the amino and carboxy termini of the peptide were

non-standard yet maintained by water molecule mediated hydrogen bonding to conserved HLA-B*3501 heavy chain residues.

The crystal structure of H2-D^b reveals a hydrophobic ridge which crosses the binding cleft and causes a compensatory arch in the backbone of bound peptides (Young et al, 1994). The presence of this ridge is thought to set a lower limit of 9 amino acids on D^b binding peptides. L^d and D^b share the amino acids which contribute to the hydrophobic ridge (Trp-73 and Tyr-156) and hence octamer peptides might be expected not to form standard N- or C-terminal interactions within the L^d binding cleft. The finding that 7 mer peptide P91A 15-21 binds to L^d demonstrates the dispensability of P8 leucine and is consistent with an altered C- terminal interaction with L^d. The molecular model of L^d which was used was based on the crystal structures of HLA-A*0201, HLA-A*6801and HLA-B*2705. A molecular model based on the structure of H2-D^b would be of value in further evaluating the interaction of the P91A peptide with the H2-L^d molecule. The structure of the L^d-P91A complex and its potential involvement in T cell selection is further discussed in Chapter 6.2 and 6.3.

Figure 4.1: The sequences of P91A peptides used to help define the exact nature of the P91A epitope.

(A) Amino terminal truncated peptides

(B) Carboxy terminal truncated peptides and tum^+ form of P91A 15-24

(C) Amino acid substituted peptide variants

(D) P91A 15-22 position two variants and AQAAALDL which has amino acid substitutions of the first four residues.

Figure 4.1

A

P91A 12-24	I S T Q N H R A L D L V A
P91A 13-24	S T Q N H R A L D L V A
P91A 14-24	T Q N H R A L D L V A
P91A 15-24	Q N H R A L D L V A
P91A 16-24	N H R A L D L V A
P91A 17-24	H R A L D L V A
P91A 18-24	R A L D L V A

B

P91A 15-24	Q N H R A L D L V A
P91A 15-23	Q N H R A L D L V
P91A 15-22	Q N H R A L D L
P91A 15-21	Q N H R A L D
P91A 15-24(17R)	Q N R R A L D L V A

C

P91A 15-24(15A)	A N H R A L D L V A
P91A 15-24(16A)	Q A H R A L D L V A
P91A 15-24(17A)	Q N A R A L D L V A
P91A 15-24(18A)	Q N H A A L D L V A
P91A 15-24(19I)	Q N H R I L D L V A
P91A 15-24(20A)	Q N H R A A D L V A
P91A 15-24(21A)	Q N H R A L A L V A
P91A 15-24(22A)	Q N H R A L D A V A
P91A 15-24(23A)	Q N H R A L D L A A
P91A 15-24(24I)	Q N H R A L D L V I

D

P91A 15-22	Q N H R A L D L
P91A 15-22(16P)	Q P H R A L D L
P91A 15-22(16A)	Q A H R A L D L
P91A 15-22(16Q)	Q Q H R A L D L
	A Q A A A L D L

Figure 4.2: Stabilisation of L^d in the presence of P91A N- and C-terminal truncated peptides. (A) N-terminal truncated peptides (figure 4.1 A) and (B) C-terminal truncated peptides, (figure 4.1 B) were incubated for 18 hours at $37^0$with 2 x 10^5 P1.HTR cells/well in 96-well flat bottom microtiter plates. Following this period cells were harvested washed twice in ice cold PBS and stained for 30 minutes on ice with the FITC directly conjugated anti-H2-D^b/L^d specific monoclonal antibody 28-14-8S (ATCC HB-27), (Ozato et al, 1980). Cells were subsequently washed twice in ice cold PBS and analysed on a Beckton Dickinson FACScan using Cell Quest software. The tum^+ form of P91A 15-24 which has arginine (R) at position three and is an L^d non binder is included as a negative control in (B).

Figure 4.2

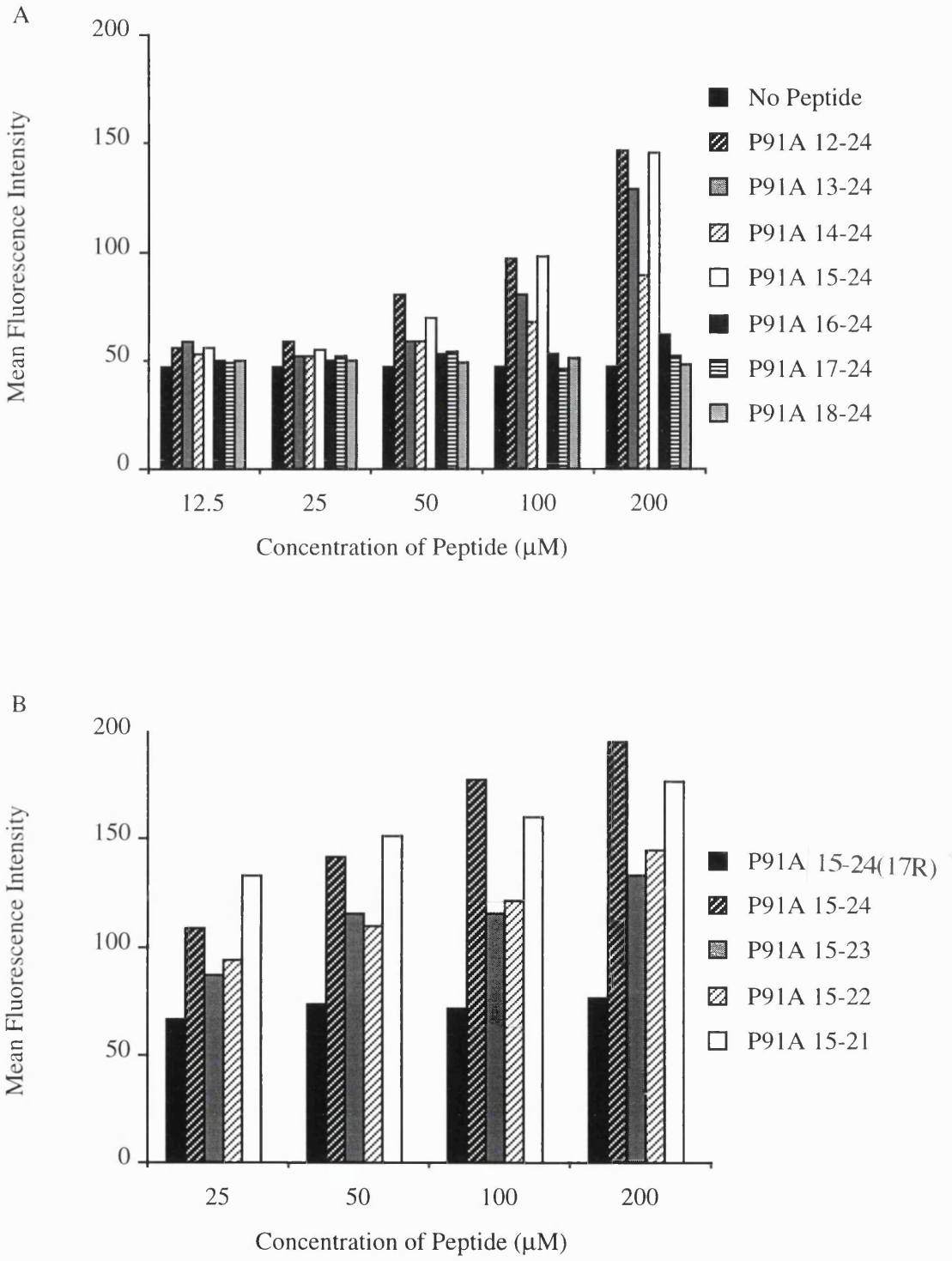

A
200
150
100
50
0
Mean Fluorescence Intensity
No Peptide
P91A 12-24
P91A 13-24
P91A 14-24
P91A 15-24
P91A 16-24
P91A 17-24
P91A 18-24
12.5
25
50
100
200
Concentration of Peptide (μM)
B
200
150
100
50
0
Mean Fluorescence Intensity
P91A 15-24(17R)
P91A 15-24
P91A 15-23
P91A 15-22
P91A 15-21
25
50
100
200
Concentration of Peptide (μM)

Figure 4.3: Stimulation of the P91A specific T cell clone P91.6 by N- and C-terminal truncated peptides. (A) Stimulation of P91.6 by P91A 12-24 and N-terminal truncated peptides, (figure 4.1A). (B) Stimulation of P91.6 by P91A 15-24 and C-terminal truncated peptides, (figure 4.1B). The tum^+ form of P91A 15-24 which has arginine (R) at position three is shown not stimulate P91.6.

Figure 4.3

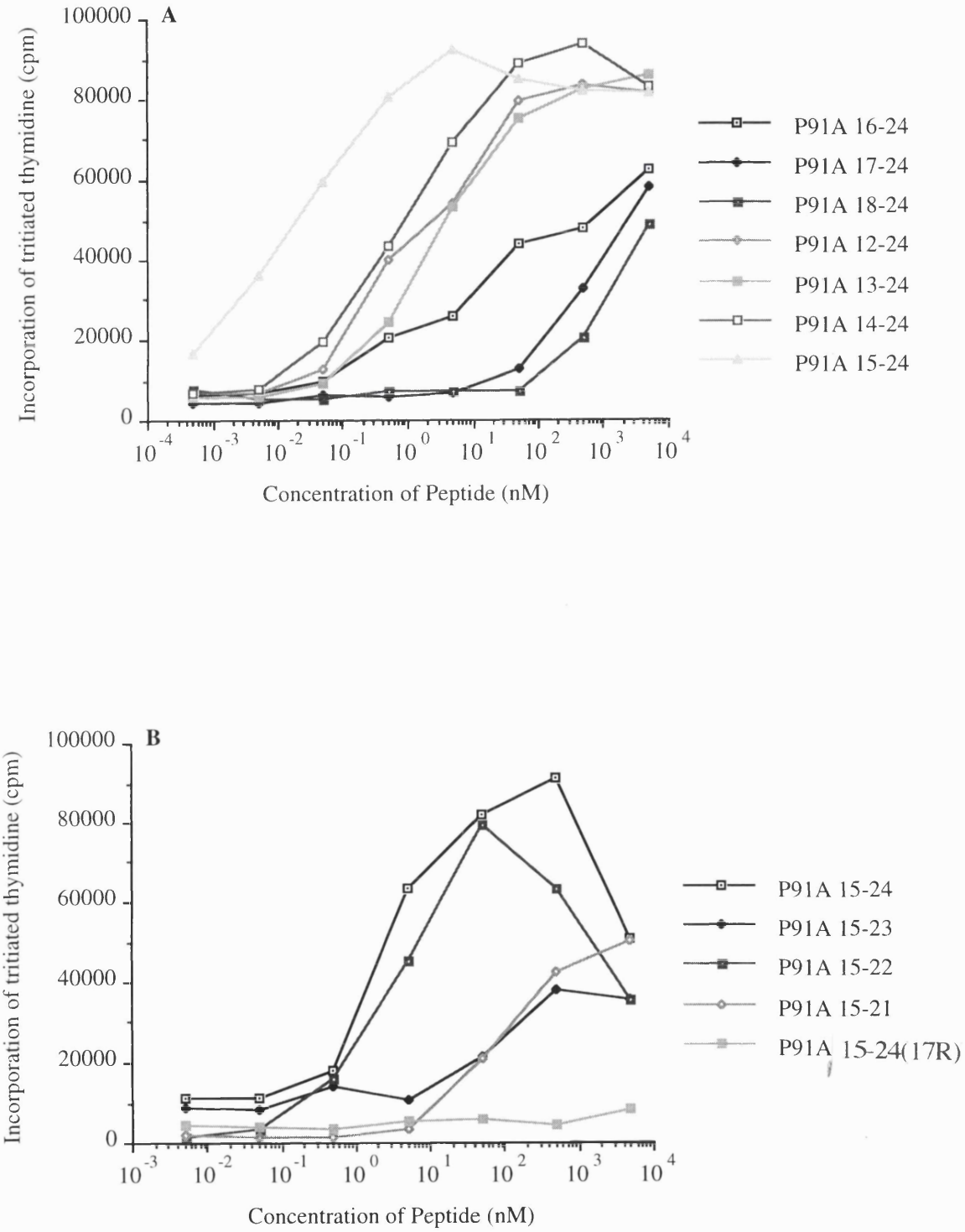

A
100000
80000
60000
40000
20000
0
Incorporation of tritiated thymidine (cpm)
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4}
Concentration of Peptide (nM)
P91A 16-24
P91A 17-24
P91A 18-24
P91A 12-24
P91A 13-24
P91A 14-24
P91A 15-24
B
100000
80000
60000
40000
20000
0
Incorporation of tritiated thymidine (cpm)
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4}
Concentration of Peptide (nM)
P91A 15-24
P91A 15-23
P91A 15-22
P91A 15-21
P91A 15-24(17R)

Figure 4.4

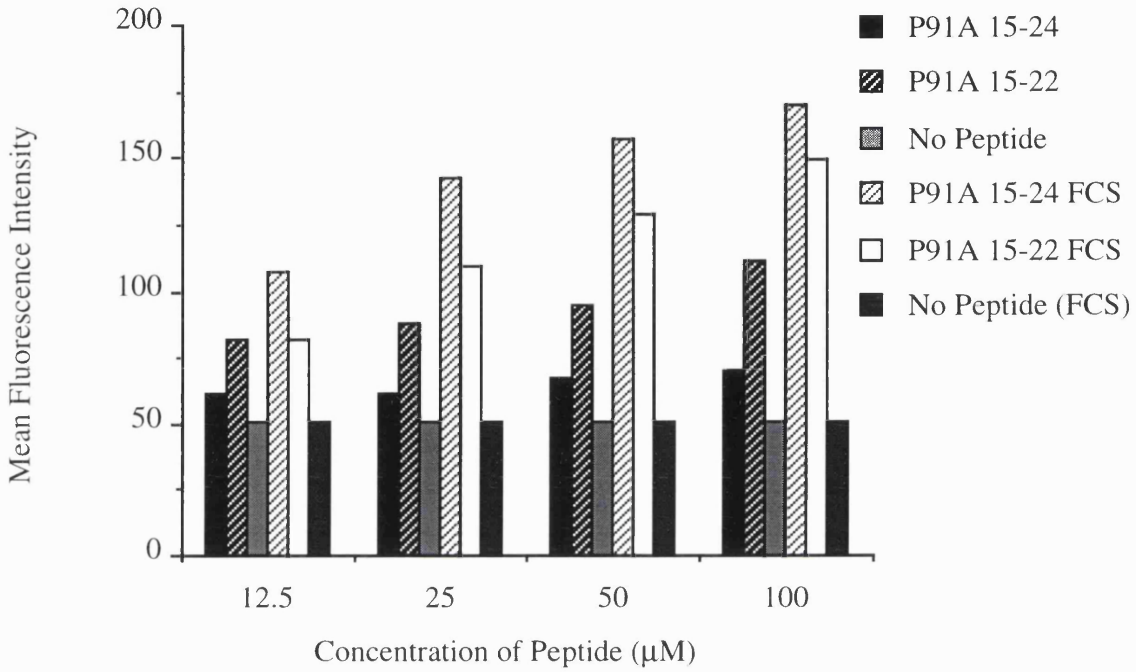


Figure 4.4: The involvement of extracellular processing in the generation of P91A L^d binding peptides. P1.HTR cells were incubated for 18 hours with P91A peptides 15-24 and 15-22 in RPMI supplemented with 10% FCS or the serum free complete medium HL1, (Hycor). After this period cells were harvested washed in ice cold PBS and stained with the monoclonal antibody 28-14-8S.

Figure 4.5

Mechanism of spontaneous cyclisation of N-terminal glutamine residues

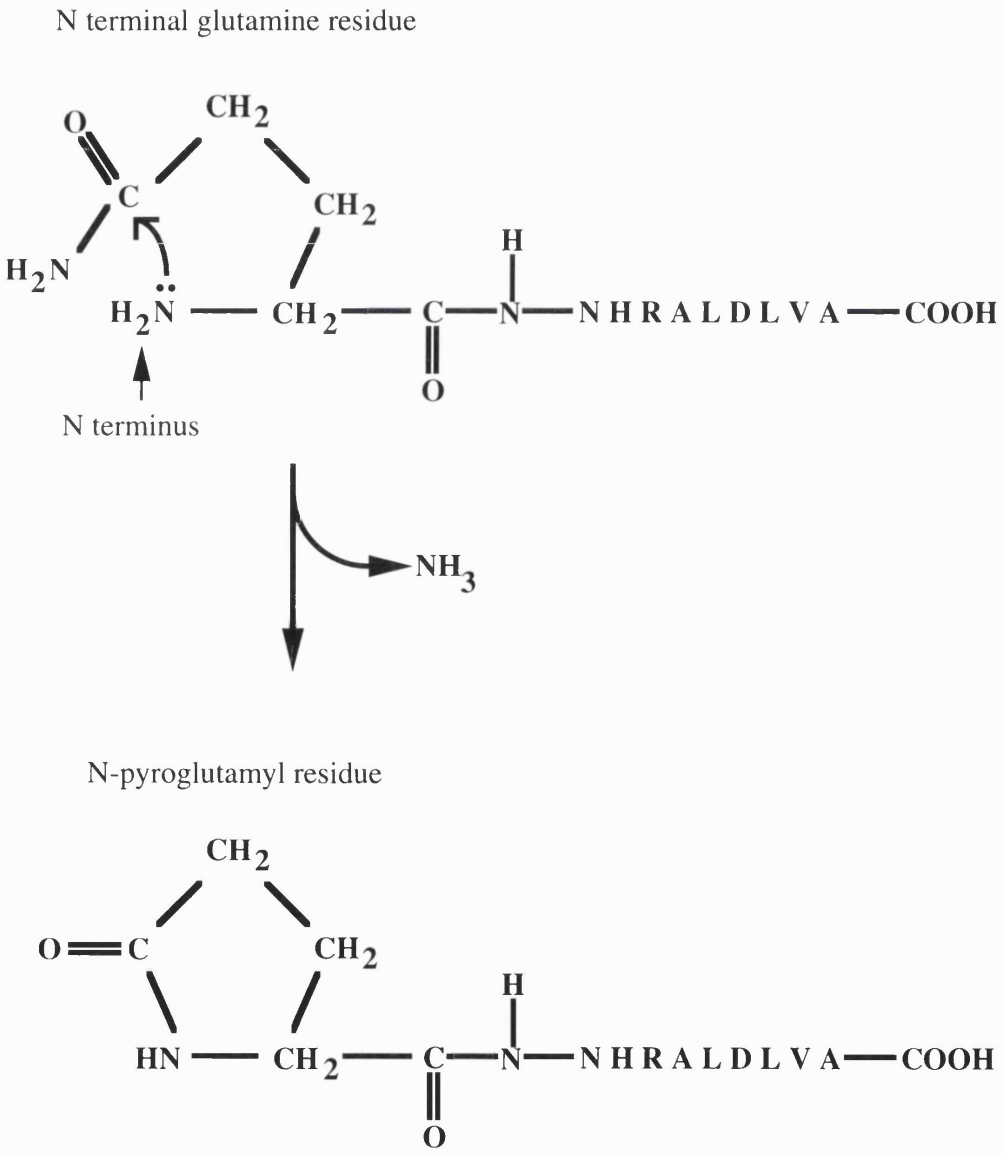


Figure 4.6: Stabilisation of surface L^d and stimulation of P91.6 by a P91A peptide with a non standard N-terminal residue. (A) Surface stabilisation of L^d by P91A 15-24, 15-22 and the pyroglutamyl form of P91A 15-24, (P91A 15-24-pyro). (B) Proliferation of P91.6 in the presence of P91A 15-24, 15-24 (17R) or P91A 15-24-pyro. The Q-1 peptide is a negative control peptide which fails to bind to L^d.

