IDENTIFICATION OF REGULATORY REGIONS THAT DETERMINE EXPRESSION OF THE MURINE CD8 LOCUS

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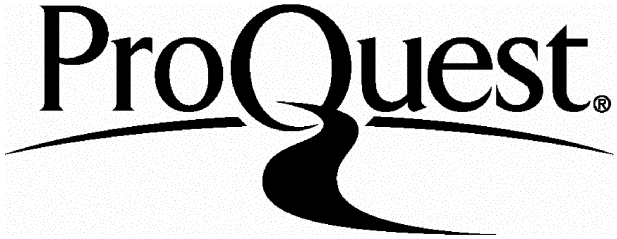

ABSTRACT

The coreceptors CD4 and CD8 play a crucial role during thymocyte development and T cell effector function and their expression is developmentally regulated. To determine the molecular mechanisms underlying the regulation of CD8 gene expression, the murine CD8 gene locus was cloned and analysed for deoxyribonuclease (DNaseI) hypersensitivity. Such analysis revealed three thymocyte specific DNaseI hypersensitive regions (cluster II, III and IV). To characterise the role of different clusters of hypersensitive sites in CD8 gene regulation in the context of the endogenous chromosomal location, we deleted selected regions from the mouse genome by homologous recombination.

Deletion of cluster III (either all three sites or just sites 1 and 2), which is located in the intergenic region between the CD8α and β genes and directs expression of a reporter transgene in mature CD8 T cells only, affected expression of CD8αα homodimers on intraepithelial (IEL) T cells, both on γδTCR and αβTCR subsets. Surprisingly, none of the thymocyte or peripheral αβTCR CD8αβ T cell subsets were affected by this mutation, which indicated differential activation of these elements within the various T cell subsets.

Deletion of cluster II, which is located immediately upstream of the CD8α gene, had two main effects: it affected the levels of expression of the CD8 gene and caused an abnormal subset distribution in the thymus with fewer DP and CD8 SP cells and an increase in cells with a CD4 SP phenotype. Staining with several maturation markers, showed that the increased numbers of thymocytes falling within the CD4 SP gate were immature cells. It is concluded that removal of regulatory sequences present in cluster II disturbs the normal developmental chromatin remodelling of the CD8 locus and as a consequence the expression of the CD8α gene.

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ABBREVIATIONS

BCR	B cell receptor
bp	Base pair
BrdU	5-Bromo-2-deoxyuridine
BSA	Bovine serum albumin
°C	Centigrade
CII	Cluster II
CIII	Cluster III
CIV	Cluster IV
CD3	Cluster of differentiation antigen 3
CD4	Cluster of differentiation antigen 4
CD5	Cluster of differentiation antigen 5
CD8	Cluster of differentiation antigen 8
CD69	Cluster of differentiation antigen 69
cDNA	Complementary DNA
cm	Centimetre
dCTP	Deoxy-cytidine-triphosphate
ddH_2O	Double distilled water
DMEM	Dulbecco's modification of Eagles medium
DMSO	Dimethylsulphoxide
DN	$CD4^-CD8^-$ double negative
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonuclease I
DNaseI-HSS	DNaseI hypersensitive site
DP	$CD4^+CD8^+$ double positive
DTE	Dithioerythritol
E.coli	Escherichia coli
EDTA	Ethylene-diamine-tetraacetate
EF	Embryonic fibroblasts
ES	Embryonic stem cells

FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluoresceine isocyanate
FTOC	Foetal thymic organ culture
HBS	Hepes buffered saline
HBSS	Hank's balanced salt solution
HLH	Helix-loop-helix
HSA	Heat stable antigen
HSV-tk	Herpes simplex virus thymidine kinase
IEL	Intraepithelial lymphocytes
Ig	Immunoglobulin
kb	Kilobase
kDa	Kilodalton
LCR	Locus control region
LIF	Leukaemia inhibitory factor
MAR	Matrix attachment region
2-ME	2-Mercaptoethanol
MEL	Mesenteric Lymphocytes
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major histocompatibility complex
ml	Millilitre
µl	Microlitre
mRNA	Messenger ribonucleic acid
ng	Nanogram
NK	Natural killer cells
nm	Nanometre
O.D.	Optical density
PBS	Phosphate buffered saline
PE	Phycoerythrine
PEG	Polyethylene glycol
PEV	Position effect variegation

RBE	Recombinase binding element
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	Revolutions per minute
RT	Room temperature
SA	Streptavidin
SDS	Sodium-dodecyl-sulphate
sIg	Surface Ig
SP	$CD4^+CD8^-$ or $CD4^-CD8^+$ single positive
SSC	Sodium chloride, sodium citrate
TAE	Tris-sodium acetate EDTA electrophoresis buffer
TBE	Tris-borate EDTA electrophoresis buffer
TCR	T cell receptor
3'UT	3' untranslated
u.v.	Ultraviolet
w/v	Weight per volume

PUBLICATIONS

Some of the work described in this thesis resulted in two publications:

1. Hostert, A., Garefalaki, A., Mavria, G., Tolaini, M., Roderick, K., Norton, T., Mee, P. J., Tybulewicz, V. L., Coles, M., and Kioussis, D. (1998). Hierarchical interactions of control elements determine CD8alpha gene expression in subsets of thymocytes and peripheral T cells. Immunity *9*, 497-508.

2. Garefalaki, A., Coles, M., Hirschberg, S., Mavria, G., Norton, T., Hostert, A., and Kioussis, D. Variegated expression of CD8α resulting from *in situ* deletion of regulatory sequences. Submitted for publication December 2001.

INTRODUCTION

The Immune System

The evolution of the immune system in mammals has been driven by the need for protection against infection by potentially dangerous organisms such as viruses, bacteria, fungi and other parasites. Defects in the immune response almost always lead to higher susceptibility to infection. To fulfil these protective functions the immune system must be capable of recognising pathogens and foreign molecules called antigens and, therefore, distinguish 'foreign' from 'self'. There are two types of host defence against infectious agents, innate immunity, which is present at all times and is non specific, and the adaptive specific immune response, which is induced by antigen and gives rise to long-lasting protection against disease.

Innate immunity participates in the early phases of host defence that protect the organism during the four or five days it takes for the adaptive immunity to mature. The elements of the innate immune system act directly on the pathogen with no need for induction. One of the main components of innate immunity is the complement, which can act directly on bacteria. Some pathogens are resistant to direct attack and they are recognised by phagocytic cells, such as macrophages and neutrophils, and are ingested and destroyed by them. Furthermore, macrophages release chemical signals, which induce the liver to produce proteins (acute phase proteins) which bind to bacteria and activate complement.

The adaptive immune response is mediated mainly by white blood cells, which express receptor molecules on their cell surface capable of recognising foreign antigen. These cells, known as lymphocytes, are present in almost all tissues of the body and circulate constantly through the blood. Two main functional types of antigen-specific lymphocytes have been distinguished. B lymphocytes develop into cells known as plasma cells that secrete antibodies. T lymphocytes develop into effector cells that either kill other cells that are infected with intracellular pathogens or help the function of other cells of the immune system including macrophages and B lymphocytes. All lymphocytes derive from stem cells in the bone marrow. From there T lymphocytes migrate to the thymus to undergo maturation and acquire antigen specificity, while B cells mature in the bone marrow. Those two organs (bone marrow and thymus) are the central lymphoid organs, where lymphocyte development takes place. Mature, antigen specific

lymphocytes migrate from these tissues via the blood to the peripheral lymphoid organs, which are the lymph nodes, spleen and Peyer's patches. Most of the cell interactions leading to immune responses against antigen occur in the peripheral lymphoid organs.

Although both cell types are capable of recognising antigen via cell surface receptors, they do so in a different manner. B cells recognise whole or 'native' antigen, whereas T cells recognise antigen only in the form of short peptides (8-20 amino acids) presented on cell surface Major Histocompatibility Complex (MHC) molecules (Zinkernagel and Doherty, 1979, Buus et al., 1986, Braciale, 1992).

Major Histocompatibility Complex (MHC) molecules

T cells can detect the presence of intracellular pathogens because peptide fragments derived from the pathogens' proteins bind to specialised molecules, called MHC molecules, that deliver these foreign peptides to the cell surface. There are two different classes of MHC molecules, the MHC class I and MHC class II molecules, and they are both encoded in the same gene locus in chromosome 17 in mouse (Haskins et al., 1983, Stern and Wiley, 1994, Hood et al., 1983, Swain, 1983). The MHC class I cell surface glycoprotein consists of an α or heavy chain that spans the membrane and a smaller chain, the β2-microglolulin, which is not encoded in the MHC locus (Bjorkman et al., 1987, Rammensee et al., 1993). MHC class II molecules consist of a complex of an α and a β chain, both of which span the membrane (Kaufman et al., 1984, Brown et al., 1993, Stern et al., 1994, Babbitt et al., 1985). The two different classes of MHC molecules bind peptides from different sources inside the cell for presentation at the cell surface (Brodsky and Guagliardi, 1991). MHC class I molecules are expressed on all body cells and bind peptides from pathogens that replicate in the cytosol, such as viruses and bacteria. MHC class II molecules are expressed primarily on cells of the immune system and bind peptides derived from proteins degraded in intracellular vesicles (Peters et al., 1991, Tulp et al., 1994, Davidson et al., 1991).

B cells

B cells arise and undergo maturation in the bone marrow in mammals and their main function is to produce antibodies. Antibodies, also called immunoglobulins (Ig), are composed of four polypeptide chains, two heavy chains and two light chains (Edelman et al., 1969) which are linked to each other by disulphide bonds. Each chain is subdivided into a variable and a constant part (Hilschmann and Craig, 1965). The variable part determines antigen specificity, whereas the constant part defines immunoglobulin class. The immunoglobulins are divided into five classes, IgM, IgD, IgA, IgG and IgE, according to their constant region and B cells express different classes of immunoglobulins depending on their developmental stage (Wienands et al., 1990). As mentioned above, B cells recognise antigen in its native form and they do so via the B cell receptor (BCR). The BCR consists of surface immunoglobulin (sIg) (immunoglobulin with a short cytoplasmic tail), which is the product of alternative mRNA splicing of the Ig heavy chain, in association with two disulphide-linked transmembrane polypeptides, the Igα and Igβ molecules. The dependence of different immunoglobulin classes on the Igα/Igβ heterodimer for surface transport is very different (Venkitaraman et al., 1991, Hombach et al., 1990, Campbell and Cambier, 1990). The cross-linking of surface immunoglobulins by antigen leads to transmembrane signalling. Upon antigen recognition, the surface immunoglobulin is endocytosed, which allows the internalisation of antigen and its subsequent processing and presentation to T cells. T helper cells will then recognise peptide fragments of the processed antigen and will deliver signals that are sufficient to activate B cells. Following activation, the B cells differentiate into plasma cells, which produce antibodies specific for the recognised antigen.

T cells

In contrast to B cells, T cells recognise antigen in the form of short peptides (8-20 amino acids) presented on MHC class I or class II molecules. This recognition is mediated, like in the B cells, by a cell surface receptor, called the T cell receptor (TCR) (Haskins et al., 1983, Reinherz et al., 1983, Meuer et al., 1983). Every T cell bears about 30000 T cell receptor molecules on its surface. Each receptor consists of two polypeptide chains, the TCR α and β chains, bound to one another by a disulphide bond

(Dembic et al., 1986, Kappler et al., 1983). T cell receptors, like immunoglobulins, have variable and constant regions. However, the main difference between B and T lymphocyte receptors is that the cell surface immunoglobulin molecule that serves as B cell receptor has two identical antigen recognition sites, while the TCR is smaller and has a single recognition site. The T cell receptor is stably associated with a complex of proteins on the cell surface, called the CD3 complex (Brenner et al., 1985, Weiss and Stobo, 1984, Allison et al., 1984). The complex consists of 5 distinct proteins, the CD3γ, CD3δ, CD3ε, CD3ζ and CD3η chains (Samelson et al., 1985) which are involved in TCR complex assembly and signal transduction (Koning et al., 1990). The ζ chain exists in the TCR complex primarily as a disulphide-linked homodimer (ζζ) or in a minority of receptors as a ζη heterodimer. The η chain differs from ζ chain only in its C-terminal exon as a result of alternative splicing of the ζ mRNA (Irving and Weiss, 1991, Clayton et al., 1991). It is believed that the CD3ε subunit can associate with the γ or the δ chain of the CD3 complex to form γε or δε heterodimers (Blumberg et al., 1990). There is evidence that components of the CD3 complex are phosphorylated on serine and tyrosine residues upon T cell activation with antigen (Samelson et al., 1986, Oettgen et al., 1985).

The generation of diversity of the TCR occurs by somatic rearrangements of gene segments that encode the variable parts of the receptor polypeptides (Kronenberg et al., 1986). The TCRβ chain gene locus consists of gene segments encoding the variable (V), joining (J), diversity (D) and constant (C) regions (Tunnacliffe et al., 1985, Toyonaga et al., 1985, Concannon et al., 1986, Tillinghast et al., 1986, Patten et al., 1984). On the other hand, the TCRα chain is encoded by variable (V), joining (J) and constant (C) gene segments (Winoto et al., 1985, Yoshikai et al., 1985, Jouvin-Marche et al., 1990). For both the TCRα and TCRβ chains somatic recombination occurs between one of each of the V, J and in the case of the β chain the D gene segments. Recombination is mediated by recombination signal sequences located upstream or downstream of each of the gene segments (Tonegawa, 1983, Hesse et al., 1989). The large number of V, D and J gene segments, together with the imprecise joining of the different segments leads to the very high diversity of the TCR.

There is an alternative type of T cell receptor made up of different polypeptides designated γ and δ chains. The γδ heterodimer, like the αβ heterodimer, consists also of variable (V) and constant (C) regions and is associated with the CD3 complex on the cell surface (Raulet, 1989). The V domains are encoded by variable(V), diversity (D) and joining (J) gene segments in the δ chain and variable (V) and joining (J) gene segments in the γ chain, which assemble by gene rearrangements to form the complete γ and δ chains. Because of the small number of V regions in the γ and δ genes there is a restricted V gene diversity in the γδ receptor. On the other hand, γδ receptors have an increased junctional variability, which can compensate for the few V regions. T cells bearing γδ T cell receptors are the first TCR bearing cells to appear in ontogeny on day 14 of gestation in the murine foetal thymus. They are a distinct lineage of cells and little is known about their function (Constant et al., 1994). In peripheral lymphoid tissues, only a very small percentage (1-5%) of CD3 positive cells express γδ T cell receptors instead of αβ receptors. However, in epithelial tissues, especially in the epidermis and small intestine of the mouse most T cells express γδ T cell receptors (Takagaki et al., 1989, Rocha et al., 1992). The mechanism that controls the lineage choice between the αβ or γδ T cell receptor is not known. There is one report that suggests that reduced Notch activity favours the γδ over the αβ T cell fate (Washburn et al., 1997). T cells can be divided into two groups on the basis of their cell surface marker expression. Two such markers are the cell surface glycoproteins CD4 and CD8. During antigen presentation, CD4 and CD8 molecules associate on the T cell surface with components of the T cell receptor and for this reason they are called coreceptors. CD4 is expressed on 70% of the peripheral mature T cells and these recognise antigenic peptides in the context of MHC class II (Meuer et al., 1982). These cells, known as T helper cells are thought to activate macrophages and to stimulate B cells that have bound antigen to differentiate into antibody producing plasma cells. The remaining 30% of T cells express the CD8αβ heterodimer, are cytotoxic T cells and recognise antigen in the context of MHC class I (Swain, 1983).

T Cell Development

T cells develop in the thymus, a lymphoid organ consisting of two lobes that is located in the upper thorax, just above the heart. Each of the lobes consists of a large number of bone marrow derived cells together with epithelial cells and developing thymocytes. The blood vessels divide each lobe into numerous lobules with an outer cortical region called the thymic cortex and an inner medulla. The tightly packed cortex contains mainly cortical epithelial cells with long branched dendritic processes rich in MHC class II expression and a large number of immature thymocytes that are in close contact with those cells (Wekerle et al., 1980, Kyewski et al., 1982, Kyewski and Kaplan, 1982). The medulla, less tightly packed with thymocytes than the cortex, contains MHC class I and MHC class II positive epithelial cells with longer processes than those found in the cortex (van Ewijk, 1991).

The first precursor T cells derived from haemopoietic stem cells in the foetal liver or the bone marrow in the adult, migrate to populate the thymus at day eleven of embryonic development (Owen and Ritter, 1969), where they develop into mature T cells. The developmental stages of thymocyte maturation can be followed by the expression of cell surface markers such as the CD4 and CD8 coreceptors and the TCR. Prothymocytes enter the thymus as cells negative for expression of CD4 and CD8 and are called double negative (DN). Expression of a rearranged TCR β chain in conjunction with a pre-α TCR chain then delivers a signal to the DN cells to proceed in their differentiation pathway (Fehling et al., 1995, Groettrup et al., 1993). Upregulation of one of the CD4 or CD8 coreceptors gives rise to $CD4^+CD8^-$ or $CD4^-CD8^+$ intermediate populations respectively between the DN and $CD4^+CD8^+$ double positive (DP) cells (Guidos et al., 1989, Guidos et al., 1989, Nikolic-Zugic and Bevan, 1990). These immature αβ TCR negative, coreceptor single positive (SP) cells are present in various ratios depending on the inbred mouse strain (Hugo et al., 1991). The other coreceptor is then upregulated and the cells become DP, as they express both CD4 and CD8 (Fowlkes and Pardoll, 1989). Approximately 80% of all thymocytes are DP and express the αβ TCR and it is these cells that undergo the processes of positive and negative selection (Schwartz, 1989).

Positive and negative selection

As mentioned above, TCR specificity is generated by random recombination between TCR gene segments. This can give rise to two types of TCRs, that are either capable or incapable of recognising self MHC. Those cells that are capable of recognising self in terms of MHC via their TCR are positively selected (Swat et al., 1992, Benoist and Mathis, 1989) whilst the other thymocytes, incapable of recognising self MHC die by apoptosis. The randomly generated TCRs could not only be incapable of recognising self, but also capable of strongly recognising self peptides generated from endogenous proteins and presented on MHC. These cells could therefore recognise autoantigens and would be potentially dangerous since they might be able to mount an immune response against self. These cells are clonally deleted by a process called negative selection (Fowlkes et al., 1988, Kappler et al., 1987, Kappler et al., 1988, Smith et al., 1989).

One model to explain positive and negative selection is the affinity model. This model suggests that thymocytes whose TCR binds MHC/peptide with high affinity are eliminated by negative selection, whereas low affinity interaction results in positive selection (Sprent et al., 1988). Only a small percentage of the DP thymocytes complete the selection process (Egerton et al., 1990) and differentiate into MHC class II or MHC class I restricted mature thymocytes having downregulated the CD8 or the CD4 coreceptor respectively (von Boehmer et al., 1989). These CD4 or CD8 SP cells migrate out of the thymus to populate the peripheral lymphoid organs and establish the T cell pool.

Lineage commitment

The coreceptor phenotype is correlated with the specificity of the TCR for MHC class. Two main models have been proposed that address how TCR and coreceptor specificity are matched. The stochastic model proposes that DP cells randomly turn down expression of either CD4 or CD8, with no regard for TCR specificity, but only those cells that have a TCR capable of recognising an MHC molecule in conjunction with the appropriate coreceptor (MHC class II and CD4 or MHC class I and CD8) survive (Chan et al., 1993, Davis et al., 1993). Those cells that have a mismatched TCR and coreceptor recognition for MHC die. In contrast, the instructive model suggests that TCR specificity

for MHC determines which of the two coreceptors is downregulated (Borgulya et al., 1991, Robey et al., 1991). There is also evidence that provides support for a model including both stochastic and instructive mechanisms. According to this model, thymocyte commitment to the CD8 lineage requires MHC class I dependent instructional signals, whereas thymocyte commitment to the CD4 lineage is MHC independent and may occur by default (Suzuki et al., 1995, Benveniste et al., 1996, Lucas et al., 1995). One report suggested that an activated form of Notch during thymic development biases the CD4/CD8 lineage decision in such a way that the development of CD8 T cells is favoured over the development of CD4 T cells (Robey et al., 1996). Recently it has been proposed that the amount of $p56^{lck}$ signal brought by the coreceptor into the TCR complex could play an important role in CD4/CD8 lineage commitment (Hernandez-Hoyos et al., 2000, Basson et al., 1998, Bommhardt et al., 1997).

Extrathymic T cell differentiation

The development of most of the T lymphocytes found in peripheral lymphoid organs occurs in the thymus, as described previously. However, T cells are observed in athymic mice (Rocha et al., 1992), or in mice with genetic defects preventing the major T cell differentiation pathway in the thymus. These observations have led to the hypothesis that T cell differentiation also occurs extrathymically (Poussier and Julius, 1994), in particular in the gut and in the liver. T cells of extrathymic origin differ in phenotype and functional behaviour from T lymphocytes resulting from the main thymic differentiation pathway and are rare in peripheral lymphoid organs. However, they are abundant in sites such as the gut epithelium, where they may have an important role in local immune responses (Rocha et al., 1995). While thymic and intestinal epithelia are both endoderm derived, their functional maturation does not occur in parallel. In mice, intraepithelial T lymphocytes (IEL) appear relatively late in life and increase in numbers with aging (Rocha et al., 1992, Klein, 1996).

Furthermore, IEL may express either type of TCR, $\alpha\beta$ or $\gamma\delta$ (Guy-Grand et al., 1991). The distribution of the two cell types varies in different organs, between species and, within the same species, between different strains (Rocha et al., 1992). The thymus independent IEL do not undergo the processes of negative and positive selection that have been described for thymocytes previously (Rocha et al., 1992). In contrast to

conventional T cells, such as those isolated from lymph nodes or spleen, IEL located in epithelial cell layers exhibit a markedly different expression pattern of the CD4 and CD8 coreceptor molecules. While in mesenteric lymph node cells (MEL) the ratio of CD4 to CD8 cells is 3:1, this is almost reversed in IEL with 70% expressing CD8 and less than 10% expressing CD4 (Maloy et al., 1991).

The CD8 molecule expressed on thymocytes and on thymus derived peripheral T cells is comprised of two disulphide linked polypeptide chains, CD8α or CD8α' and CD8β (see below). However, the CD8α chain can be expressed on the membrane in the absence of CD8β in the form of CD8αα homodimers. In contrast to CD8+ MEL, which almost exclusively express the CD8αβ heterodimer, the αβTCR positive IEL express either CD8αα homodimers, or CD8αβ heterodimers on their surface (Guy-Grand et al., 1991). The IEL that belong to the γδTCR lineage express almost exclusively CD8αα homodimers (Poussier and Julius, 1994) (Rocha et al., 1992). The IEL that express CD8αα homodimers are thought to be extrathymically derived (Rocha et al., 1994). The expression of CD8αα homodimers on IEL indicates that the CD8α and CD8β genes can be differentially regulated in this T cell subset.

It has been shown, that the deficiency in MHC class I expression results in lack of mature CD8 T cells, because of a failure in positive thymic selection (Zijlstra et al., 1990, Koller et al., 1990). In contrast, the frequency and phenotype of γδ TCR IEL in these mice is unaffected by the absence of MHC class I expression, indicating that γδ T cells do not require interactions with class I molecules for their maturation (Correa et al., 1992, Das and Janeway, 1999).

Molecular Biology of CD4 And CD8

The surface molecules CD4 and CD8 undergo a complex pattern of expression in the thymus. Thymocytes pass through a process of selection and finally they leave the thymus to populate peripheral lymphoid organs. Mature T cells express the CD4 and CD8 coreceptors in a mutually exclusive manner. To understand that complex pattern of expression, it is very important to know the structure, genomic organisation and function of the CD8 and CD4 molecules.

The cell surface molecule CD8

The mouse CD8 molecule is a cell surface glycoprotein expressed on thymocytes and T cell subsets. CD8 is primarily expressed as a heterodimer composed of two polypeptides, the Lyt-2 (CD8α) and Lyt-3 (CD8β) (Cantor and Boyse, 1975) (Walker et al., 1984). The two polypeptide chains are linked with disulphide bonds to form the heterodimer (Kirszbaum et al., 1989, Ledbetter et al., 1981). The two chains are encoded by two separate genes, CD8α/Lyt-2 (CD8α) and CD8β/Lyt-3 (CD8β) which are tightly linked to each other (Itakura et al., 1972) and to the Ig-κ light chain locus (Gottlieb, 1974, Gibson et al., 1978). The CD8α and CD8β genes are located on murine chromosome 6, they are 36kb apart from each other and they are organised in the same transcriptional orientation (Ledbetter and Seaman, 1982, Gorman et al., 1988).