Figure 4.6

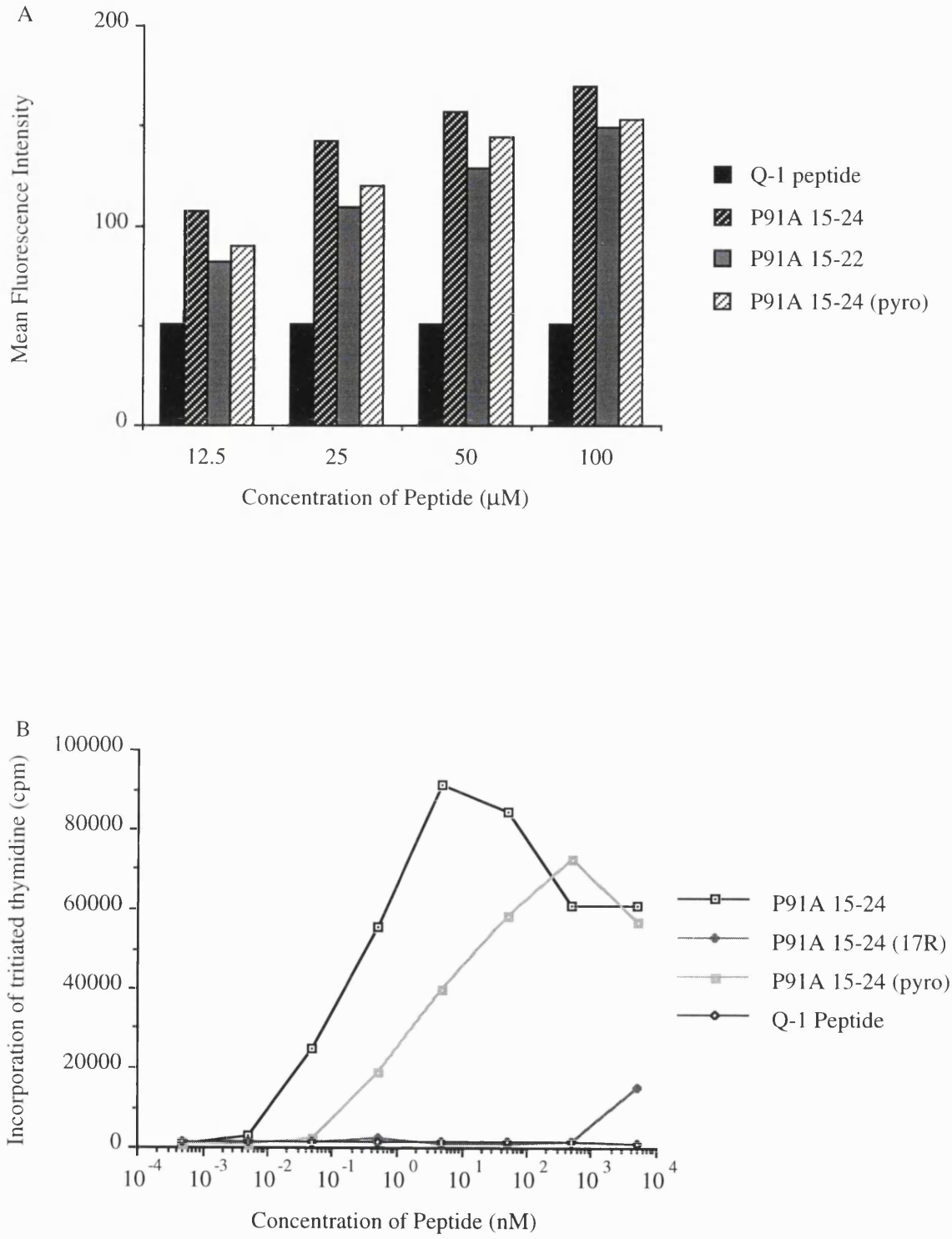
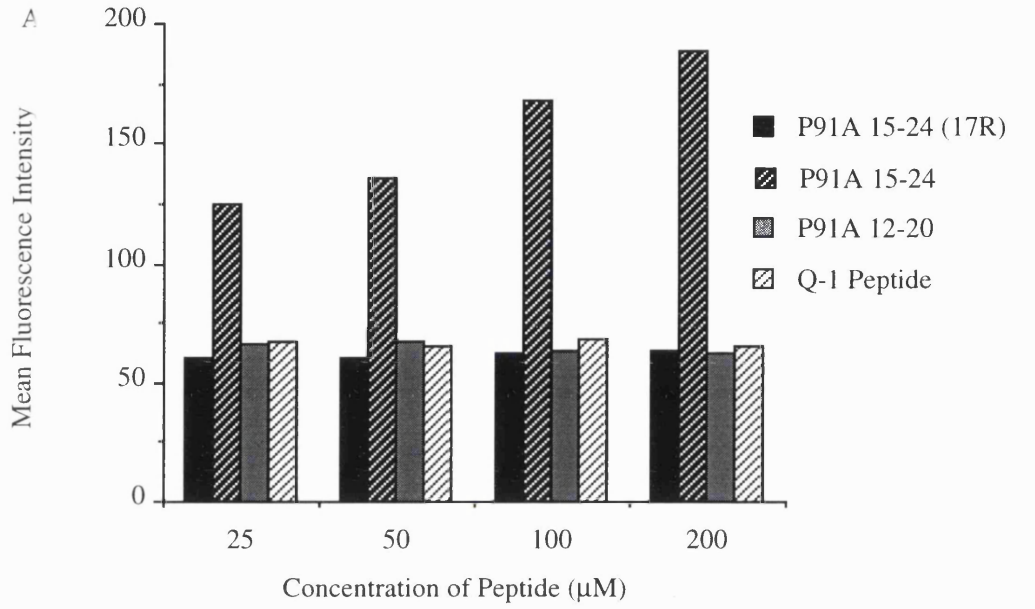

A
200
100
0
Mean Fluorescence Intensity
12.5
25
50
100
Concentration of Peptide (μM)
Q-1 peptide
P91A 15-24
P91A 15-22
P91A 15-24 (pyro)
B
100000
80000
60000
40000
20000
0
Incorporation of tritiated thymidine (cpm)
10 -4
10 -3
10 -2
10 -1
10 0
10 1
10 2
10 3
10 4
Concentration of Peptide (nM)
P91A 15-24
P91A 15-24 (17R)
P91A 15-24 (pyro)
Q-1 Peptide

Figure 4.7: A P91A peptide previously predicted to be the natural P91A epitope fails to bind to L^d or stimulate P91.6. (A) Surface stabilisation of L^d in the presence of P91A 15-24, P91A 15-24 (17R) or a predicted candidate P91A epitope, P91A 12-20, (Corr et al, 1992). The Q-1 peptide is a negative control L^d non binding peptide. (B) Stimulation of P91.6 with the same panel of peptides.

Figure 4.7


A
Mean Fluorescence Intensity
200
150
100
50
0
25
50
100
200
Concentration of Peptide (μM)
P91A 15-24 (17R)
P91A 15-24
P91A 12-20
Q-1 Peptide

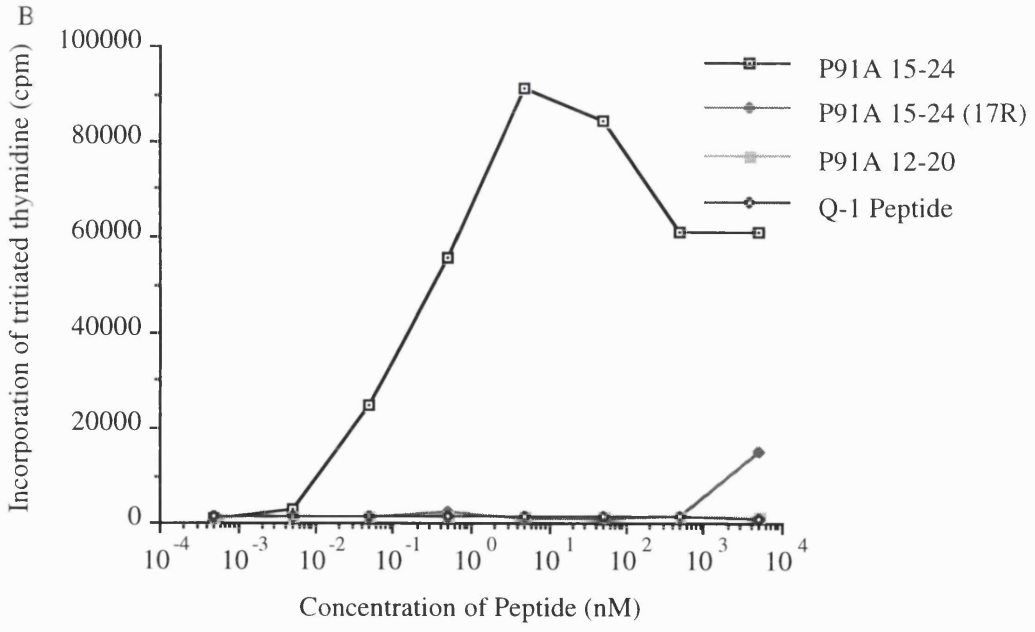

B
Incorporation of tritiated thymidine (cpm)
100000
80000
60000
40000
20000
0
10 -4
10 -3
10 -2
10 -1
10 0
10 1
10 2
10 3
10 4
Concentration of Peptide (nM)
P91A 15-24
P91A 15-24 (17R)
P91A 12-20
Q-1 Peptide

Table 4.1

Name	Sequence	Predicted Binding Energy (kcals/mol)
P91A 12-15	**I S T Q** N H R A L D L V A	-128.328
P91A 13-16	I **S T Q N** H R A L D L V A	+181.527
P91A 14-17	I S **T Q N H** R A L D L V A	-146.251
P91A 15-18	I S T **Q N H R** A L D L V A	-155.474
P91A 16-19	I S T Q **N H R A** L D L V A	+356.891
P91A 17-20	I S T Q N **H R A L** D L V A	+42.598
MCMV	**Y P H F** M P T N L	-135.779
P91A 22-24	I S T Q N H R A L D **L V A**	-5.912
P91A 21-23	I S T Q N H R A L **D L V** A	-5.763
P91A 20-22	I S T Q N H R A **L D L** V A	-12.524

Table 4.1: Predicted energies of binding of P91A N- terminal tetrapeptides and C-terminal tripeptides. N-terminal tetrapeptides and C-terminal tripeptides were modelled into the binding cleft of the L^d molecule. Following geometry optimisation a series of binding energies are obtained which reflect the interaction of the P91A peptidewith the L^d binding groove. Negative values reflect energy liberated on binding and positive values indicate additional energy which must be added to the system to promote binding. Molecular modelling was carried out in conjunction with Dr. Chris Thorpe and Dr. Paul Travers, Department of Crystallography, Birkbeck College, University of London.

Figure 4.8: Molecular modelling of P91A P2 and P3 residues into the L^d binding groove. P2 residues (A) asparagine (P91A 15-22 P2 residue), (B) proline (L^d P2 motif residue) and (C) serine (secondary L^d motif residue) modelled into the L^d B pocket. (D) P3 residue histidine (tum^- P3 residue) and (E) P3 residue arginine (tum^+ P3 residue) modelled into the L^d D pocket. Molecular modelling was carried out in conjunction with Dr. Chris Thorpe and Dr. Paul Travers, Department of Crystallography, Birkbeck College, University of London.

Figure 4.8

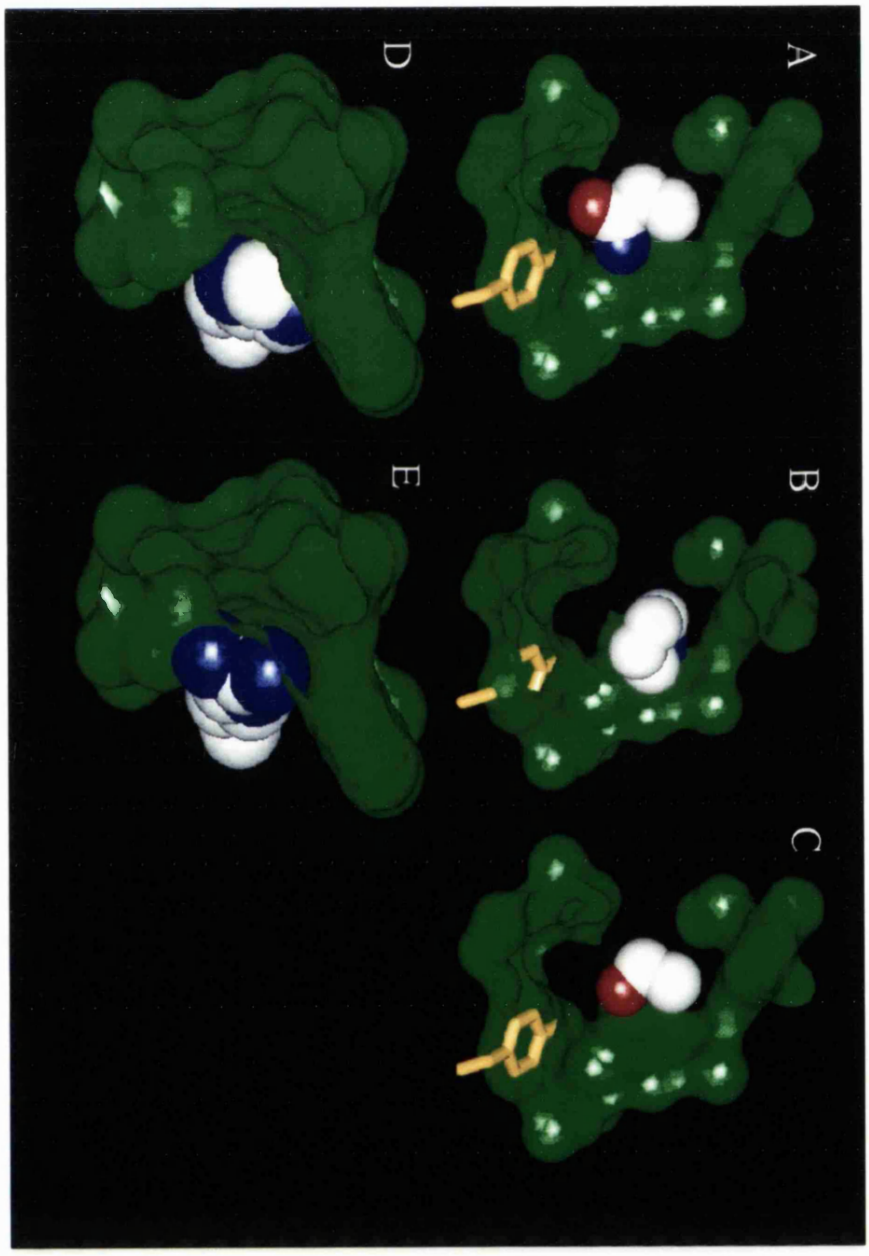


Figure 4.9: Definition of MHC and TCR contact residues within the P91A epitope.

(A) Stabilisation of surface L^d by amino acid substituted peptides.

(B) Proliferation of P91.6 in response to amino acid substituted peptides

(C) Proliferation of high affinity clone 3B in response to amino acid substituted peptides.

Residues in P91A 15-24 were serially substituted with alanine. Where an alanine residue was represented in the native P91A 12-24 peptide isoleucine was used as the substituting residue. Sequences of amino acid substituted variants are shown in figure 4.1C. P91A 15-22 (21A) was used in preference to P91A 15-24 (21A) due to increased solubility of the former peptide. The proliferative response of clone 3B to the L^d non binding peptide P91A 15-22 (21A) was not assessed.

Figure 4.9 A

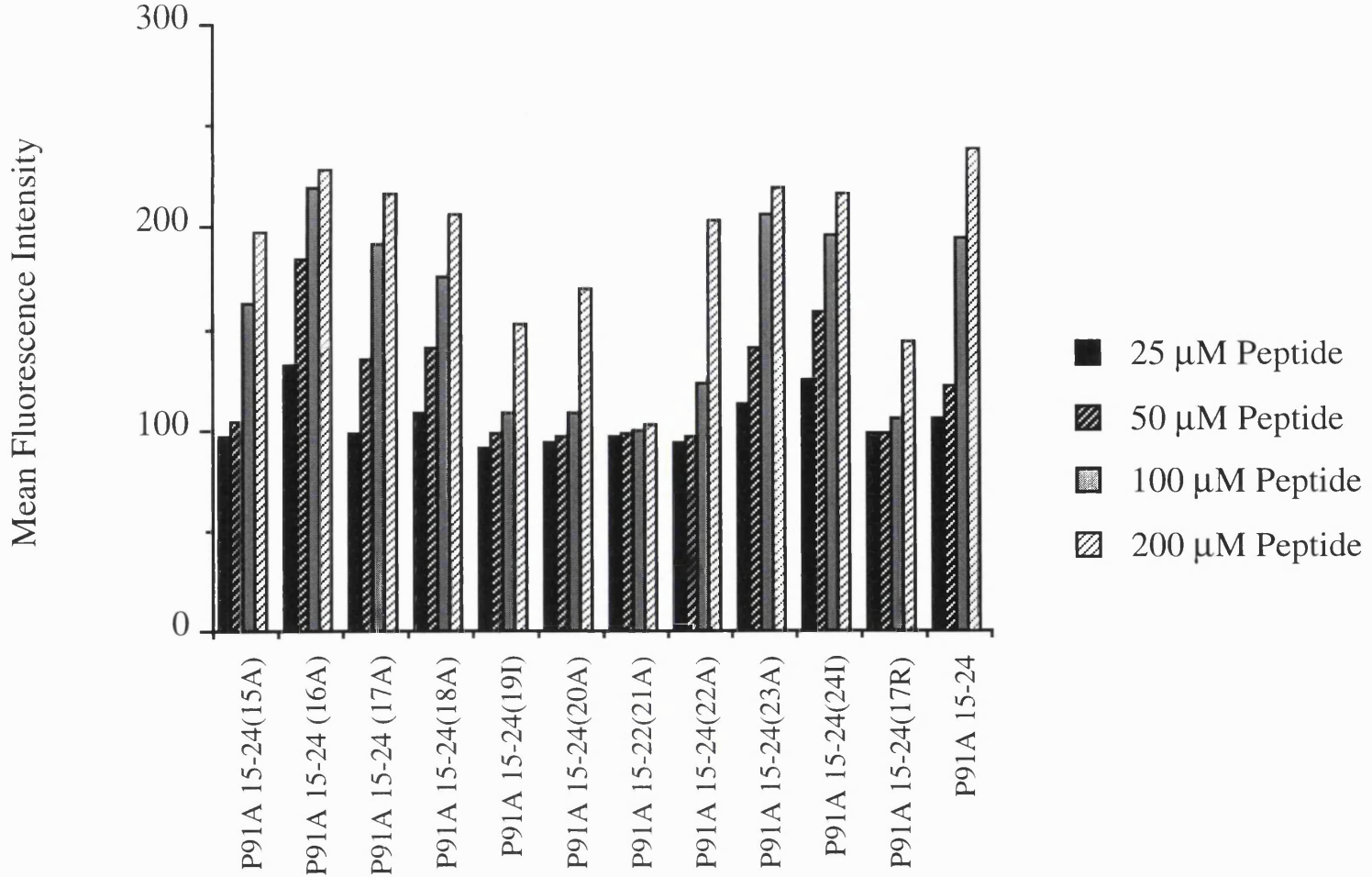

Mean Fluorescence Intensity
300
200
100
0
P91A 15-24(15A)
P91A 15-24 (16A)
P91A 15-24 (17A)
P91A 15-24(18A)
P91A 15-24(19I)
P91A 15-24(20A)
P91A 15-22(21A)
P91A 15-24(22A)
P91A 15-24(23A)
P91A 15-24(24I)
P91A 15-24(17R)
P91A 15-24
25 µM Peptide
50 µM Peptide
100 µM Peptide
200 µM Peptide

Figure 4.9 B

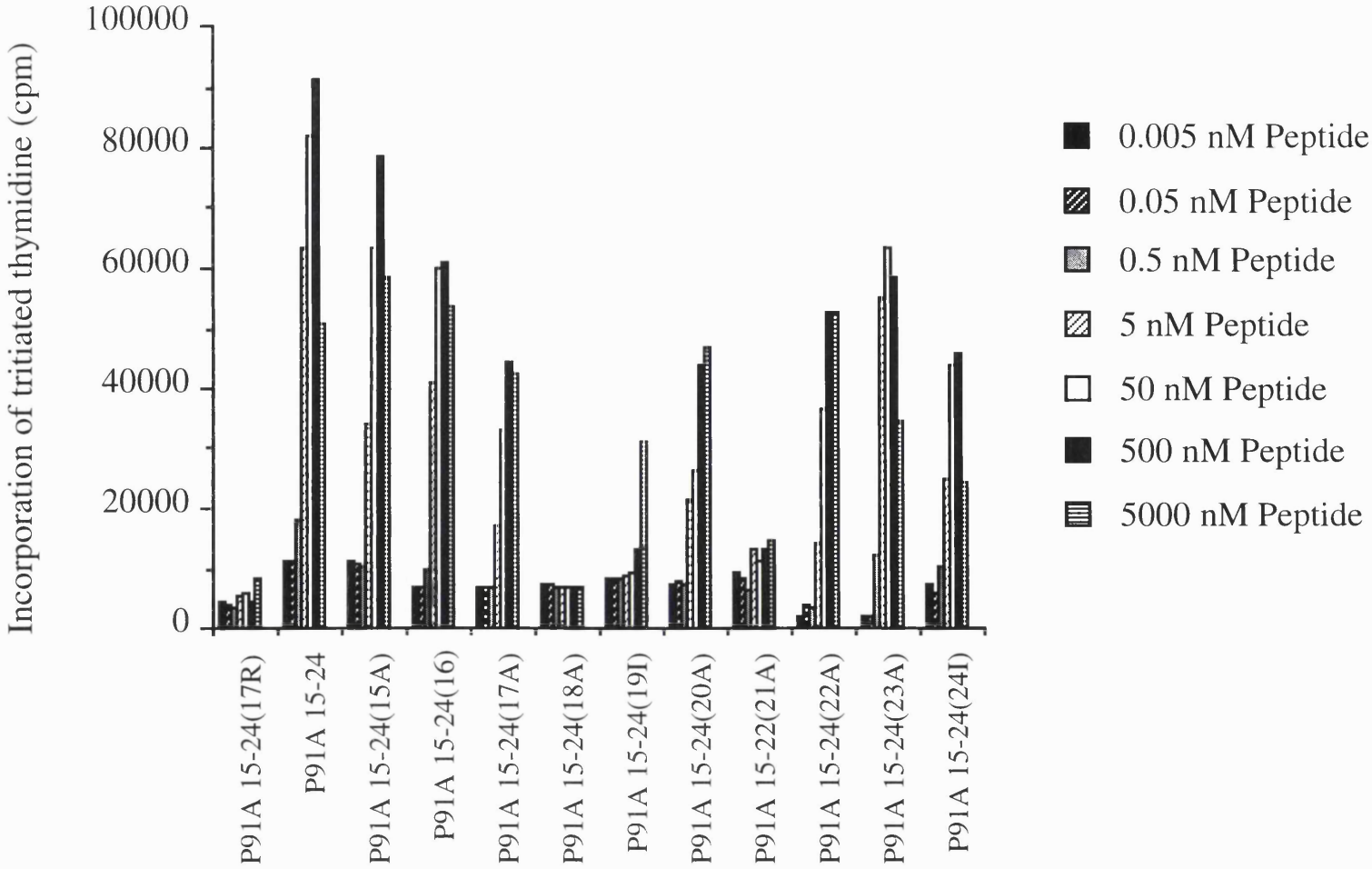

Incorporation of tritiated thymidine (cpm)
100000
80000
60000
40000
20000
0
P91A 15-24(17R)
P91A 15-24
P91A 15-24(15A)
P91A 15-24(16)
P91A 15-24(17A)
P91A 15-24(18A)
P91A 15-24(19I)
P91A 15-24(20A)
P91A 15-22(21A)
P91A 15-24(22A)
P91A 15-24(23A)
P91A 15-24(24I)
0.005 nM Peptide
0.05 nM Peptide
0.5 nM Peptide
5 nM Peptide
50 nM Peptide
500 nM Peptide
5000 nM Peptide