The CD8α/Lyt-2 antigen is found in two polypeptide forms, 38kDa (α) and 34 kDa (α') (Walker et al., 1984) and each one of the two α chains can form a heterodimer with the β chain (Jay et al., 1982). The CD8β/Lyt-3 antigen, on the other hand, is found as one polypeptide chain, smaller than the CD8α/Lyt-2, 30kDa in size. The disulphide bonding between the CD8α and CD8β polypeptides occurs between cysteine residues located in the extracellular domains of the CD8α and CD8β chains (Kirszbaum et al., 1989). Both polypeptides are glycosylated with three N-linked glycan units having been identified on the CD8α subunit and one N-linked glycan unit on the CD8β (Rothenberg and Triglia, 1983, Luscher et al., 1985).

The CD8 molecule is predominantly expressed as a heterodimer composed of an α and a β chain. However, the CD8α chain can be expressed on the cell surface in the absence of CD8β (Blanc et al., 1988, Gorman et al., 1988) by forming CD8αα

homodimers. In contrast, the CD8β protein is expressed on the surface only in association with the CD8α chain. Conventional T cells, such as those isolated from the lymph nodes or spleen, express CD8αβ heterodimers on their surface, whereas intraepithelial lymphocytes (IEL) located in epithelial cell layers express both CD8αα homodimers and CD8αβ heterodimers on their surface (Guy-Grand et al., 1991).

Both genes encoding the CD8α and CD8β chains have been found to have two allelic forms, Lyt-2.1 and Lyt-2.2 for the CD8α and Lyt-3.1 and Lyt-3.2 for the CD8β gene (Boyse and Old, 1971). There is serologic difference between the two allelic forms of each of the CD8α and CD8β genes. This is due to nucleotide differences between the alleles, which results in amino acid substitutions in the extracellular domains of the CD8α and CD8β molecules (also see below).

The human CD8 is also a transmembrane molecule that, like the murine CD8, consists of an α (Leu-2) (Kavathas et al., 1984) and a β chain (Johnson, 1987), which form a heterodimer. Both chains have homology to the immunoglobulin variable region light chain family (Littman et al., 1985, Sukhatme et al., 1985).

The CD8α structure and genomic organisation

Initially the human CD8α cDNA was cloned from L cells and subsequently the murine CD8α was isolated. The predicted amino acid sequence showed that both the human and the mouse CD8α molecules are members of the immunoglobulin super gene family (Zamoyska et al., 1985). Sequence analysis showed that both molecules have a high structural and sequence homology, thus being 56% and 61% identical at the amino acid and nucleotide level respectively (Nakauchi et al., 1985).

The genomic organisation of the murine CD8α gene was determined by Nakauchi et al (1987) and Liaw et al (1986) and it was found to consist of five exons and four introns and spans approximately 4.4 kilobases (Liaw et al., 1986). The exon organisation corresponds approximately to the functional domains of the protein. Thus, exon I encodes the fused leader peptide and immunoglobulin (Ig) variable region-like domain, exon II the hinge-like region, exon III the transmembrane domain and exons IV and V encode the cytoplasmic tail (Nakauchi et al., 1987).

Alternative splicing to include or exclude exon IV results in two forms of mRNA and accounts for the difference in size between the α and α ' chains of the CD8 described

above (Zamoyska et al., 1985, Tagawa et al., 1986). Thus, the two chains differ in their cytoplasmic tails as a result of a 31bp deletion in the α' relative to the α chain cDNA sequence (Liaw et al., 1986). Although mRNA encoding the α' chain is efficiently translated in thymocytes and mature T cells, mature T cells retain the α' protein in a late cellular compartment and do not express it on the cell surface (Zamoyska and Parnes, 1988).

The genomic organisation of the human CD8α gene has also been determined and is very similar to that of the mouse. The human CD8α gene spans approximately 8kb on chromosome 2 and is organised into six exons which encode separate functional domains of the protein. Exon I encodes the 5' untranslated region and leader peptide, exon II the Ig V-like region, exon III the hinge-like region, exon IV the transmembrane domain and exons V and VI the cytoplasmic tail of the protein (Norment et al., 1989). Alternative splicing is also possible in the human CD8α gene, as was previously described for the murine CD8α before. Exclusion of nucleotide sequences from exon VI, due to alternative splicing, results in a transcript, which encodes a secreted form of the protein. This transcript accounts for approximately 15% CD8α mRNA in human T cell leukaemia lines and in normal human tissues (Norment et al., 1989).

The CD8β structure and genomic organisation

The genomic organisation of the murine CD8β (Ly-3) gene was determined by several groups (Blanc et al., 1988, Gorman et al., 1988, Nakayama et al., 1989). It spans approximately 14kb on chromosome 6 in mouse, it is present in a single copy in the genome and consists of six exons and five introns. As in the case of CD8α, the exons correlate well with the putative functional domains of the protein. Exon I encodes the leader peptide, exon II encodes the Ig variable region-like and joining segment-like region (Johnson and Williams, 1986), exon III encodes the hinge-like region, exon IV the hydrophobic transmembrane domain, exon V part of the cytoplasmic tail and finally exon VI encodes the remainder of the cytoplasmic tail and the 3' untranslated region (Nakayama et al., 1989). The organisation of exons corresponding to functional domains of the protein is very similar to other members of the Ig super gene family (Liaw et al., 1986), as was shown for the CD8α gene.

There is a human gene homologous to the mouse CD8β gene. The human CD8β gene was mapped to chromosome 2, like the human CD8α gene (Spurr et al., 1988). Three different CD8β cDNAs have been cloned and they appear to have different cytoplasmic tail lengths due to alternative splicing (Norment and Littman, 1988). In contrast to the single copy CD8β gene locus in mouse, humans appear to carry a duplicated CD8β gene (Nakayama et al., 1992). CD8β1 has been mapped to chromosome 2, however the chromosomal location of CD8β2 is still to be determined. Structurally, the CD8β1 consists of nine exons, whilst CD8β2 was found to contain seven. The two genes are 98.5% identical in the coding region. The human and the mouse CD8β chain are 50.7% homologous at the amino acid level (Johnson, 1987).

Serological differences between CD8α and CD8β alleles

As described above, both the CD8α and CD8β genes have two serologically defined alleles. In the case of CD8α the two described alleles are the Lyt-2.1 and Lyt-2.2 (Gorman et al., 1988). A single nucleotide change at amino acid position 78 results in an amino acid substitution from methionine (Lyt-2.2) to valine (Lyt-2.1). This change is responsible for the serologic difference between the two CD8α alleles (Liaw et al., 1986). The two alleles of the CD8α gene are highly conserved, not only in the protein coding region, but also in the 5' untranslated region and the introns.

The two alleles of the CD8β are called Lyt-3.1 and Lyt-3.2. Comparison of the sequence of Lyt-3.1 and Lyt-3.2 has revealed a single nucleotide difference leading to an amino acid substitution in the extracellular domain of the protein. Specifically, the change from serine at amino acid position 77 in Lyt-3.1 to arginine in Ly-3.2 is responsible for the serological difference between the two alleles of the CD8β gene (Nakayama et al., 1989).

Different inbred mouse strains bear different alleles of the CD8α and CD8β genes. Specifically for the CD8α gene, C57Bl/10 and 129/Sv mice express the CD8α/Lyt-2.2 allele, whereas CBA/Ca and DBA/2 mice express the CD8α/Lyt-2.1 allele. Advantage was taken of the serological difference of the CD8α alleles in the work described in this thesis. Deletion mutations were generated in mice of 129/Sv genetic background (that express the CD8α/Lyt-2.2 allele). In order to analyse the expression of a murine CD8α gene, that has a regulatory region mutated, in the presence of a functional

allele, the mutant mice were crossed to CBA/Ca or DBA/2 wild type mice which carry and express the CD8α/Lyt-2.1 allele. Thus, using specific monoclonal antibodies it is possible to distinguish the mutant gene encoded protein from that of the wild type gene using FACS analysis. (CBA/Ca x 129/Sv)F1 wild type mice were used as controls in these stainings as they carry and express both the CD8α/Lyt-2.1 and the CD8α/Lyt-2.2 alleles.

The cell surface molecule CD4

The CD4 molecule was first identified in rat (Williams et al., 1977, Bernstein et al., 1980), subsequently in human (Reinherz et al., 1979, Terhorst et al., 1980) and in mouse (Dialynas et al., 1983). CD4 is a glycoprotein of 55kDa which was found to immunoprecipitate as a single polypeptide chain (Chan et al., 1988, Classon et al., 1986). The protein is 431 amino acids and is preceded by a 26 amino acid signal peptide. The extracellular portion of CD4 consists of 368 amino acids which is followed by a 25 amino acid hydrophobic transmembrane portion. CD4 contains a 38 amino acid cytoplasmic tail, which is composed of highly basic amino acids. The amino terminal extracellular portion of CD4 can be subdivided into several domains. Approximately 100 amino acids make up the Ig-V like region, which is followed by a short sequence that resembles the Ig-J segments. This is then followed by a further Ig-J like (J') (Tourvieille et al., 1986) and Ig-V like domain (V') (Clark et al., 1987).

CD4 is encoded by a single non rearranging gene on mouse chromosome 6 (Field et al., 1987). The gene encoding CD4 is transcribed mainly in a subset of T cells, but also in the brain, where a shorter transcript was found (Gorman et al., 1987). The structural organisation is similar to that of other members of the immunoglobulin gene superfamily (Maddon et al., 1985, Littman and Gettner, 1987, Tourvieille et al., 1986), except of the presence of an intron in the middle of the sequence encoding the amino-terminal immunoglobulin-like homology unit.

The structure of the gene encoding CD4 was determined by restriction mapping and sequencing of genomic clones. It is composed of 10 exons separated by nine introns and spans 26 kb of DNA (Gorman et al., 1987). The intron-exon organisation corresponds well with the identified domains of the polypeptide. The CD4 gene has a large 8.6kb intron in the 5' untranslated region. Exon I encodes the 5' untranslated

portion, exon II encodes the leader sequence, exon III and IV encode the V-like domain, exon V encodes the J, V' and J' regions, exon VI and VII the connecting peptide, exon VIII the transmembrane domain, exon IX part of the cytoplasmic domain and exon X the remainder of the cytoplasmic domain and the 3' UT region (Gorman et al., 1987).

The human CD4 gene is located in chromosome 12 (Isobe et al., 1986, Kozbor et al., 1986) and its structure was found to be very similar to that of the mouse (Maddon et al., 1987).

CD4 And CD8 In T Cell Development And Function

T cells can be divided into two groups on the basis of their cell surface expression of the CD4 and CD8 coreceptors, which initially was correlated with T cell function. Thus, CD4 positive cells were thought to be of T helper and CD8 positive cells of cytotoxic phenotype (Cantor and Boyse, 1977, Reinherz et al., 1979). However, later CD4 and CD8 expression was correlated with a recognition capacity for peptides presented by MHC class I and class II molecules, respectively. Therefore, most CD8 positive cells are MHC class I restricted, whereas most CD4 positive cells are MHC class II restricted (von Boehmer et al., 1989).

Adhesion of CD4 and CD8 to MHC class II and class I respectively is thought to be due to direct binding between the coreceptor and the MHC molecule (Doyle and Strominger, 1987, Norment et al., 1988). In both cases the adhesion could be blocked by monoclonal antibodies against the coreceptor or the MHC molecule (MacDonald et al., 1988). Mutation analysis of MHC class I molecules suggested that CD8 and MHC class I interact at an exposed acidic side loop of the α3 domain of the MHC class I molecule (Salter et al., 1990). Thus, one CD8α molecule binds with one MHC class I molecule, at the loop located at the top and side of the Ig-like domain of the CD8α (Giblin et al., 1994, Gao et al., 1997). In the case of CD4 the binding occurs in the β2 domain of MHC class II (Cammarota et al., 1992, Konig et al., 1992).

The effect of CD8 on TCR-peptide/MHC interaction has been investigated. It was found that CD8αβ heterodimers have higher affinity for MHC class I than CD8αα homodimers (Garcia et al., 1996). It was also shown that in the absence of CD8, the TCR-peptide/MHC class I complex dissociates rapidly. Thus, the CD8 increases the time

of interaction between a TCR and a peptide/MHC complex. This means that the coreceptors increase the avidity of TCR-peptide/MHC antigen interaction (Moretta et al., 1984, Marrack et al., 1983).

A role for CD4 and CD8 in intracellular signalling

Both CD4 and CD8 were found to interact via their cytoplasmic tails with the lymphocyte restricted cytoplasmic tyrosine kinase $p56^{lck}$, which suggests a role of CD4 and CD8 in intracellular signalling (Veillette et al., 1988, Barber et al., 1989). In particular, the cytoplasmic domains of CD4 and CD8α chains associate with $p56^{lck}$ and this association triggers T cell activation by transducing transmembrane signals via $p56^{lck}$ (Turner et al., 1990, Veillette et al., 1988). The CD8β tail does not contain an interaction motif for $p56^{lck}$ (Itano et al., 1994). Recent data also suggest that interaction of $p56^{Lck}$ with the CD4 and CD8 coreceptors is required for positive and negative selection (Trobridge et al., 2001).

It has been shown that antibody mediated cross-linking of the TCR with either CD4 or CD8 leads to intracellular tyrosine phosphorylation (Gilliland et al., 1991) and enhanced Ca^{2+} mobilisation (Ledbetter et al., 1988). These effects are not observed if cross-linking is carried out with CD4 or CD8 coreceptors that lack $p56^{lck}$ binding sites (Collins et al., 1992). The signalling function of CD8 during T cell activation was evident from experiments which investigate the role of CD8α cytoplasmic tail (Zamoyska et al., 1989, Letourneur et al., 1990). The tailless CD8α', which does not have the $p56^{lck}$ binding site, was less efficient at restoring antigen responsiveness of a T cell hybridoma than the full length CD8α molecule. Furthermore, mice that carried a CD8α transgene with mutated cysteine residues in the $p56^{lck}$ binding site, showed a reduction in the number of CD8 positive cells, but no difference in the cytotoxic activity of these cells (Chan et al., 1993). On the other hand, mice that carried a tailless CD8α chain had a much more severe phenotype both in numbers of CD8 cells and in the capacity to generate anti-viral cytotoxic T cells (Fung-Leung et al., 1993). Thus, the ability to restore antigen responsiveness was found to correlate with the ability of CD8α to interact with $p56^{lck}$ (Zamoyska et al., 1989).

The cytoplasmic tail of CD4 also appears to be important. A tailless form of CD4 can substitute in vivo for wild type CD4, but a reduced number of CD4 cells is produced in these mice (Killeen et al., 1993). In addition, overexpression of a CD4 transgene that lacked a cytoplasmic tail, was found to cause a disruption in normal development of thymocytes (Davis and Littman, 1995).

A role for CD4 and CD8 in T cell development

Several experiments that inactivate the endogenous CD4 and CD8 genes have been carried out, which show an important role of the coreceptors in thymocyte development.

CD8α knock-out mice

Mutant mice lacking expression of CD8α on the cell surface were generated by disrupting the coding sequence by inserting the neomycin resistance gene in exon1 of the CD8α gene (Fung-Leung et al., 1991). As mentioned before, surface expression of CD8β is dependent on the presence of the CD8α chain (Blanc et al., 1988, Gorman et al., 1988). Therefore, disruption of the CD8α gene leads to a complete absence of CD8 cell surface expression.

In the mice homozygous for the disrupted CD8α gene, surface expression of CD8α was not detected on thymocytes and lymph node cells. The $CD4^-CD8^-$ (DN) population appeared to be unchanged in these mice, but the population that would correspond to the $CD4^+CD8^+$ (DP) stage seemed to have merged with the $CD4^+CD8^-$ subpopulation. Furthermore, cytotoxic response of T lymphocytes from the mutant mice against viral antigens was dramatically decreased.

Thus, CD8 positive cells are not present in peripheral lymphoid organs, but the CD4 T lymphocyte population is unaltered. CD8 is necessary for the maturation and positive selection of class I restricted cytotoxic T lymphocytes, but is not required on any of the intermediate thymocyte populations ($CD4^+CD8^+TCR^{low}$ or $CD4^-CD8^+$ TCR^-) during the development of functional class II MHC restricted helper T cells.

CD8β transgenic and knock-out mice

Several experiments investigating the role of the CD8β chain in thymocyte development and T cell effector function have been reported (Crooks and Littman, 1994, Itano et al., 1994, Nakayama et al., 1994). In one of these experiments, transgenic mice were generated that expressed a CD8β chain that had its cytoplasmic tail deleted (Itano et al., 1994). Overexpression of the mutant CD8β gene caused efficient competition with the endogenous wild type CD8β chain and the mice were expressing mainly CD8αβ heterodimers that contained the mutant CD8β chain. In these transgenic mice, an inhibition of CD8 T cell development was observed. The cells that did develop were found to express CD8 in the form of CD8αα homodimers.

Mice deficient for CD8β gene expression were generated by replacement of exon 2 and part of intron 3 in the CD8β gene with the neomycin resistance gene (Crooks and Littman, 1994). This modification resulted in the deletion of 37% of the coding sequence of the mature protein, including most of the immunoglobulin-like domain and all of the J-like region (Nakayama et al., 1989). In mice lacking the CD8β chain, the $CD4^+CD8\alpha^+$ (DP) thymocytes appeared to develop normally, suggesting that CD8β is dispensable for early development of thymocytes and that CD8α expression is independent of the presence of CD8β. The CD4 SP thymocytes of these mice also appeared normal. In contrast, a significant reduction in the number of CD8 positive cells was observed in these mice. The CD8 positive thymocytes present in the mutant mice express normal levels of TCR and other maturation markers, such as CD69 and heat stable antigen (HSA). In addition, the CD8α level on CD8 positive thymocytes was the same in mutant and wild type animals. The CD8 positive T cells that complete maturation in mutant mice for CD8β, have normal cytotoxic activity (Crooks and Littman, 1994). Thus, CD8β is required for efficient selection processes during thymocyte maturation, but not for antigen-specific responses by mature CD8 cytotoxic T cells.

CD4 knock-out mice

In mice with a disrupted CD4 gene, expression of CD4 was not detected on thymocytes or lymph node cells. In addition, the development of class II MHC-restricted helper T cells was greatly reduced in the mutant mice. In contrast, maturation of CD8 T cells was normal, with CD8 cells being present in normal numbers in the thymus and

having normal cytotoxic activity against viruses. However, in the periphery of the mutant mice the CD8 single positive cells expanded to occupy the compartment that would otherwise have been occupied by CD4 positive cells. This result indicated that expression of CD4 on progenitor cells and $CD4^+CD8^+$ (DP) thymocytes is not obligatory for the selection of $CD4^-CD8^+$ cells (Rahemtulla et al., 1991, Killeen et al., 1993). Although T helper cell development is affected in the mutant mice, some T helper cells with $CD4^-$ $CD8^-$ phenotype exist.

Regulation Of Gene Expression

Differentiation of specific cell types in multicellular organisms necessitates developmentally controlled regulation of gene expression. The eventual structural and functional phenotype of a cell is dependent on the activation or silencing of specific genes. Most genes are actively transcribed during interphase and it has been suggested that during this stage in the cell cycle the *cis*-acting elements and their associated genes are more accessible to the transcriptional machinery. It is thought that the selective transcription of genes is regulated by the interaction of proteins binding to gene regulatory DNA elements, but is also influenced by chromatin structure.

Eukaryotic gene structure

Eukaryotic genes are arranged into coding and non-coding DNA sequences, exons and introns respectively (Berget et al., 1977, Jeffreys and Flavell, 1977). In addition to the protein coding DNA, other important gene regulatory elements are involved in gene regulation. The three main types of regulatory sequences which have been defined are promoters, enhancers and silencers, but additional elements exist, such as Matrix Attachment Regions (MARs) and Locus Control Regions (LCRs). All these elements mediate their function by means of protein factors that recognise specific DNA sequences to which they bind. The specificity of transcription factors for certain regulatory elements ensures the differential expression of genes in different cell types and developmental stages (Clevers and Ferrier, 1998, Georgopoulos, 1997).

Promoters

The promoters are regions of DNA that can be found 5' of most functional genes. They specifically interact with RNA polymerase and play an essential role in defining the start site of transcription (Moreau et al., 1981). In many cases their function is mediated via an AT rich sequence known as TATA box, which is typically located 25 to 30 nucleotides upstream of the transcription initiation site (Mathis and Chambon, 1981). Some promoters of eukaryotic genes, however, lack a TATA box motif but initiate transcription properly (Maniatis et al., 1987). In such promoters a DNA element called the initiator overlaps the precise transcription start site (Weis and Reinberg, 1992, Gill, 1994). The TATA-less promoters regulate a large variety of cellular and viral genes. One common property of many TATA-less promoters is the presence of multiple copies of the GC box, which binds transcriptional activator Sp1 (Ptashne, 1988).

The basal transcriptional machinery

In order for transcription of a gene to occur, the basal transcriptional machinery has to assemble at the core promoter elements. Binding of the transcriptional initiation complex occurs at the TATA box or the initiator (Sawadogo and Roeder, 1985). In addition to RNA polymerase II, the basal transcription factors that are required for transcription initiation are TFIIA, -B, -D, -E, -F and -H (Weinmann, 1992, Conaway and Conaway, 1993). More transcription factors are involved in regulating gene expression, one main subset being the Helix-Loop-Helix (HLH) proteins, that are thought to be involved in lineage determination and cell type differentiation (Zhuang et al., 1994, Bain et al., 1994).

Enhancers

Enhancers can be located 5' or 3' of the start site or inside of the respective gene. They are regulatory elements first identified in viruses, that were shown in cell transfection assays to increase the level of transcription regardless of their orientation with respect to the gene (Banerji et al., 1981). Enhancers are associated with many eukaryotic genes and are capable of functioning at considerable distance from them (Serfling et al., 1985).

Several models for enhancer function have been proposed. Originally, it was proposed, that the enhancer works as an entry site for transcription factors which then slide along the DNA (Wasylyk et al., 1984). The structural model, on the other hand, proposes that the surrounding chromatin structure is changed by enhancers and that this allows access of transcription factors. Finally, there is the prevailing model that proposes that enhancers exert their influence by acting as binding sites for transcription factors, which subsequently interact with promoter bound factors of the transcriptional machinery (Ptashne, 1988).

Silencers

They were first identified in yeast and they have properties opposite to those of a transcriptional enhancer (Brand et al., 1985, Shore and Nasmyth, 1987). The mechanism of action is not clear, but silencers are thought to change the structure of adjacent chromatin (Pirrotta, 1997), possibly by influencing DNA methylation or activity of histone deacetylases (Struhl, 1998), or they may function by directly influencing components involved in initiating transcription of the relevant gene. They have been shown to operate in either orientation and their effects are independent of their position with respect to the affected promoter. Numerous vertebrate genes have been shown to contain negative regulatory elements that may silence gene expression in a tissue or developmental specific manner, including the immunoglobulin genes (Imler et al., 1987) and the CD4 gene (Sawada et al., 1994, Siu et al., 1994).

Matrix Attachment Regions (MARs)

MARs are AT-rich stretches of DNA that have high affinity for the nuclear matrix and they are involved in the transcriptional regulation of gene expression. They are typically 200-300 bases in length, they contain topoisomerase II cleavage sites and occur on average of one every 30 kb of eukaryotic DNA. Furthermore, they have been increasingly observed near enhancer and promoter regions of genes (Banan et al., 1997). However, their ability to regulate transcription of specific genes is questioned, as they have been shown to be able to bind to nuclear matrices of a wide variety of cells. Some cell type-specific MAR-binding proteins have been described that associate with specific

MAR sites and they might contribute to specific repression or activation of adjacent genes (Banan et al., 1997, Alvarez et al., 2000).

Locus Control Regions (LCRs)

LCRs have been identified for several genes, including the human β-globin locus (Grosveld et al., 1987), the human CD2 locus (Greaves et al., 1989), the αδ TCR locus (Diaz et al., 1994) and HLA class I genes (Chamberlain et al., 1991) and have been shown to direct transgene expression at levels comparable with the endogenous chromosomal locus, regardless of the integration site. LCRs are required to establish an open chromatin structure and, thus, allow accessibility of additional regulatory proteins to the locus (Kioussis and Festenstein, 1997). This process takes place at the level of chromatin remodelling. Furthermore, LCRs are thought to provide proper tissue specific regulated transcription of genes (Grosveld et al., 1987).