Figure 4.9 C

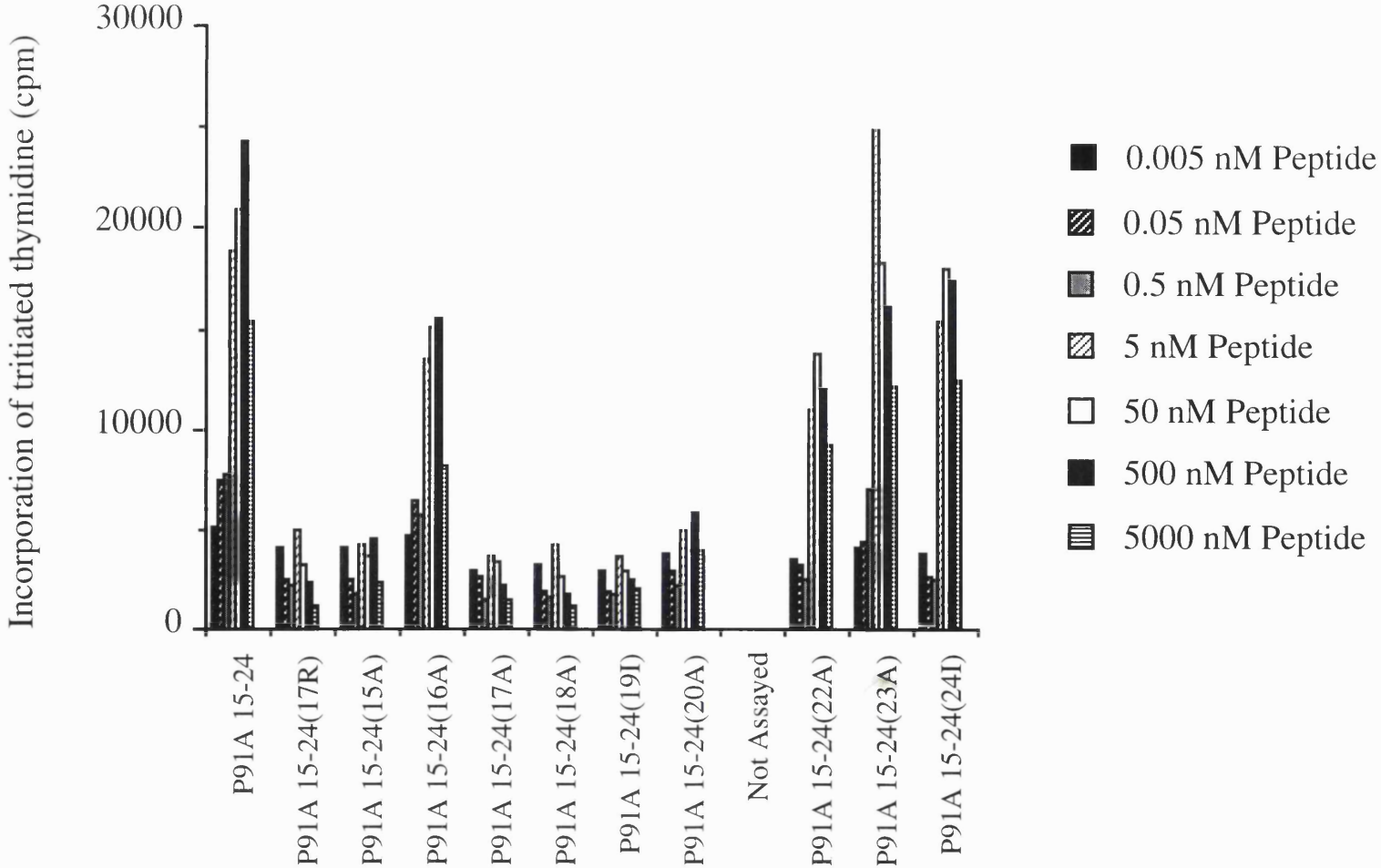

Incorporation of tritiated thymidine (cpm)
30000
20000
10000
0
P91A 15-24
P91A 15-24(17R)
P91A 15-24(15A)
P91A 15-24(16A)
P91A 15-24(17A)
P91A 15-24(18A)
P91A 15-24(19I)
P91A 15-24(20A)
Not Assayed
P91A 15-24(22A)
P91A 15-24(23A)
P91A 15-24(24I)
0.005 nM Peptide
0.05 nM Peptide
0.5 nM Peptide
5 nM Peptide
50 nM Peptide
500 nM Peptide
5000 nM Peptide

Table 4.2

The Definition of MHC and TCR contact residues within the P91A Epitope

Peptide	L^d Binding[a)]	T cell stimulation[b)] P91.6	3B
A N H R A L D L V A	+++	+++	-
Q **A** H R A L D L V A	++++	+++	+++
Q N **A** R A L D L V A	+++	+++	-
Q N H **A** A L D L V A	+++	-	-
Q N H R **I** L D L V A	-	-	-
Q N H R A **A** D L V A	-	+	+
Q N H R A L **A** L[c)]	-	-	ND[d)]
Q N H R A L D **A** V A	+	++	+++
Q N H R A L D L **A** A	+++	+++	+++
Q N H R A L D L V **I**	+++	+++	+++

a) MHC binding relative to P91A 15-24 (100μM); ++++ > 100%, +++ 70-100%, ++ 35-70%, + 5-35%, - <5%.

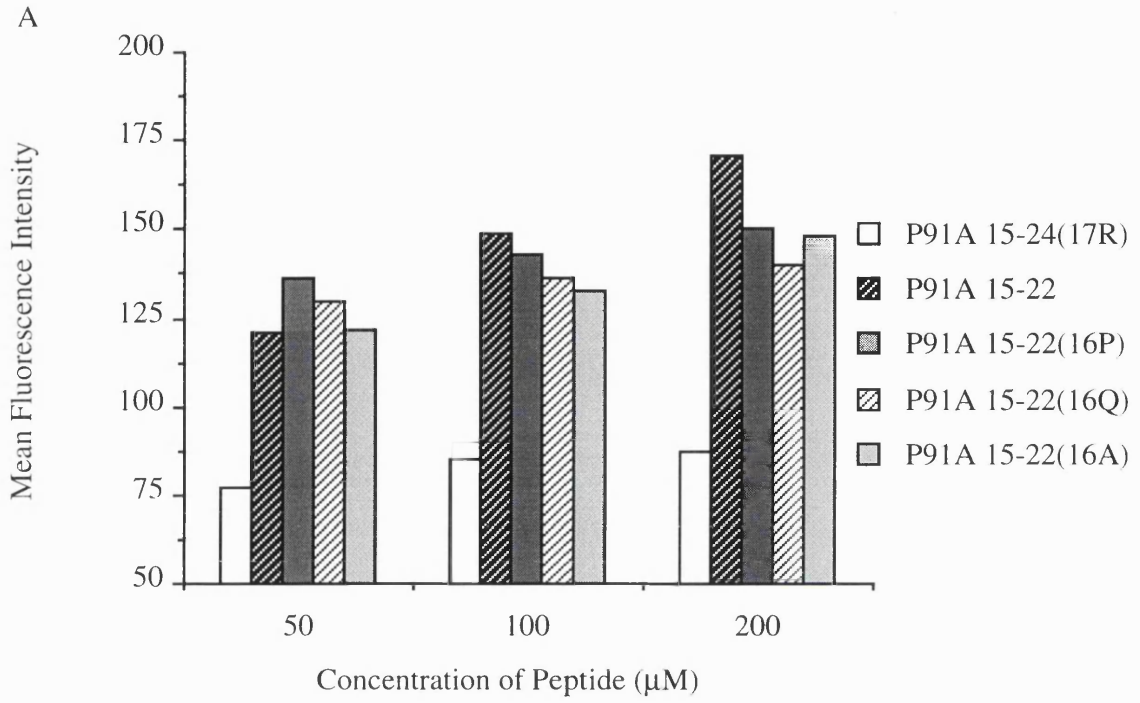
b) T cell stimulation relative to P91A 15-24 (60nM); +++ > 100%, ++ 35-70%, + 5-35%, - <5%.

c) Q N H R A L A L was used because Q N H R A L A L V A was insoluble.

d) ND: not done.

Figure 4.10: Stabilisation of L^d by P91A 15-22 P2 variants and a multiple amino acid substituted P91A 15-22 peptide variant. (A) Stabilisation of L^d on the surface of P1.HTR cells by P91A 15-22 variants which have proline, glutamine or alanine at position two, (figure 4.1D). (B) Stabilisation of L^d on the surface of L^d transfected RMA-S cells in the presence of P91A 15-24 (17R) (QNRRALDLVA), P91A 15-22 (QNHRALDL) or the variant peptide AQAAALDL which has alanine or glutamine substitutions of the first four residues.

Figure 4.10


A
Mean Fluorescence Intensity
200
175
150
125
100
75
50
50
100
200
Concentration of Peptide (μM)
P91A 15-24(17R)
P91A 15-22
P91A 15-22(16P)
P91A 15-22(16Q)
P91A 15-22(16A)

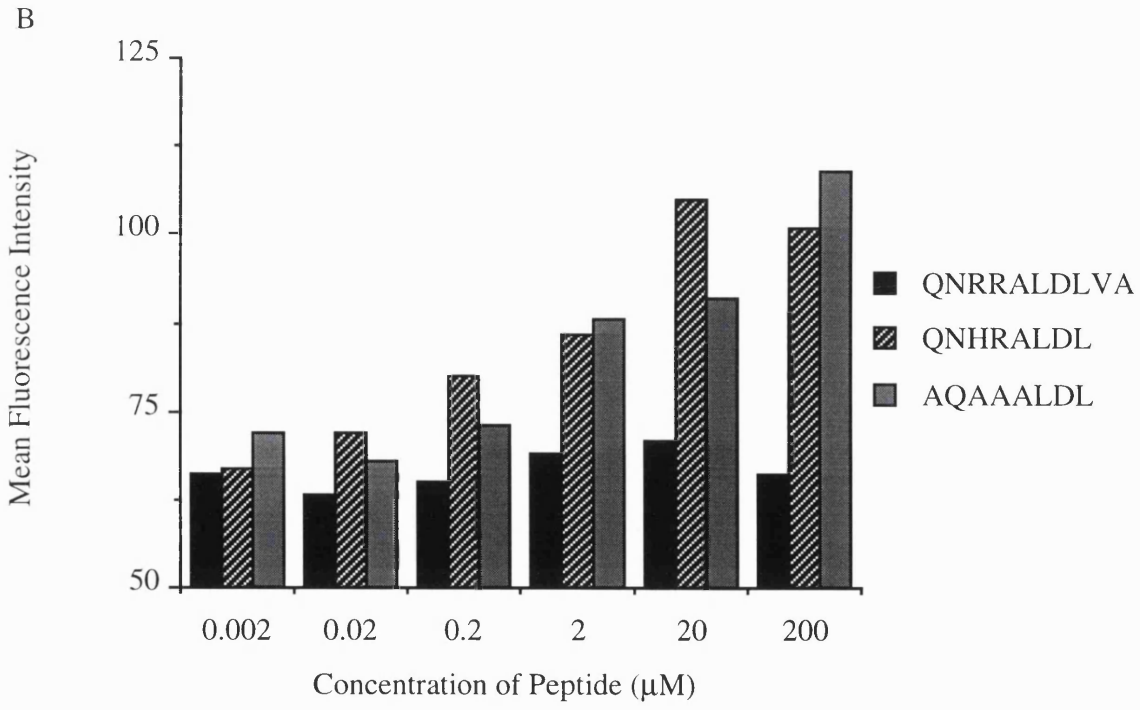

B
Mean Fluorescence Intensity
125
100
75
50
0.002
0.02
0.2
2
20
200
Concentration of Peptide (μM)
QNRRALDLVA
QNHRALDL
AQAAALDL

Figure 4.11: Co-elution of the naturally processed P91A peptide with the candidate P91A epitope P91A 15-22 (QNHRALDL). (A) Stimulation of P91.6 with HPLC separarted fractions of acid extracted peptides from P91A transgenic spleen cells. (B) The elution profile of the synthetic peptide P91A 15-22 indicates that the biological active material and synthetic peptide P91A 15-22 elute in the same fraction. Peptide elution was carried out by Dr. Hans Stauss, Department of Immunology, Imperial College of Science, Technology and Medicine, Hammersmith Campus, University of London.

Figure 4.11

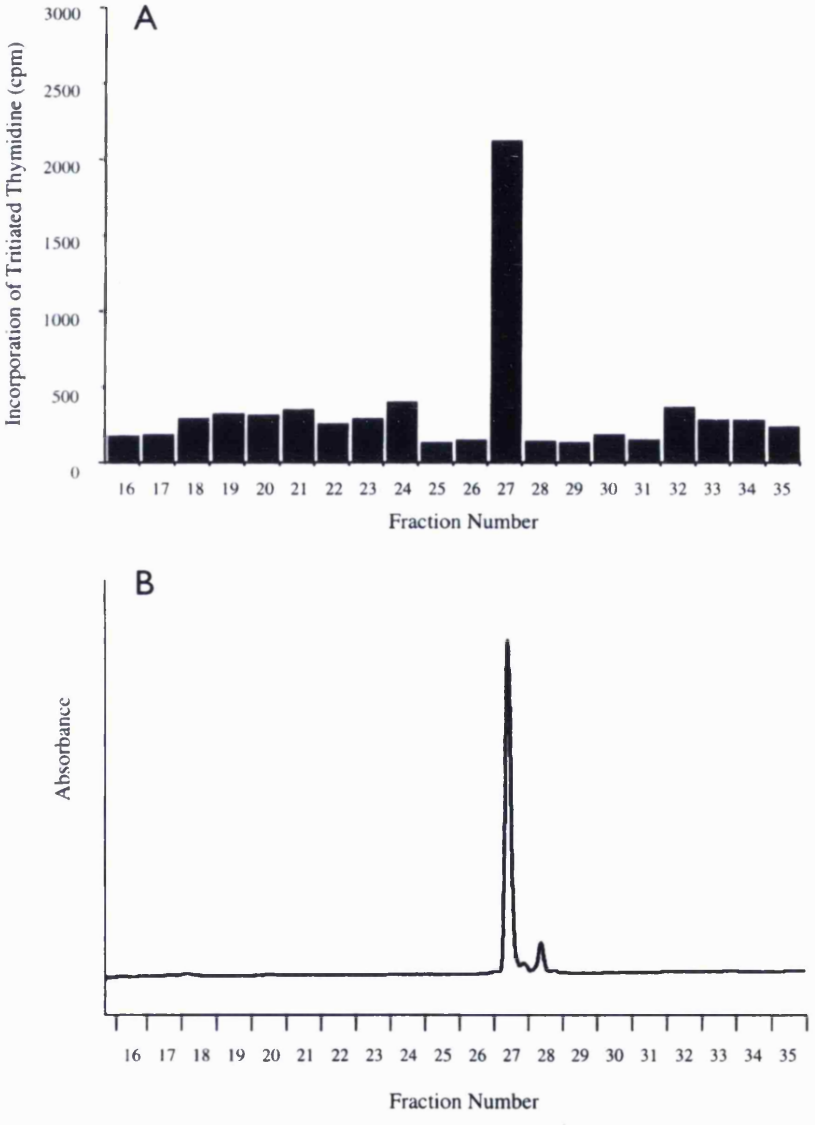
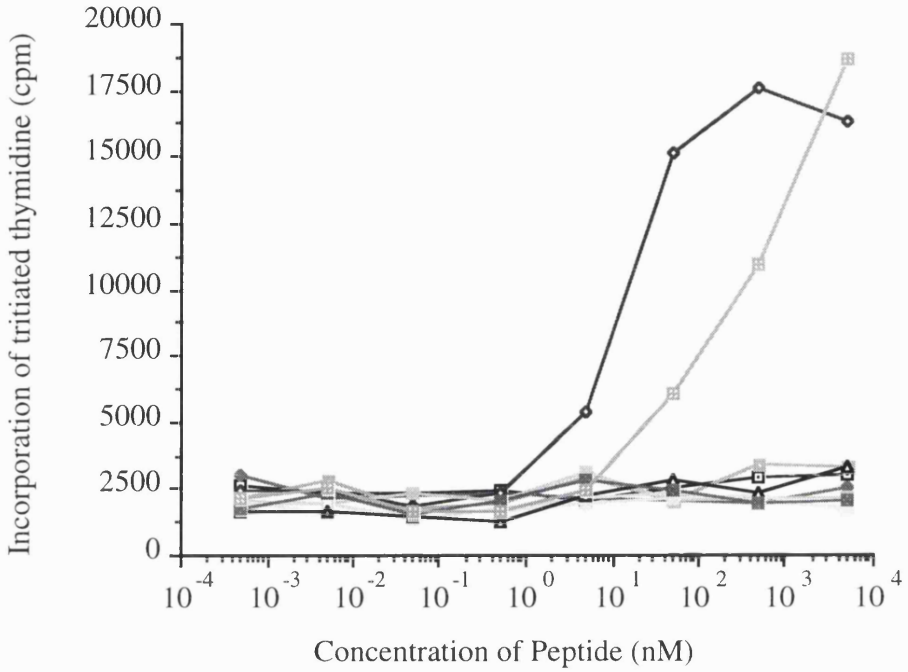

A
Incorporation of Tritiated Thymidine (cpm)
3000
2500
2000
1500
1000
500
0
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
Fraction Number
B
Absorbance
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
Fraction Number

Figure 4.12



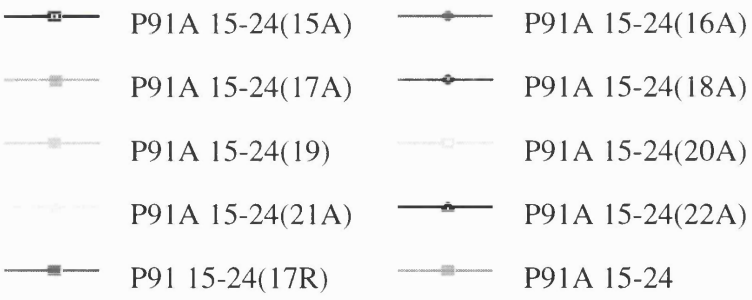


Figure 4.12: Stimulation of low affinity clone 4A with P91A 15-24 amino acid substituted peptide variants. Proliferation of clone 4A to the P91A variant P91A 15-24(18A) is 10-100 fold greater than to P91A 15-24.

Figure 4.13: Amino acid sequence of the CDR3 regions of P91A transgenic and non-transgenic clones and the P91.6 T cell clone. (A) CDR3α regions and (B) CDR3β regions. ▭ Residues encoded by TCR V segments. ▭ Residues encoded by J segments. ▭ Residues encoded completely by D segments. Unboxed residues are encoded by N-nucleotides and by codons composed of nucleotides derived from different germline segments. Residues are assigned to a germline segment if the nature of the amino acid is unequivocally determined by the first two germline encoded nucleotides. Amino acids in bold type are TCR framework residues.

Figure 4.13 A

Clone		Sequence of TCR α chain CDR 3 regions	
4A	TCRA V8 S13	**C A** L S D R G T G Y Q N F F **F G**	J41
4G	TCRA V4 S4	**C A** L Q G T G S K L S **F G**	J49
3B	TCRA V3 S7	**C A** V N T G Y Q N F Y **F G**	J41
3E	TCRA V3 S7	**C A** V N T G Y Q N F Y **F G**	J41
16	TCRA V4 S4	**C A** L G T G Y Q N F Y **F G**	J41
19	TCRA V3 S5	**C A** L S D Y S N N R L T **L G**	J7
P91.6	TCRA V18S $^{P91.6}$	**C A** Y N T N T G K L T **F G**	J21

Figure 4.13 B

Clone		Sequence of TCR β chain CDR 3 regions	
4A	TCRB V8 S3	**C A S** S D R N T E V F **F G**	J1 S1
4G	TCRB V1 S1	**C A S** S Q D R V E Q Y **F G**	J2 S6
3B	TCRB V10 S1	**C A S** R D R G L E Q Y **F G**	J2 S6
3E	TCRB V8 S3	**C A S** S D G N E Q Y **F G**	J2 S6
16	TCRB V8 S3	**C A S** S D G P T E V F **F G**	J1 S1
19	TCRB V8 S3	**C A S** S D G N E Q Y **F G**	J2 S6
P91.6	TCRB V8 S2	**C A S** G G G T G A Y N S P L Y **F A**	J1 S6

5.0 Intracellular processing of subgenic P91A protein fragments: the generation of the P91A epitope in the endoplasmic reticulum.

Aspects of central and peripheral tolerance to the P91A epitope have been examined using P91A transgenic mice (Chapter 2.2.1 and 2.2.2). T cells have been cloned which recognise the P91A epitope and have been shown to vary in their affinity for the P91A-L^d complex (Chapter 2.2.1). The nature of T cell receptors has been determined for both high and low affinity P91A specific T cell clones and attempts been made to generate a P91A specific low affinity TCR transgenic mouse (Chapter 3). Additionally, the exact nature of the P91A epitope which is generated *in vivo* has been determined and TCR and MHC contact residues defined (Chapter 4). The presented work therefore investigates aspects of P91A cellular immunology and continues through an examination of the T cell receptors involved in P91A recognition to the identification of the natural P91A epitope. In this chapter, aspects of P91A processing and epitope generation are investigated.

5.1 Introduction

It is generally accepted that the majority of MHC class I binding peptides are generated in the cytosol and translocated into the lumen of the ER where they associate with class I heavy chain/β_2m and are exported to the cell surface. This mechanism for generating class I binding peptides involves the systematic proteolysis of cytosolic proteins to generate peptides of optimal length for transport into the ER which retain the capacity to bind to MHC class I molecules.

The proteasome, in its various guises (20S core protein or associated with regulator complexes), has emerged as the proteolytic complex responsible for generating the majority of class I binding peptides (Chapter 1.2.1). Although much of the evidence implicating the role of the proteasome in antigen processing is indirect, recent

experiments using peptide aldehyde inhibitors of the proteasome suggest a central role for the proteasome in generating class I binding peptides (Rock et al, 1994 and Hughes et al, 1996). Unfortunately, peptide aldehyde inhibitors are not proteasome specific and have been shown to inhibit lysosomal cysteine proteases and calpains (Sasaki et al, 1990). More recently, the Streptomyces metabolite lactacystin has been shown to bind to the 20S proteasome and specifically inhibit proteasome activity (Löwe et al, 1995 and Fenteany et al, 1995). Lactacystin forms a covalent bond to the active site of the β subunit MB1 and in doing so inhibits both the trypsin-like and chymotrypsin-like activities of the proteasome (Fenteany et al, 1995). This inhibition results in a profound block in cytosolic antigen processing and presentation and confirms the importance of the proteasome in generating MHC class I binding peptides (Cerundulo et al, 1997). For further discussion of the proteasome's involvement in the generation of class I binding peptides see Chapter 1.2.