Chromatin Structure And Gene Regulation

The interaction of the transcriptional machinery with the various DNA binding sites described above does not take into account the compaction of nucleosomal DNA and the steric constraints imposed on it by nucleosomal packaging of DNA. There is evidence of interaction between transcription factors and histone proteins in the regulation of gene expression (Croston and Kadonaga, 1993). For example, mutations in histone genes have major effects on transcriptional activation and repression (Grunstein, 1990). Therefore it is important to examine the packaging of DNA into nucleosomal structures and the role that this has in gene regulation.

Histones and nucleosome structure

Eukaryotic DNA is organised into a regularly repeating protein-DNA complex called the nucleosome (Kornberg, 1977). The nucleosome is a complex of DNA and proteins called histones and it is considered the basic unit of chromatin. Nucleosomes consist of two of each of the histone molecules H2A, H2B, H3 and H4 and approximately 160-200 bp of DNA (Wolffe, 1992). The core histones contain a large number of lysine and arginine amino acid residues that can be modified by acetylation

and this has important consequences in the structure of the nucleosome and, therefore, the interaction of transcription factors with their recognition site in chromatin (Lee et al., 1993). Except of the four core histones mentioned above, most eukaryotic cells contain also the linker histone H1 (Wolffe, 1992). The DNA is wrapped around the histones to form the nucleosomes, which are connected with each other by linker DNA of 20 to 40 bp. Tandem arrays of nucleosomes are held together and are stabilised by the binding to histone H1 to form the chromatin fibre (Widom, 1989). Subsequently, the chromatin fibre is organised into loops and is further folded to a very compact structure.

DNaseI hypersensitive sites

Transcriptionally active genes show an increased sensitivity to DNaseI and this is thought to indicate a more accessible chromatin structure (Garel and Axel, 1976). The molecular basis for this difference and whether the sensitivity occurs before or is a consequence of transcription is unknown. These DNaseI hypersensitive sites are a feature of nearly all active genes and coincide with *cis* acting regulatory elements, such as enhancers (Landry et al., 1993), promoters, silencers (Sawada and Littman, 1991), MARs (Gerasimova and Corces, 1996) and LCRs (Grosveld et al., 1987, Greaves et al., 1989). They can be constitutive, tissue specific, developmentally regulated and inducible.

Position Effect Variegation (PEV)

Eukaryotic genomes are divided into two cytologically distinct entities, euchromatin and heterochromatin. Euchromatin contains most of the single copy DNA, it decondenses during interphase and replicates throughout S phase. Heterochromatin, on the other hand, is found mainly in centromeric and telomeric regions of chromosomes, it contains few genes and it is rich in highly repetitive sequences. In addition, it is constitutively condensed throughout the cell cycle and replicates late in S phase. Thus, genes that are in heterochromatic regions or close to the junctions with heterochromatin are compacted and are inactive. The variable, but heritable, inhibition of euchromatic gene activity when they are brought close to heterochromatin due to chromosomal rearrangements, is called Position Effect Variegation (PEV) (Karpen, 1994) and was first characterised in *Drosophila* (Weiler and Wakimoto, 1995). PEV has also been described in transgenic mice systems, where the site of integration of the transgene results in

different expression levels depending on the surrounding chromatin (Festenstein et al., 1996). When the transgene is integrated close to a heterochromatic region, some of the cells are found to express the transgene, whereas in some others the transgene is inactive. One of the functions of LCRs is to overcome such position effects and thus, direct expression of linked genes irrespective of the chromosomal site of integration in transgenic mice. Deletion of specific sequences of an LCR can result in PEV (Kioussis and Festenstein, 1997).

Regulation Of Expression Of The CD4 Gene

The regulation of expression of the two coreceptors has been the subject of numerous studies (Ellmeier et al., 1999). In the case of CD4 gene regulation, three main regulatory elements are involved, a promoter, an enhancer and a silencer. The combined activity of these and additional elements results in the regulated expression of the CD4 gene during T cell development.

CD4 promoter

The minimal murine CD4 promoter, which does not have a TATA box, has been mapped within a 101bp fragment immediately 5' from the transcription initiation site by using transient transfection assays. The CD4 promoter functions only in T cells, thus it partly contributes to the tissue specificity of the CD4 gene. However, the presence of the CD4 promoter linked the CD4 cDNA in transgenic mice is not sufficient to direct expression of the transgene in mice, suggesting that other regulatory elements are necessary for CD4 expression (Salmon et al., 1996). Sequence analysis revealed the presence of two Myb transcription factor binding sites within the 101bp fragment, which are both necessary for full activity of the promoter (Siu et al., 1992, Allen et al., 2001). Similarly to the murine promoter, the human CD4 gene promoter does not have a classical TATA box and has a tissue specific transcriptional activity (Salmon et al., 1993).

CD4 enhancer

DNaseI hypersensitivity site analysis within 80kb of the murine CD4 locus led to the identification of a *cis*-acting sequence that displayed enhancer activity in transiently transfected T cell lines, but not in B cell lines or fibroblasts (Sawada and Littman, 1993). This enhancer, which is located approximately 13 kb upstream of the transcriptional start site of the CD4 gene, is responsible for CD4 gene expression in double positive thymocytes and helper T cells. However, when this enhancer was linked to various reporter genes, it was found to be active both in CD4 and CD8 SP T cell lines, indicating that subset specificity of CD4 expression is not mediated by this enhancer (Sawada and Littman, 1991). A human CD4 enhancer has also been identified and it is located 6.5 kb upstream of the human CD4 gene (Blum et al., 1993). Experiments using human CD4 gene constructs showed that sequences contained within or closely linked to the human CD4 gene are sufficient to reconstitute the appropriate regulation of human CD4 expression on all thymocytes and mature peripheral T cell subsets.

The murine CD4 enhancer was found to contain three nuclear binding sites (CD4-1, CD4-2 and CD4-3). The T and pre B cell specific nuclear factor LEF was shown to bind to one of these site (CD4-2), whereas the other two (CD4-1 and CD4-3) were found to contain E-box motifs. Mutational analysis of the protein binding sites indicated that CD4-1 and CD4-2 are not essential for transcriptional activity in T cells, but mutation of both E-boxes within the CD4-3 sequence abolished enhancer activity (Sawada and Littman, 1993).

CD4 silencer

Several early studies suggested that T cell subset specific CD4 expression is subject to negative regulation (Sawada and Littman, 1991). Whereas human CD4 transgenes with intact intron/exon organisation were specifically expressed in CD4, but not CD8, SP cells (Blum et al., 1993), another transgene lacking introns 1-4 was expressed in both lineages (Sawada et al., 1994). In addition, the presence of DNaseI hypersensitive sites in the first intron of the murine gene suggested that this region was involved in subset specific expression of the CD4 gene (Sawada and Littman, 1991). When the intronic element was deleted, the CD4 enhancer and promoter were active in both $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ subsets and also in DN thymocytes. Thus, initially the

CD4 silencer was thought to function at three different stages of T cell development to inhibit CD4 gene expression. First, it functions to inhibit CD4 gene expression in non-T haemopoietic cells, second, it inhibits expression of CD4 in the $CD4^-CD8^-$ population and as the thymocytes mature to $CD4^+CD8^+$ T cells, the silencer ceases function permitting the expression of CD4. After the T cell selection processes, the silencer either remains non-functional leading to the $CD4^+$ $CD8^-$ T helper cells, or it resumes function to inhibit the enhancer, leading to the $CD4^+CD8^-$ cytotoxic T cells (Siu et al., 1994, Sawada et al., 1994).

The murine CD4 intronic element was narrowed down to a 434 bp fragment that could repress reporter transgene expression in CD8 lineage cells. This activity was independent of orientation or location relative to the transcribed region (Siu et al., 1994, Sawada et al., 1994). Footprinting analysis using T cell nuclear extracts identified three functional sites (sites I, II and III) within the 434 bp silencer. Deletion of any single site had no effect on silencer activity in transgenic mice. However, multiple deletions of these sites indicated that combinations of sites are required for silencer activity, suggesting that there is redundancy in the mechanism of silencer function (Duncan et al., 1996).

A lineage specific silencer was also identified in the first intron of the human CD4 gene, by using a human CD4 transgene in which exons 2-10 were replaced with cDNA sequences (Donda et al., 1996). The human CD4 silencer is contained within a 484 bp fragment and two protein binding sites were detected by footprinting analysis.

Additional CD4 transcriptional control elements

Although the main elements responsible for proper developmental and subset specific expression of the CD4 gene are the promoter, the enhancer and the silencer, several reports suggest that additional elements may be involved. A second enhancer was identified approximately 24 kb upstream of the murine CD4 gene (Wurster et al., 1994). Its activity appeared restricted to mature T cells, however, the cell type specificity of this enhancer remains unclear, since there is expression in transgenic mice of a linked reporter gene not only in T cells, but also in B cells and macrophages.

Furthermore, when the murine proximal promoter was linked to the human CD4 promoter and the human CD4 cDNA (no intronic sequences were included), it was found to direct expression of the transgene to mature CD4 and CD8 T cells, but not in double positives. This experiment suggested that there are different elements responsible for CD4 expression in immature and mature T cells (Salmon et al., 1996), thus a third enhancer element located in the 3' flanking region of the CD4 gene might be involved in expression of the CD4 gene in double positive thymocytes (Adlam et al., 1997). Another report suggested that there are two distinct silencing mechanisms that control CD4 expression during development. One of them is active in the DN thymocytes and the second is active later, in the CD8 SP thymocytes and peripheral CD8 SP T cells (Uematsu et al., 1997).

Regulation Of Expression Of The CD8 Gene

The CD8 molecule is expressed predominantly as a CD8αβ heterodimer on thymus derived T cells, or as CD8αα homodimer on extrathymically derived intraepithelial lymphocytes. This partially overlapping, but distinct pattern of expression of the CD8α and β genes, which are closely linked at a distance of 36 kb (Gorman et al., 1988), suggests that their expression must be both coordinately and independently regulated. This was further suggested from transfection experiments using thymoma lines, which demonstrated differential regulation of the CD8α and CD8β genes (Hwang et al., 1993, Gu and Gottlieb, 1992).

CD8β promoter

The murine CD8β promoter, that does not have a typical TATA box, has been analysed for the presence of *cis*-regulatory elements (Kawachi et al., 1996). Its activity is restricted to T cells, however it has not been determined whether the activity is specific for the CD8 lineage. The CD8β promoter has three copies of the GC box positioned close to each other. Deletion analysis revealed that one of these GC boxes is a binding site for the ubiquitously expressed transcriptional activator Sp1 (Dynan et al, 1985).

CD8α promoter and *cis*-regulatory elements

The TATA-less human CD8α promoter contains a decameric sequence that resembles the cyclic AMP response element (Gao and Kavathas, 1993) and contains DNA binding sites for transcription factors that have been implicated in T cell specific gene expression. DNaseI-HSS mapping has been carried out on the human CD8α (hCD8α) and the mouse CD8α gene and it identified several tissue specific DNaseI-HSS (Hambor et al., 1993, Landry et al., 1993). This led to the identification of potential transcriptional control elements located within the last intron of the hCD8α gene (Hambor et al., 1993, Hanke et al., 1995). There is evidence that this enhancer element is involved in NK cell specific expression of CD8 in humans (Kieffer et al., 1996). Deletion of the 5' end of this enhancer element indicated that it contained a negative regulatory element (Hanke et al., 1995). Sequence comparison between the last introns of the murine and the human CD8α showed that the murine CD8 gene lacks a region homologous to the human enhancer, as well as protein binding motifs that are present in the human enhancer element (Kieffer et al., 1996).

Although human CD8α gene expression is thought to be mainly controlled at the transcriptional level, there is one report that suggested that it is regulated at least in part by post-transcriptional mechanisms in mature peripheral blood CD4 T cells (Gao et al., 1996).

The murine CD8 gene locus was cloned from cosmid and P1 libraries (Hostert et al., 1997) and mice transgenic for the insert of one of the isolated P1 clones, P1-5, were generated. The analysis of these mice showed that the 80 kb injected fragment, spanning the whole CD8 locus including 2 kb upstream of the CD8β gene and 25 kb downstream of the CD8α gene, carries sufficient information to direct subset-specific and developmentally regulated expression of the transgenic CD8 gene locus. This suggested that the major regulatory elements for expression of the CD8α and β genes are localised within this large genomic fragment. A similar result was also reported for the human CD8β gene in transgenic mice generated with a 95 kb genomic fragment encompassing the human CD8β locus (Kieffer et al., 1997). In both cases, a variegating expression pattern was observed due to influence from surrounding genomic regions of the transgene integration site. This is an indication that an LCR is not involved in regulating expression of the endogenous CD8α and β genes.

DNaseI hypersensitive site (HSS) analysis over the CD8 gene locus was performed (shown in figure 1) and four clusters (I, II, III and IV) of DNaseI-HSS were identified (Hostert et al., 1997). Three of the clusters (clusters II, III and IV) were found to be specific for T cells, while cluster I (located 20 kb downstream of the CD8α gene) was also present in DNaseI treated liver nuclei. The role of individual DNaseI-HSS within the 80 kb murine CD8 locus was characterised further by using a combination of HSS or clusters to prepare reporter constructs and analysing them for enhancer activity in transgenic mice (Hostert et al., 1997, Hostert et al., 1998, Ellmeier et al., 1997, Ellmeier et al., 1998, Zhang et al., 1998).

DNaseI-HSS cluster III

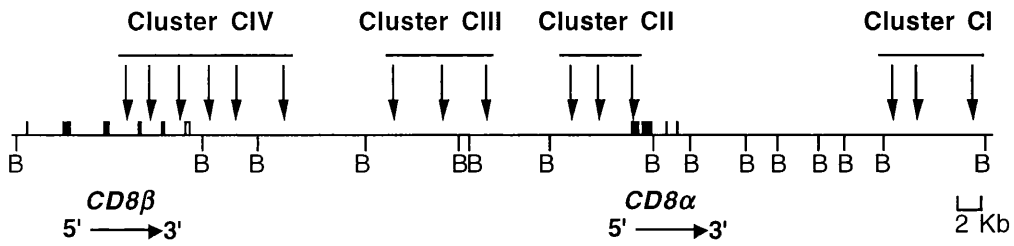
Cluster III consists of three DNaseI-HSS and is located in the intergenic region between CD8α and β genes, approximately 16 kb upstream of the murine CD8α gene (Hostert et al., 1997). It was the first *cis*-acting element shown to have CD8 lineage restricted enhancer activity. In transgenic mice, it was found to be sufficient to direct expression of a linked reporter gene in mature CD8 SP thymocytes and peripheral CD8 SP T cells (Hostert et al., 1997, Ellmeier et al., 1997). In contrast, expression of the transgenic reporter was absent from immature DP thymocytes. Furthermore, cluster III was also able to direct expression of the reporter gene in extrathymically derived CD8αα IEL of both γδTCR and αβTCR lineages (Ellmeier et al., 1997). Reporter constructs containing DNaseI-HSS CIII-1 or CIII-2 alone, or a combination of the two, were also able to direct expression of the reporter in mature CD8 SP thymocytes and peripheral CD8 SP T cells, suggesting that there is redundancy in the activity of regulatory elements lying within cluster III (Hostert et al., 1997).

DNaseI-HSS cluster II

Cluster II consists also of three DNaseI-HSS and is located immediately upstream of the CD8α gene (Hostert et al., 1997). This cluster contains the promoter of the CD8α gene, which is believed to coincide with the third DNaseI-HSS of the cluster, and two possible upstream regulatory elements. The presence of cluster II alone linked to a reporter gene in transgenic constructs was not sufficient to direct expression of the transgene in any thymocyte or T cell subset. However, in conjunction with cluster III

Figure 1: Organisation of the CD8 gene locus (Hostert et al, 1997)

Map of the murine CD8 gene locus showing the location of the CD8α and CD8β genes (filled boxes: translated exons, open boxes: untranslated exons). Restriction enzyme sites are indicated by vertical bars (B, BamHI) and the location of DNaseI-HSS clusters is shown by vertical arrows and labeled CI to CIV.


Cluster CIV
Cluster CIII
Cluster CII
Cluster CI
B
CD8β
5' → 3'
CD8α
5' → 3'
2 Kb

DNaseI-HSS, cluster II restored transgene expression in the immature $CD4^+CD8^+$ DP thymocytes, while retaining subset specific expression in mature T cells (Hostert et al., 1998). Furthermore, DNaseI-HSS CII-2 was absent in DNaseI treated splenocyte nuclei, suggesting that a possible element for expression in immature thymocytes may lie in this region.

From an earlier study cluster II was shown to contain binding sites for the T lymphocyte-restricted transcription factor GATA-3 and was found to be functional in a transient transfection assay using a minimal c-fos promoter linked to a CAT reporter gene (Landry et al., 1993). However, it is unlikely that GATA-3 is a major mediator of subset specific expression of CD8, because it is also expressed in CD4 SP T cells (Zheng and Flavell, 1997). Further work on potential CD8 regulatory elements (Lee et al., 1994) identified an element with negative regulatory function on CD8α gene expression in a transfected thymoma cell line, that is located close to DNaseI-HSS CII-1. Furthermore, a more recent work indicated a role of this region in regulating the association of the CD8 locus with the nuclear matrix (Banan et al., 1997, Alvarez et al., 2000).

DNaseI-HSS cluster IV

Cluster IV is located in the vicinity of CD8β gene and consists of six DNaseI-HSS mapping both within introns and 3' flanking sequences of the CD8β gene (Hostert et al., 1997). The generation of transgenic mice with genomic fragments containing all DNaseI-HSS of cluster IV, demonstrated the presence of other CD8 locus regulatory elements that direct expression in DP thymocytes and mature CD8 SP T cells (Ellmeier et al., 1998). This was independently confirmed by generating transgenic mice with constructs in which DNaseI-HSS from cluster IV were tested in combination with either cluster III (Hostert et al., 1998), cluster II or both (Zhang et al., 1998). In addition, transgenic mice that contained a combination of cluster IV and cluster II DNaseI-HSS and, thus, expressed the transgene only in DP thymocytes, had an increased population of $CD4^-CD8^-$ cells in the periphery, but had normal cytotoxic activity (Zhang et al., 2001, Zhang et al., 2001).

By testing combinations of DNaseI-HSS from cluster IV, it was possible to identify two distinct elements within the cluster. One of them contains DNaseI-HSS CIV-4 and CIV-5 and directs expression of a reporter gene both in DP and in CD8 SP

thymocytes and peripheral CD8 SP T cells. However, this element does not direct expression of the linked reporter in CD8αα homodimer-expressing IEL, both of αβTCR and γδTCR lineages. In contrast, reporter gene expression was detectable on CD8αβ homodimer-expressing IEL. The second element contains only DNaseI-HSS CIV-3 and displays an enhancer activity, which is restricted to DP thymocytes (Ellmeier et al., 1998).

Gene Targeting Technology

The elucidation of the principle mechanisms controlling gene regulation has been the aim of numerous studies and it is very important for defining the fundamental aspects of cell differentiation and lineage commitment. However, due to the complexity of the eukaryotic genome, the study of gene function and regulation has been a difficult and slow process. Gene targeting, the introduction of site-specific modifications into the genome by homologous recombination, has been proved to be an invaluable technique in the field of mouse genetics and allowed the analysis of various aspects of gene function *in vivo*.

Homologous recombination

Homologous recombination has been mainly used to generate null mutations of genes. This can be done either by insertion of a selectable marker into an exon critical for gene function and thus causing gene disruption or by directly deleting DNA segments responsible for the gene expression. However, removal of large genomic regions can be dangerous, as it can lead to unintended loss of unidentified genes residing in introns or of regulatory elements responsible for the expression of other genes. Furthermore, homologous recombination technique can be used to delete regulatory regions of genes in order to study their function (Fiering et al., 1995).

Homologous recombination in mammalian cells between an artificial targeting vector and an endogenous gene was first achieved in 1985 for the β-globin locus (Smithies et al., 1985). Subsequently, the technique was used to target embryo derived stem (ES) cells. These cells are derived from pluripotent, undifferentiated cells of the

inner cell mass of pre-implantation blastocysts and can contribute efficiently to both somatic and germline tissues after reintroduction into the blastocysts. Usage of ES cells offered the advantage that mice with the desired mutation could be generated, following inactivation of the chosen gene by gene targeting (Thomas and Capecchi, 1987).

In order to introduce a designed mutation into the germline of mice, a targeting vector containing the desired mutation is introduced into ES cells by electroporation. In most cells the targeting vector inserts randomly into the ES cell genome. However, in a few cells homologous recombination between the targeting vector and the cognate DNA sequence occurs and mutations are transferred to the genome. The cells that have undergone homologous recombination are cloned and maintained as pure populations. The clones that have a euploid karyotype are injected into 3.5-day blastocysts, which are, subsequently, injected into the uteri of foster mothers and are allowed to develop. This process gives rise to chimeric animals in that they are composed from cells derived from both the donor stem cells and the host blastocyst. Breeding of the chimeric mouse to a wild type mouse results in mice heterozygous for the mutation upon germline transmission (Capecchi, 1989).

To enrich for clones that have undergone homologous recombination, as opposed to random integration, a strategy for positive and negative selection of the clones has been developed (Mansour et al., 1988). For negative selection, a thymidine kinase (tk) gene from Herpes Simplex Virus (HSV) is inserted at the end of the linearised targeting vector. Cells that have undergone homologous recombination will have lost the tk gene, whereas cells in which the construct integrated randomly, can be eliminated by using the drug gancyclovir. For positive selection, a selectable marker is used in the construct, such as the neomycin resistance gene. Cells that have undergone homologous recombination will be resistant to G418 and will survive the selection process.

An important issue that should be considered is the genetic background on which the mutation will be studied. The genetic background of the majority of ES lines is 129, however, the inbred 129 substrains show considerable genetic variation (Simpson et al., 1997). It has also been reported that use of the same genetic background for the targeting vector and the ES cells used for the targeting experiment increases the frequency of homologous recombination (te Riele et al., 1992). In addition, the frequency of homologous recombination can be influenced by the length of the homology used in the

vector. Finally, the frequency seems to be locus dependent, possibly due to differences in chromatin structure and thus accessibility of the locus for the enzymatic machinery involved.

Cre/loxP system

Conventional gene targeting leads to inactivation or modification of a gene in all tissues of the body from the onset of development throughout the whole lifespan. More recently, methods have been developed aimed at controlling gene deletion in a time- and/or tissue-specific manner. These conditional gene targeting approaches are particularly useful in cases where complete gene inactivation leads to a lethal phenotype that prevents a more detailed analysis. Moreover, if a given gene has a widespread pattern of expression, tissue-specific gene inactivation may define physiological roles of the gene product in a certain tissue, without compromising other functions in the organism. Control of gene targeting in a time-dependent manner allows the analysis of functions at different time points in development.

Selective deletion of gene segments has been achieved by the use of site-specific recombinases, that cleave DNA at specific target sequences. The most commonly used recombinases are Cre from the bacteriophage P1 and FLP from yeast, that recognise loxP and FRT sites, respectively (Kilby et al., 1993, Argos et al., 1986). Cre recombinase is a 38 kDa recombinase (Sternberg et al., 1986), that promotes intra and inter-molecular recombination at specific 34 bp sites, the loxP sites (Matsusaka et al., 2000, Sternberg and Hamilton, 1981). These sites are composed of two,14 bp long, recombinase binding elements (RBEs) arranged as two inverted repeats surrounding a central crossover region. Two recombinase subunits bind to each loxP site (one to each RBE) and they catalyse the recombination reaction. Because the crossover sequence is asymmetric, it provides directionality to the site (Van Duyne, 2001). Recombination between two head-to-tail loxP sites leads to the excision of the intervening DNA, leaving a single loxP site (Sauer and Henderson, 1988). However, if the two loxP sites are organised in the opposite orientation, inversion of the intervening sequence occurs (Ramirez-Solis et al., 1995).

Deletion of desired gene segments can be achieved by flanking the gene with loxP sites and in a second step transiently expressing the Cre recombinase by transfecting

the ES cells with a Cre expressing plasmid (Gu et al., 1993). Alternatively, deletion can be mediated by breeding the loxP 'carrying' mice to transgenic strains in which a Cre transgene is expressed either constitutively or in a cell type or developmentally specific manner (Gu et al., 1994). The latter can be achieved if the transgenic mice used express Cre recombinase under the control of specific promoters. Several such transgenic lines have been generated so far (O'Gorman et al., 1997, Wagner et al., 1997, Meyers et al., 1998, Lakso et al., 1996, Rickert et al., 1997). In order to control the function of the Cre gene in a time-dependent manner and thus, avoid any non-desired deletion mediated by Cre, inducible systems can be used (Gossen and Bujard, 1992).

The Cre/loxP system can also be used to delete selection markers left in the mouse genome after gene targeting. It has been observed that transcription from the strong promoters driving the introduced selection markers may interfere with the expression of neighbouring genes (Fiering et al., 1995, Rijli et al., 1994, Mei et al., 2000). Therefore, selection marker cassettes should be removed after homologous recombination.

PROJECT AIMS

Regulatory elements that determine the tissue and T cell subset specific expression of the CD8 locus had been previously identified. The function of such elements, which coincide with DNaseI hypersensitive sites, was initially examined by using transgenic mice that carried reporter gene constructs with linked combinations of hypersensitive sites. The result indicated a differential developmental and lineage specificity role for each of the regulatory elements.

The aim of the project is to delineate the role of these DNaseI hypersensitive regions by deleting them from the endogenous locus. For this purpose the locus was targeted in ES cells and resultant mutant mice were analysed for CD8 expression in the different T cell subsets.

MATERIALS AND METHODS

General

Chemicals and reagents

Restriction endonucleases, T4 DNA ligase, Klenow DNA polymerase, protein K, DNaseI, alkaline phosphatase and Rnase A were obtained from Boehringer Mannheim and New England Biolabs. Hybond-N+ nylon membranes were purchased from Amersham. Chemicals were obtained from BDH, Sigma Chemical Company and Pharmacia Biotech. Antibodies were purchased from Becton Dickinson Life Technologies (GIBCO-BRL), Pharmacia Biotech and Sigma Chemical Company. Tissue culture plastics were obtained from Corning-Costar, Becton Dickinson and Nalge Nunc International. Fetal calf serum (FCS) and other media supplements were purchased from Globepharm limited, GIBCO-BRL and Sigma Chemical Company.

Bacteriological

Bacteriological cultures

Liquid cultures were grown in L-Broth supplemented with 100μg/ml ampicillin. For agar plates L-Broth was supplemented with 1.5% (w/v) bactoagar. Bacteria were frozen in 1 ml aliquots of L-Broth supplemented with 1/10 volume of 10x Hogness.

L-Broth:

1% (w/v) bactotryptone; 0.5% (w/v) bactoyeast extract;

1% (w/v) NaCl in ddH_2O.

Hogness (10x):

36mM KH_2PO_4; 13mM K_2HPO_4; 20mM NaCitrate;

10mM $MgSO_4$; 40% (w/v) Glycerol.

Competent bacteria and cell transformation

For competent bacteria a single colony of Escherichia coli (E.coli) DH5α bacteria was picked from agar plates and grown (250rpm in a New Brunswick Model G25 orbital shaker) overnight to stationary phase at 37°C in 10ml of L-Broth. 500ml of L-Broth was inoculated with 5ml of the overnight culture and grown with shaking at 37°C until an optical density of 0.5 at 600nm was reached. Bacteria were then pelleted in 50ml falcon tubes (Falcon, Beckton Dickinson) at 1285g, 4°C for 20 minutes and gently resuspended in 50ml of transformation buffer. Cells were left on ice for 10 minutes and then frozen in 400µl aliquots in sterile eppendorf tubes in a dry ice/methanol bath and stored at –70°C.

For transformation of bacteria, ligation reaction containing 100ng of DNA was mixed with 20µl of 5X KCM buffer and made up to 100µl final volume with sterile ddH_2O. 100µl of competent cells were added and left on ice for 20 minutes. Following incubation at RT for a further 10 minutes 1ml of L-Broth was added and cells were grown for 60 minutes at 37°C. 100µl of this culture was then plated out onto L-agar plates supplemented with ampicillin.

Transformation Buffer:

L-Broth; 10% (w/v) PEG (3350 MW); 5% (v/v) DMSO;

10mM $MgCl_2$; 10mM $MgSO_4$.

5X KCM:

500mM KCl; 150mM $CaCl_2$; 250mM $MgCl_2$.

Small scale plasmid DNA isolation

Bacteria of 10ml overnight culture of L-Broth supplemented with ampicillin were pelleted by centrifugation at 3500rpm for 10 minutes at 4°C. The bacterial pellet was then resuspended in 200µl 1X glucomix, transferred to an eppendorf tube and incubated at room temperature (RT) for 5 minutes. Bacteria were then lysed with 400µl of Lysis Mix and incubated on ice for 10 minutes. This was followed by the addition of 200µl 5X KOAc pH4.8 and gentle mixing. Debris was pelleted for 10 minutes at 13000rpm in a benchtop centrifuge and plasmid DNA was precipitated from the supernatant with 0.6 volumes of isopropanol at RT and collected by centrifugation at 13000rpm. The DNA pellet was then resuspended in 100µl ddH_2O and stored at –20°C.

Glucomix (10X):

250mM Tris-HCl, pH8.0; 500mM Glucose; 100mM EDTA.

Lysis Mix:

200mM NaOH; 1% (w/v) SDS.

5M KOAc, pH4.8:

3M with respect to potassium; 5M to acetate; adjusted to pH4.8 with glacial acetic acid.

Large scale plasmid DNA isolation

Bacteria were harvested from 1litre over night culture by centrifugation at 4000rpm for 30 minutes, at 4°C. The pellet was resuspended by vortexing in 40ml 1X glucomix and incubated for 5 minutes at RT. Bacteria were then lysed with 80ml of Lysis Mix for 10 minutes and chromosomal DNA and protein was precipitated for 10 minutes on ice by the addition of 40ml 5M KOAc. Debris was removed by centrifugation for 20 minutes at 4000rpm. The supernatant was filtered through 2 layers of cheesecloth and plasmid DNA was precipitated at RT by the addition of 0.6 volumes of isopropanol and harvested by centrifugation for 20 minutes at 5000rpm. The pellet was dissolved in 9ml of ddH_2O. 10g of CsCl and 0.5ml of 10mg/ml ethidium bromide were added to the DNA solution. Samples were then transferred into Quickseal Ultracentrifuge Tubes (Beckman) and centrifuged at 55000 rpm for 15 hours in a Beckman VTi65.2 rotor. Banded plasmid DNA was recovered using a hypodermic needle and syringe and ethidium bromide was removed by repeated extraction using butanol saturated with ddH_2O. The DNA solution was then diluted with 3 volumes of ddH_2O and DNA was precipitated with 2.5 volumes of ethanol. centrifugation at 8000rpm the DNA pellet was resuspended in 1ml ddH_2O. Then, RNase A was added to the DNA to a final concentration of 20mg/ml and it was incubated at 37°C for 30 minutes. The DNA was then extracted once with equal volume of phenol:chloroform:isoamylalcohol and precipitated by adding 3M NaOAc to 0.1M final concentration and 2.5 volumes of ethanol. The DNA pellet was washed with 70% ethanol, resuspended in 1ml ddH_2O and stored at –20°C.

DNA

Genomic DNA preparation

Five mm of tail tissue was incubated at 55°C overnight in 500µl tail mix supplemented with 200µg/ml of proteinase K in a shaking water bath. The DNA was then extracted twice by shaking for 10 minutes with equal amount of phenol:chloroform followed by centrifugation at 13000rpm in a benchtop centrifuge for a further 10 minutes. This was followed by a similar extraction with chloroform. DNA was then precipitated with equal volume of isopropanol. The DNA was immediately spooled out using a pasteur pipette which had previously been sealed in a bunsen burner. The DNA was washed with 70% ethanol, air dried briefly and then resuspended in ddH_2O overnight at 4°C. DNA concentration was then determined by spectrophotometry.

Tail mix:

50mM Tris-HCl, pH8.0; 10mM EDTA; 1% (w/v) SDS.

Phenol:Chloroform:

Phenol:Chloroform:Isoamylalcohol (25:24:1 v/v) equilibrated with 10mM Tris-HCl, pH7.5.

DNA restriction digests

Restriction digests were carried out according to the manufacturers' directions. DNA was incubated under the appropriate conditions for the restriction enzyme used. For digests with more than one enzyme, which had not the same buffer requirements, DNA was digested in consecutive single digests. The DNA was phenol:chloroform extracted and ethanol precipitated between these digests. For enzymes that required the same restriction buffer, both enzymes were used simultaneously in the same reaction.

Agarose gel electrophoresis

Agarose gels were prepared in 1X TBE and run horizontally and submerged in 1X TBE buffer. This buffer was supplemented with 5mg/ml of ethidium bromide and run

at 4V/cm2. DNA was visualized and photographed under short wave u.v. light. DNA size markers used were BstEII digested Lambda (l) DNA (sizes (kb): 14.1, 8.5, 7.4, 6.2, 5.7, 4.8, 4.3, 3.7, 2.3, 1.9, 1.37, 1.26, 0.7, 0.2). DNA digests were supplemented with 1/5 volumes of Orange G loading buffer before loading.

TBE (10X):

890mM Tris-HCl; 890mM Boric Acid; 20mM EDTA.

Orange G:

20% Ficoll; 110mM Tris-HCl, pH7.5; 0.25% (w/v) orange G.

Extraction of DNA from agarose gels

DNA digests were run in agarose gels prepared in 1X TAE and bands were visualized under long wave u.v. light. The DNA was extracted from the gel using a GeneCleanII kit (Anachem) following the manufacturers instructions.

50X TAE:

2M Tris-Acetate; 100mM EDTA; pH8.0.

Blotting And Hybridisation

Southern blot

10µg of genomic DNA was digested in a final volume of 50µl overnight in the appropriate conditions for the restriction enzyme using 40 units of enzyme. To check completion of digestion, 3µl of this reaction was mixed with 0.5µg of lambda DNA in a second eppendorf tube and incubated in parallel. The next morning the lambda DNA samples were run on a 0.8% agarose gel to determine the extend of digestion. Digests were then electrophoresed slowly (2V/cm2) overnight in a 0.7% agarose gel followed by photography under short wave u.v. light. The gel was then prepared for blotting by submerging it for 40 minutes in Blot I solution. The gel was then incubated for a further 50 minutes in Blot II solution. It was then set up for overnight blotting according to Southern (1975). DNA was then fixed onto the membrane by baking at 80°C for 2 hours.

Blot I solution:

0.5M NaOH; 1.5M NaCl.

Blot II solution:

0.5M Tris-HCl, pH7.5; 3M NaCl.

DNA probe labeling

DNA probes were radioactively labeled by random priming using a random priming kit (Pharmacia). 50ng of DNA resuspended in ddH_2O was denatured at 100°C for 5 minutes, cooled immediately on ice for 5 minutes and incubated in a final volume of 50µl with 10µl of Random Priming Buffer, 5µl of [a32P]-dCTP (370MBq/ml, Amersham) and 1µl of labeling grade klenow enzyme at 37°C for 30 minutes. Unincorporated nucleotides were removed using a Pharmacia 50G nick column according to the manufacturers' instructions. The collected probe was denatured by boiling at 100°C for 5 minutes before use in hybridisation reactions.

Filter hybridisation

Filter membranes were pre-hybridised at 65°C for at least 2 hours in 25ml of hybridisation buffer in either Hybaid bottles placed in a rotating Hybaid oven or in perspex boxes submerged in a shaking waterbath. 50ng of labeled probe was added to the hybridisation buffer and left to hybridise overnight at 65°C. Following hybridisation, nylon filters (Hybond N+, Amersham) were washed in a final stringency of 0.1X SSC; 0.1% (w/v) SDS at 65°C changing wash solutions every 20 minutes. Filters were then prepared for autoradiography.

SSC (20X):

3M NaCl; 0.3M NaCitrate.

Hybridisation buffer:

10X Denhardts; 3X SSC; 0.1% (w/v) SDS; 10% (w/v) Dextran Sulphate;

50mg/ml denatured salmon sperm DNA.

100X Denhardts:

2% (w/v) BSA; 2% (w/v) Ficoll 400; 2% (w/v) Polyvinyl pyrollidone.

Autoradiography

Washed nylon membranes from hybridisations were wrapped in Saran Wrap and exposed to Kodak Xomat-AR photographic film at –70°C using intensifying screens.

Membrane stripping

Radioactive probe was removed from hybridisation membranes by immersion of the membrane into a boiling solution of 0.1X SSC; 0.1% (w/v) SDS and letting the solution cool to RT. Complete removal of probe was confirmed by exposure of the membrane to photographic film.

λ phage library screening

A λ phage mouse genomic library (λFIX, Stratagene) was screened with an appropriate probe. The host bacterial strain (XL-1 BLUE) was grown at 37°C until the OD_{600} was 0.6 and they were resuspended in 10mM $MgSO_4$ to a final OD_{600} of 0.5. The bacteria were stored in 10mM $MgSO_4$ buffer at 4°C for up to a week. The titre of the phage library was calculated by making serial dilutions of the library in SM buffer and platting out on 10cm plates, so that 10-100 plaques were formed on a plate. 200μl of the host bacteria were mixed with 50μl of each phage dilution and were incubated for 15 minutes at 37°C. Top Agar was kept in the liquid state at 48°C. Subsequently, 3ml of Top Agar were added to the bacteria and phage, they were mixed gently by inversion and were plated out on a 10cm plate that had been dried well. The plates were incubated at 37°C for 8-10 hours and the plaques were counted.

A total of 1.2 million plaques were screened and 20cm x 20cm square culture plates were used. 1.2ml of host bacteria were mixed with the appropriate amount of phage library stock (to have 150000 plaques per plate), they were incubated 15 minutes at 37°C and 22ml of Top Agar was added and the mixture was plated out on plates. The plates were incubated at 37°C for 10 hours. Subsequently, the plates were cooled at 4°C for at least one hour and the plaques were transferred onto nitrocellulose filters by smoothly placing them on the agar surface. The exact position of the filter on the plate was marked with water insoluble ink. The first filter was peeled off the agar surface after

90 seconds. A replica filter was produced by placing a second pre-wetted filter on the agar surface for 3 minutes. The filters were immersed successively in denaturing solution (4 minutes), neutralizing solution (4 minutes), wash solution (4 minutes) and finally 3 x SSC. The filters were let to air dry and were baked for 2 hours at 80°C. The plates were stored at 4°C.

The filters were hybridised in 200ml of hybridisation buffer (described above) with 600ng of ^{32}P labeled DNA probe in a perspex box at 65°C overnight, after a 2 hour pre-hybridisation period in the same volume of hybridisation buffer. Subsequently, the filters were washed and exposed for 5 days to Kodak film as described before. The autoradiograph was aligned with the corresponding agar plate and agar around each positive clone was removed. The phage was eluted in 1ml SM buffer for 2 hours at RT. Serial dilutions of the phage for each positive clone were used for a second and a third screening round with the same probe, until a single positive plaque was picked from each clone.

Four positive clones were identified and used for phage DNA preparation. 250000 plaque forming units from each positive clone were plated in one 20cm x 20cm plates. The plates were incubated at 37°C for 10 hours and the phage was harvested in 50ml SM buffer after shaking at 4°C for 2 hours. A phage DNA extraction Kit (Qiagen) was used for the isolation of phage DNA according to the manufacturers' instructions.

SM buffer:

2.9gr NaCl; 1gr $MgSO_4$ $7H_2O$; 2.5ml gelatin 2%; 25ml Tris-HCl 1M pH7.5;

make up volume to 500ml with ddH_2O.

Top Agar:

L-Broth; 0.7% agarose; 10mM $MgSO_4$.

Denaturing solution:

0.5M NaOH; 1.5M NaCl.

Neutralizing solution:

0.5M Tris-HCl, pH7.5; 3M NaCl.

Wash solution:

3 x SSC; 0.1%SDS.

Tissue Culture

Expansion and trypsinisation of ES cells

D3 (Doetschman, 1985) or PC3 (O'Gorman et al 1997) embryonic stem cells from 129/Sv mice were maintained in the undifferentiated state by growth on a feeder layer of γ-irradiated primary embryonic fibroblasts using culture medium supplemented with leukaemia inhibitory factor (LIF) (Mansour, 1988). The ES cells were grown to confluency on 6cm culture dishes in 5ml of ES medium. The culture dishes were pre-coated with 3 ml of 0.1% Gelatin for 30 minutes and were containing a monolayer of $2x10^6$ embryonic fibroblasts. The culture medium was changed every day and when the cells reached confluency they were washed with 5 ml of PBS and then 0.5 ml of trypsin was added to them. The cells were put in 37°C for 3 minutes and then they were pipetted up and down vigorously with a pasteur pipette ensuring that a single cell suspension was obtained. Then, the cells were spun out through medium, resuspended in 5 ml of ES medium and 1/10 of them was transferred to a fresh 6 cm culture dish with a feeder layer.

ES Medium:

450ml Dulbecco's Modified Eagle's Medium (DMEM) with 0.11G/L NaPyruvate, pyridoxine; 75ml Foetal calf serum (FCS); 5ml Glutamine 100X stock at 200mM; 2.5ml Penicillin/ Streptomycin 200X stock at 10^4 units penicillin and 10mg/ml streptomycin; 5ml Non essential amino acid (MEM) 100X stock; 3.5μl β-mercaptoethanol; 50μl Leukemia inhibitory factor (LIF) 10^7 units/ml.

Phosphate buffered saline (PBS):

1.4M NaCl; 27mM KCl; 81mM Na_2HPO_4; 15mM KH_2PO_4; adjusted to pH 7.5 with HCL

Trypsin:

To 1 litre of ddH_2O add: 2.5gr Trypsin powder; 0.4gr EDTA; 7.0gr NaCl; 0.3gr $Na_2HPO_4.12H_2O$ or 0.22gr $Na_2HPO_4.7H_2O$; 0.24gr KH_2PO_4; 0.37gr KCl; 1.0gr D-glucose; 3.0gr Tris; 1ml Phenol Red 0.5%; adjusted to pH7.6 and filtered.

Preparation of embryonic fibroblasts

To make G418 resistant embryonic fibroblasts (EFneo), a β2m-/- or an IL3-/- male mouse was mated to a wild-type female. The pregnant female was sacrificed at day 13 or 14 of gestation and the embryos were dissected out free of extraembryonic membranes in sterile conditions. Each embryo was washed in 70% ethanol and subsequently in PBS and soft organs and viscera (liver, gut, brain) were dissected out. The remaining carcass was cut in small pieces and was thrown into 2ml/embryo of trypsin in 15ml tube (FALCON). The tube was left at 4°C for 2-18 hours and then at 37°C for 10 minutes. The embryonic tissue was pipetted up and down every 5 minutes and equal volume of EF medium was added. The big pieces of embryonic tissue were left to settle for 2 minutes, the supernatant was removed and plated out in one 162cm^2 tissue culture flask in 50ml of EF medium. The flask was incubated at 37°C and 5% CO_2. The medium was changed the next day and the fibroblasts were left to grow for 2 further days until they reached extreme confluency. Then, the fibroblasts from one flask were split 1/5 and passed into 5 new 162cm^2 flasks. When they reached confluency after 3 days, the fibroblasts from each flask were split 1/5 and were passed into five 162cm^2 flasks again. They were left to grow for further 3 days and then the fibroblasts from all 25 flasks were collected, γ-irradiated (3500 rads) and were frozen in EF medium containing 12% DMSO at 4x10^6 cells/vial or 20x10^6 cells/vial. The total yield of fibroblasts is 400-600x10^6 irradiated EFs/embryo. The irradiated EFs were used to form a monolayer on the culture dish in which the ES cells were grown. One vial of 4x10^6 cells is enough for 1x10cm plate or 2x6cm plates.

EF Medium:

450ml Dulbecco's Modified Eagle's Medium (DMEM) with 0.11G/L NaPyruvate, pyridoxine; 50 ml Foetal calf serum (FCS); 5ml Glutamine 100X stock at 200mM; 2.5ml Penicillin/ Streptomycin 200X stock at 10^4 units penicillin and 10mg/ml streptomycin.

Transfection and selection of ES cells

The ES cells were grown to 50-100% confluency. The DNA used for the transfection was a $CsCl_2$ banded plasmid, it was linearised, ethanol precipitated and redissolved in sterile TE at concentration 2mg/ml. 10cm and 6cm culture dishes with $4X10^6$ and $2X10^6$ embryonic fibroblasts respectively were prepared in advance to receive the electroporated cells. The ES cells were trypsinised, spun out through medium and resuspended in Hepes buffered saline (HBS) containing $7X10^{-4}$% 2-mercaptoethanol (2-ME) at a concentration of 10^7cells/ml. The linearised targeting construct was added to the stock of cells at a final concentration of 45µg/ml or 50µg/ml and 0.8ml of this mix was used for each electroporation. The electroporations were done with a BIORAD Gene Pulser at 400Volts and 25µF using 0.4cm electrode gap microcuvettes (BIORAD). The cells were transferred then to a 15 ml Falcon tube containing 3.4 ml medium and plated out onto 4X10cm plates (0.9ml/plate) for double selection and 1X6cm plate (0.4ml) for G418 selection. Selection was initiated 24hrs later at a concentration of 250µg/ml or 300µg/ml G418 and 2µM (=0.51µg/ml) Gancyclovir.

The selective medium was changed every 3 days and 576 resistant colonies were picked after 10 days onto a 96-well plate, in 20µl PBS using P-200 Gilson pipette and a yellow tip. 6 days later the clones were transferred to 24-well plates and expanded for another 5 days. At confluency, half of each well was frozen in ES medium containing 12% DMSO and the other half was used to make DNA.

Hepes buffered saline (HBS):

137mM NaCl; 5mM KCl; 0.7mM Na_2HP0_4; 6mM dextrose;

21mM Hepes, pH7.1.

G418 stock:

50mg/ml in PBS stored up to a month at 4°C.

Gancyclovir stock:

1mg/ml in PBS stored frozen.

Karyotyping of ES cells

The cells were grown to 50-75% confluency, the medium was changed and 2 hours later Demecolcine (Sigma) was added at a final concentration of 20ng/ml to arrest the cells in metaphase. Two hours later the cells were trypsinised for 3 minutes at 37°C, spun out through ES medium for 5 minutes at 1200rpm and washed one more time in PBS. They were then resuspended in 5ml KCl 0.56% (Hypotonic solution) and let stand at room temperature for 6 minutes following centrifugation at 750rpm for 5 minutes. The pellet was resuspended carefully in 5ml fixative by adding it dropwise and flicking thoroughly, left at room temperature for 5 minutes and spun down for a further 5 minutes. The fixing procedure was done another 3 times and finally the cells were resuspended in 1ml fixative and dropped from 30cm height on a microscope slide that was washed before in 5% Acetic Acid in ethanol. The chromosome spreads were stained with 10% Giemsa's (DBH) for 20 minutes and chromosomes were count in high power microscope.

Fixative:

Methanol: Acetic Acid 3:1

Injection of ES cells into blastocysts and generation of mutant mice

The cells were grown to 70-80% confluency, then trypsinised for 3 minutes at 37°C, spun out through 5ml ES medium at 1200rpm for 5 minutes and resuspended in 5ml Injection medium. 3.5-day blastocysts were collected from C57Bl/6 females and kept in blastocyst medium. 10-15 ES cells were injected into each blastocyst and 10-12 blastocysts were transferred into the uterus of each foster mother. Chimeric males were bred to C57Bl/6 or DBA/2 females and germline transmission was determined by agouti coat colour.

Injection medium:

Dulbecco's modification of Eagle's medium with 20mM Hepes,

without glutamine and bicarbonate (ICN Biomedicals); 10% Feotal calf serum;

1.5% Penicillin/streptomycin/ glutamine.

Blastocyst medium:

Dulbecco's modification of Eagle's medium; 10% Foetal calf serum;

1.5% Penicillin/ streptomycin/ glutamine.

Isolation of mouse small intestinal intraepithelial lymphocytes

The mice were sacrificed and the small intestine was dissected from 0.5cm below stomach to 1cm above cecum. The intestine was washed by flushing into it 40ml cold CMF solution with a 20-ml syringe. Fat, connective tissue and Peyer's patches were removed from the intestine and then it was cut longitudinally and laterally into 0.5cm pieces. The intestinal pieces were washed 2 times in 40ml cold CMF solution to remove remaining mucus and then incubated in 20ml CMF/FCS/DTE for 20 minutes shaking at 37°C, following vortexing for 15 seconds at maximum setting. When the intestinal pieces settled, the supernatant was transferred to another tube and the rest was treated once more as described above. The combined supernatant was centrifuged 7 minutes at 1200rpm, 20°C and the cell pellet was resuspended in 10ml RPMI and added into a nylon wool column, that was washed with 20ml RPMI earlier. The cells were spun down for 10 minutes at 1200rpm, resuspended in 3 ml RPMI and added to a 44%-64% percoll (Pharmacia) gradient. The gradient was centrifuged at 3000rpm at 20°C for 10 minutes. The interphase between the two layers of the gradient with the IEL was transferred to a new tube, diluted 1:3 in RPMI and centrifuged for 8 minutes at 2500rpm, 20°C to pellet. The IEL were then counted and stained with antibodies.