Although the proteasome is undoubtedly the major cytosolic protease involved antigen processing there is evidence to suggest that accessory proteases may be involved in the production of class I epitopes and that proteasome independent peptide production can occur. Much of the information concerning non-proteasome mediated proteolysis of antigens has been obtained by studying antigen processing in TAP deficient cell lines. A discussion of non-proteasome mediated generation of class I restricted peptides follows.

Export of mature MHC class-I -peptide complexes from the ER to the cell surface occurs only in the presence of suitable MHC binding peptides. The majority of these peptides are generated in the cytosol and translocated into the ER in a TAP1/2 dependant manner. TAP deficient cell lines T2 (human) and RMA-S (murine) fail to translocate peptides into the ER due to either complete absence of the TAP 1 and 2 molecules (T2) or the presence of a truncated TAP 2 product (RMA-S), (Momburg et al, 1992 and Yang et al, 1992). As a result of this block to peptide transport, TAP

deficient cell lines have low levels of surface MHC class I expression. Examination of peptides eluted from class I molecules present on the surface of RMA-S and T2 has revealed a very restricted set of peptides which are generated from the hydrophobic signal sequences responsible for mediating co-translational translocation of secreted and ER resident proteins into the ER (Henderson et al, 1992, Crowley et al, 1993 and Gilmore, 1993). Following translocation, the signal sequence, which does not form part of the mature protein, is removed by the ER resident enzyme, signal peptidase. Signal sequence peptides so formed are outwith the size range of class I binding peptides and must undergo further processing before optimal class I binding peptides are generated. The presence of class I bound signal sequence derived peptides in TAP deficient cell lines provides evidence for at least two types of proteolytic activity within the ER; signal peptidase and additional amino or carboxy peptidases responsible for peptide trimming.

Under normal circumstances MHC class I molecules are not predominately loaded with signal sequence derived peptides. One assumes that this is due to the competition of TAP transported peptides for binding to class I heavy chains. The loading of signal sequence derived peptides onto class I molecules may be of only minor importance and limited physiological value but serves to illustrate that non-proteasome proteolysis can in certain circumstances generate class I binding peptides. Furthermore, it illustrates that association of class I heavy chain with the TAP heterodimer is not an essential prerequisite for peptide binding. This mechanism for by passing the TAP transporter has been shown to be generally effective and has been used to target minimal T cell epitopes to the ER in TAP deficient cell lines (Anderson et al, 1991 and Elliott et al, 1995).

The liberation of class I epitopes from signal sequences is likely to involve limited proteolytic activity (signal peptidase, amino and carboxy peptidases). However, evidence has gradually accumulated which suggests that ER resident proteases may

be capable of more complex antigen processing. Both the tum$^-$ antigen P91A and the bacterial enzyme β-galactosidase when targeted to the ER are processed and presented as peptides to cytotoxic T cells (Rammensee et al, 1989 and Godelaine et al, 1993). Furthermore, the HIV envelope protein (gp120), which is subject to co-translational translocation into the ER, is processed and presented to CD8+ CTL in TAP deficient (T2) cells (Hammond et al, 1993). These studies suggest that more extensive proteolytic activity may exist within the ER and that this activity may be capable of generating epitopes from ER resident proteins.

A recent examination of antigen processing in T2 cells has provided further evidence for extensive proteolysis in the ER. In this study, an amino-terminal extension of 40 amino acids and a carboxy-terminal extension of 120 amino acids could be removed from an ER targeted carboxy terminal fragment of the Influenza A nucleoprotein to generate a D^b restricted epitope (Elliott et al, 1995). The proteases involved in the processing of the flu nucleoprotein fragment were not identified but were not inhibited by peptide aldehyde inhibitors or the proteasome specific inhibitor lactacystin (Cerundulo et al, 1997). Interestingly, the peptide aldehyde inhibitor N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) has been previously shown to block the trimming of signal sequence peptides in the TAP deficient cell line .174, resulting in decreased cell surface expression of HLA-A2 (Hughes et al, 1996). The failure of LLnL to inhibit degradation of the flu nucleoprotein fragment suggests that alternative ER resident proteases are involved in this process.

It is likely that the trimming of peptides in the ER is an integral part of antigen processing, since the proteasome generates and the TAP complex transports peptides which can be 17 amino acids or more in length (Momburg et al, 1994a, Schumacher et al, 1994a, Kuckelkorn et al, 1995 and Yang et al, 1996). The fate of peptides which are present in the ER very much depends on whether they associate with class I heavy chain or not. Translocated peptides which do not bind to class I molecules can be

quickly returned to the cytosol where they can be degraded or transported back into the ER (Roelse et al, 1994). Those peptides which fail to bind to class I molecules and are not recirculated to the cytosol may undergo further processing in the ER. The nature and efficacy ER resident proteases is unclear. However, one might predict that the judicious activity of amino and carboxy peptidases would be of benefit in generating optimal class I binding peptides from longer peptides which have been translocated into the ER.

Very little is known about ER resident proteases and their role in antigen processing. Leader peptidase may have a specific and restricted role to play in antigen processing but is unlikely to be involved in more extensive proteolysis. Serine, cysteine and calcium dependent proteases have all been identified in association with the ER (Wikstrom et al, 1991, Tsao et al, 1992 and Wileman et al, 1993) and have been shown to be involved in the degradation of incompletely assembled or misfolded proteins and glycoproteins (Wileman et al, 1990). This is of interest since most of the studies investigating antigen processing in the ER have examined the fate of aberrantly expressed truncated chimeric proteins. Furthermore, proteins which are inappropriately expressed in the ER have been shown to have half-lives in the order of minutes, even though they are relatively stable when expressed in their native compartment (Stoller et al, 1989). Chimeric test antigens which are targeted to the ER are therefore likely to be susceptible to proteolysis and targeted degradation. This may facilitate epitope generation and give a false impression of the capacity of ER resident proteases to generate epitopes from correctly folded full length glycosylated proteins.

The role of glycosylation in protecting proteins from degradation in the ER remains unclear. Glycoprotein derived CTL epitopes have been described but the role of ER proteases in their generation is unclear. Epitopes from influenza haemaglutinin or measles F protein, both of which are ER targeted glycosylated proteins, are generated

only in the presence of a functional TAP complex (Elliott et al, 1995; van Binnendijk et al, 1992). This suggests that ER targeted proteins may escape into the cytosol and be subject to proteasome mediated proteolysis. Furthermore, in the absence of a functional TAP complex ER proteolysis would seem to be incapable of generating the small amount of peptide required to sensitise cells for CTL mediated kill. In a parallel set of experiments Elliott and colleagues show that the D^b restricted influenza $NP_{366-374}$ epitope cannot be generated from full length ER targeted flu-nucleoprotein whereas truncated forms of flu NP which lack potential glycosylation sites are processed in the ER to generate the CTL epitope (Elliott et al, 1995). Glycosylated proteins may therefore be sheltered from the class I associated processing pathway by their limited exposure to cytosolic proteases and their relative resistance to proteolysis in the ER.

Taken together, these data show that the ER contains multiple proteases which are capable, in certain circumstances, of generating antigenic peptides from larger proteins and protein fragments.

5.1.1 Processing and presentation of the P91A epitope

Previous experiments have shown that cells transfected with promoterless fragments of the P91A gene can generate the P91A epitope and are lysed by P91A specific CTL (De Plaen et al, 1988; Chomez et al, 1992). The same observation has been made for two other genes coding for tum^- antigens (Szikora et al, 1990; Sibille et al, 1990). These data suggest that in certain circumstances transfected subgenic fragments of DNA can be transcribed under the influence of a resident promoter located near the site of fragment insertion. Transcription initiated by a resident promoter is likely to result in the production of a chimeric RNA message (part resident gene, part transfected gene). If the transfected fragment of DNA contains 3' intronic sequence and integrates into an intron of a resident gene then it is possible that RNA splicing to

the splice acceptor site of the transfected fragment may occur. This may result in the production of an in frame message which can be translated into a chimeric protein and enter the class I processing pathway.

Although this is one method through which promoterless fragments of DNA can be transcribed and translated it cannot account for the transcription and translation of transfected subgenic DNA fragments which lack splice acceptor sites. When this issue was examined using P91A subgenic fragments it was observed that local translation could be initiated at an ATG codon located 18 bases 3' of the P91A epitope, figure 5.1 A (Chomez et al, 1992). While this does not address the issue of transcription initiation it does suggest that translation initiation can occur at internal ATG codons present in subgenic DNA which is integrated into a resident gene; whether this integration is in frame with the reading frame of the resident gene or not. This finding provides support for the notion that start codons located near to the start of sequences coding for antigenic peptides can initiate translation. More specifically, it indicates that the ATG codon located at position 9 of P91A exon 4 is capable of initiating translation and that the resultant truncated protein fragment can be processed to generate the P91A epitope. The role of internal potential start codons in initiating translation under normal conditions is unclear. If it occurs then it is likely to be an inefficient process. Having said this, it has been suggested that a single MHC-peptide complex can sensitise target cells for CTL mediated kill (Sykulev et al, 1996)). If this is the case then an inefficient process may be all that is required to generate sufficient epitope to sensitise cells.

As discussed in section 5.1, the role and extent of antigen processing in the ER is unclear. Nevertheless, constructs which target P91A to the ER as part of either secreted or ER resident proteins have been shown to sensitise target cells for CTL mediated lysis (Godelaine et al, 1993). In these experiments constructs were transfected into P1.HTR cells which have functional TAP 1 and 2 molecules (Van Pel

et al, 1985). The role of processing in the cytosol and import of peptides into the ER could therefore not be fully assessed. Furthermore, the potential role of the internal start codon ATG9 in translation and production of a cytosolic truncated product was not considered.

5.2 Results and discussion

5.2.1 The generation of the P91A epitope from ER targeted and cytosolic truncated P91A protein fragments.

To further assess whether processing in the ER is capable of generating the P91A epitope a series of constructs based upon P91A exons 4 and 5 were engineered, figure 5.1B. All construct fragments were generated by PCR amplification of P91A cDNA and H2-K^k genomic DNA and cloned into the eukaryotic expression vector pcDNAI (Invitrogen). Constructs were manually sequenced prior to use.

Constructs A and B consist of P91A exons 4 and 5 preceded by the H2-K^k ER signal sequence and followed by the c-myc tag coding sequence (Evan et al, 1985). Constructs C and D consist of P91A exons 4 and 5 followed by the c-myc tag coding sequence. Constructs A and C retain the ATG9 internal start codon which is 5' of the P91A epitope, figure 5.1A. Constructs B and D have had ATG9 replaced with ACG9. Constructs A and B encode a 148 amino acid protein which is targeted to the ER (hereafter referred to as P91A(A) and P91A(B)). Constructs C and D encode a 122 amino acid protein which is released into the cytosol (hereafter referred to as P91A(C) and P91A(D)) Translation initiated at ATG9 of constructs A and C may result in the production of a truncated protein product which is 113 amino acids in length and is not targeted to the ER. All constructs have a c-myc tag at the carboxy terminus which can be detected using the monoclonal antibody 9E-10 (Evan et al, 1985; Campbell et al, 1992).

Constructs A-D and the control plasmid pcDNAI were transiently transfected into the recipient cell line P1.pyt. P1.pyt is a stable transfectant of P1.HTR which expresses the polyoma large T antigen (Scott et al, 1992). Polyoma large T antigen induces episomal replication of pcDNA I and therefore increase the level of construct encoded protein expressed in transfected cells. Transfectant populations were cultured for 72 hours after which their ability to stimulate the P91A specific T cell clone P91.6 was assessed. As shown in figure 5.2, all four constructs appear to result in epitope generation as assessed by proliferation of P91.6. Interestingly P1.pyt cells transfected with constructs A and B are as effective at stimulating P91.6 as those transfected with constructs C and D. These data support the notion that the P91A epitope can be efficiently processed out of a larger protein fragment in the endoplasmic reticulum. The finding that P1.pyt cells transfected with construct B stimulate P91.6 as effectively as those transfected with construct A, indicates that internal initiation of translation is not required for epitope generation and that substitution of methionine with threonine does not affect epitope generation.

Although these results are suggestive of processing in the ER the involvement of processing in the cytosol has not been excluded. It is possible that the construct encoded protein levels following episomal replication are in excess of that which the cell can transport efficiently into the ER. If this is the case, then the ER targeted proteins P91A(A) and P91A(B) may leak into the cytosol, be degraded by the proteasome and be transported via TAP1/2 into the ER. The involvement of processing in the cytosol was addressed by transfecting constructs P91A(A)-(D) into an RMA-S cell line expressing H2-L^d and polyoma large T antigen (RMA-S-L^d.pyt).

5.2.2 Stable transfection of RMA-SL^d with polyoma large T antigen

The RMA-S cell line has a point mutation in the TAP2 gene resulting in a premature stop codon (Yang et al, 1992). As a result of this defect the TAP1/2 heterodimer fails

to form and transport of antigenic peptides into the ER is severely restricted. This defect can be overcome by directly targeting antigenic peptides and in certain cases longer proteins to the ER (Anderson et al, 1992, Bacik et al, 1994, Elliott et al, 1995). RMA-SL^d is a derivative of RMA-S which has been transfected with the L^d gene under its own regulatory elements (Udaka et al, 1992). This cell line was a kind gift from Dr. K. Udaka. Although transfection conditions for RMA-S have been published (Udaka et al, 1992) the electroporation equipment available in our laboratory did not permit exact replication of the conditions used.

In order to establish optimal conditions for transfection, RMA-S L^d cells were electroporated at various voltages (constant capacitance 125μF) with the chloramphenicol acetyl-transferase (CAT) plasmid (pcDM8-CAT). After a period of 72hrs culture, transfection efficiency was assessed by standard CAT assay. As shown in figure 5.3A, transfection of RMA-S L^d at 200, 250 and 300 volts fails to result in detectable enzyme activity. Optimal transfection conditions have been established for P1.HTR (Scott et al, 1992). In the present study transfection of P1.HTR cells with pcDM8-CAT using these optimised conditions also fails to result in detectable enzyme activity. In contrast, transfection of P1.pyt using identical conditions results in easily detectable enzyme activity as shown by the formation of both the mono-acetylated variants of chloramphenicol, figure 5.3A. The failure to detect enzyme activity in pcDM8.CAT transfected P1.HTR makes it possible that a low level of enzyme activity may also be undetectable in RMA-S L^d transfected cells.

In an attempt to overcome this potential problem RMA-S L^d and P1.HTR were co-transfected with pcDM8-CAT and polyoma large T antigen. Polyoma large T antigen should drive episomal replication of pcDM8-CAT in cells which take up both plasmids. RMA-S L^d cells were transfected at voltages ranging from 200-450 volts (constant capacitance 125μF). P1.HTR and P1.pyt were transfected using previously defined optimal conditions; 350 volts and 125μF. As before, transfection of P1.pyt

with pcDM8-CAT results in detectable enzyme activity and the production of both mono-acetylated forms of chloramphenicol, figure 5.3B. Additionally, co-transfection of pcDM8-CAT and polyoma large T antigen into P1.HTR cells now results in weak but detectable enzyme activity. These data suggest that co-transfection of polyoma large T antigen with pcDM8-CAT results in increased enzyme production in co-transfected cells and hence increases the sensitivity of the assay. An examination of the transfection conditions for RMA-S L^d now reveals that optimal transfection occurs at 250-300 volts.

In order to create a TAP deficient recipient cell line capable of supporting episomal replication, RMA-S L^d was co-transfected with polyoma large T antigen and the hygromycin resistance gene. Stable transfectants were selected, cloned and expanded in the presence of hygromycin. The expression of polyoma large T antigen was indirectly assessed by CAT assay, figure 5.4. RMA-S L^d transfectants 19 and 20 display associated enzyme activity which is equivalent to or greater than that of CAT transfected P1.pyt cells. Transfectant 15 displays no associated enzyme activity and is likely to have integrated the hygromycin resistance gene in the absence of polyoma large T antigen. RMA-S L^d transfected with pcDM8-CAT displays no associated enzyme activity. These data indicate that RMA-S L^d.pyt transfectants 19 and 20 have stably integrated and express polyoma large T antigen. Transfectant 19 was chosen as the recipient cell line for the transient transfection of P91A constructs A-D.

5.2.3 Generation of the P91A epitope in the TAP deficient cell line RMA-S L^d.pyt

P91A Constructs A-D and control plasmid pcDNA I were transiently transfected into RMA-S L^d.pyt. Following 72 hours of culture the ability of transfectants to stimulate the T cell clone P91.6 was assessed. As shown in figure 5.5, all transfectants appear

to generate the P91A epitope and stimulate proliferation P91.6 T cell clone. This is an interesting if unpredicted result.

The proliferation of P91.6 to P91A(A) and P91A(B) transfectants is strong evidence that processing of P91A(A) and P91A(B) occurs in the endoplasmic reticulum. Following removal of the H2-K^k signal peptide, the P91A epitope lies 14 amino acids from the amino terminus and 97 amino acids from the carboxy terminus of P91A(A) and P91A(B). Proteases in the ER must therefore remove a nine amino acid N-terminal extension and a long carboxy terminal extension in order to generate the P91A epitope. It has previously been suggested that carboxy terminal processing in the ER is inefficient. This would not seem to be the case in the present example.

P91A(C) and P91A(D) transfectants both induce proliferation of P91.6. This is an unexpected result since peptides generated in the cytosol from P91A(C) and P91A(D) should not be transported in the ER. The trivial explanation for this result is that cytosolic peptides liberated into the extracellular environment during the course of the proliferation assay stabilise MHC class I molecules on feeder spleen cells and RMA-S L^d.pyt. P91A -L^d complexes so formed could then be capable of stimulating P91.6. This possibility is being investigated using supernatant from transfected cells to stimulate P91.6 or to sensitise L^d expressing target cells for CTL mediated lysis.

Although the above mechanism may explain the observed result it is also possible that a physiological mechanism is responsible for transporting cytosolic peptides from the cytosol into the ER in RMA-S L^d.pyt transfectants. RMA-S cells have been shown to present peptides from numerous cytosolic proteins, all be it at much lower efficiency than the TAP expressing parental line RMA (Esquivel et al, 1992, Hosken and Bevan, 1992). Furthermore, it is also unclear whether TAP1 retains partial function in RMA-S and is capable of low efficient transport of certain peptides. Additionally, T2 cells which are deficient in both TAP1 and 2 have been shown to present cytosolic

peptides derived from transfected minigenes and whole cytosolic proteins (Zweerink et al, 1993, Snyder et al, 1997). Together, these results suggest the existence of a TAP independent mechanism of peptide transport into the ER. In the present experiment, it is possible that high levels of P91A(C) and P91A(D) construct derived protein combined with a lack of competition from TAP transported peptides, favours the production, transport and loading of the P91A peptide onto empty H2-L^d molecules in the ER. The physiological importance of such a route under normal circumstances is unclear.

Taken together these results suggest that the processing of larger protein fragments can occur in the ER and that a TAP independent mechanism of peptide import into the ER exists. At present these results require substantiation and validation. The localisation of construct encoded P91A proteins is being examined by confocal microscopy, stable transfectants are currently being produced and processing will be further examined using protease inhibitors.

Figure 5.1: Localisation of the P91A epitope coding sequence within P91A exon 4 and the structure of P91A constructs used for transfection. (A) DNA and amino acid sequence of P91A exon 4. The location of a potential start codon (ATG) upstream of the P91A epitope coding sequence is indicated. (B) Diagramatic representation of P91A constructs used for transfection. * indicates the presence of a start codon or potential start codon.