CMF solution (10X):

10% Hank's balanced salt solution (HBSS) Ca^{2+} and Mg^{2+} free;

10% 10X HEPES-bicarbonate buffer; 2% FCS.

CMF/FCS/DTE solution:

CMF with 10% FCS, 15.4mg DTE (in 100ml)

HEPES-bicarbonate buffer (10X):

10mM HEPES; 250mM sodium bicarbonate; adjust pH to 7.2 with HCl.

Flow Cytometry

3-Colour flow cytometry

For flow cytometry 10^6 cells were stained in 100µl of PBA at 4°C in the dark with the following reagents in appropriate combinations: Fluoresceine isocyanate (FITC)-conjugated anti-βTCR (H57.597, Pharmingen); FITC-conjugated anti-δTCR (GL3, Pharmingen); FITC-conjugated anti-CD8α (YTS169); FITC-conjugated anti-CD8β (53-5.8, Pharmingen); FITC-conjugated anti-CD8α/Lyt2.1 (49-11.1); phycoerythrine (PE)-conjugated anti-CD8α (CT-CD8, Caltag); PE-conjugated anti-CD8β (CT-CD8β, Caltag); PE-conjugated anti-CD4 (H129.19, Pharmingen); tricolor (TC)-conjugated anti-CD4 (CT-CD4, Caltag); TC-conjugated anti-CD8α (CT-CD8α, Caltag); biotin-conjugated anti-CD8α/Lyt2.2 (2.43); biotin-conjugated anti-CD8β; biotin-conjugated anti-CD69 (H1.2F3, Pharmingen); biotin-conjugated anti-CD5 (53-7.3, Pharmingen); biotin-conjugated anti-HSA. Cells stained with biotin-conjugated antibodies were subsequently stained with a second layer of streptavidin (SA)-red 670 (GIBCO-BRL). The cells were washed in ice cold PBS-Azide and harvested by centrifugation for 7 minutes at 1200rpm, 4°C. When required, red blood cells were lysed by incubating samples in RBC lysis solution for 5 minutes at RT. Following red blood cells lysis the samples were diluted with an equal volume of ice cold PBS-Azide and cells were collected by centrifugation as described above. Three colour analysis was performed with a Becton Dickinson FACScan laser instrument using CellQuest software.

PBS (10X):

1.4M NaCl; 27mM KCl; 81mM Na_2HPO_4; 15mM KH_2PO_4; adjusted to pH7 with HCl.

PBS-Azide:

1X PBS; 0.02% (w/v) Sodiumazide.

PBA:

PBS-Azide; 1% BSA.

RBC lysis solution (1X):

17mM Tris-HCl pH7.2; 144mM NH_4Cl.

4-Colour flow cytometry

For 4-colour flow cytometry 10^6 cells were stained in 100µl PBA at 4°C in the dark with the following combination of antibodies: FITC-conjugated anti-CD8α/Lyt2.1 (49-11.1); PE-conjugated anti-βTCR (H57.597, Pharmingen); Biotin-conjugated anti-CD8α/Lyt2.2 (2.43); APC-conjugated anti-CD4 (RM4-5APC, Pharmingen). The cells were stained as described above first with the biotin-conjugated antibody and subsequently with the FITC, PE and APC conjugated antibodies together with Streptavidin PE-Cy7 as second layer antibody. Four colour analysis was performed with a Becton Dickinson FACS Calibur laser instrument using CellQuest software.

BrdU labeling

For in vivo BrdU labeling, the mice were injected once i.p. with BrdU at 1mg/ml. At the appropriate time the mice were sacrificed and 10^6 cells were stained by usual procedure using TC-conjugated anti-CD8α (CT-CD8, Caltag) and PE-conjugated anti-CD4. The cells were washed in Wash Solution, spun down for 1 minute at 2000rpm and resuspended in 25µl of ice cold 0.15M NaCl. Then, 70µl of ice cold 95% ethanol were added while mixing the cells and they were incubated on ice for 30 minutes. The cells were washed with 100µl of Wash Solution, they were resuspended in 100µl of Solution I and incubated 30 minutes at room temperature and then 30 minutes in the cold. Following that, the cells were washed, spun down and resuspended in 200µl of Solution II. After 30-45 minutes incubation at 37°C the cells were washed, spun down and the pellet was resuspended in 35µl of Solution III. Then, 5µl of anti-BrdU-FITC (Becton Dickinson) were added and the cells were incubated at room temperature for 30-60 minutes. Finally, the cells were washed and resuspended in 100µl PBS and acquired in a Becton Dickinson FACScan laser instrument.

Wash Solution:

PBS (no Azide); 2% FCS.

Solution I:

PBS; 1% Paraformaldehyde; 0.01% Tween-20.

Solution II:

0.15M NaCl; 4.2mM $MgCl_2$; 10µM HCl; 50 units/ml DNase I.

Solution III:

PBS; 5% FCS 0.5% Tween-20

7AAD staining

For 7AAD staining 10^6 cells were stained, as described above, with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8α diluted in SB. The cells were washed with PBS-Azide, they were resuspended in 100μl SB/ 0.3% Saponin/ 7AAD and incubated in the dark at 37°C for 30 minutes. Then, the cells were left at 4°C for at least 30 minutes and they were acquired as they were in a Becton Dickinson FACScan laser instrument. 7AAD is measured on the same laser channel as the fluorochrome tricolor.

SB:

PBS; 2% FCS; 0.1% NaAzide.

7AAD:

1mg/ml in 50% DMSO

Foetal Thymic Organ Culture (FTOC)

Foetal thymic lobes were isolated from day 15 embryos, obtained from timed matings of $CII^{+/\Delta 1,2}$ heterozygous males with CBA/Ca female mice. The lobes were transferred onto nucleopore polycarbonate filters (Costar Corporation, Cambridge, MA) and cultured at 37°C, 5% CO_2 in RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated FCS, 2mM L-glutamine and 50μM 2-mercaptoethanol. The lobes were allowed to develop for further one or three days. Thymocytes from these foetal lobe cultures were harvested for flow cytometric analysis by gently disrupting the thymic lobes manually in 1.5-ml Eppendorf tubes. Subsequently, they were stained with anti-CD8α/Lyt-2.1, anti-CD4 and anti-CD8α/Lyt-2.2 and three colour FACS analysis was carried out.

RESULTS

Deletion of cluster III DNaseI-HSS CIII-1 and CIII-2

The cluster III DNaseI-HSS located in the intergenic region between the CD8α and CD8β genes figure 1 directs reporter gene expression to mature T cell subset (Hostert et al., 1997). In order to address the function of cluster III in situ, we generated mice that lack two of the three sites of cluster III DNaseI-HSS (CIII-1 and CIII-2).

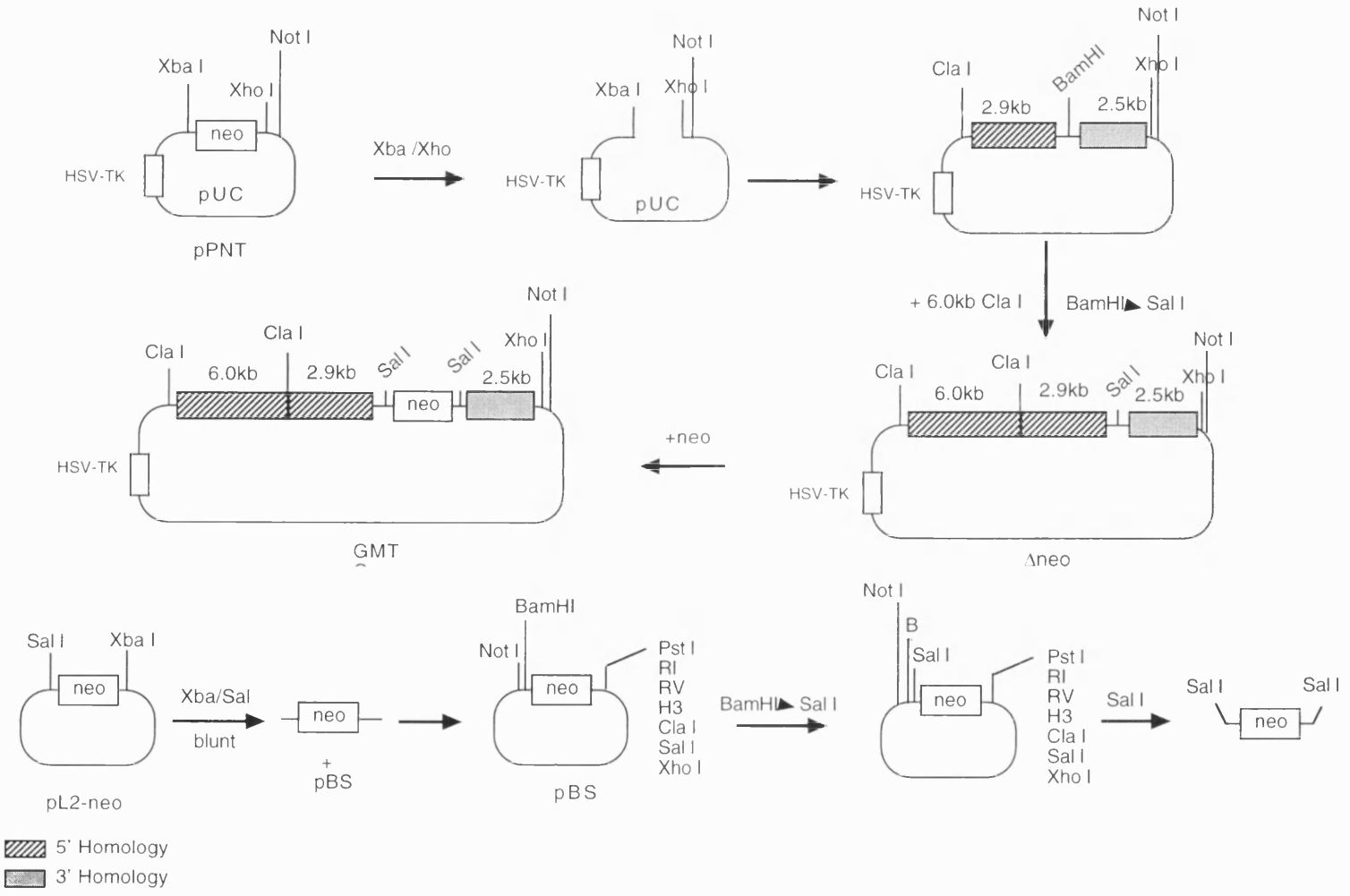
Generation of $CIII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice

In order to delete DNaseI-HSS CIII-1 and CIII-2 of cluster III from the mouse genome, a targeting construct was introduced into embryonic stem cells by homologous recombination. For the generation of the targeting construct (figure 2) the following steps were taken: in order to include an artificial Hind III site for screening purposes, the loxP-flanked neomycin gene was excised from plasmid pL2-neo as a 1.2kb XbaI-SalI fragment, blunted and ligated into the SmaI site of pBluescript that had its BamHI site mutated to SalI. The neomycin gene was then isolated as a SalI fragment and ligated into pΔneo. This was constructed by ligation of 2.9kb ClaI-BamHI fragment upstream of cluster III (upstream homology) and 2.5kb BamHI-SalI fragment downstream of cluster III (downstream homology) into the 5.4kb XbaI-XhoI fragment, lacking the original neo gene, of the pPNT vector (Tybulewicz et al., 1991). A further 6.0kb BamHI-ClaI fragment upstream homology was ligated into the vector as a ClaI fragment. Finally, the SalI fragment containing neo was ligated between upstream and downstream homology to give the final targeting construct, GMTC.

The targeting construct contained the neomycin resistance gene (positive selection) and the Herpes Simplex Virus thymidine kinase (HSV-tk) gene (negative selection). The vector was designed so that when replacement of the endogenous sequence by the exogenous one occurred via homologous recombination, the HSV-tk gene was not transferred into the targeted site. Cell lines in which the homologous recombination occurred were therefore neo^r, HSV-tk^-. On the other hand, random integrations of the targeting vector into the recipient cell genome resulted in neo^r, HSV-tk^+ cells and therefore sensitive to the drug gancyclovir. By selection for cells containing a functional neo^r gene (G418) and against cells that contained a functional HSV-tk gene (gancyclovir), cells in which homologous recombination occurred were enriched (Mansour et al., 1988).

Figure 2: Generation of the GMTC targeting construct

All the relevant restriction enzyme sites used for the cloning steps are shown. The neomycin resistance gene (neo) and the herpes simplex virus thymidine kinase (HSV-tk) are indicated by open boxes and the upstream and downstream homologies of the targeting vector are shown by filled boxes. The generation of the GMTC targeting construct is described in detail in the text.


Not I
Xba I
Xho I
neo
HSV-TK
pUC
pPNT
Xba /Xho
Not I
Xba I
Xho I
HSV-TK
pUC
Not I
Cla I
BamHI
Xho I
2.9kb
2.5kb
HSV-TK
+ 6.0kb Cla I
BamHI▶ Sal I
Not I
Cla I
Cla I
6.0kb
2.9kb
Sal I
2.5kb
Xho I
HSV-TK
Δneo
+neo
Not I
Cla I
Cla I
Sal I
Sal I
Xho I
6.0kb
2.9kb
neo
2.5kb
HSV-TK
GMT
Sal I
Xba I
neo
pL2-neo
Xba/Sal
blunt
neo
+
pBS
BamHI
Not I
neo
Pst I
RI
RV
H3
Cla I
Sal I
Xho I
pBS
BamHI▶ Sal I
Not I
B
Sal I
neo
Pst I
RI
RV
H3
Cla I
Sal I
Xho I
Sal I
Sal I
Sal I
neo
5' Homology
3' Homology

The construct was linearised with NotI and introduced into D3 embryonic stem cells of 129/Sv origin (Doetschman et al., 1985) by electroporation (Rahemtulla et al., 1991). For each electroporation $0.8x10^7$ ES cells were used and the DNA concentration was 45μg/ml. Selection was initiated 24hrs later at a concentration of 250 or 300μg/ml G418 and 2μM (0.51μg/ml) gancyclovir. The targeting event replaces an 8kb BamHI genomic fragment containing the cluster III DNaseI-HSS CIII-1 and CIII-2 with the neomycin resistance gene. The homologous recombination event is shown in figure 3A. Because of the artificially introduced HindIII site in the 3' end of the neomycin gene, the 4.8kb wild type HindIII fragment is converted to a smaller 4.2kb mutant fragment, as detected by the 1.7kb XbaI-ScaI probe. This probe is located downstream of the targeted region, 3' of the homology of the targeting vector (figure 3A).

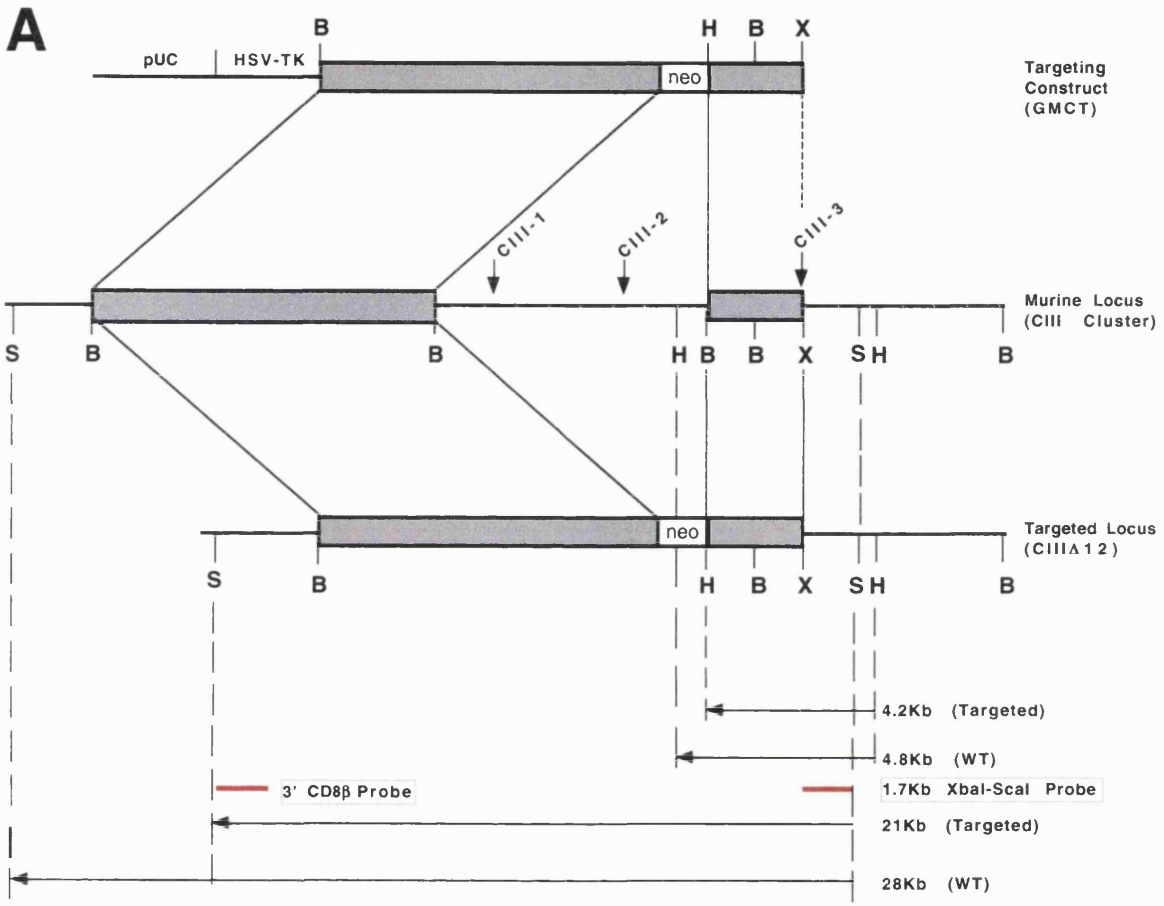
Of 576 resistant clones screened by Southern blot analysis and using the 1.7kb XbaI-ScaI probe, six were found to have undergone homologous recombination and the integrity of the locus was verified with an additional probe (3'CD8β probe) located upstream of the targeted region (outside the homology) and shown in figure 3A. Because of the 8kb BamHI deleted fragment, the 28kb ScaI wild type fragment is converted to a smaller 21kb mutant fragment, as detected with the 3' CD8β probe. In figure 3B is shown the Southern blot of two positive clones (number 169 and 171), run next to 129/Sv wild type DNA.

Two of the clones (number 169 and 171) that had a euploid karyotype (chromosome number of 40) were used to generate chimeric mice by injection into 3.5-day blastocysts derived from C57Bl/6 females and subsequent transfer into the uteri of foster mothers. Chimeric males were bred to C57Bl/10 or DBA/2 females and germline transmission was determined by agouti coat colour. Heterozygous mice that carried the $CIII^{\Delta 1,2}$ deletion mutation were identified by Southern blot analysis and were bred to generate the homozygous mutant mice for the deletion. Mice heterozygous and homozygous for this mutation are designated $CIII^{+/\Delta 1,2}$ and $CIII^{\Delta 1,2/\Delta 1,2}$, respectively. figure 4A shows the Southern blot analysis of wild type 129/Sv, $CIII^{+/\Delta 1,2}$ heterozygous and $CIII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice and confirms the predicted conversion of the 4.8kb wild type HindIII fragment to a smaller 4.2kb mutant fragment, as detected with the 1.7kb XbaI-ScaI probe shown in figure 3A.

Figure 3: Deletion of DNaseI-HSS CIII-1 and CIII-2 by homologous recombination

(A) Diagram of the murine CD8 gene locus showing the targeting construct, DNaseI-HSS cluster CIII and the targeted allele. The neomycin gene (open box, labeled neo) was flanked by 8.5kb 5' and 2.5kb 3' homologous DNA sequences (filled boxes). Restriction enzyme sites are shown by vertical bars and are labelled B, BamHI; H, HindIII; X, XbaI; S, ScaI. Vertical arrows indicate cluster CIII DNaseI-HSS and the probes (1.7kb XbaI-ScaI and 3' CD8β genomic fragments) used to detect homologous recombination events are shown as solid horizontal bars. Horizontal arrows illustrate the expected HindIII fragments hybridising with the 1.7kb XbaI-ScaI probe (4.8kb wild type allele, 4.2kb targeted allele) and the expected ScaI fragments hybridising with the 3'CD8β probe (28kb wild type allele, 21kb targeted allele).

(B) Southern blot analysis of two clones (169, 171) that had undergone homologous recombination, run along with wild type 129/Sv DNA. The sizes of wild type and mutant fragments, the restriction enzymes and the probes used are shown.


A
pUC
HSV-TK
neo
Targeting Construct (GMCT)
CIII-1
CIII-2
CIII-3
Murine Locus (CIII Cluster)
Targeted Locus (CIIIΔ12)
4.2Kb (Targeted)
4.8Kb (WT)
3' CD8β Probe
1.7Kb XbaI-ScaI Probe
21Kb (Targeted)
28Kb (WT)

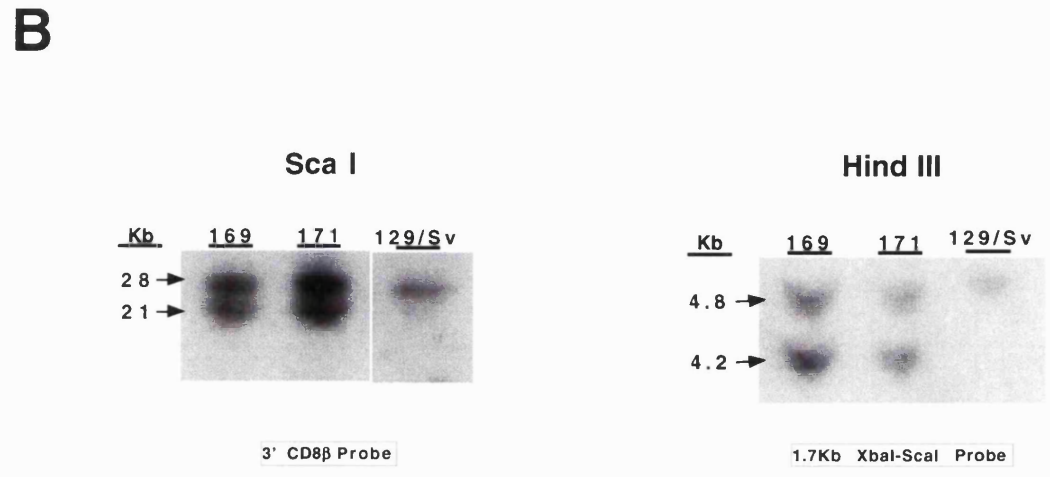
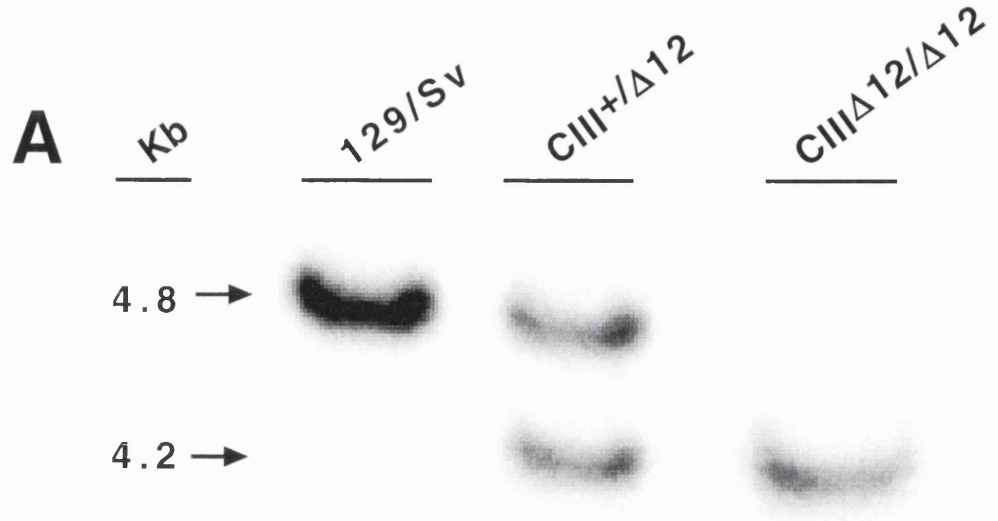
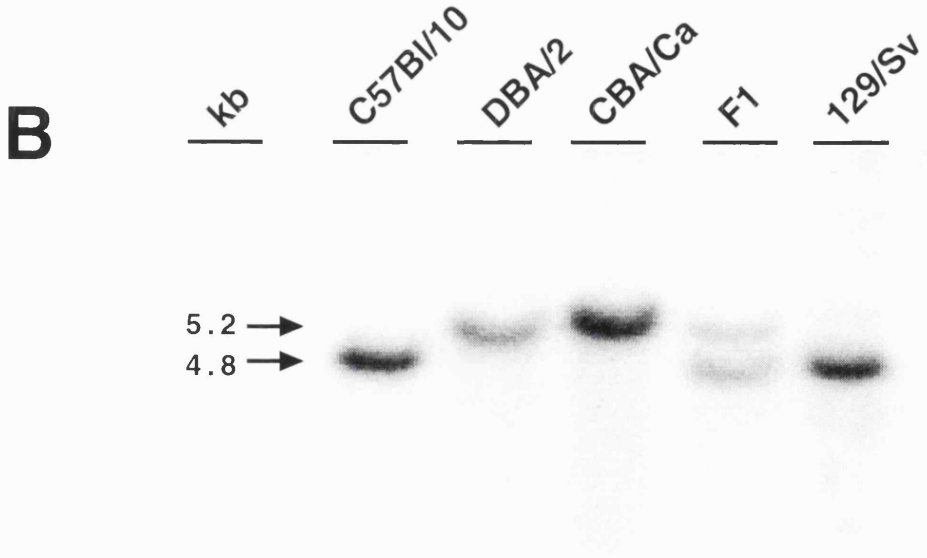

B
Sca I
Kb
169
171
129/Sv
28
21
3' CD8β Probe
Hind III
Kb
169
171
129/Sv
4.8
4.2
1.7Kb XbaI-ScaI Probe

Figure 4: HindIII restriction site polymorphism in the CD8 locus between mouse strains

(A) Southern blot analysis of 129/Sv wild type, $CIII^{+/\Delta 1,2}$ heterozygous and $CIII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice. The introduction of an artificial HindIII site with the neomycin gene results in a reduction of a wild type 4.8kb HindIII fragment to a mutant 4.2kb HindIII fragment.