Figure 5.1 A

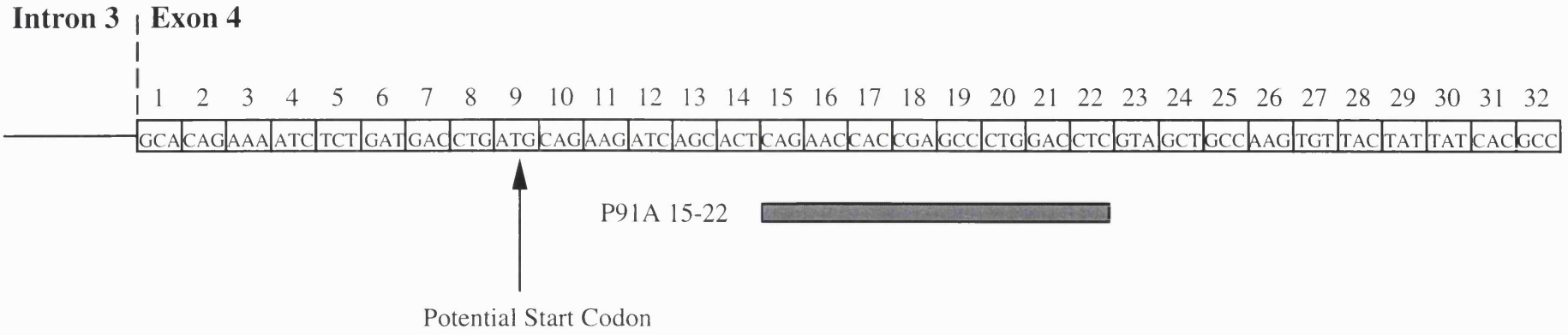


Figure 5.1 B

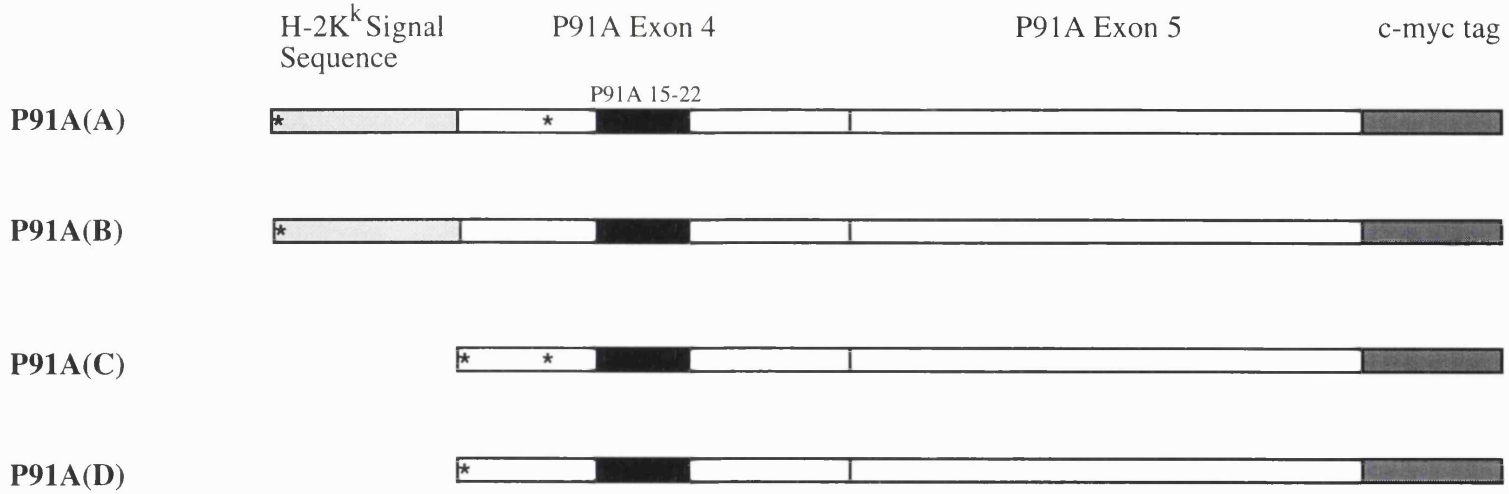


Figure 5.2

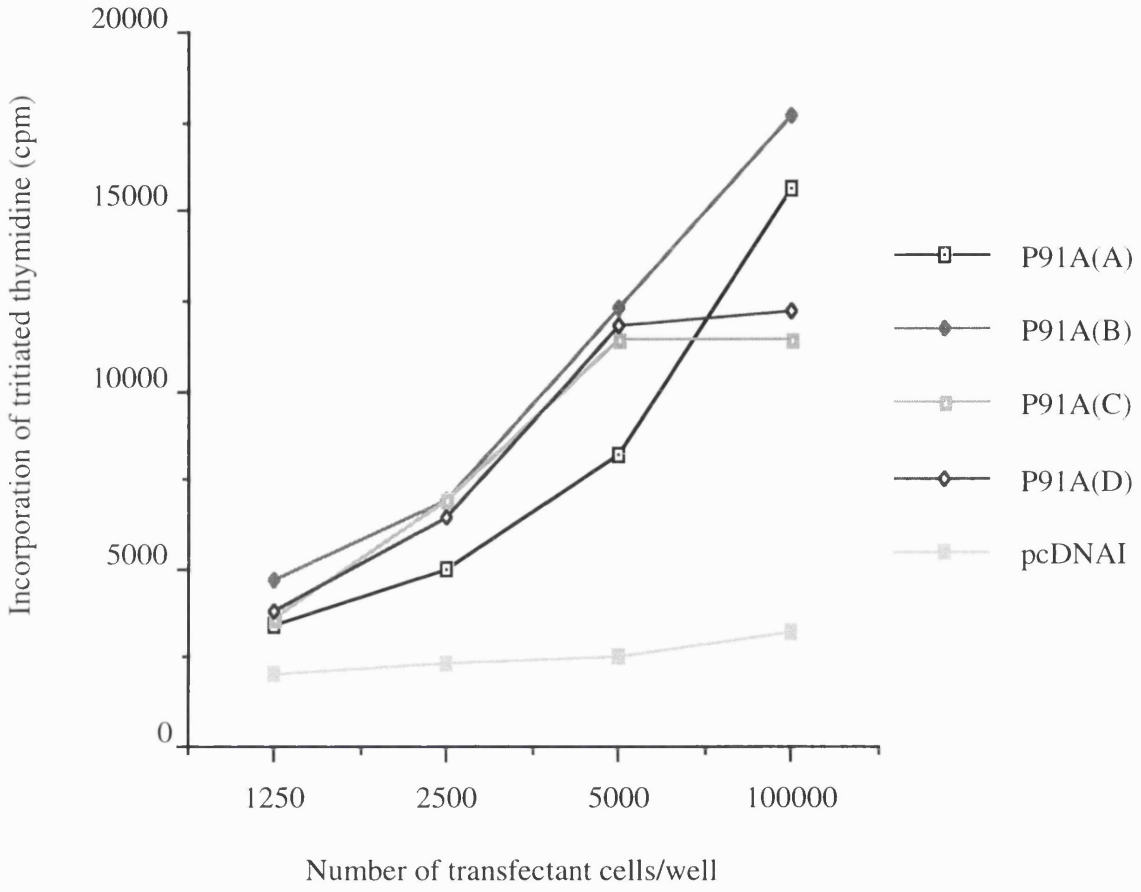
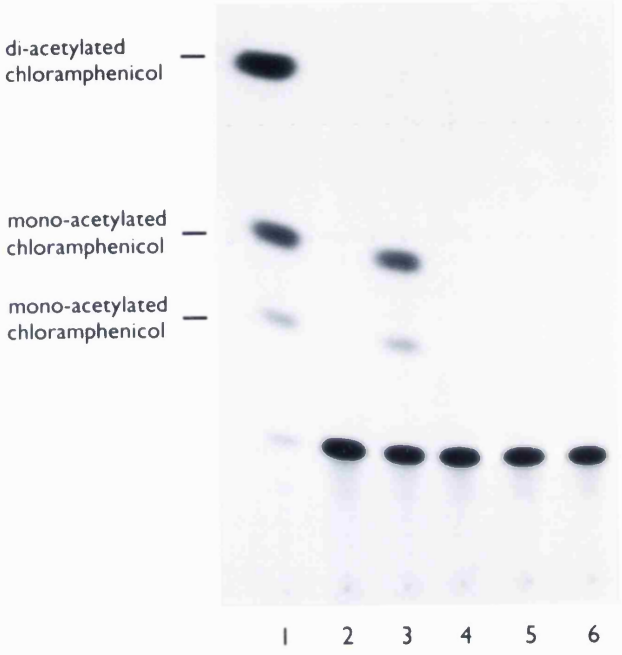


Figure 5.2: Expression of the P91A epitope in transfected P1.pyt cells. P91A constructs P91(A), (B), (C), (D) and the control plasmid pcDNAI were transfected into the recipient cell line P1.pyt and proliferation of P91.6 assessed.

Figure 5.3: Establishing optimal transfection conditions for an RMA-S cell line. (A) Lane 1: production of mono-acetylated and di-acetylated forms of ^{14}C chloramphenicol in the presence of added chloramphenicol acetyl-transferase. Lane 2: P1.HTR cells, lane 3: P1.pyt cells, lanes 4, 5 and 6 RMA-SL^d cells transfected with pcDM8-CAT. P1.HTR and P1.pyt transfection conditions: 350 volts, 125 μF. RMA-SL^d transfection conditions: lane 4: 200 volts, 125 μF, lane 5: 250 volts, 125 μF, lane 6: 300 volts, 125 μF. (B) Lanes 4-9: co-transfection of RMA-SL^d with polyoma large T antigen and pcDM8-CAT. Lane 3: co-transfection of P1.HTR with polyoma large T antigen and pcDM8-CAT. Lane 2: transfection of P1.pyt cells with pcDM8-CAT. Lane 1: production of mono-acetylated and di-acetylated forms of ^{14}C chloramphenicol in the presence of added chloramphenicol acetyl-transferase. All transfections performed at constant capacitance (125 μF) and at the following voltages: lane 2: 350 volts, lane 3: 350 volts, lane 4: 200 volts, lane 5: 250 volts, lane 6: 300 volts, lane 7: 350 volts, lane 8: 400 volts, lane 9: 450 volts.

Figure 5.3

A


di-acetylated
chloramphenicol
mono-acetylated
chloramphenicol
mono-acetylated
chloramphenicol
1 2 3 4 5 6

B

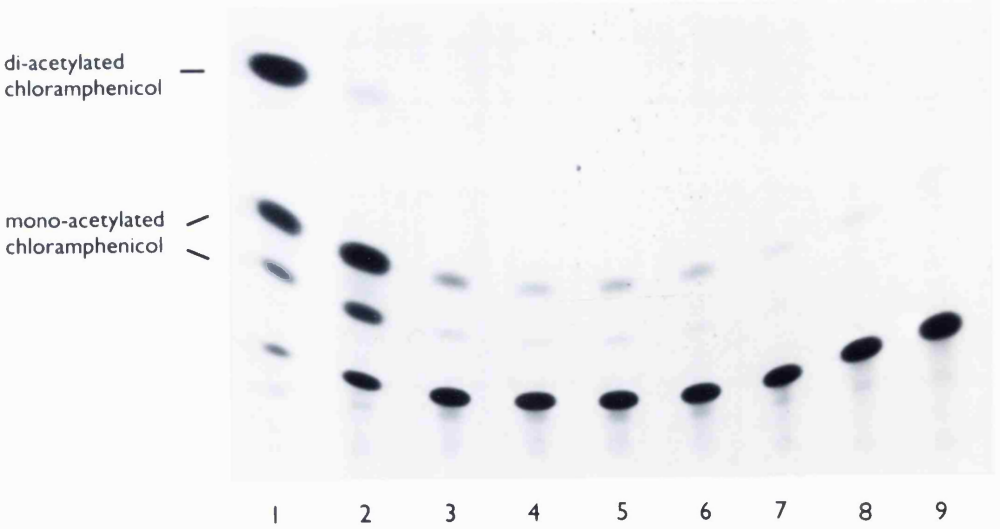

di-acetylated
chloramphenicol
mono-acetylated
chloramphenicol
1 2 3 4 5 6 7 8 9

Figure 5.4: Stable transfection of RMA-SL^d with polyoma large T antigen.
Lane 1: production of mono-acetylated and di-acetylated forms of ^{14}C chloramphenicol in the presence of chloramphenicol acetyl-transferase. Lane 2: P1.HTR cells, lane 3: P1.pyt cells, lane 4: RMA-S L^d transfectant 20, lane 5: RMA-S L^d transfectant 19, lane 6: RMA-S L^d transfectant 15, lane 7: untransfected RMA-S cells.

Figure 5.4

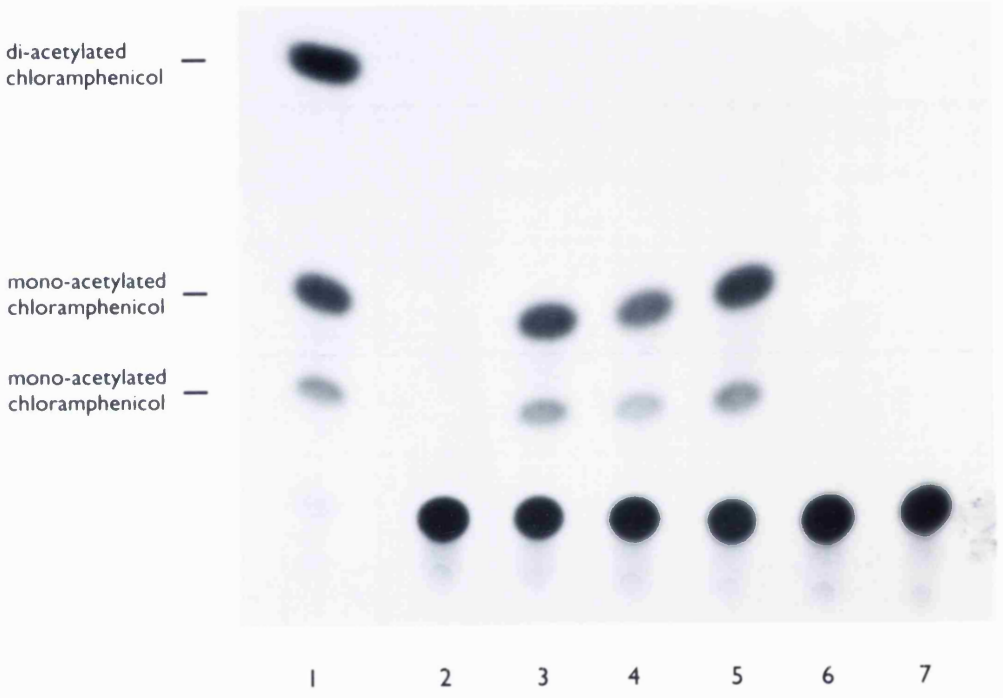

di-acetylated chloramphenicol
mono-acetylated chloramphenicol
mono-acetylated chloramphenicol
1
2
3
4
5
6
7

Figure 5.5

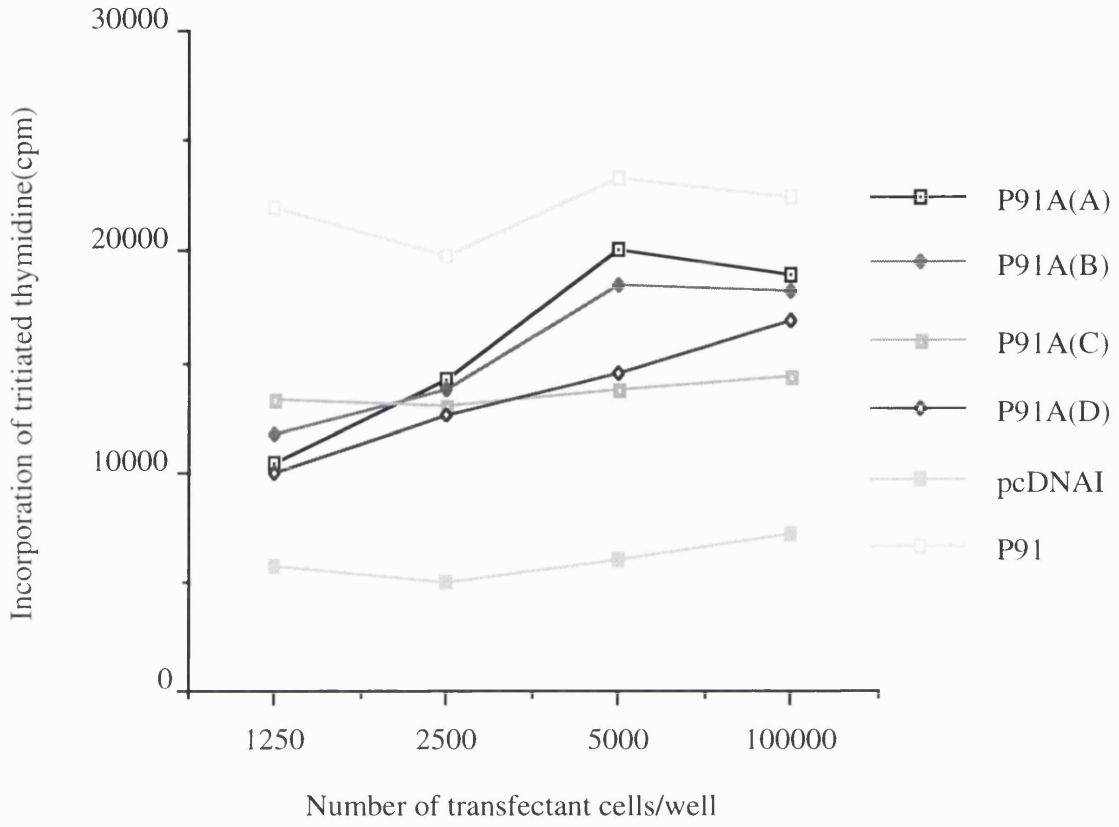


Figure 5.5: Expression of the P91A epitope in transfected RMA-S L^d.pyt cells. P91A constructs P91A(A), (B), (C), (D) and control plasmid pcDNAI were transfected into the recipient cell line RMA-S L^d.pyt and proliferation of P91.6 assessed. Stimulation of P91.6 by the P91A tum- cell line P91 acts as a positive control.

6.0 Discussion

6.1 P91A transgenic mice can be used to investigate thymic negative selection

Transgenic approaches to studying T cell selection have focused on the MHC molecule, T cell receptor or superantigen as transgene (Sha et al, 1988a and b and 1990; Teh et al, 1989; Berg et al, 1989a and b; Bohme et al, 1990; Ishikawa et al, 1991; Mamalaki et al, 1992; Golovkina et al, 1992; Chervonsky et al, 1995). These methods either fix the T cell repertoire to a single specificity or examine gross alterations in selection which are driven by the introduction of a novel MHC molecule or superantigen. Although selection in TCR or MHC transgenic models will ultimately involve MHC bound peptide, the *in vivo* involvement of specific peptides in positive and negative selection has not been examined in transgenic models. More recently, selection has been examined on MHC class II molecules in which a single peptide has been covalently integrated into the antigen binding groove and in H2-M deficient mice in which invariant chain derived CLIP peptides predominate in the antigen binding groove (see section 6.3), (Ignatowicz et al, 1996, Fung-Leung et al, 1996). P91A transgenic DBA/2 mice differ from non-transgenic DBA/2 mice in the expression of a single H2- L^d binding peptide (P91A 15-22, QNHRALDL) and offer the opportunity to study thymic selection of the unmanipulated T cell repertoire on a defined T cell epitope.

The major initial aim of this project was to examine peripheral T cell responses to the P91A peptide and altered peptide ligands in P91A transgenic and non-transgenic mice. In doing so, it was hoped to establish specific features of positive and negative selection of P91A specific T cells in P91A transgenic mice and establish more general characteristics of thymic selection. P91A transgenic and non-transgenic mice are identical but for the expression of a single H2-L^d binding peptide in P91A transgenic

mice. This peptide is defined in the present work as P91A 15-22 (QNHRALDL). The wild type P91A 15-22 peptide if generated in non-transgenic mice fails to bind to L^d due to the presence of an arginine residue at peptide position 3 (QNRRALDL). Previous work in this and other laboratories has indicated that the P91A gene is expressed in all peripheral tissues examined and in thymic sub-populations (thymic epithelial cells , thymic dendritic cells and MHC class-II negative stromal cells). Furthermore, the P91A specific T cell clone P91.6 recognises P91A transgenic but not non-transgenic thymocytes. Taken together these results suggest that the P91A epitope is expressed on P91A transgenic thymic cell types involved in repertoire selection and would be expected to direct negative and potentially positive selection. Although selection is by definition an intra-thymic event, thymocytes which are selected to survive and escape deletion will ultimately arrive in the periphery and distribute themselves throughout secondary lymphoid tissue. An examination of peptide specific T cell responses in P91A transgenic and non-transgenic mice can therefore be related to thymic selection events.

6.1.1 P91A transgenic mice provide a clear example of negative selection of peptide specific $CD8^+$ T cells

Following *in vitro* culture with P91A peptide, specific T cell lines and clones were derived from P91A transgenic and non-transgenic mice. Although initially interpreted as suggesting a failure of negative selection in P91A transgenic mice a more detailed analysis of peptide responsiveness revealed a differential sensitivity to peptide in that T cells from transgenic mice required 100-1000 fold more P91A peptide to elicit half maximal proliferation than T cells derived from non-transgenic mice. These data suggested that high affinity P91A specific T cells had been deleted in P91A transgenic mice leaving low affinity P91A reactive cells to escape into the periphery. Although this is the most likely interpretation of these results the outcome of an interaction between two cells, be they thymocyte and thymic dendritic cell or T cell

clone and peptide pulsed splenocyte, may more accurately reflect the overall avidity of the interaction (as dictated by levels of cellular adhesion/co-stimulatory molecules e.g., LFA-1, CD2, CD27, CD40-Ligand, CD44, VLA-4, CD28, TCR and CD8) rather than the affinity of the TCR for the MHC-peptide complex (Armitage et al, 1992, Hintzen et al, 1994, Cai and Sprent, 1996, Rodrigues et al, 1992, Spits et al, 1986, Koopman et al, 1990). In the present study a comprehensive analysis of adhesion and co-stimulatory molecules expressed on P91A transgenic and non-transgenic T cell clones has not been attempted. Although this may be of value, the correlation of clone sensitivity with transgenic/non-transgenic origin focuses attention on the TCR/MHC-peptide complex as the likely source of differential peptide sensitivity. The work presented is interpreted to suggest that T cell receptors derived from P91A transgenic low affinity T cell clones are, as the name suggests, low affinity receptors. Nevertheless, alterations in the levels of TCR or CD8 expression can alter peripheral responsiveness to peptide and thymic selection.

Studying the negative selection of T cells reactive with Mls-1^a (Mtv-7 in combination with H2-E) Wallace et al, found a differential requirement for the CD4 co-receptor in the negative selection of thymocytes expressing certain Vβ chains. Furthermore, the dependency on CD4 for deletion correlated *in vitro* and *in vivo* with poor reactivity to the Mtv-7/H2-E complex (Wallace et al, 1992;) . The level of CD8 expression has also been shown to determine the outcome of thymic selection (Robey et al, 1992). Thymocytes expressing the 2C TCR are positively selected in 2C TCR transgenic $H2^b$ mice. However, coexpression of a CD8 transgene and the 2C TCR in double transgenic $H2^b$ mice is sufficient to convert positive to negative selection indicating that increasing the overall avidity of the interaction can be sufficient to alter the outcome of thymic selection. T cell receptor density has also been correlated with negative selection in TCR transgenic mice (Homer et al, 1993). In this study, two lines of TCR transgenic mice were developed which differ in T cell surface density of Vβ11 and crossed with mice which express Mtv-8/H2-E (Vβ11 confers reactivity to

Mtv-8/H2-E). T cells expressing Vβ11 at high density were deleted while those expressing <10% of normal TCR density were not subject to negative selection. The simplest interpretation of this result is that a quantitative threshold for negative selection based on TCR surface density has been established. Taken together these data strongly suggest that the outcome of intrathymic selection events can be dictated by the level of T cell receptor and/or CD4/CD8 coreceptor expression.

In the P91A model system, TCR or coreceptor levels have not been artificially altered. The ability to select T cells in P91A transgenic and non-transgenic mice should therefore be equivalent. Having said this, CD4+CD8+ thymocytes have a normally distributed expression of CD4 and CD8 coreceptor molecule and although the distribution is tight it remains possible that thymocytes which would have otherwise undergone negative selection on the basis of an interaction with the P91A epitope escape deletion by expressing a marginally lower level of CD8. Such a cell would be inactive in the periphery if it retained this lower level of CD8. Furthermore, peripheral activation of T cells generally requires a higher level of antigen (MHC-peptide or MHC-superantigen) than that required to induce negative selection. CD8 coreceptor levels were measured on P91A transgenic and non-transgenic clones and found to be equivalent. This suggests that differences in the level of CD8 expression do not explain the differential sensitivity of P91A transgenic and non-transgenic T cell clones to P91A peptide.