(B) Southern blot analysis of C57Bl/10, DBA/2, 129/Sv and (C57Bl/10 x CBA/Ca) F1 wild type mice. DNA from these mice was digested with HindIII and the Southern blot was hybridised with the 1.7kb XbaI-ScaI probe. There is a difference in the detected HindIII fragment as a result of a polymorphism in this restriction enzyme site in the CD8 locus between mouse strains.


A
Kb
129/Sv
CIII+/Δ12
CIIIΔ12/Δ12
4.8
4.2


B
kb
C57Bl/10
DBA/2
CBA/Ca
F1
129/Sv
5.2
4.8

Hind III restriction site polymorphism in the CD8 locus between mouse strains

In wild type 129/Sv mice there is a 4.8kb HindIII genomic fragment within the 3' end of cluster III DNaseI-HSS, as detected by the 1.7kb XbaI-ScaI probe (shown in figure 3A). The same is true for C57Bl/10 wild type mice. However, in DBA/2 and CBA/Ca wild type mice the HindIII fragment detected by the above probe is 5.2kb, thus 0.4kb bigger than the corresponding fragment in 129/Sv and C57Bl/10 mice.

Figure 4B shows the Southern blot analysis of wild type C57Bl/10, DBA/2, CBA/Ca, (C57Bl/10 x CBA/Ca) F1 and 129/Sv mice and confirms the described difference in the wild type HindIII fragment, as detected with the 1.7kb XbaI-ScaI probe. The C57Bl/10 mice have the 4.8kb fragment, whereas the CBA/Ca mice have the 5.2kb fragment. It is therefore expected, that the (C57Bl/10 x CBA/Ca) F1 mice will have both fragments, as shown in the figure. This suggests that there is a polymorphism in one of the HindIII sites in cluster III DNaseI-HSS in the CD8 locus between mouse strains. This polymorphism could be used for identification of the genetic background of mice by Southern blot analysis and by using the 1.7kb XbaI-ScaI probe.

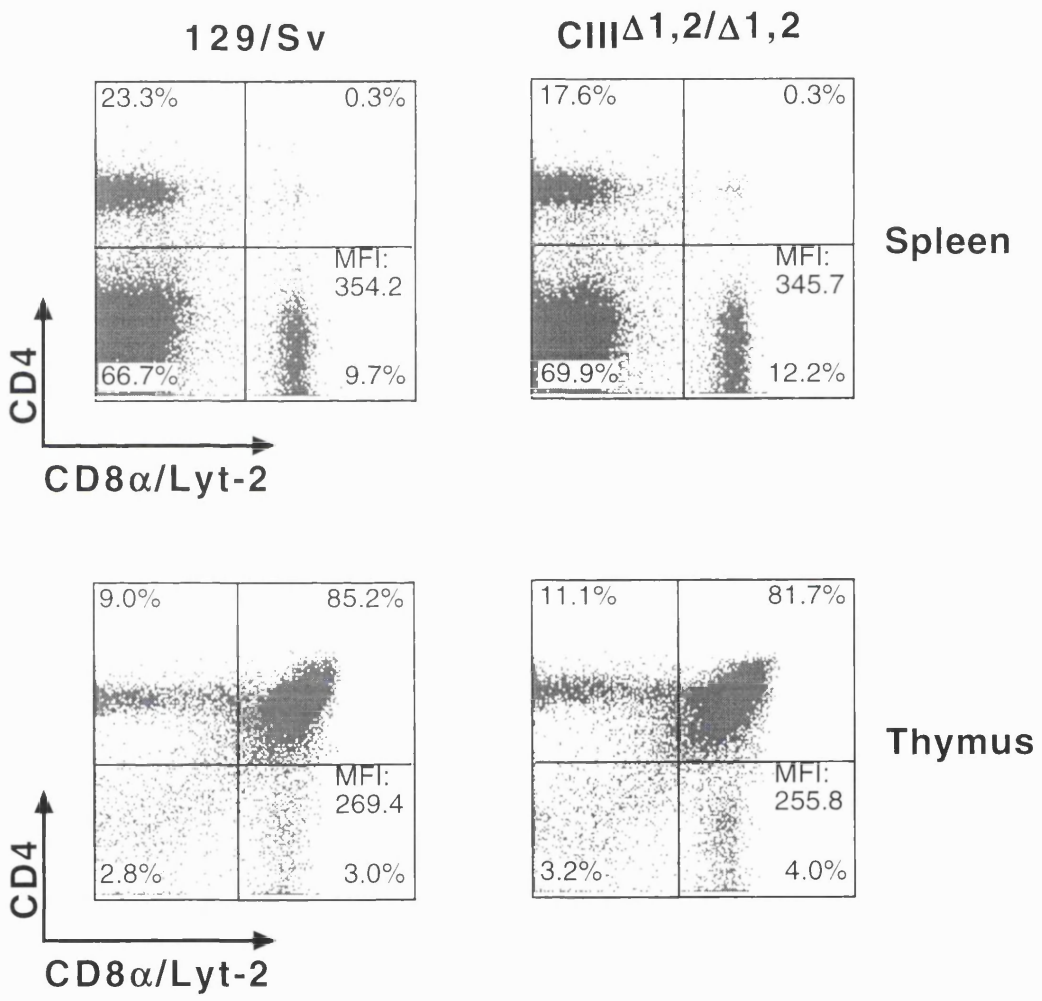
Analysis of CIII$^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice

Thymocytes and peripheral T cell subsets are not affected by deletion of cluster III DNaseI-HSS CIII-1 and CIII-2

In order to examine the effect of the CIII$^{\Delta 1,2}$ deletion on CD8α gene expression, splenocytes and thymocytes were isolated from wild type 129/Sv and CIII$^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice and stained with anti-CD4 and anti-CD8α/Lyt-2. Figure 5 shows the FACS profiles of splenocytes (top panel) or thymocytes (bottom panel) isolated from 129/Sv wild type control mice (left-hand side) and CIII$^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice (right-hand side) stained with the above combination of antibodies. Cells were analysed by plotting CD8α/Lyt-2 against CD4 and electronically gating the different cell populations. The percentages of cells falling in each gate, as well as the mean fluorescence intensity (MFI) of the CD8α/Lyt-2 antibody are shown in the dot-plots. This analysis showed that FACS profiles of T cells and thymocyte subsets were similar for both wild type and mutant mice with no significant differences in either percentages of subsets or CD8α mean fluorescence intensity between the mice analysed.

Figure 5: Thymocytes and peripheral αβTCR T cell subsets are not affected by deletion of CIII

FACS analysis of 129/Sv wild type and $CIII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice. Splenocytes and thymocytes were isolated and stained with anti-CD8α and anti-CD4 antibodies. T cell and thymocyte subsets were resolved by plotting CD4 against CD8α and analysed for CD8α expression. Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants and mean fluorescence intensities (MFI) for CD8α are also shown. Both percentages of cells and levels of expression of CD8α are very similar between mutant and wild type control mice.


129/Sv
CIII Δ1,2/Δ1,2
23.3%
0.3%
MFI:
354.2
66.7%
9.7%
17.6%
0.3%
MFI:
345.7
69.9%
12.2%
Spleen
CD4
CD8α/Lyt-2
9.0%
85.2%
MFI:
269.4
2.8%
3.0%
11.1%
81.7%
MFI:
255.8
3.2%
4.0%
Thymus
CD4
CD8α/Lyt-2

In addition, the expression of the CD8β gene was not altered by this mutation (data not shown).

Thus, the deletion of two of cluster III DNaseI-HSS had no effect on expression of the CD8α in the CD8αβ SP thymocyte compartment. This could be due to the fact that this mutation deleted only two of DNaseI-HSS of cluster III and thus left behind the third DNaseI-HSS of this cluster. It is possible that the third DNaseI-HSS alone is sufficient to direct expression of the CD8α gene on T cells of thymic origin. Another possible explanation is that additional *cis*-acting regulatory elements are involved in the regulation of CD8 expression in thymus derived T cells, such as elements in the CD8β gene region, which are able to compensate for the loss of cluster III activity.

$CIII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice lack CD8α gene expression on both $\gamma\delta TCR^+$ and $\alpha\beta TCR^+$ CD8αα intraepithelial T cells

Intraepithelial lymphocytes (IEL) are either of αβTCR or γδTCR lineage. The ones that are of αβTCR lineage express CD8αα homodimers or CD8αβ heterodimers on their surface, whereas the ones that are of γδTCR lineage express mainly CD8αα homodimers. The IEL that express CD8αα homodimers are thought to be extrathymically derived (Rocha et al., 1992). Studies of the methylation state of cytosines of an approximately 1 kb portion of the CD8β gene 5' regulatory region indicate that $CD8\alpha\alpha^+$ IEL have not previously expressed the CD8β gene (Hamerman et al., 1997).

Mice that carry cluster III transgene constructs express the reporter transgene in CD8αα gut intraepithelial lymphocytes (IEL) (Ellmeier et al., 1997). In order to examine the effect of the $CIII^{\Delta 1,2}$ deletion on CD8α gene expression in these cells, IEL were isolated from $CIII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice (carrying the CD8α/Lyt-2.2 allele) and $CIII^{+/\Delta 1,2}$ heterozygous mutant mice (carrying one CD8α/Lyt-2.1 allele and one CD8α/Lyt-2.2 allele). The heterozygous mice were generated by crossing $CIII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice (carrying the CD8α/Lyt-2.2 allele) to DBA/2 wild type mice (carrying the CD8α/Lyt-2.1 allele). The CD8α/Lyt-2 allelic difference is detectable at the protein level using specific monoclonal antibodies. This allows expression of the $CIII^{\Delta 1,2}$ mutated allele to be distinguished from the wild type DBA/2 allele. DBA/2 wild type mice were used as non-transgenic controls, as well as (DBA/2 x 129/Sv) F1 mice since

they carry both the CD8α/Lyt-2.1 (DBA/2 encoded) and CD8α/Lyt-2.2 (129/Sv encoded) allele.

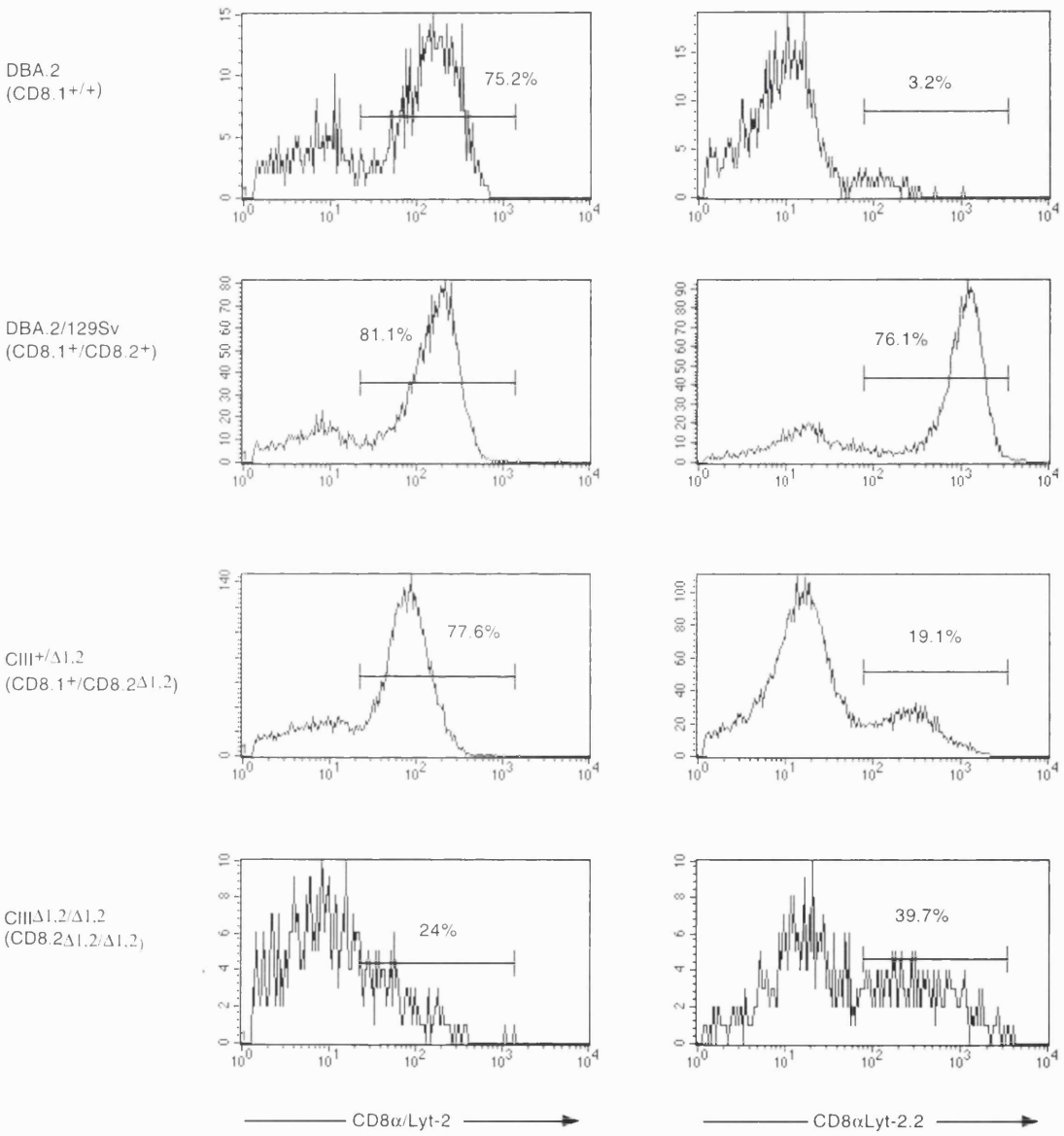
IEL isolated from the above mice were stained with anti-γδTCR, anti-CD8α/Lyt-2.2 and anti-CD8α/Lyt-2. As mentioned before, $\gamma\delta TCR^+$ IEL express only CD8αα homodimers. Thus, $\gamma\delta TCR^+$ cells were gated and analysed for CD8α/Lyt-2 and CD8α/Lyt-2.2 expression. The results are shown in the histograms shown in figure 6 with the percentages of positive cell populations seen in the histograms. This analysis showed that 77.6% of $\gamma\delta TCR^+$ CD8αα IEL isolated from $CIII^{+/\Delta 1,2}$ heterozygous mutant mice were found to express CD8α at levels comparable to those found in DBA/2 and (DBA/2 x 129/Sv) F1 control mice when stained with an antibody that recognises both CD8α alleles (pan CD8) (figure 6, left-hand side). In contrast, only 24% of $\gamma\delta TCR^+$ CD8αα IEL isolated from $CIII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice were found to fall in the CD8α positive gate and their level of expression was 10-fold lower compared to the control mice.

IEL isolated from the same mice were also stained with anti-αβTCR, anti-CD8β and anti-CD8α/Lyt-2 or with anti-αβTCR, anti-CD8β and anti-CD8α/Lyt-2.2 antibodies. αβTCR IEL express CD8αα homodimers or CD8αβ heterodimers on their surface. In order to assess CD8α expression on αβTCR IEL that have CD8αα homodimers on their surface (and are thought to be extrathymically derived), cells were gated for αβTCR expression and were analysed by plotting CD8β against CD8α/Lyt-2 or CD8α/Lyt-2.2. Subsequently, $CD8\beta^-$ cells were gated and analysed for CD8α/Lyt-2 and CD8α/Lyt-2.2 expression in the histograms shown in figure 7. The percentages of positive cell populations are shown in the histograms. Similarly to the γδTCR IEL, $\alpha\beta TCR^+$ $CD8\beta^-$ IEL expressing CD8αα homodimers showed a significant reduction in both percentages and levels of CD8α expression in $CIII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice compared to the wild type control and the $CIII^{+/\Delta 1,2}$ heterozygous mutant mice when they were stained with an antibody that recognises total CD8α (figure 7, left-hand side).

The intraepithelial lymphocytes that are of αβTCR lineage express CD8αα homodimers or CD8αβ heterodimers on their surface. The ones that express CD8αβ heterodimers are thought to have gone through normal development in the thymus, like most peripheral T cells. In order to assess CD8α expression on IEL that have CD8αβ

Figure 6: Cluster III (CIII-1 and CIII-2) mutant mice lack CD8α gene expression on γδTCR$^+$CD8αα intraepithelial T cells

Intraepithelial T cells were isolated from murine gut of wild type DBA/2 (top histograms), wild type (DBA/2 x 129/Sv) F1 (second line histograms), CIII$^{+/\Delta 1,2}$ heterozygous (third line histograms) and CIII$^{\Delta 1,2/\Delta 1,2}$ homozygous (bottom histograms) mice. IEL were stained with anti-γδTCR, anti-CD8α/Lyt-2 and anti-CD8α/Lyt-2.2 specific antibodies. γδTCR$^+$ T cells were gated and analysed for CD8α/Lyt-2 expression (left-hand side histograms) or CD8α/Lyt-2.2 expression (right-hand side histograms). The percentages of positive cell populations are shown in the histograms. Mutant mice show a considerable reduction in the CD8α expression in γδTCR$^+$ T cells compared to the wild type controls.


DBA.2
(CD8.1+/+)
75.2%
3.2%
DBA.2/129Sv
(CD8.1+/CD8.2+)
81.1%
76.1%
CIII+/Δ1.2
(CD8.1+/CD8.2Δ1.2)
77.6%
19.1%
CIIIΔ1.2/Δ1.2
(CD8.2Δ1.2/Δ1.2)
24%
39.7%
CD8α/Lyt-2
CD8αLyt-2.2

heterodimers on their surface αβTCR$^+$ cells were gated and were analysed by plotting CD8β against CD8α/Lyt-2. Subsequently, CD8β$^+$ cells were gated and analysed for CD8α/Lyt-2 expression. This analysis showed that there is normal CD8α expression on αβTCR CD8αβ IEL (data not shown), as was shown also for T cells that migrated from the thymus to populate peripheral lymphoid organs.

In our CIII$^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice we observed a dramatic reduction of the CD8α gene expression in both γδTCR and αβTCR CD8αα IEL. This suggests that in the presence of an active CD8β gene, cluster III activity is redundant in CD8αβ T cells. In IEL, however, which have not activated the CD8β gene, cluster III becomes important for CD8α gene expression.

Cluster III expression control is restricted to the *cis* allele

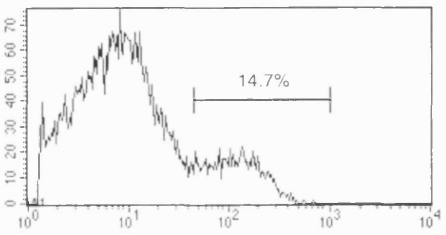
It is possible that regulatory elements can exert their effects in *trans* via a diffusible product (RNA or protein) and therefore a mutation in one allele may disturb the expression of the other. In order to address whether the influence of the deletion of two of the DNaseI-HSS of cluster III is restricted to the *cis* CD8α allele, CIII$^{+/\Delta 1,2}$ heterozygous mutant mice carrying a wild type CD8α/Lyt-2.1 allele and a mutant CD8α/Lyt-2.2 allele were analysed. IEL from these mice were isolated and stained with antibodies specific for γδTCR, CD8α/Lyt-2 and CD8α/Lyt-2.2 as described above. In CIII$^{+/\Delta 1,2}$ heterozygous mutant mice, CD8α/Lyt-2.2 expression was absent (19.1%) from γδTCR$^+$ CD8αα IEL (figure 6, CIII$^{+/\Delta 1,2}$ histogram, right-hand side), whereas CD8α/Lyt-2.1 expression from the other allele was not affected (76.1%) (figure 6, CIII$^{+/\Delta 1,2}$ histogram, left-hand side). The DBA/2 wild type control mouse appeared negative for CD8α/Lyt-2.2, as it carries the CD8α/Lyt-2.1 allele.

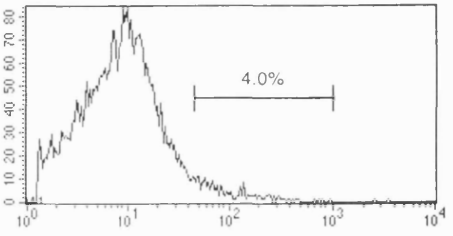
Similarly, in CIII$^{+/\Delta 1,2}$ heterozygous mutant mice, αβTCR$^+$ CD8αα IEL showed a reduction in CD8α/Lyt-2.2 expression (9.1%) (figure 7, CIII$^{+/\Delta 1,2}$ histogram, right-hand side), whereas CD8α/Lyt-2.1 expression from the other allele was not affected (29.5%) (figure 7, CIII$^{+/\Delta 1,2}$ histogram, left-hand side). This result indicates that deletion of cluster III DNaseI-HSS CIII-1 and CIII-2 is unable to affect CD8α gene transcription in *trans* in heterozygous mice.

Figure 7: Cluster III (CIII-1 and CIII-2) mutant mice lack CD8α gene expression on αβTCR$^+$CD8αα intraepithelial T cells

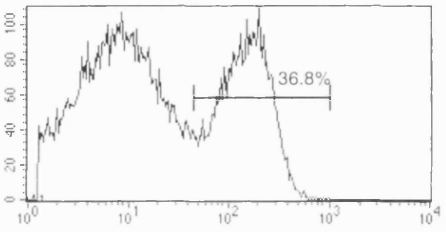
Intraepithelial T cells were isolated from murine gut of wild type DBA/2 (top histograms), wild type (DBA/2 x 129/Sv) F1 (second line histograms), CIII$^{+/\Delta 1,2}$ heterozygous (third line histograms) and CIII$^{\Delta 1,2/\Delta 1,2}$ homozygous (bottom histograms) mice. IEL were stained with anti-αβTCR, anti-CD8β, anti-CD8α/Lyt-2 and anti-CD8α/Lyt-2.2 specific antibodies. αβTCR$^+$ and CD8β$^-$ T cells were gated and analysed for CD8α/Lyt-2 expression (left-hand side histograms) or CD8α/Lyt-2.2 expression (right-hand side histograms).The percentages of positive cell populations are shown in the histograms. Mutant mice show a considerable reduction in the CD8α expression in αβTCR$^+$CD8αα T cells compared to the wild type controls.

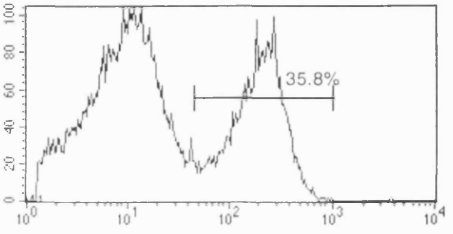
DBA.2
(CD8.1$^{+/+}$)



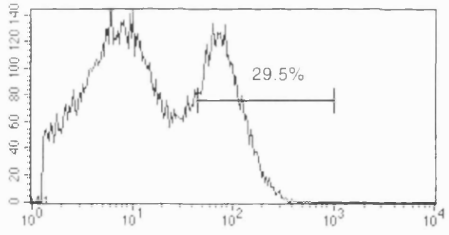


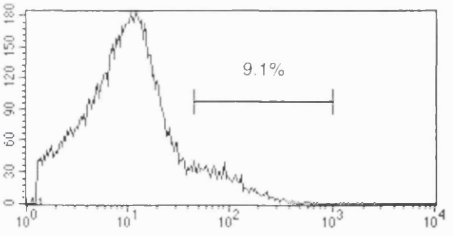
DBA.2/129Sv
(CD8.1^{+}/CD8.2^{+})



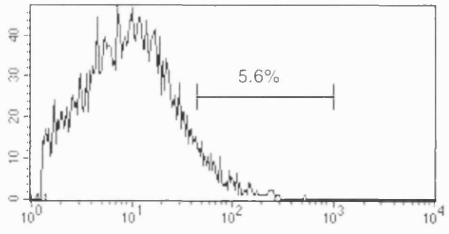


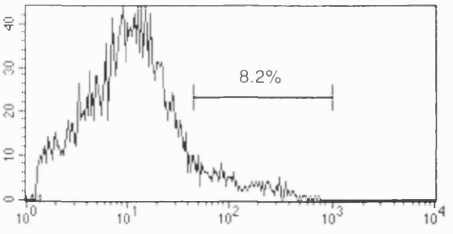
CIII$^{+/\Delta 1,2}$
(CD8.1^{+}/CD8.2$^{\Delta 1,2}$)





CIII$^{\Delta 1,2/\Delta 1,2}$
(CD8.2$_{\Delta 1,2/\Delta 1,2}$)





CD8α/Lyt-2 →

CD8α/Lyt-2.2 →

Deletion of cluster III DNaseI-HSS CIII-1, CIII-2 and CIII-3

The deletion of two of the three DNaseI-HSS of cluster III described above does not affect the expression of the CD8α gene in thymocytes or mature T cells from $CIII^{\Delta 1.2/\Delta 1.2}$ homozygous mutant mice. From transgenic mice analysis (Ellmeier et al., 1997, Hostert et al., 1997) it was shown that sites CIII-1, CIII-2 alone or in combination are sufficient to direct expression of a linked reporter gene to the mature CD8 SP T cell subset. Therefore it is possible that the third DNaseI-HSS CIII-3 (present in the mutant mice described above) alone is also sufficient to direct subset specific expression of the reporter gene. In order to investigate further the function of cluster III, we generated mice that lack all three DNaseI-HSS of cluster III.

Isolation and characterisation of λ phage clones

For the generation of mice that lack all three DNaseI-HSS of cluster III from their genome, a targeting construct was introduced into embryonic stem cells of 129/Sv origin by homologous recombination. Use of isogenic DNA to generate the targeting construct increases the frequency of homologous recombination events. The genomic fragments spanning the region between cluster III and cluster II, as well as the DNaseI-HSS of cluster II coming from 129/Sv genetic background had not been cloned previously in the lab.

For the purpose of obtaining these fragments, a 129/Sv genomic λ phage library (λFIX, Stratagene) was screened with a 1.3kb XbaI-BglII genomic fragment located in the region between cluster III and cluster II (figure 8). This led to the isolation of 4 independent phage clones, PHAGE 1 (insert size of 15kb), PHAGE 2 (insert size of 18kb), PHAGE 3 (insert size of 18kb) and PHAGE 4 (insert size of 20kb) (figure 8). Restriction enzyme digests and Southern blot analysis showed that the genomic insert of PHAGE 1 spanned the region between cluster III and cluster II and contained a 6kb diagnostic BamHI fragment from this region. PHAGE 2 covered most of the 6kb BamHI fragment and downstream sequences. In contrast to the first two clones, PHAGE 3 spanned the cluster III and upstream sequences and, finally, the genomic insert of PHAGE 4 covered most of the 6kb BamHI fragment and the cluster III.

Figure 9 shows the Southern blot analysis of the four phage clones digested with BamHI, BamHI-SalI, SalI, BglII and BglII-SalI and hybridised with the XbaI-BglII probe. In the λ FIX library, the genomic inserts are cloned in the SalI site of the phage

vector. This means that the SalI fragment hybridising with the probe was the whole insert of each clone. In PHAGE 1 the hybridising BamHI fragment was identical in size with the fragment coming from a BamHI-SalI double digest, which suggested that this clone contained the full 6kb BamHI fragment located between cluster III and cluster II. In contrast, in PHAGE 2,3 and 4 the fragment resulting from the BamHI-SalI double digest was smaller than the one coming from the single BamHI digest. This showed that only a part of the 6kb BamHI genomic fragment was contained in these clones. In the cases where the hybridising BamHI fragment was bigger in size than the SalI fragment (PHAGE 2 and 3), this was a so called end fragment and contained phage sequences along with CD8 genomic sequences.

In order to investigate whether any one of the phage clones contained the 8.5kb BamHI fragment spanning the cluster II and the first three exons of the CD8α gene, the blot was stripped and re-hybridised with the 0.6kb B/E probe shown in figure 8. This analysis showed (figure 10) that only PHAGE 1 and PHAGE 2 hybridised with this probe and therefore their inserts extended downstream of the 6kb BamHI fragment. Of the two clones, in PHAGE 2 the hybridising BamHI fragment was identical in size with the fragment coming from a BamHI-SalI double digest, which suggested that this clone contained the full 8.5kb BamHI fragment spanning the cluster II and the first exons of the CD8α gene. Both the 6kb BamHI fragment from PHAGE 1 and the 8.5kb BamHI fragment from PHAGE 2 were cloned in the pBluescript vector and used for the generation of targeting constructs (see below).

Figure 8: Diagram of isolated λ phage clones

Map of the murine CD8 gene locus showing the location of the CD8α and CD8β genes (filled boxes: translated exons, open boxes: untranslated exons). Restriction enzyme sites are indicated by vertical bars (B, BamHI; X, XbaI; Bl, BglII; E, EcoRI) and the location of DNaseI-HSS clusters is shown by vertical arrows and labeled CI to CIV. A λ phage genomic library was screened using a 1.3kb X/Bl genomic fragment and a 0.6kb B/E genomic fragment as probes. The two probes are indicated as horizontal bars. Four genomic clones were identified, PHAGE 1 (15kb insert size), PHAGE 2 (18kb insert size), PHAGE 3 (18kb insert size) and PHAGE 4 (20kb insert size).

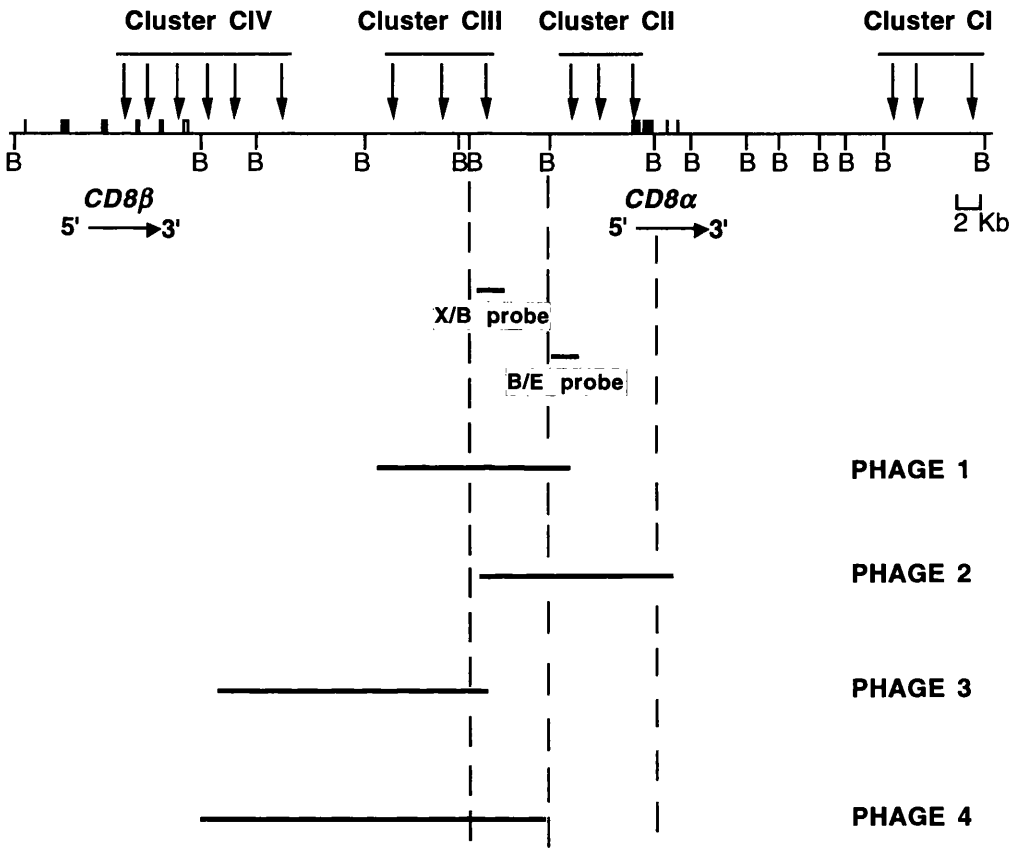
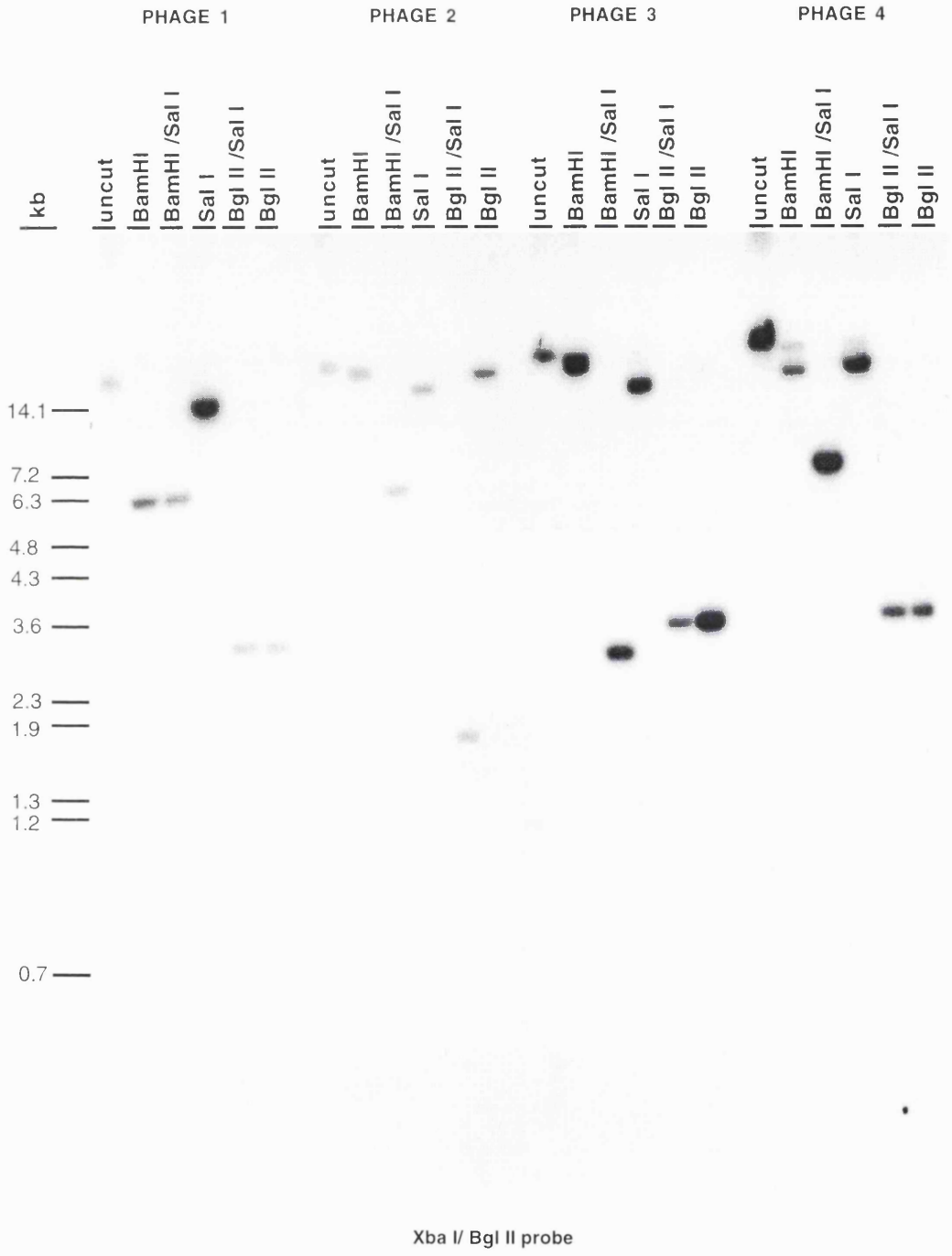

Cluster CIV
Cluster CIII
Cluster CII
Cluster CI
B
CD8β
5' 3'
CD8α
5' 3'
2 Kb
X/B probe
B/E probe
PHAGE 1
PHAGE 2
PHAGE 3
PHAGE 4

Figure 9: Characterisation of λ phage clones

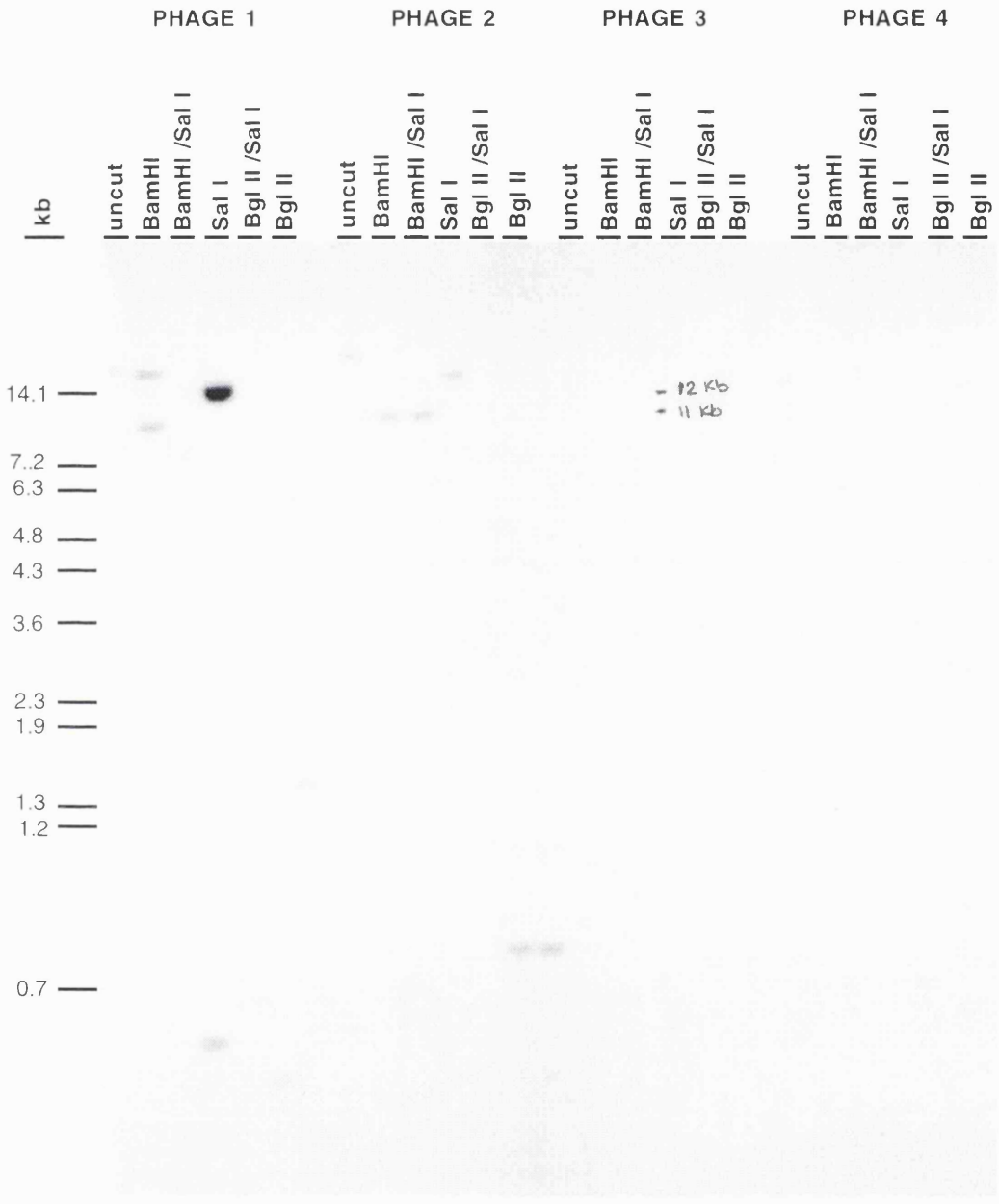
Southern blot analysis of four phage clones digested with BamHI, BamHI-SalI, SalI, BglII and BglII/SalI and hybridised with the 1.3kb XbaI-BglII probe.



Xba I/ Bgl II probe

Figure 10: Characterisation of λ phage clones

Southern blot analysis of four phage clones digested with BamHI, BamHI-SalI, SalI, BglII and BglII/SalI and hybridised with the 0.6kb B/E probe.


PHAGE 1
PHAGE 2
PHAGE 3
PHAGE 4
kb
uncut
BamHI
BamHI /Sal I
Sal I
Bgl II /Sal I
Bgl II
14.1
7..2
6.3
4.8
4.3
3.6
2.3
1.9
1.3
1.2
0.7
12 Kb
11 Kb
B/E probe

Generation of CIII $^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice

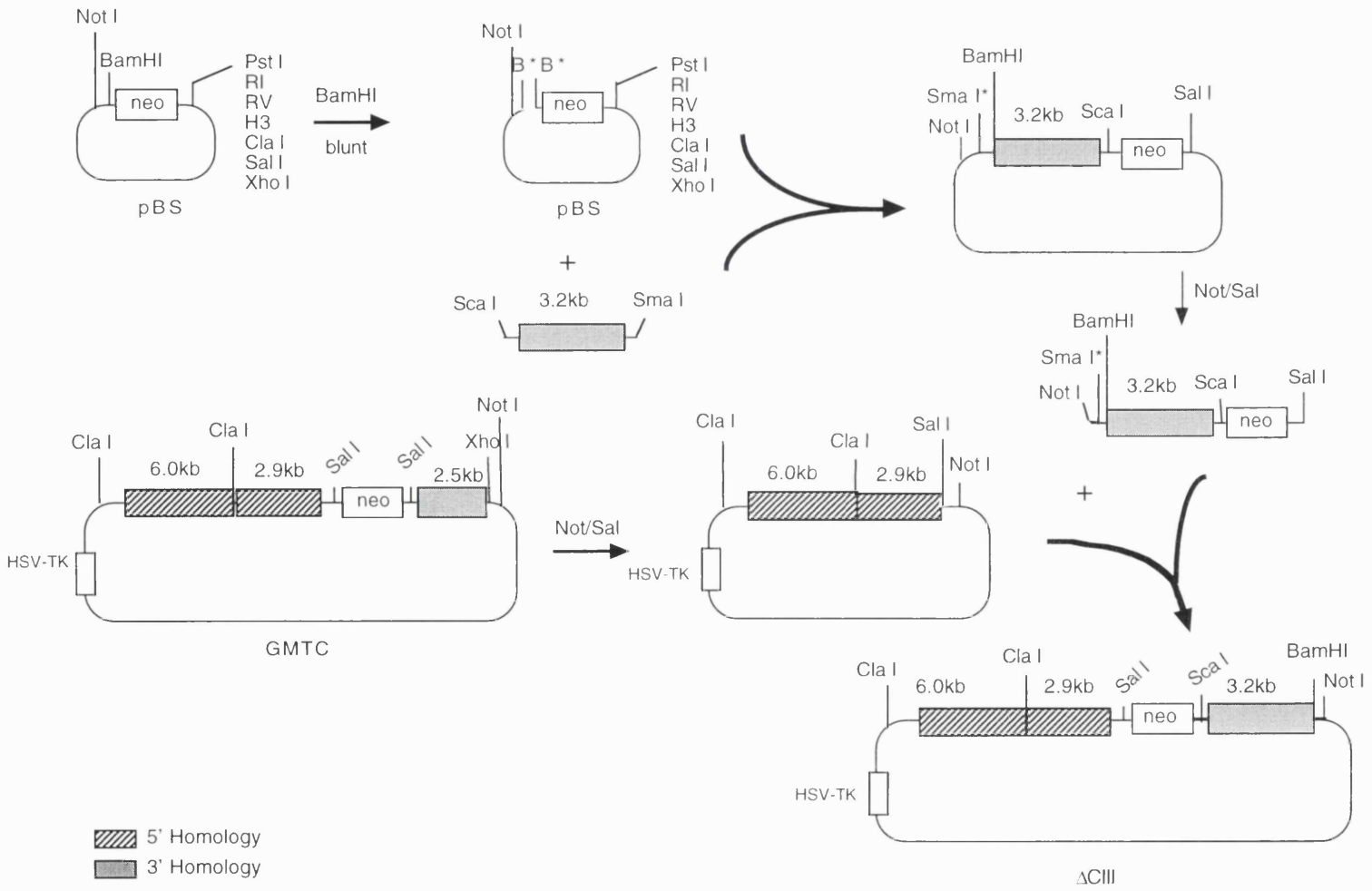
In order to delete DNaseI-HSS CIII-1, CIII-2 and CIII-3 of cluster III from the mouse genome, a targeting construct was introduced into embryonic stem cells by homologous recombination. For the generation of the targeting construct (figure 11) the following steps were taken: from the 129/Sv genomic λ phage library screening (described above), a 6kb BamHI genomic fragment located between cluster III and cluster II was isolated and cloned into the pBluescript vector. A 3.2kb ScaI-BamHI genomic fragment (downstream homology) was excised from this plasmid as ScaI-SmaI fragment, blunted and ligated into the blunted BamHI site of a vector that contained the neomycin gene. This vector was constructed as follows: the loxP-flanked neomycin gene was excised from plasmid pL2-neo as a 1.2kb XbaI-SalI fragment, blunted and ligated into the SmaI site of pBluescript.

For upstream homology, the same 8.9kb genomic fragment was used as in the GMTC targeting vector described in the previous section. For this purpose, the GMTC targeting vector was digested with SalI and NotI restriction enzymes and the 14.3kb fragment containing upstream homology sequences and the HSV-tk gene was isolated. Then, the 4.6kb fragment (downstream homology + neo gene) was excised from the plasmid described above as NotI-SalI fragment and ligated into the 14.3kb fragment to give the final targeting construct, ΔCIII. The targeting construct contained the neomycin resistance gene for positive selection and the HSV-tk gene for negative selection, as described before.

The construct was linearised with NotI and introduced into D3 embryonic stem cells from 129/Sv origin (Doetschman et al., 1985) by electroporation. For each electroporation $0.8x10^7$ ES cells were used and the DNA concentration was 45μg/ml. Selection was initiated 24hrs later at a concentration of 250μg/ml G418 and 2μM (0.51μg/ml) gancyclovir. The targeting event replaces a 12kb genomic fragment containing the cluster III DNaseI-HSS CIII-1, CIII-2 and CIII-3 with the neomycin resistance gene. The homologous recombination event is shown in figure 12A. Because of an EcoRV site introduced in the 3'end of the neomycin gene, the 13.5kb wild type EcoRV fragment is converted to a smaller 6.8kb mutant fragment, as detected by the 0.6kb XbaI probe. This probe is located downstream of the targeted region, 3' of the homology of the targeting vector (figure 12A). Figure 12B (right-hand side) shows a

Figure 11: Generation of the ΔCIII targeting construct

All the relevant restriction enzyme sites used for the cloning steps are shown. B, BamHI. The neomycin resistance gene (neo) and the herpes simplex virus thymidine kinase (HSV-tk) are indicated by open boxes and the upstream and downstream homologies of the targeting vector are shown by filled boxes. The asterisk indicates restriction sites that are destroyed. The generation of the ΔCIII targeting construct is described in detail in the text.


Not I
BamHI
Pst I
RI
RV
H3
Cla I
Sal I
Xho I
neo
pBS
BamHI
blunt
B*B*
Sca I
3.2kb
Sma I
Sma I*
Not I
Sal I
Not/Sal
Cla I
6.0kb
2.9kb
2.5kb
HSV-TK
GMTC
ΔCIII
5' Homology
3' Homology

Southern blot analysis of one positive clone (67) and 129/Sv genomic DNA digested with EcoRV and probed with the 0.6kb XbaI probe.