As discussed above, differences in TCR level can set a quantitative threshold for negative selection. Once again this type of effect has been primarily observed in TCR transgenic mice. What is its relevance to selection of the peripheral T cell repertoire? One tends to assume that peripheral T cells will have a similar level of TCR expression. This may not always be the case. It has been demonstrated that certain Vα chains are incapable of dimerising efficiently with particular Vβ chains (Kuida et al, 1991). If this situation arises then surface TCR levels will be reduced in the presence

of a relative excess of CD3 components in the ER. In a different scenario a proportion of human and murine T cell clones and peripheral T cells have been shown to express two different TCR αβ heterodimers (Padovan et al, 1993 and 1995, Schittek et al, 1989, Davodeau et al, 1995, Malissen et al, 1988, Couez et al, 1991). In humans it has been estimated that approximately 1% of peripheral T cells express two β chains and up to a third of human peripheral T cells have been predicted to express two α chains (Padovan et al, 1993 and 1995, Davodeau et al,1995). The percentage of cells expressing two α chains has however been questioned (Alam et al, 1995; Elliott and Altmann, 1995). Alam and colleagues found the percentage of thymocytes expressing dual TCRα chains to be greater in TCR low thymocytes than in TCR high thymocytes and proposed that T cells expressing two species of TCR were absent from the peripheral T cell pool. Two factors should be held in mind when considering this data. Firstly, in TCR low thymocytes sequential rearrangements of the TCRA locus may occur on one chromosome. This might tend to increase the percentage of cells expressing two TCRα chains if the product of the first rearrangement persists long enough for cell surface expression to be detected after the second rearranged TCRα chain appears on the cell surface. Within the confines of the experimental system employed this possibility could not be formally ruled out. Secondly, insufficient numbers cells may have been acquired when examining TCR high thymocytes to detect small numbers of dual TCRα chain expressing cells. Aside from these data, it nevertheless remains likely that a significant proportion of peripheral T cells will express more than a single species of TCR αβ heterodimer. The expression of two α chains and a single β chain or two β chains and a single α chain may lead to preferential pairing of α and β chains. If this occurs then the structurally disfavoured αβ heterodimer may be expressed on the cell surface at a lower level. Furthermore, if two functional α and β chains are present and can form stable heterodimers one might expect a lower surface density of either receptor due to a competition for available CD3 components in the ER. In either case, a thymocyte which would otherwise be subject to negative selection may escape deletion by having a lower level of a specific

TCR either alone or in combination with a higher level of a second TCR αβ heterodimer.

It has been further reasoned that positive/negative selection of a thymocyte expressing two T cell receptors on a single T cell may enhance the escape of potentially autoreactive T cells into the periphery. This does not seem to follow since there is no reason to believe that a thymocyte will preferentially utilise one TCR species for selection purposes carrying the other along as passenger. It is more likely that both TCR will be subject to selective pressures. It is however possible that a dual TCR expressing thymocyte will express a potentially autoreactive TCR below a threshold sufficient to induce negative selection and escape into the periphery on the basis of positive selection on the alternatively expressed TCR. The involvement of dual TCRα chain expressing T cells in auto-immune disease has been examined in mice which can only rearrange the TCR A locus on a single chromosome. Three disease models have been examined; experimental allergic encephalitis, systemic lupus erythematosis and insulin dependent diabetes mellitus. In all cases the inability to rearrange and express two TCR α chains was shown to have no direct effect on disease progression (Elliott and Altmann, 1995, 1996).

P91A specific T cells from P91A transgenic and non-transgenic mice were stained with an anti-CD3 monoclonal antibody and shown to have equivalent levels of TCR. Once again it would appear that differential sensitivity to peptide of P91A specific T cell clones cannot be accounted for on the basis of surface receptor (TCR or CD8) quantity. This is not to imply that the CD8 coreceptor is acting equivalently in the recognition of the P91A epitope by transgenic and non-transgenic T cell clones. On the contrary, it may well be the case that the interaction of transgenic clones with the P91A epitope is highly dependent on CD8 coreceptor engagement. It has previously been demonstrated that the CD8 dependency of cytotoxic T cells inversely correlates with the concentration of peptide required to induce half maximal lysis (Alexander et

al, 1991). In the present study the differential sensitivity of transgenic and non-transgenic clones is assessed by proliferation. It has recently been demonstrated that qualitatively different T cell responses can be elicited by different concentrations of peptide which result in increased or decreased T cell receptor occupancy (Valitutti et al, 1996). More specifically, cytotoxicity could be detected at low peptide concentrations while higher peptide concentrations (3 to 6 logs) were required to induce proliferation. It would be interesting to assess the CD8 dependency of P91A specific transgenic and non-transgenic T cell clones in both proliferation and cytotoxicity assays, the prediction being that P91A specific clones derived from transgenic mice would be more likely to have a dependence on CD8.

The work in this thesis strongly suggests that P91A specific T cell receptors expressed on transgenic and non-transgenic T cells differ in their affinity for the H2-L^d-P91A peptide complex. Perhaps the most direct evidence for this is the finding that the substitution of a major T cell receptor contact residue in the P91A peptide results in increased proliferation of transgenic clone 4A yet completely abrogates the response of non-transgenic clone 3B and the P91A tum^- specific T cell clone P91.6. The P91A (15-22) peptide has an arginine residue at position 4. This is a large positively charged residue located in the centre of the P91A peptide and is predicted by molecular modelling to have a long TCR accessible solvent exposed side chain. Substitution of this residue with alanine, glutamine, glutamic acid or lysine fails to alter binding to L^d and in all but one clone analysed (in this and other studies) P4 arginine has been shown to be critical for T cell recognition (Robinson and Lee, 1996, Alexander-Miller et al, 1994, this thesis). These data suggest that P91A reactive thymocytes may be deleted, at least in part, on the basis of the ability of their expressed TCRs to interact with the P4 arginine residue. Extending this argument, it may be that transgenic clone 4A escapes deletion on the basis of a failure of it's TCR to interact productively with thymic expressed P91A epitope as result of electrostatic or steric clash with the P4 arginine residue. The finding that P4 alanine is preferred at

this position for the stimulation of transgenic clone 4A would tend to support such a notion.

The crystal structures of two TCR/MHC-peptide complexes reveal a deep central pocket formed by CDR3α and CDR3β (Garcia et al, 1996, Garboczi et al, 1996). The amino acid composition of this pocket will be hypervariable since it is encoded by V(D)J junctional sequence. This pocket exists in an otherwise relatively flat contact surface and has been shown to accommodate the tyrosine side chain of the HTLV-1 Tax peptide. Although purely speculative, an examination of the CDR3α and CDR3β sequences of transgenic clone 4A reveals the presence of two non-germline encoded arginine residues not present in any other sequence examined which may converge in a CDR3 pocket and be incompatible with the binding of the P91A P4 arginine residue. The proliferation data using amino acid substituted peptides therefore supports the notion that the differential sensitivity of transgenic and non-transgenic T cell clones is primarily dictated by the nature of the interaction between their respective T cell receptors and the H2-L^d-P91A peptide complex and additionally, implies that in line with the prevailing model of T cell selection that P91A specific T cells escape negative selection in P91A transgenic mice by expressing T cell receptors which are of low affinity for H2-L^d-P91A.

Although the data presented support an affinity based model for negative selection in P91A transgenic mice it must be noted that the affinity of the interaction between transgenic or non-transgenic T cell receptors and H2-L^d-P91A has not been measured. It would certainly be of interest to establish the kinetic features of transgenic and non-transgenic TCR binding to the H2-L^d-P91A peptide complex and correlate this information with biological responses. In the present work proliferation of T cell clones is assessed and interpreted to reflect differences in TCR affinity. The biological responses of a variety of T cell clones have previously been assessed and shown to correlate with the kinetic parameters of the TCR interaction with the MHC-

peptide complex (Matsui et al, 1994, Sykulev et al, 1994 and 1995, Lyons et al, 1996). The overall affinity of the TCR for MHC-peptide has been directly correlated with the epitope density required to induce half maximal lysis of target cells; with lower affinity T cells shown to require a higher epitope density to induce equivalent lysis (Sykulev et al, 1995). Furthermore, this relationship has been shown to be linear for T cell receptors with affinities below $5 \times 10^6 M^{-1}$. The contention that overall affinity of the TCR for MHC-peptide correlates with biological response has been used to propose that alloreactive T cells may have a higher intrinsic affinity for ligand than T cells recognising syngeneic MHC and foreign peptide (Sykulev et al, 1994). Although it is possible that such a situation may exist the data suggesting this derives from an examination of only two T cell clones. An analysis of further allogeneic and syngeneic interactions is required before generalities can be made.

The affinity of an interaction is the composite function of an association phase and dissociation phase whereby a low association (on-rate) or fast dissociation (off-rate) may both produce a low affinity interaction. The biological activity of agonist, weak agonist and antagonist peptides has been shown to be more closely related to the rate of dissociation of the TCR/MHC-peptide complex, with TCR dissociation rates faster for MHC-antagonist peptide complexes than for MHC-agonist complexes (Matsui et al, 1994, Lyons et al, 1996). Interestingly the on-rate of an antagonist peptide can be equivalent to or higher than an agonist peptide, its biological activity being dictated by a much faster off-rate. Furthermore, antagonist peptides with increased dissociation rates have been shown to promote positive selection of TCR transgenic thymocytes in foetal thymic organ culture while agonist peptides with slow off-rates tend to induce negative selection.

Although the bulk of kinetic data appears to correlate with the biological responses of T cell clones there is some evidence that the kinetics of the TCR/MHC-peptide interaction do not exactly reflect the biological activity of certain MHC-peptide

ligands (Al-Ramadi et al, 1995). In such cases peptides have been shown to efficiently induce CTL lysis yet have low or undetectable affinity for the MHC-peptide ligand. Such findings may reflect the sensitivity of the technique (surface plasmon resonance) used to measure affinity. Furthermore, in cases where the biological activity is high but affinity is low a dependence on CD8 has been shown to be necessary for lysis. Taken together these data suggest that the biological activity of T cell clones directly correlates with definable kinetic parameters. In light of these findings and the data presented it is reasonable to suggest that P91A specific T cells present in P91A transgenic mice have T cell receptors which are of lower affinity than those of P91A specific non-transgenic T cells.

6.1.2 P91A transgenic mice can be used to investigate the scope of negative selection

P91A transgenic mice can be used to examine the overall diversity and flexibility of the T cell repertoire. P91A specific thymocytes which are reactive with P91A will be deleted on the basis of a high avidity/affinity interaction with H2-L^d-P91A in the thymus. However, T cells specific for related peptides may or may not be subject to negative selection in P91A transgenic mice. Within the P91A epitope position two (P2-N) appears to be neither concerned with MHC or TCR interaction and substitution of this residue with alanine, glutamine or proline fails to alter binding efficiency or T cell recognition (Robinson and Lee, 1996, Alexander-Miller et al, 1994, this thesis and data not shown).This suggests that peptides with specific altered P2 residues will bind well to the L^d molecule and be recognised by T cells largely on the basis of interactions with alternative peptide residues (e.g. P4-R). The question therefore arises; can T cells in P91A transgenic mice recognise such substituted peptide variants? It might be predicted that negative selection of P91A specific T cells in P91A transgenic mice would delete the associated repertoire of T cells capable of recognising P91A P-2 variants. That is, negative selection of P91A specific

thymocytes is cross selective for T cells of related specificity. Alternatively, it may be that the repertoire is sufficiently diverse to distinguish between the subtle differences in the P91A epitope and P2-variants and select cells on this basis. These issues are currently being investigated using peptide pulsed dendritic cells to immunise transgenic and non-transgenic mice.

P4-R is a major TCR contact residue for P91A specific T cells and substitution of this residue with alanine has been shown to destroy stimulatory capacity. Consequently T cells with specificity for peptides with P91A P4-A would be expected to escape negative selection in P91A transgenic mice. Arginine is a positively charged residue. P91A peptides with an alternative positively charged residue at this position (e.g. lysine) have not been tested for their ability to stimulate high affinity P91A specific T cell clones. It is possible that these will retain the ability to stimulate high affinity P91A specific T cells. If this is the case then one could ask whether the repertoire is sufficiently diverse in P91A transgenic mice to recognise P4-variants of P91A which retain the capacity to stimulate P91A specific T cells. This and the experiments outlined above would help establish the fine specificity and ultimate flexibility and diversity of the peripheral T cell pool.

6.2 Interaction of the P91A 15-22 peptide with the H2-L^d binding groove and possible implications for positive selection of thymocytes

The P91A epitope is defined in the present work as the octamer P91A 15-22 (QNHRALDL). Multiple complementary approaches have been used to define the P91A epitope; T cell stimulation and L^d stabilisation with N- and C-terminal truncated synthetic peptides, molecular modelling of candidate peptides within the L^d binding groove and elution of the naturally processed P91A epitope. Further analysis has identified major TCR (P1-Q, P3-H and P4-R) and MHC (P5-A, P6-L, P7-D and

P8-A) contact residues which appear to be situated within opposing halves of the peptide. Of more specific interest, substitution of the P2-asparagine residue with alanine, glutamine or proline fails to alter peptide binding indicating that this peptide position is not an anchor site for the P91A peptide. In this respect the P91A 15-22 octamer peptide differs from the majority of peptides which bind to L^d (P2-proline is an essential MHC contact residue for L^d binding peptides) and resembles the p2Ca peptide (LSPFPFDL) which is derived from mouse 2-oxoglutarate dehydrogenase and sensitises H2-L^d expressing targets for allospecific kill by the T cell clone 2C (Udaka et al, 1993, Udaka et al, 1992). Amino acid substitution of the p2Ca peptide has identified MHC contact residues (P6-F, P7-D and P8-L) which like P91A 15-22 distribute towards the carboxy terminal end of the peptide. Although similar, the exact dependence on anchor residues differs between p2Ca and P91A 15-22 in that P7-D is a major contact residue for P91A 15-22 but is of lesser importance for p2Ca (Al-Ramadi et al, 1995). Nevertheless both peptides are of identical length, alike in their lack of requirement for a specific P2 residue and differ from all other known L^d binding peptides. p2Ca and P91A 15-22 therefore represent a sub-class of peptides which utilise an alternative strategy to bind to L^d.

As discussed in Chapter 4, L^d is a member of a structural subgroup of MHC class I molecules consisting of D^b, D^f, D^q, D^{lvl}, K^d, L^d and L^q (Young et al, 1994). These molecules share conserved structural features the most prominent of which is the presence of a cross groove hydrophobic ridge. Interestingly, the presence of such a ridge dictates that peptides bound to this sub-group of class I molecules must arch out of the binding groove towards their C-terminus. This has been visualised in the case of the flu nucleoprotein peptide ($NP_{366-374}$) crystallised in complex with H2-D^b (Young et al, 1994). Herein lies a problem, for it has been suggested that the presence of a hydrophobic ridge precludes the binding of peptides which have fewer than nine residues. Both the P91A 15-22 and p2Ca peptides are octamers and would therefore

be presumed to be incapable of binding to L^d. This is obviously not the case. How then might P91A 15-22 and p2Ca overcome this potential obstacle and bind to L^d?

It has previously been demonstrated that a class I binding peptide may extend out of the binding groove at its N-terminus (Collins et al, 1994). This suggests that specific hydrogen bonds between conserved A pocket tyrosine residues and the peptide terminal amine group may not be essential for binding. Furthermore, truncated peptides which fail to either bind in the A or F pockets have been co-crystallised with HLA-A2 (Bouvier and Wiley, 1994). Taken together, these findings indicate that peptides may bind to MHC class I molecules in the absence of specific N- and C-terminal interactions. In addition, although not directly comparable the non-classical class I molecule H2-M3 has been shown to bind N-formylated peptides with the P1 residue side chain and formyl group accommodated in the B pocket (Wang et al, 1995b). Once again this suggests that peptides may bind to class I molecules utilising alternative strategies in the absence of specific N-terminal interactions with conserved A pocket tyrosine residues. Is it possible that P91A 15-22 and p2Ca bind to L^d in the absence of specific N-terminal interactions within the A pocket? If this were to occur then the N-terminal residue of P91A 15-22 and p2Ca might bind within pocket B and the register of the peptide be shifted towards the right (F pocket end of the groove). There is some evidence to suggest that this may be the case.

The naturally processed and presented form of the 2C epitope is the octamer peptide p2Ca (Udaka et al, 1992). However, the nonamer QL9 (QLSPFPFDL) which has an extension of one amino acid at the N-terminus is 50-100 fold more efficient at sensitising target cells for lysis by the T cell clone 2C (Sykulev et al, 1995). Furthermore, the affinity of the 2C TCR for p2Ca may be 10-100 fold lower than for the QL9-L^d complex (Sykulev et al, 1995). These data imply that QL9 is a stronger epitope than p2Ca for the 2C T cell clone and that p2Ca may be the preferred natural epitope due to processing constraints. A similar situation may exist for the P91A

epitope. The nonamer peptide P91A 14-22 has been shown to bind and stabilise surface L^d in the apparent absence of extracellular processing, indicating that both nonamer and octamer P91A peptides might bind to L^d (Alexander-Miller et al, 1994). This could occur by one of two means; an additional residue could be accommodated by a conformational alteration in the peptide backbone, or a nonamer peptide could extend into and fill an otherwise empty A pocket. Evidence from the crystal structure of D^b might suggest the latter since an octamer peptide would be predicted not to be capable of interacting with both A and F pockets. It is possible that there is an unusually high degree of peptide flexibility in the N-terminal half of p2Ca/QL9 and P91A 14/15-22 peptides when bound to L^d. All human and many mouse class I binding peptides have a P2 anchor position which interacts with pocket B. This anchor tends to decrease peptide flexibility towards the N-terminus. P91A and 2C peptides have dispensed with this requirement and have concentrated their MHC contact sites towards the C-terminus, consequently N-terminal flexibility may be greater than normal for these peptides in complex with L^d. If this is the case then an additional amino acid may be accommodated through backbone flexibility in the N-terminal half of the peptide. It would be interesting to construct a model L^d based on the crystal structure co-ordinates of D^b and examine the interaction of P91A and 2C nonamer and octamer peptides. Ultimately crystallisation of L^d in association with ocatmer and nonamer peptides will help resolve this binding paradox.

Does the binding of an alternative sub-population of peptides to L^d confer any selective advantage in $H2^d$ mice? This is not an easy question to answer. The surface expression of L^d is thought to be lower than that of K^d and D^d due to a lack of suitable L^d binding peptides (Lie at al, 1991). This suggests that L^d may be functioning at suboptimal capacity as a restriction molecule in the periphery. If this is the case then any alteration in the L^d molecule which permits an increased diversity of peptides to bind would have a selective advantage. It is therefore possible that a novel binding mechanism has evolved to increase the diversity of peptides which L^d

can present and been selected for on this basis. This might suggest that L^d has not yet realised its full potential as a restricting molecule and that given time its mode of selecting and binding peptides will improve with a resultant increase in surface expression. The $H2^d$ locus is likely to exist within outbred mouse populations where it may be subject to selective pressure. One might question the level of selective pressure which will operate to drive further evolution of the L^d molecule. $H2^d$ mice have two other class I molecules (D^d and K^d) and many other inbred mouse strains do not have a H2-L product. Although it is very difficult to ascertain, it has not been reported that mice with only K and D class I molecules are at any disadvantage compared to mouse strains which have K, D and L products. Furthermore, mice which lack an H2-E molecule do not appear immune compromised when compared to strains which express both H2-A and H2-E products. It therefore appears that MHC molecules may have developed to a stage where their capacity for binding peptides is so powerful that any further evolution in their structure may be at best very slow.

Does the use of an alternative binding strategy by certain L^d associated peptides have any effect on thymic selection events? The answer to this question is undoubtedly yes for the negative selection of potentially autoreactive cells. More interestingly it is possible that positive selection may be altered on the basis of an interaction with L^d in complex with peptides which have an alternative binding strategy. The exact nature of the positively selecting ligand for any given peripheral T cell is still unclear. However, at least in the case of TCR transgenic mice the presence of the MHC molecule to which the T cell is restricted in the periphery is required for positive selection in the thymus. It is interesting to speculate that T cells specific for L^d-P91A or L^d-p2Ca may be selected in the thymus on the basis of an interaction with an MHC-peptide complex in which the selecting peptide utilises the same or similar binding strategy to that of the epitope in the periphery. This idea is not so far fetched when one considers that both the P91A and 2C epitopes induce a conformational change in L^d as recognised by the monoclonal antibody B22/249 (Solheim et al,

1993b). Residues affecting B22/249 reactivity map to a loop of the α1 domain as well as the α1 helix situated immediately above the B pocket. It is therefore likely that P91A and 2C induce structural alterations in the L^d α1 helix above pocket B and as such may alter the spectrum of T cells capable of interacting the L^d molecule. The presence of a conformational change in L^d supports the notion that T cells selected on L^d and non standard motif peptides may have specificity in the periphery for this subgroup of L^d epitopes and that T cells selected on L^d and standard motif peptides may recognise this category of L^d epitopes. This possibility is at present unverified.

6.3 Positive Selection in P91A transgenic mice

At the outset of this work little was known concerning the involvement of peptide in the positive selection of thymocytes. Using K^{bm} mutant strains of mice in which residues in the floor of the K^b binding groove are altered, Nikolic-Zugic and Bevan initially proposed a critical role for peptides in the positive selection of CD8+ T cells specific for H2-K^b in complex with the ovalbumin peptide $Ova_{258-276}$ (Nikolic-Zugic and Bevan, 1990). In this study the K^{bm8} mutant, which has amino acid substitutions in the floor of the binding groove, failed to select T cells which could recognise the H2-K^b-$Ova_{258-276}$ complex. Furthermore, this failure to select $Ova_{258-276}$ specific CTL appeared to correlate with a failure of the mutant molecule to bind the $Ova_{258-276}$ peptide. Negative selection of $Ova_{258-276}$ specific CTL on the K^{bm8}-$Ova_{258-276}$ complex was ruled out by examining selection in $H2^b$ X $H2^{bm8}$ F1 mice, in which $Ova_{258-276}$ specific CTL were selected. Together these data support a role for self peptides in the selection of thymocytes . In addition, the ability of four different H2-K^b variants to select $Ova_{258-276}$ specific T cells correlated with their ability to present $Ova_{258-276}$ peptide, suggesting that a self peptide mimic may have been involved in positive selection. In the same year, Sha and colleagues correlated mutations in the binding groove of K^b, which were predicted to alter peptide binding, with decreased positive selection of 2C TCR bearing thymocytes in transgenic mice (Sha et al, 1990).

These early data suggested an involvement for peptide in the positive selection of thymocytes, however the nature of peptides capable of mediating positive selection remained unknown.

It was initially thought that P91A transgenic mice might be used to explore the involvement of peptide in positive selection. The ability to use P91A transgenic and non-transgenic mice to examine positive selection hinges on the assumption that the P91A epitope in P91A transgenic mice directs positive selection of a sub-population of thymocytes which may not be subject to positive selection in non-transgenic mice. If this is the case then T cells might be expressed in the periphery of transgenic mice which fail to be selected in non-transgenic mice. By defining MHC and TCR contact residues of the P91A epitope it was hoped to establish a panel of altered peptides which would retain the ability to bind to L^d but fail to stimulate high affinity P91A specific T cell clones. Such peptides might fall into the category of antagonist peptides as defined for CTL (Jameson et al, 1993). Furthermore, T cells which recognise peptides of this type may be positively selected in P91A transgenic mice on the basis of an interaction with the P91A epitope and be absent in non-transgenic mice due to a failure of positive selection.

In light of recent reports, it seems unlikely that such a focused system of selection will operate in P91A transgenic mice. Hu and colleagues, have isolated and sequenced peptides from a mouse thymic epithelial cell line and shown them to be capable of inducing positive selection of thymocytes in TAP^- foetal thymic organ culture (Hu et al, 1997). Not all peptides were equally able to induce positive selection, however in the case of one peptide, CD8+ cells were selected to 62% of the level selected in TAP^+ foetal thymic organ cultures. Although in this study repertoire diversity was not examined, the finding that thymic eluted peptides were capable of inducing positive selection of TCR transgenic thymocytes derived from two independent TCR transgenic mice might suggest that the repertoire of thymocytes

selected on a single peptide is diverse. Further evidence that the T cell repertoire is diverse when selected on a single peptide has been obtained using H2-M deficient mice (H2-M$^-$), in which class II associated invariant chain peptide (CLIP) is bound in the antigen binding groove and in transgenic mice expressing MHC class II molecules preloaded with a covalently bound peptide (A^{b}Eαp transgenic mice), (Grubin et al, 1997, Tourne et al, 1997, Surh et al, 1997, Ignatowicz et al, 1997). In the latter case the expression of a single unique MHC-peptide complex is achieved by crossing mice onto an invariant chain knockout, class II knockout background (A^{b}Eαp/class II$^-$/Ii$^-$ mice). Irrespective of which system is examined, a surprisingly large number CD4+ cells are positively selected in mice expressing a single MHC-peptide specificity: 20%-50% of the number of CD4+ T cells that are normally present in these mice. It is however false to suggest that this represents 50% of a normal repertoire since cells positively selected on a single peptide will only be negatively selected on a single peptide in H2-M$^-$ or A^{b}Eαp/class II$^-$/Ii$^-$ mice. This lack of negative selection has been shown to result in approximately two thirds of CD4+ cells in H2-M$^-$ or A^{b}Eαp/class II$^-$/Ii$^-$ mice displaying reactivity to the same MHC molecule complexed with a wild-type spectrum of self peptides (Surh et al, 1997). Furthermore, in radiation bone marrow chimeras in which recipient thymic epithelial cells are H2-M$^-$ and donor thymic dendritic cells are H2-M$^+$ the number of CD4+ cells has been shown to be reduced five fold, reflecting negative selection on MHC-class II-peptide complexes expressed on bone marrow derived cells (Surh et al, 1997). Despite this finding, CD4+ cells from H2-M$^-$, A^{b}Eαp/class II$^-$/Ii$^-$ and wild-type mice proliferate equivalently to alloantigen and soluble test antigens (keyhole limpet haemocyanin and fowl γ globulin) and have similar Vβ chain usage (Surh et al, 1997). Taken together, these data suggest that a broad peripheral T cell repertoire is selected in both H2-M$^-$ and A^{b}Eαp/class II$^-$/Ii$^-$ mice.

Although the repertoire may be diverse when selected on a single peptide, it is not complete. In six out of six TCR transgenic mice, thymocytes that are positively

selected in mice expressing wild-type class I molecules are not selected in H2-M$^-$ or $A^bE\alpha$p/class II$^-$/Ii$^-$ mice (Surh et al, 1997, Tourne et al, 1997, Grubin et al, 1997). In addition, peptides extracted from MHC class I molecules expressed on a thymic epithelial cell line have been shown to preferentially select different TCR transgenic thymocytes (F5 V P14) in foetal thymic organ culture (Hu et al, 1997). Together, these data suggest that a single specificity of MHC-peptide complex is incapable of selecting all TCR specificities. Furthermore, a closer examination of T cell receptor usage by peptide specific T cells reveals a different spectrum Vβ usage in $A^bE\alpha$p/class II$^-$/Ii$^-$ and wild-type mice (Ignatowicz et al, 1997). The presence of a restricted repertoire is further suggested by conserved Jβ usage in T cells derived from $A^bE\alpha$p/class II$^-$/Ii$^-$ mice responding to pigeon cytochrome C: while a range of J segments were utilised by T cells derived from wild-type mice in the recognition cytochrome C, TCRB J2 S6 was used exclusively by T cells derived from $A^bE\alpha$p/class II$^-$/Ii$^-$ mice (Ignatowicz et al, 1997). Finally, using TCR β chain transgenic H2-M$^-$ mice, Sant'Angelo and colleagues have analysed the pre and post selection repertoire of thymocytes and found distinct differences between H2-M$^-$ and H2-M$^+$ mice. More specifically sequence analysis of the TCR CDR3α region in pre and post selection thymocytes from TCR β chain transgenic H2-M$^-$ mice reveals restricted CDR3 length and amino acid composition in post selection thymocytes. In addition, CDR3α length is severely restricted in TCR β chain transgenic H2-M$^-$ mice compared to non-transgenic H2-M$^-$ mice. Together these data indicate that selection of thymocytes in TCR β chain transgenic H2-M$^-$ is diverse but restricted by the selecting peptide. This data should not however be over interpreted as supporting a model of thymocyte positive selection in which the selecting peptide selects thymocytes which have related specificity in the periphery since restriction in CDR3α length and sequence was observed in a situation where the β chain of the selecting receptor was fixed.

Based on the above observations, it is likely the P91A epitope will function to positively select a sub-population of thymocytes in P91A transgenic mice. It is however also likely that this population of thymocytes will overlap with thymocyte populations selected on other endogenous MHC-peptide ligands. If this is the case then attempts to examine positive selection in P91A transgenic mice using peptide immunisation strategies are likely to be unsuccessful.

In more general terms, it may be that T cell receptors expressed on a specific thymocyte interact with MHC molecules and a spectrum of self peptides. If this is the case then what is the ultimate role of peptide in positive selection? The peripheral repertoire of T cells should be capable of recognising and responding to antigenic peptides in the context of self MHC. It is unlikely, though not impossible, that certain peptides expressed in the thymus specifically select T cells which are important for recognising key pathogen derived epitopes. What is more likely is that T cells are selected for optimal recognition of self MHC. If this is the case then peptide may play a dominant role in maintaining the conformation of the antigen binding groove but play a minor role in contacting the TCR during positive selection. Alternatively, peptides involved in positive selection may actually destabilise the interaction of the TCR and MHC-peptide complex. Such a possibility has been suggested for the escape/selection of low affinity P91A transgenic clone 4A. Which mechanism operates, and both may operate for selection of different T cell receptors or the same T cell receptor selected on different MHC-peptide complexes, will depend in part on the intrinsic affinity of the TCR for the MHC molecule in the absence of bound peptide. In regard to this point, certain TCR α and β chains have been shown to be preferentially expressed on CD4+ or CD8+ T cells, suggesting preferential interaction with and selection on MHC class II or class I molecules (Sim et al, 1996). This effect occurs in the presence of MHC bound peptide but may not be dependent on it. In addition, Vβ6, Vβ10, Vβ2 and Vα11 expressing thymocytes have been shown to be preferentially selected on H2-E (Elliott, 1997). How might these results be

interpreted? One possibility is that the TCR α and β chains which are preferentially selected on specific MHC products confer critical kinetic parameters on the overall TCR heterodimer when interacting with the selecting ligand. In keeping with recent data this would mean that the TCR dissociation rate would be greater for preferred TCR chains preferentially selected on MHC class I or II and that thymocytes would be selected on the basis of an optimal dominant instability of the TCR/MHC-peptide complex (Alam et al, 1996; Lyons et al, 1996). If this is the case then the involvement of peptide may be of lesser importance, contributing little to the overall kinetics of the interaction.

There is some data to suggest that positive selection of thymocytes involving preferred TCR chains may involve a primary destabilising interaction of the TCR with the selecting molecule. Vβ11$^+$ thymocytes are partially deleted by the chimeric class II molecule DRαEβ in complex with Mtv-8 and -9 whilst Vα11$^+$ cells are positively selected (Elliott, 1997). Interestingly, in Vβ11$^+$ cells which escape negative selection Vα11$^+$ cells are over represented suggesting that a TCR chain which promotes positive selection is capable of counteracting negative selection. Once again in view of current kinetic models of T cell selection these data support a dominant destabilising interaction of the TCR with the MHC-peptide complex. Although MHC specific effects can alter the T cell repertoire in CD4$^+$ and CD8$^+$ subsets and may be less dependent on MHC bound peptide it remains likely that the majority of thymocytes will have a dependence on peptide for positive selection. The exact nature of this dependence will vary for different T cell receptors in combination with specific MHC-peptide complexes.

7.0 Materials and Methods

7.1 Mice and *in vivo* techniques

7.1.1 Mice

DBA/2 mice (6 - 8 weeks old) were obtained from OLAC (Bicester, UK). BALB/c, B10.BR and BALB/c nude mice were obtained from the Specific Pathogen Free (S.P.F.) unit of the Clinical Research Centre (CRC), Harrow and maintained at the Biological Services Unit, Hammersmith Hospital. A DBA/2 P91A transgenic mouse colony was maintained in the non-SPF unit of the CRC, Harrow and subsequently at the Biological Services Unit, Hammersmith Hospital. Attempts to breed P91A mice to homozygosity were unsuccessful. In general DBA/2 P91A transgenic mice were poor breeders and produced small litters which displayed high levels of neonatal mortality. Non-transgenic DBA/2 mice housed under the same conditions were also poor breeders. In an attempt to improve breeding characteristics, DBA/2 P91A transgenic mice were crossed with BALB/c mice. In general F1 litters were larger than DBA/2 litters and there was a lower level neonatal mortality. At present DBA/2 mice are being back-crossed to BALB/c in an attempt to increase the vigour of this transgenic line.

7.1.2 Immunisation of Mice

Spleen cell suspensions were prepared in Balanced Salt Solution (BSS) in the absence of Foetal Calf Serum (FCS) and filtered through sterile nylon gauze. Cell suspensions were adjusted to 1 x 10^8 cells/ml and mice injected intraperitoneal (i/p) with 1 x 10^7 cells (100µl).

7.1.3 Skin Grafting

Surgical Equipment:

Sterile instruments; two pairs of rat tooth forceps, one pair straight fine scissors, scalpel handles, one glass "hockey stick" forceps, pair of fine curved scissors, pair of watchmaker's forceps, fine narrow forceps, clamping scissors.

Additional equipment:

Sterile plastic Petri dishes, tail removing board, scales/mouse balance, 1ml syringes, retainers, perspex mouse grafting board, electric clippers, plaster wetting tray, heating corturoy, surgical clips, surgical clip gun.

Plaster of Paris bandages (Smith & Nephew) were cut into 20cm x 2.5cm strips.

Tulle gras was prepared by cutting fine linen bandage into appropriate sized squares layering them into a heat-proof container and melting paraffin wax over them in a microwave oven. This was followed by sterilisation for one hour at 140°C in a hot air oven. Tulle was cut into 2cm x 2cm squares and placed into a sterile plastic Petri dish prior to use.

Solutions:

Chlorhexidine (Hibitane), Smith & Nephew. Sterile isotonic saline. Pentobarbitone Sodium, 60mg/ml (Sagatal, RMB Animal Health Limited) diluted 1:10 with sterile isotonic saline. Spray dressing, (Smith & Nephew). Plaster wetting fluid - 2.5% Cetavolon (Zeneca). Balanced Salt Solution (BSS).

Preparation of donor skin:

Donor mice were killed by cervical dislocation and placed on the tail removing board. Tails were sterilised with chlorhexidine. The tip of the tail was held with a pair of rat tooth

forceps and a ring incision made around the base of the tail and continued along the dorso mid-line for about 2-3 inches. Tail skin was removed and placed dermis side down in a sterile plastic Petri dish. Tail skin was subsequently flattened out, cut into 1cm x 1cm squares and moistened with a small volume of sterile saline.

Preparation of recipient mice:

Mice were anaesthetised using 60μg/g body weight pentobarbitone sodium given as an intraperitoneal injection and secured to grafting boards using retainers. Mice were shaved over the chest area and surgically prepared. The surgical site was dressed with spray dressing.

Grafting:

The graft bed was prepared by cutting and removing an elliptical area of skin from the flank of the mouse. Graft beds were slightly larger than the size of the graft to be used. Donor skin graft was carefully positioned in the graft bed and a square of tulle placed directly over the grafted area. A Plaster of Paris bandage was used to hold the graft in place. Following surgery mice were placed on clean wood chips in a warm area and recovery from anaesthesia monitored.

Scoring the skin grafts:

Plasters were carefully removed after 7-10 days. Skin grafts were scored every day for the first month and every 2-3 days thereafter. Rejection was registered when less than 10% of the original graft remained viable. Median Survival Time (MST) of skin grafts were calculated; that is the time taken for 50% of skin grafts to be rejected.

7.2 *In vitro* cellular immunology techniques

7.2.1 Tissue culture reagents

Foetal calf serum (FCS) was heat inactivated at 56°C for 1 hour in a water bath and stored at -20°C.

RPMI 1640 medium (with bicarbonate buffer) and Dulbecco's Modified Eagles Medium (DMEM) (Gibco, BRL) were supplemented with 10% FCS, L-glutamine (2mM), HEPES pH 7.2 [N-{2-Hydroxyethyl]-piperazine-N'- 2-ethanesulphonic acid]) (10mM), penicillin (100i.u./ml), streptomycin (100µg/ml) and 2-ß-mercaptoethanol ($5x10^{-5}$M). DMEM was additionally supplemented with sodium bicarbonate (4.4%, v/v) and for cultures involving the T cell clone P91.6, L-Arginine HCl (0.55mM) and L-Asparagine (0.24mM) were added. Serum free medium was obtained from Hycor (UK) and supplemented with glutamine (2mM).

Additional tissue culture reagents:
Bromodeoxyuridine (BrdU), (Sigma) used at a final concentration of 100µg/ml.
Hypoxanthine/Aminopterin/Thymidine, obtained as a 50 x solution (50 x HAT), (Gibco, BRL).
Hypoxanthine and Aminopterin (HA) obtained as 100 x stock solutions, (Gibco, BRL).
^{51}Cr sodium chromate in BSS (1 µCi/µl), (Amersham, UK)
^{3}H tritiated thymidine in BSS (0.5 µCi/µl), (Amersham, UK)
G418 sulphate (Geneticin) (Gibco, BRL) prepared as stock solution in (PBS) and used at a final concentration of 1mg/ml.
Hygromycin prepared as a stock solution in RPMI an used at a final concentration of 0.75µg/ml

Peptides:

Peptides were synthesised on an Applied Biosystems automated peptide synthesizer (model 431A). Peptides with an N-terminal glutamine were synthesized and purified as their N-Fmoc derivatives to prevent cyclization of the terminal glutamine to pyroglutamic acid under acidic conditions. The protective Fmoc group was removed by piperidine prior to use. Peptides were purified by HPLC on a C8 reverse-phase column using a gradient of acetonitrile in 0.1% v/v trifluoroacetic acid (TFA). The dominant peak was collected and amino acid composition determined by analysis of samples hydrolyzed by exposure to 6M HCl for 24 hours at 110° C. The average mass of ions ($M + H^+$) derived from the peptides by secondary ionization mass spectrometry was measured in a VG ZAB SE mass spectrometer. Peptides were resuspended in PBS and frozen at -80°C. The sequences of peptides used are shown in Figure 4.1.

Cytokines:

Supernatant from secondary mixed lymphocyte cultures (2° MLC-sup) was prepared as follows and used in the refeeding of the T cell clone P91.6. Briefly, 5×10^7 C57 Bl/6 spleen cells were cultured for 14 days with 1×10^8 irradiated (2000 rads) DBA/2 spleen cells in 40 ml of complete DMEM supplemented with arginine and asparagine. Following this period stimulated C57 Bl/6 blasts were harvested and recultured for 24 hours with fresh DBA/2 spleen cells in 30ml complete DMEM. Supernatant was subsequently harvested, filtered through a 0.22μM filter and stored at -20°C. 2° MLC-sup was incorporated into P91.6 refeeding cultures at a final concentration of 12.5% - 50% (actual concentration being dependent on the titrated activity of the prepared batch).

Supernatant from concanavalin A (ConA) stimulated rat spleen cells (ConA-sup) was prepared as follows and used in the derivation and standard refeeding of T cell clones. Single cell suspensions of spleen cells from 12 wk old Lewis rats were incubated at 1×10^8 cells/40ml in complete RPMI 1640 supplemented with 1μg/ml ConA. Following 24 hours of culture supernatant was harvested filtered through a 0.22μM filter and stored at

-20°C. Rat splenocyte blasts were cultured for a further 24 hours in RPMI 1640 and a second batch of supernatant prepared as before. The activity of ConA-sup was titrated prior to use. In general day 1 ConA-sup was used at a final concentration of 1%-3% in cultures.

Human recombinant interleukin-2 (hrIL-2) was obtained as a freeze dried powder, prepared as a stock solution in BSS (5000iu/ml) and used at a final concentration of 5-20iu/ml in cultures

7.2.2 Cell lines

Tumour cell lines:

EL4, RMA-S and all RMA-S derivative cell lines were cultured in RPMI 1640 using $25cm^2$ or $75cm^2$ tissue culture flasks.

Thymidine Kinase negative (tk^-) P1.HTR derivative cell lines (Van Pel et al, 1985) and the tk^- P91A tum^- cell line P91.1, (Uyttenhove et al, 1980) were maintained in $25cm^2$ or $75cm^2$ tissue culture flasks in complete DMEM supplemented with 0.1mg/ml BrdU for selection against tk+ revertant cells. P1.HTR and P91.1 have been selected for resistance to 8-azaguanine (AzG^r). As a result neither cell line can utilise the salvage pathway of DNA synthesis and therefore fail to proliferate in the presence of aminopterin (which blocks *de novo* DNA synthesis). By contrast, AzG sensitive cells use the salvage pathway of DNA synthesis and therefore utilise exogenous hypoxanthine and thymidine in the presence of aminopterin. It is this feature which results in the selective death of P1.HTR and P91.1 in the presence of HAT or HA.

T cell lines and clones:

P91A specific T cell lines and clones were derived from P91A transgenic and non transgenic mice following the method of Alexander et al, 1991. Briefly, 5 X 10^6 responder spleen cells/well were cultured in 24 well Linbro plates (Flow Laboratories, ICN Biomedicals) in complete RPMI 1640 and 50μM P91A 12-24 peptide . Following one

week of culture, cells were harvested and replated into 24 well plates at 3 X 10^6 cells/well in the presence of 25μM P91A 12-24 peptide. Lines were subsequently maintained by weekly restimulation of 2 X 10^5 cells/well in the presence of 1 X 10^5 irradiated syngeneic spleen cells, 6μM P91A 12-24 peptide and 1%-3% ConA-sup. T cell lines 4 and 14 were derived from P91A transgenic DBA/2 X BALB/c F1 mice. T cell lines 1 and 3 were derived from a DBA/2 and BALB/c mouse respectively. T cell clones were derived from T cell lines by limiting dilution. T cells were harvested from T cell cultures and plated into flat bottom 96 well plates at 0.3 cells/well in the presence of 5 X 10^5 irradiated syngeneic spleen cells, 6μM P91A 12-24 peptide and 1%-3% ConA-sup. T cells were restimulated weekly by replacing 100μl of medium with fresh medium containing 5 X 10^5 irradiated syngeneic spleen cells, 1-3% ConA-sup and 6μM P91A 12-24 peptide. Clones were subsequently expanded into 24 well Linbro plates and 1 X 10^5 cells/well restimulated every 7-10 days with 4 X10^6 irradiated syngeneic spleen cells/well, 6μM P91A 12-24 peptide and 1%-3% ConA-sup.

For the purposes of RNA extraction low affinity clone 4A was cultured in 75 cm^2 tissue culture flasks (Falcon) in the presence of irradiated BALB/c nude feeder spleen cells. Following sequential rounds of restimulation 2 X 10^8 T cells were recovered.

The P91.6 T cell clone was a kind gift from Dr. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium) and was maintained as follows. T cells were plated into 24 well Linbro plates at 5 X 10^4 cells/well in the presence of 4 X 10^6 irradiated syngeneic spleen cells, 1 X 10^5 P91.1 cells or 6μM P91A 12-24 peptide, complete DMEM supplemented with arginine and asparagine, 1 X HAT (if P91.1 cells were used) and 12.5-50% 2° MLC-sup. P91.6 was restimulated every 7-10 days.

7.2.3 Cytotoxicity assays

Effector cells (T cell lines or clones) were harvested, washed twice in BSS containing 2% FCS and resuspended in complete RPMI 1640 (10% FCS). Target cells were harvested, washed twice in BSS (2% FCS) and resuspended in 100µl of BSS (2% FCS) containing 100µCi ^{51}Cr sodium chromate. Target cells were gently agitated for 90 minutes at 37°C after which time they were washed three times in BSS (2% FCS) and resuspended at 1 X 10^5 cells/ml in complete RPMI 1640 (10% FCS). Effector cells were dispensed in a volume of 100 µl into round bottomed microtitre wells and four serial one in three dilutions carried out. An appropriate number of target cells (the exact number being dependent on the maximum effector target ratio required and the numbers of T cells recovered from the cultures) were uniformly added to all wells. Control wells were set up in triplicate to assess lysis of target cells in the presence of 100µl 5% Triton-X100 (maximum release) or in the presence of 100µl RPMI 1640 (10% FCS) (spontaneous release). Plates were centrifuged at 1000 rpm for 1 minute and incubated at 37°C in a humidified 5% CO_2 incubator for 3 hours.

At the end of this period 100µl of supernatant was harvested from each well into appropriate plastic tubes. Tubes were sealed with paraffin wax and γ emission counted using an LKB 1272 Clinigamma Counter, (Pharmacia). Results were expressed as counts per minute (cpm) and percent specific lysis determined as follows:

$$\% \text{ Specific Lysis} = \frac{\text{Experimental (cpm)} - \text{Spontaneous (cpm)}}{\text{Maximum (cpm)} - \text{Spontaneous (cpm)}}$$

In all experiments spontaneous lysis was less than 10%.

7.2.4 Proliferation assays

T cell were harvested from T cell lines and clones washed and resuspended in complete RPMI 1640 (10% FCS). Syngeneic spleen cells were prepared as a single cell suspension in complete RPMI and irradiated (2000 rad). T cells (10^4 cells/well) and spleen cells (5 X 10^5 cells/well) were co-cultured in 96 well flat-bottom microtitre plates with serial dilutions of peptide (5μM - 0.5pM), or appropriate stimulator cells and 1-3% ConA-sup or 5-20iu hrIL-2. After 60 hours cells were pulsed for 12 hours with 0.5μCi tritiated thymidine/well. Incorporation of tritiated thymidine was measured in a Wallac beta plate counter and the arithmetic means of triplicates calculated. When testing HPLC-eluted fractions, 10μl of eluted material was used to stimulate the P91A-specific T cell clone P91.6.

7.2.5 Electroporation of mammalian cell lines

P1.HTR, RMA-S and there derivative cell lines were electroporated using the method of Chu and co-workers (1987) as modified by I. Campbell (ICRF; personal communication). Cells were harvested in the log phase of growth when culture cell viability was 100% and washed three times in hepes buffered saline (HeBs; 20mM Hepes pH 7.05, 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM Dextrose). Cells (1.2 X10^7/ml in 0.25ml) were aliquoted into 0.4cm BioRad electroporation cuvettes (BioRad Laboratories, Hemel Hempstead, UK) and sterile plasmid DNA (12.5μg) added. Cells were pulsed at various voltages constant capacitance (125μF) and left at room temperature for 10 minutes before seeding into complete medium. Where stable transfectants were required cells were plated into 96 flat bottom well plates (5 X 10^5 cells/plate) and placed on selection (0.75μg/ml hygromycin or 1mg/ml G418-sulphate). Cells that were transiently transfected were harvested for assay after 72 hours.

7.2.6 Flow cytometry

MHC stabilisation assay:

P1.HTR cells were placed in 96-well flat bottom microtitre plates at 2 x 10^5 cells/well and test peptides added. Following an 18 hour culture period cells were harvested and washed twice in ice cold PBS containing 1% normal rat serum and 0.02% azide. Cells were stained for 30 minutes on ice with the anti-H2-D^b/L^d directly FITC conjugated monoclonal antibody 28-14-8S (ATCC HB-27), (Ozato et al, 1980) and subsequently washed twice in ice cold PBS (0.02% azide). Cells were resuspended in PBS (0.02% azide) and analysed on a Becton Dickinson FACScan using Cell Quest software.

FACS analysis of T cell clones:

T cells were harvested 7 days following stimulation and washed twice in ice cold PBS containing 1% normal rat serum, 0.02% azide. Cells were stained on ice for 1 hour with KT-3 (anti-CD3), (Tomonari, 1988) or KT-15 (anti-CD8), (Tomonari and Spencer, 1990). Following this period cells were washed three times in ice cold PBS/azide and the second layer antibody, FITC conjugated goat anti-mouse Fc added. After one hour further staining on ice, cells were washed three times in ice cold PBS/azide. Fluorescence was assessed in the presence or absence of specific antibody.

7.2.7 Elution of peptides

P91A transgenic spleen cells (10^9) were lysed in 4ml 0.7% TFA followed by ultra-sonication (20 bursts. Branson sonifier and vortexed for 3 minutes. The lysate was microfuged (Sigma 2K15, 15000 rpm, 4°C, 30 minutes). The supernatant was collected and transferred to a 10-kDA cut-off Centricon filter and centrifuged at 5000 x g for three hours at 4°C. The filtrates were used for separation on a C2/18 reverse-phase HPLC column (SuperPac Pep-S, 250 x 4mm, particle size 5nm, pore size 100Å, Pharmacia). For peptide loading, buffer A (0.1% TFA in H_2O) was used. The gradient 0-60% acetonitrile at

1% per minute and at a flow rate of 1ml per minute. Fractions of 1ml were collected and dried by vacuum centrifugation (Savant, SpeedVac). The fractions were resuspended in 100 ml PBS and after 5 minutes sonication in a water bath, the samples were stored at -20°C. Synthetic peptides were loaded and eluted under identical conditions.

7.3 Molecular Biology Techniques

7.3.1 Preparation of DNA and RNA

Preparation of Genomic DNA:

5 X 10^6 T cells were harvested washed twice and resuspended in 2mls of PBS. 2mls of 2 X lysis buffer (20mM NaCl, 20mM Tris HCl pH 8.0, 4mM EDTA, 1% SDS, 150µg/ml proteinase K [Böehringer, Mannheim]) was added and cells gently mixed for 1 hour at room temperature. The lysate was subsequently incubated overnight at 37°C and the following day extracted twice with an equal volume of Tris-HCl equilibrated Phenol (0.1M Tris-HCl pH 7.4 and 0.1% 8-hydroxyquinoline) (Rathburn, UK) and chloroform (BDH, UK). Following the final chloroform extraction the aqueous layer was removed and the DNA precipitated by the addition of 1/10 volume 3M sodium acetate pH 4.8 and 2 X the total volume of 100% ethanol (BDH, UK). DNA was precipitated overnight at -20°C. The following day, genomic DNA was removed using a sealed glass pasteur pipette or gently spun to the bottom of the tube and washed twice in 70% ethanol. DNA was dried at room temperature for 1-5 minutes and resuspended in TE pH 7.4 (10mM Tris-HCl pH 7.4, 1mM EDTA). DNA concentration and purity was assessed by measuring the optical density at 260nM and 280nM.

Preparation of small quantities of plasmid DNA:

Small quantities of plasmid DNA were prepared from overnight bacterial cultures (bacterial colonies were picked and expanded overnight at 37° in 3ml of L-Broth containing appropriate antibiotics) as follows. 1.5ml of the bacterial culture was poured into an

Eppendorf tube and microfuged at 12,000 rpm for 2 minutes. Supernatant was removed, 100µl of lysis Buffer (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0, 4mg/ml lysosyme) added and the mix incubated for 5 minutes at room temperature. Subsequently, 200µl of 0.2M NaOH, 1% SDS was added and the mix incubated for a further 5 minutes at room temperature. 150µl of 3M sodium acetate pH 4.8 was then added and the tube gently mixed by inversion. Following centrifugation at 12,000 rpm the supernatant was collected and extracted with 400µl of Tris equilibrated Phenol/chloroform. The aqueous layer was collected after centrifugation at 12,000 rpm for 2 minutes and 300µl removed and precipitated with 750µl of ethanol. After 1 hour precipitation on ice, tubes were spun for 10 minutes at 12,000 rpm and DNA collected and washed with 70% ethanol. The pellet was subsequently air dried for 1-5 minutes and dissolved in 50µl TE/RNase (20µg/ml).

Preparation of large quantities of plasmid DNA:

Large quantities of plasmid DNA were prepared following the manufactures instructions using QIAGEN plasmid purification kits (QIAGEN Ltd, UK). This method is based on a modified alkaline lysis procedure which is followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under low-salt conditions.

Preparation of RNA and cDNA:

1-4 X 10^6 T cells were harvested, washed twice in PBS and pelleted at the bottom of RNase free Eppendorf tubes. RNA was isolated using RNAzol B (Biotecx Laboratories, Inc. Distributed in the UK by Biogenesis Ltd). Manufacturers instructions were followed. RNA was stored at -80°C in the presence of the RNase inhibitor RNAguard (Pharmacia, UK) as per manufacturers instructions. cDNA was prepared as follows. 1µg of RNA was incubated for 10 minutes at 70°C with 200ng of random hexamers, (Pharmacia, UK). The reaction was cooled to room temperature and the following added: 2µl dithiothreitol (100mM) (BDH, UK), 2µL dNTP's (10mM) (Pharmacia, UK), 200units of Superscript reverse transcriptase (BRL, UK), 1µl RNAguard (Pharmacia, UK) and 2µl of 5 X Superscript 1st strand Buffer, (BRL, UK). The reaction volume was adjusted to 40µl with RNAse free water and incubated at 37°C for 1 hour. Following incubation at 95°C for 5

minutes 60µl of RNAse free sterile distilled water was added. 1µl of cDNA was used as template in subsequent polymerase chain reactions (PCR).

7.3.2 Southern Analysis of DNA

DNA was digested overnight at 37°C using restriction enzymes and appropriate buffers (New England BioLabs, UK). Digestion was assessed by electrophoresis through 0.7% agarose gels containing 0.5µg/ml ethidium bromide. Large 0.7% agarose (0.5µg/ml ethidium bromide) gels were prepared and samples electrophoresed slowly overnight. The following day gels were gently agitated in 500ml of denaturing solution (1.5M NaCl, 500mM NaOH) for 1 hour then transferred to neutralising buffer (1.5M NaCl, 1M Tris-HCl pH 8.0) for 1 hour. DNA was transferred overnight to Hybond-N nylon membranes (Amersham Life Science, UK) by capillary action as per manufacturers instructions. The following day, DNA was UV cross linked to filters (UV-Stratalinker 2400 (Stratagene)). Filters were incubated for 3 hours at 55°C in prehybridisation solution (0.45M NaCl, 45mM sodium citrate, 0.1% Ficoll 400, 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.5% SDS, 100µg/ml sonicated herring testes DNA, 100µg/ml salmon sperm DNA). Radioactive probes were prepared using 20ng of DNA and the Ready-To-Go Labelling Kit (Pharmacia, UK) as per manufacturers instructions and α^{32}P-dCTP (Amersham Life Sciences, UK). Unincorporated α^{32}P-dCTP was removed by centrifugation through Sephadex G50 (BDH, UK) (5 minutes at 1500 rpm) and labelled probe collected. Probes were boiled and snap cooled immediately prior to use. Filters were transferred to hybridisation bottles and 5-10ml of hybridisation solution added, (as prehyb solution plus 5% Dextran Sulphate). Labelled probe was added and filters rotated slowly overnight at 65°C. The following day filters were washed at 65°C in 0.3M sodium chloride/30mM sodium citrate/0.1% SDS for 2 hours then subsequently in 0.03M sodium chloride/3mM sodium citrate/0.1% SDS. Background levels of radioactivity were checked every 15 minutes. Filters were dried and exposed to X-OMAT AR film (Kodak, Scientific Imaging Systems Ltd, UK) at -80°C.

7.3.3 Library construction and screening

2 X 10^8 T cells from clone 4A were expanded in the presence of BALB/c nude feeder spleen cells, washed twice in PBS and snap frozen in liquid nitrogen. Cells were packaged in dry ice and a custom cDNA library prepared (Invitrogen Corporation, Sorrento Valley, San Diego). 3.2μg of mRNA was obtained and oligo dT primed cDNA prepared. cDNA was size selected (> 500bp) on agarose gels and BstX-1/EcoR 1 adapters added. cDNA was ligated into the pcDNA2 vector, transformed into TOP 10F bacteria and 1.2 X 10^6 primary recombinants obtained. The average insert length of 10 clones analysed was 1.1kb.

The library was screened as follows. Large nylon filters (22cm x 22cm) Hybond-N (Amersham, UK) were laid on to LB agar plates containing 50μg/ml ampicillin. Bacteria were plated onto filters at 2.5 X 10^5/plate. Four plates were constructed in this manner. Plates were incubated overnight at 37°C. The following day, two replica filters were lifted from each master plate, placed on to antibiotic containing LB agar plates and incubated at 37°C for 2-3 hours. Master plates were stored at 4°C. Replica filters were treated with 10% SDS for 3 minutes, denaturing solution for 5 minutes, neutralising buffer for 6 minutes. Filters were then rinsed in 0.3M sodium chloride/30mM sodium citrate for 6 minutes, air dried and baked at 80°C for 2 hours. Following this period, filters were incubated at 42°C in 50mM Tris-HCl pH 8.0/1M sodium chloride/1mM EDTA/0.1% SDS and cellular debris removed from the filters. Filters were washed in 0.3M sodium chloride/30mM sodium citrate and hybridised with TCRA and TCRB specific probes. Hybridisation was performed as for Southern blots.

7.3.4 Polymerase chain reaction (PCR)

All primer sequences are listed in Appendix 1.

Reaction conditions were as follows:

TCRAV PCR using the primers of Casanova et al (1991).

30 cycles: 94°C, 60 seconds; 55°C, 60 seconds; 72°C, 60 seconds

TCRAV and TCRBV PCR using the primers of Candeias et al (1991).

26 cycles: 94°C, 60 seconds; 50°C, 120 seconds; 72°C, 60 seconds

Multiple PCR rounds were used to amplify TCRA and TCRB transcripts (figure 3.3 B)

P91A Constructs, (P91A exons 4 and 5, and H2-K^k signal sequence)

30 cycles: 94°C, 60 seconds; 65°C, 60 seconds; 72°C, 60 seconds.

Orientation of the TCRA and TCRB transcripts in the VA hCD2 vector.

30 cycles: 94°C, 60 seconds; 55°C, 60 seconds; 72°C, 60 seconds

Reactions:

60-200ng of template DNA was added to PCR reaction buffer (10nM Tris-HCl pH 8.4, 0.1% Tween-20, 200μM dNTPs, 50mM potassium chloride, 2mM magnesium sulphate and 125ng of appropriate sense and antisense primer), 0.6U of Taq polymerase (Gibco BRL) added and the final volume adjusted to 25μl with sterile distilled water. The solution was overlaid with one drop of light mineral oil (Sigma) and the samples placed on a Techne PHC-2 or Hybaid OmniGene thermocycler. Samples were preheated to 94°C for 5 minutes, followed by 26 or 30 cycles as shown above, and finally heated to 72°C for 8 minutes.

For the purposes of subcloning, PCR products were electrophoresed on 0.7% low melting point agarose gels (Gibco, BRL) and bands excised. An equal volume of distilled water was added to each gel fragment and the samples heated to 70°C. When the agarose had melted, DNA was extracted twice with phenol and chloroform and precipitated as described

previously. For the purposes of sequencing, PCR products were cloned into the TA cloning vector (pCR Vector, version 2.2) and transformed into TA Cloning One Shot competent cells, (TA Cloning Kit, Invitrogen).

P91A constructs were prepared as follows:
P91A exons 4 and 5 were amplified using 5' and 3' primers containing Pst-1 and Xho-1 restriction sites respectively. P91A PCR products were ligated into the expression vector pcDNA 1 (Invitrogen) following double digestion of PCR products and vector with the appropriate restriction enzymes. The H2-K^k signal sequence was amplified using 5' and 3' primers containing BamH-1 and Pst-1 restriction sites respectively and cloned into pcDNA 1. In order to prepare P91A constructs A and B, figure 5.1, the H2-K^k signal sequence was excised and cloned immediately 5' of P91A exons 4 and 5 utilising the shared Pst-1 site at the 5' end of P91A fragment and at the 3' end of the H2-K^k signal sequence fragment.

In all cases vector was dephosphorylated prior to ligation using Calf Intestinal Alkaline Phosphatase (Pharmacia, UK) as per manufacturers instructions and ligations were performed using T4 DNA ligase (Ready-To-Go T4 DNA Ligase, Pharmacia, UK).Ligations were carried at vector : insert molar ratios 1 : 1, 1 : 3 and 1 : 10 using a vector concentration of 100ng.

7.3.5 Chloramphenicol acetyl-transferase assay

The method described by Gorman (1985) was used. Briefly, cells were harvested washed three times in PBS, pelleted and resuspended in 100µM 0.25M Tris HCl, pH 7.8. Cells were disrupted by three successive freeze-thaw cycles (five minutes in ethanol/dry ice bath followed by five minutes in a 37° water bath). Reactions were set up as follows; 70µl 1 M Tris HCl, ph 7.8, 35µl water, 20µl extract, 5µl ^{14}C chloramphenicol (50 mCi/mmol) (Amersham, UK), 20µl 4 mM acetyl Coenzyme A, (Sigma, UK) and incubated for 1 hour

at 37°C. Chloramphenicol was extracted in 1 ml ethyl acetate which and evaporated to a final volume of 30µl. Samples were spotted onto silica gel thin layer chromatography (TLC) plates and run in a chloroform : methanol (95 : 5) equilibrated glass TLC tank.

7.3.6 DNA sequencing

DNA was sequenced using the method of dideoxynucleotide chain termination and the T7 Sequencing Kit (Pharmacia, UK). Manufacturers instructions were followed. Sequencing reactions were electrophoresed through 6% (19:1) acrylamide:bisacrylamide gels (50% urea) at 32 Watts for three hours. Gels were dried down on to filter paper and exposed overnight to X-OMAT AR film (Kodak, Scientific Imaging Systems Ltd, UK) at room temperature.

7.4 Computational Methods

The model for H2-L^d was built using the Composer modelling package from the crystallographic structures of HLA-A2 (HLA-A*0201: 2.6-Å, co-ordinates 3hla.pdb), HLA-Aw68 (HLA-A*6801: 2.6-Å, co-ordinates 2hla.pdb), and HLA-B27 (HLA-A*2705: 2.1-Å, co-ordinates: D.Madden, Gorga, J., Strominger, J. and Wiley, D., personal communication), and the geometry optimised.

N-terminal tetra and C-terminal tripeptides were modelled into the binding cleft initially in the conformation found in HLA-B27: peptide complexes. The sequence of the remainder of the peptide was changed to poly-alanine. None of the poly-alanine or H2-L^d backbone atoms were allowed to move in the subsequent geometry optimisation procedure and only residues of H2-L^d within 6 Å of a polyleucine N-terminal tetramer or C-terminal trimer were taken into account in geometry optimisation calculations (Thorpe, 1995). The energies obtained at the convergence point of the molecular mechanics calculation for each tetra- or tripeptide give rise to a qualitative series of binding energies.

Appendix 1

(A) TCRA V specific primers (Casanova et al, 1991) (sequence 5' to 3')

TCRA V1	GCACTGATGTCCATCTTCTC
TCRA V2	AAAGGGAGAAAAAGCTCTCC
TCRA V3	AAGTACTATTCCGGAGACCC
TCRA V4	CAGTATCCCGGAGAAGGTC
TCRA V5	CAAGAAAGACAAACGACTCTC
TCRA V6	ATGGCTTTCCTGGCTATTGCC
TCRA V7	TCTGTAGTCTTCCAGAAATC
TCRA V8	CAACAAGAGGACCGAGCACC
TCRA V9	TAGTGACTGTGGTGGATGTC
TCRA V10	AACGTCGCAGCTCTTTGCAC
TCRA V11	CCCTGCACATCAGGGATGCC
TCRA V12	TCTGTTTATCTCTGCTGACC
TCRA V13	ACCTGGAGAGAATCCTAAGC
TCRA Vα34S-281	TCCTGGTTGACCAAAAAGAC
TCRA V16	TGGTTTGAAGGACAGTGGGC
TCRA V17	CATTCGCTCAAATGTGAACAG
TCRA V18	CAAATGAGAGAGAGAAGCGC
TCRA V19	GGAAAATGCAACAGTGGGTC
TCRA V20	GACATGACTGGCTTCCTGAAGGCCTTGC

TCRA C antisense primer (sequence 5' to 3')

TCRA C	GCGAATTCCAGACC TCAACTGGACCACAG

(B) TCRA V and TCRA C primers, (Candeias et al, 1991) (sequence 5' to 3')

NK121 GTCCTGACCTCGCATGCCA

NK122 GTCCTGACCTCGCATGCCA

NK123 GTCCTGACCTCGCATGC

MQ284 AGCACACGAGGGTAGCCTT

MS175 GACAGAACTTTGAATTCCTCTGCTTTTTGATGG

OE7 GGAGTCACATTTCTCAGATCC

TCRB V and TCRB C primers (Candeias et al, 1991) (sequence 5' to 3')

NW36 GTCCTGACCTCGCATGCTTC

NW37 GTCCTGACCTCGCATGCTTCA

NW38 GTCCTGACCTCGCATGC

NJ108 GGCCCCATTGCTCTTGGAATC

NJ109 CGGCACATTGATTTGGGAGTC

NJ110 TCTCGAATTCAGGCAGAGGGTGCTGTCC

OE6 CACAGCAGGTTCTGGGTTCTG

(C) Primers used in the production of P91A constructs A, B, C and D

H2-K^k signal sequence 5' primer (Sequence 5' to 3')

GCCGGATCCTAAGTAAGTAAGACCGGTGCGATGGCACCCTGCACGC
TGCTCCTGCTGTTGGCGGCCGCCCTG

H2-K^k signal sequence 3' primer (sequence 5' to 3')

CCGCTGCAGAGAGTGAGGGCCCGCGCGGGTCTGAGTCGGGGC

Constructs A and B, P91A exon four 5' primer (sequence 5' to 3')

(A) CGCCTGCAGAAAATCTCTGATGACCTGATGCAGAAG
(B) C

Constructs C and D, P91A exon four 5' primer (sequence 5' to 3')

(C) CGCCTGCAGTAAGTAAGTAAGACCGGTGCGATGGCACAG

AAAATCTCTGATGACCTGATGCAGAAG
(D) C

P91A exon five, 3' primer (sequence 5' to 3')

GCGCTCGAGTTAGTTGAGATCCTCCTCAGAGATCAATTTTTGCTCCC
CTGTGTAATAGAGGTACCTGGCCCA

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