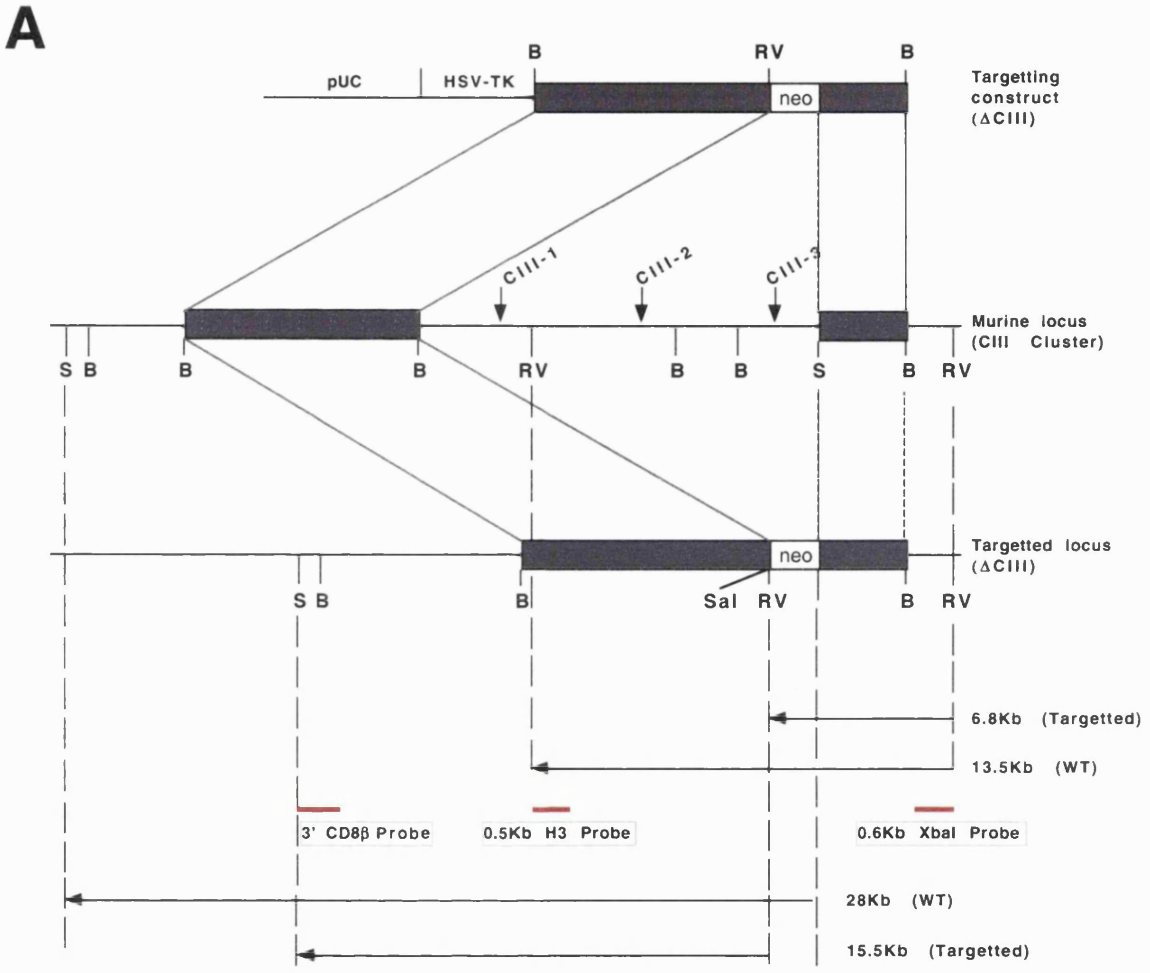
Of 576 resistant clones screened by Southern blot analysis and using the 0.6kb XbaI probe, six were found to have undergone homologous recombination and the integrity of the locus was verified with additional probes located upstream of the targeted region and outside the homology (3'CD8β probe) or within the region of homology (0.5kb HindIII probe) and shown in figure 12A. Because of a SalI site artificially introduced in the 5' end of the neomycin gene, the 28kb ScaI wild type fragment is converted to a smaller 15.5kb mutant fragment in a ScaI-SalI double digest as detected by the 3' CD8β probe (figure 12B, left-hand side). The hybridisation with a probe that recognises a sequence inside the region of homology is necessary to ensure that there are no additional random integrations of the targeting construct elsewhere in the genome. For this purpose, the same ScaI-SalI double digest was used and the Southern blot was probed with the 0.5kb HindIII probe that recognises a sequence within the region of homology of the targeting construct. The 28kb ScaI wild type fragment is converted to a smaller 15.5kb mutant fragment and no additional fragments can be detected. This indicates that there are no other random integrations of the targeting construct in the genome (figure 12B, middle).

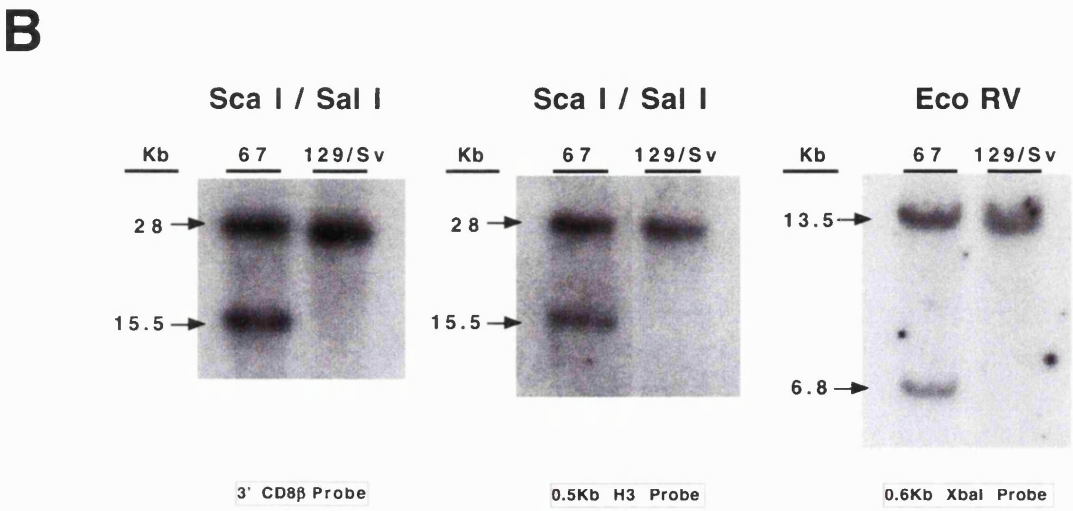
Four of the clones that had a euploid karyotype (chromosome number of 40) were used to generate chimeric mice by injection into 3.5-day blastocysts derived from C57Bl/6 females and transferring them into the uteri of foster mothers. Chimeric males were bred to C57Bl/10 females and germline transmission from one of the chimeric males was determined by agouti coat colour. Heterozygous mice that carried the $CIII^{\Delta 1,2,3}$ deletion mutation were identified by Southern blot analysis and were bred to generate mice homozygous for the deletion. Mice heterozygous and homozygous for this mutation are designated $CIII^{+/\Delta 1,2,3}$ and $CIII^{\Delta 1,2,3/\Delta 1,2,3}$, respectively. Figure 13A shows the Southern blot analysis of wild type 129/Sv, $CIII^{+/\Delta 1,2,3}$ heterozygous and $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice and confirms the predicted conversion of the 13.5kb wild type EcoRV fragment to a smaller 6.8kb mutant fragment, as detected with the 0.6kb XbaI probe shown in figure 12A.

Figure 12: Deletion of DNaseI-HSS CIII-1, CIII-2 and CIII-3 by homologous recombination

(A) Diagram of the murine CD8 gene locus showing the targeting construct, DNaseI-HSS cluster CIII and the targeted allele. The neomycin gene (open box, labeled neo) was flanked by 8.5kb 5' and 3.2kb 3' homologous DNA sequences (filled boxes). Restriction enzyme sites are shown by vertical bars and are labelled B, BamHI; RV, EcoRV; S, ScaI; Sal, SalI. Vertical arrows indicate cluster CIII DNaseI-HSS and the probes (0.6kb XbaI, 3' CD8β and 0.5kb H3 genomic fragments) used to detect homologous recombination events are shown as solid horizontal bars. Horizontal arrows illustrate the expected EcoRV fragments hybridising with the 0.6kb XbaI probe (13.5kb wild type allele, 6.8kb targeted allele) and the expected ScaI/SalI fragments hybridising with the 3'CD8β probe (28kb wild type allele, 21kb targeted allele).

(B) Southern blot analysis of one clone (67) that had undergone homologous recombination, run along with wild type 129/Sv DNA. The sizes of wild type and mutant fragments, the restriction enzymes and the probes used are shown.


A
B
RV
B
pUC
HSV-TK
neo
Targetting construct (ΔCIII)
CIII-1
CIII-2
CIII-3
Murine locus (CIII Cluster)
S B
B
B
RV
B
B
S
B
RV
Targetted locus (ΔCIII)
neo
S B
B
Sal
RV
B
RV
6.8Kb (Targetted)
13.5Kb (WT)
3' CD8β Probe
0.5Kb H3 Probe
0.6Kb XbaI Probe
28Kb (WT)
15.5Kb (Targetted)


B
Sca I / Sal I
Sca I / Sal I
Eco RV
Kb
67
129/Sv
28
15.5
Kb
67
129/Sv
28
15.5
Kb
67
129/Sv
13.5
6.8
3' CD8β Probe
0.5Kb H3 Probe
0.6Kb XbaI Probe

Analysis of CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice

Thymocytes and peripheral T cell subsets are not affected by deletion of cluster III DNaseI-HSS CIII-1, CIII-2 and CIII-3

In order to examine the effect of the CIII$^{\Delta 1,2,3}$ deletion on CD8α gene expression, lymph node cells and thymocytes were isolated from wild type 129/Sv and CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice and stained with anti-CD4 and anti-CD8α/Lyt-2. Figure 13B shows the FACS profiles of lymph node cells (top panel) or thymocytes (bottom panel) isolated from 129/Sv wild type control mice (left-hand side) and CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice (right-hand side) and stained with the above combination of antibodies. Cells were analysed by plotting CD8α/Lyt-2 against CD4 and electronically gating the different cell populations. The percentages of cells falling in each gate, as well as the mean fluorescence intensity (MFI) of the CD8α/Lyt-2 antibody are shown in the dot-plots. This analysis showed that FACS profiles of T cells and thymocyte subsets were similar for both wild type and mutant mice with no significant differences in either percentages of subsets or CD8α mean fluorescence intensity between the mice analysed.

Thus, the deletion of all three sites of cluster III DNaseI-HSS had no effect on expression of the CD8α in the CD8αβ SP compartment, just as shown for the CIII$^{\Delta 1,2}$ deletion. From transgenic mice analysis (Ellmeier et al., 1997, Hostert et al., 1997) it was shown that cluster III sites are sufficient to direct expression of a linked reporter gene to the mature CD8 SP T cell subset. However, deletion of cluster III from the mouse genome did not have an effect on the expression of the CD8α on CD8αβ SP cells. The most possible explanation is that there are additional *cis*-acting regulatory elements that are involved in the regulation of CD8 expression in thymus derived T cells, such as elements in the CD8β gene, which are able to compensate for the loss of cluster III activity.

Deletion of the neomycin gene in CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice

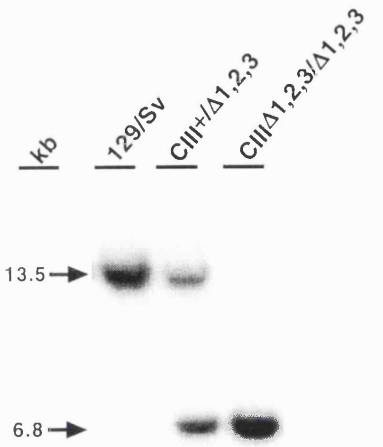
In CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice, the 12kb genomic fragment containing all three DNaseI-HSS of cluster III was removed from the mouse genome and was replaced with the neomycin resistance gene that is 1.4kb long. This deletion did not have any effect on CD8 expression in any thymocyte or T cell subset. In the mutant mice

Figure 13: Thymocytes and peripheral αβTCR T cell subsets are not affected by deletion of CIII

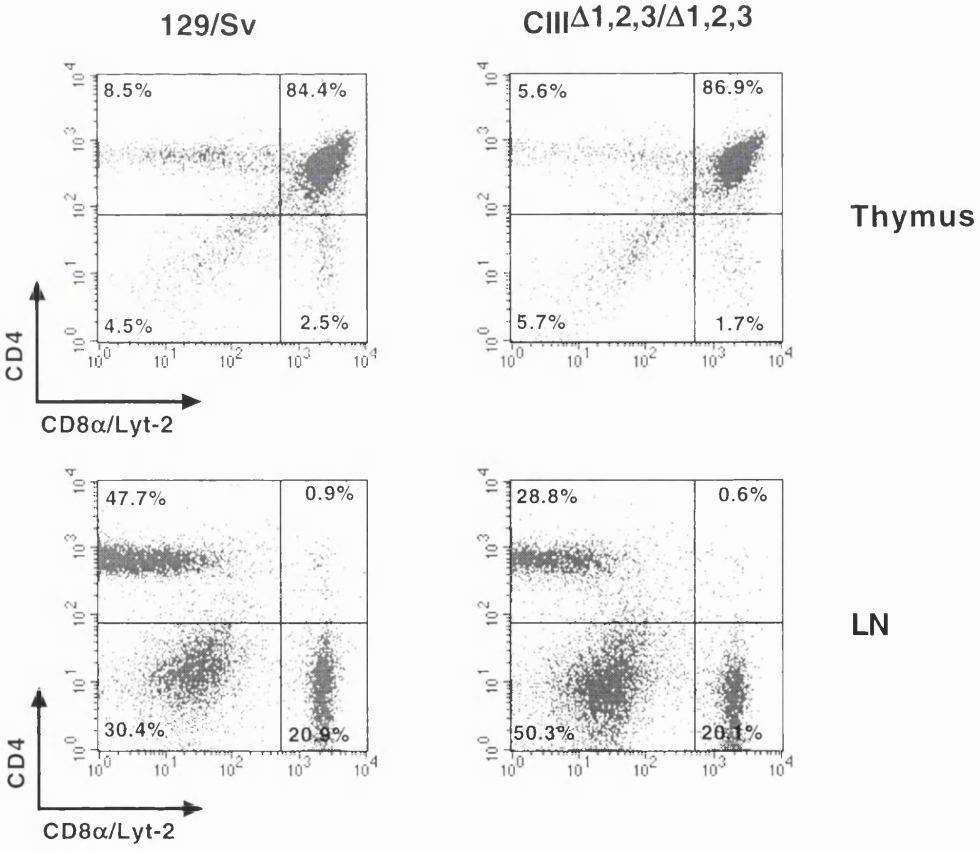
(A) Southern blot analysis of 129/Sv wild type, $CIII^{+/\Delta 1,2,3}$ heterozygous and $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice. The 13.5kb EcoRV fragment in the wild type allele is converted to a smaller 6.8kb fragment in the targeted allele.

(B) FACS analysis of 129/Sv wild type and $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice. Thymocytes and lymph node cells were isolated and stained with anti-CD8α/Lyt-2 and anti-CD4 antibodies. T cell and thymocyte subsets were resolved by plotting CD4 against CD8α/Lyt-2 and analysed for CD8α expression. Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants. Both percentages of cells and levels of expression of CD8α are very similar between mutant and wild type control mice.

A


kb
129/Sv
CIII+/Δ1,2,3
CIIIΔ1,2,3/Δ1,2,3
13.5
6.8

B


129/Sv
CIIIΔ1,2,3/Δ1,2,3
8.5%
84.4%
4.5%
2.5%
5.6%
86.9%
5.7%
1.7%
Thymus
47.7%
0.9%
30.4%
20.9%
28.8%
0.6%
50.3%
20.1%
LN
CD4
CD8α/Lyt-2

the neomycin gene is still present in the genome. It has been reported that in studies of complex regulatory elements *in vivo* the presence of the inserted selectable marker can influence the phenotype of the mutation (Fiering et al., 1995). Therefore, the selectable marker should be removed from the mouse genome.

The neomycin gene used for the generation of the ΔCIII targeting vector was flanked by Cre recombinase target (loxP) sites in an orientation that would allow deletion of the neomycin gene upon expression of Cre. This target site-specific reaction leaves a single loxP at the site of recombination. In order to delete the neomycin gene from the genome of $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice and remove any influence that this might have in the phenotype of the mutation deletion, the Cre recombinase was introduced into fertilised oocytes. For this purpose, a $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant male mouse was mated with C57Bl/10 females. The oocytes were removed from the uteri of the females and were injected with a Cre expressing plasmid. The oocytes were transferred subsequently into the uteri of foster mothers. All mice born would be heterozygous for the $CIII^{\Delta 1,2,3}$ mutation.

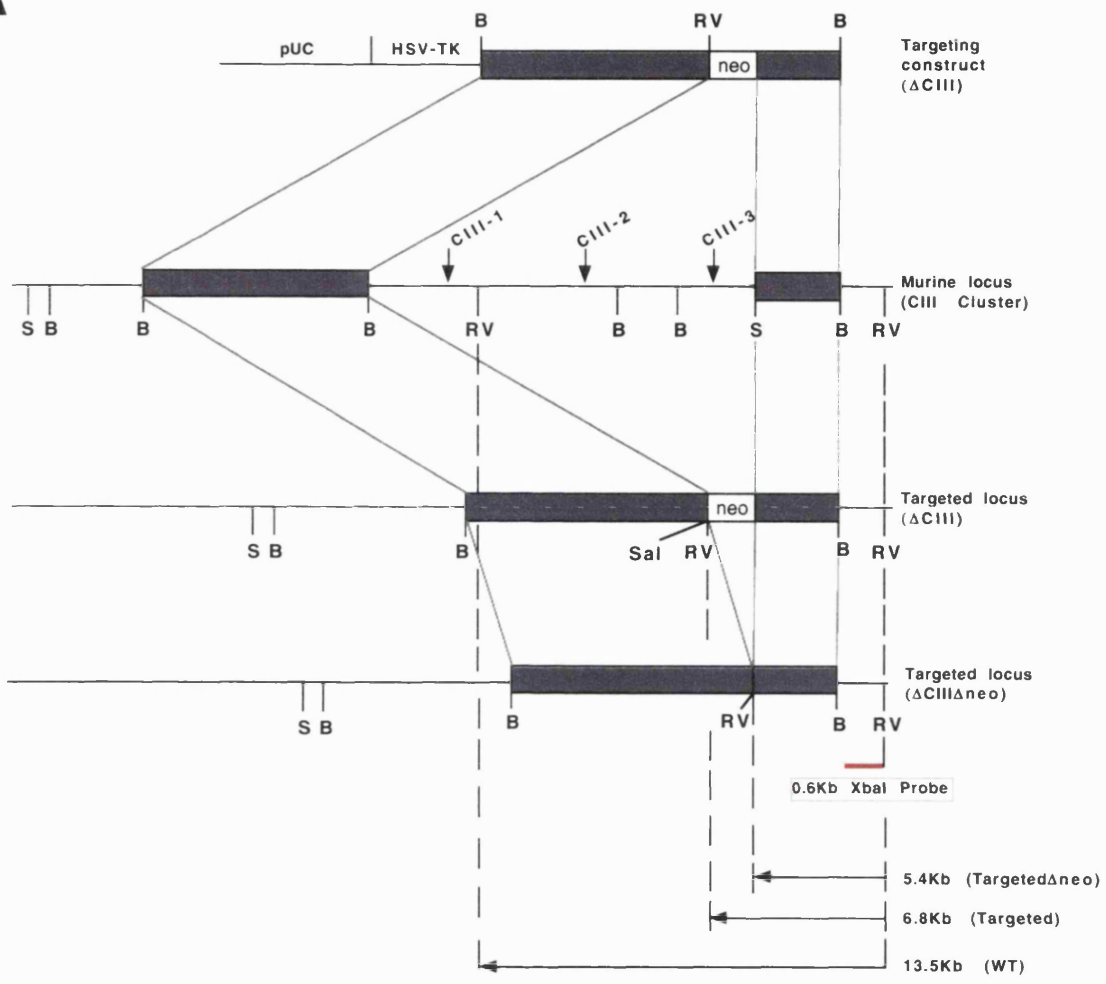
When homologous recombination occurs, the 13.5kb wild type EcoRV fragment is converted to a smaller 6.8kb mutant fragment, as detected by the 0.6kb XbaI probe, located downstream of the targeted region. If deletion of the neomycin gene is mediated by Cre recombinase, the 6.8kb EcoRV mutant fragment containing the neomycin gene and sequences of the CD8 locus will be converted to a smaller 5.4kb fragment, as detected by the same probe (figure 14A). The heterozygous mice that no longer have the neomycin gene in their genome were bred to generate homozygous mice, that are designated $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$. Figure 14B shows the Southern blot analysis of wild type 129/Sv, $CIII^{+/\Delta 1,2,3}$ heterozygous, $CIII^{+/\Delta 1,2,3}\Delta neo$ heterozygous and $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ homozygous mutant mice and confirms the predicted conversion of the 13.5kb wild type EcoRV fragment to a smaller 6.8kb mutant fragment after the homologous recombination event and to an even smaller 5.4kb fragment after deletion of the neomycin gene, as detected with the 0.6kb XbaI probe.

Figure 14: Deletion of the neomycin gene in CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice

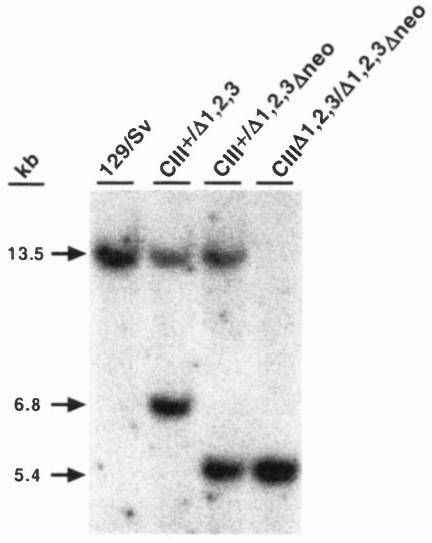
(A) Diagram of the murine CD8 gene locus showing the targeting construct, DNaseI-HSS cluster CIII, the targeted allele with the neomycin gene and the targeted allele after deletion of the neomycin gene. The neomycin gene (open box, labeled neo) was flanked by 8.5kb 5' and 3.2kb 3' homologous DNA sequences (filled boxes). Restriction enzyme sites are shown by vertical bars and are labelled B, BamHI; RV, EcoRV; S, ScaI; Sal, SalI. Vertical arrows indicate cluster CIII DNaseI-HSS and the probe (0.6kb XbaI genomic fragment) used to detect the homologous recombination event and the deletion of the neomycin gene is shown as a solid horizontal bar. Horizontal arrows illustrate the expected EcoRV fragments hybridising with the 0.6kb XbaI probe (13.5kb wild type allele, 6.8kb targeted allele and 5.4kb targeted allele after deletion of the neomycin gene).

(B) Southern blot analysis of 129/Sv wild type, CIII$^{+/\Delta 1,2,3}$ heterozygous, CIII$^{+/\Delta 1,2,3}\Delta$neo heterozygous and CIII$^{\Delta 1,2,3/\Delta 1,2,3}\Delta$neo homozygous mutant mice. The DNA was digested with EcoRV and the Southern blot was hybridised with the 0.6kb XbaI probe. When homologous recombination occurs, the wild type 13.5kb EcoRV fragment is converted to a mutant 6.8kb EcoRV fragment. After deletion of the neomycin gene, the 6.8kb mutant fragment is converted to a smaller 5.4kb fragment.

A


B
RV
B
pUC
HSV-TK
neo
Targeting construct (ΔCIII)
CIII-1
CIII-2
CIII-3
Murine locus (CIII Cluster)
S B
B
B
RV
B
B
S
B
RV
Targeted locus (ΔCIII)
neo
S B
B
Sal
RV
B
RV
Targeted locus (ΔCIIIΔneo)
S B
B
RV
B
RV
0.6Kb XbaI Probe
5.4Kb (TargetedΔneo)
6.8Kb (Targeted)
13.5Kb (WT)

B


kb
129/Sv
CIII+/Δ1,2,3
CIII+/Δ1,2,3Δneo
CIIIΔ1,2,3/Δ1,2,3Δneo
13.5
6.8
5.4

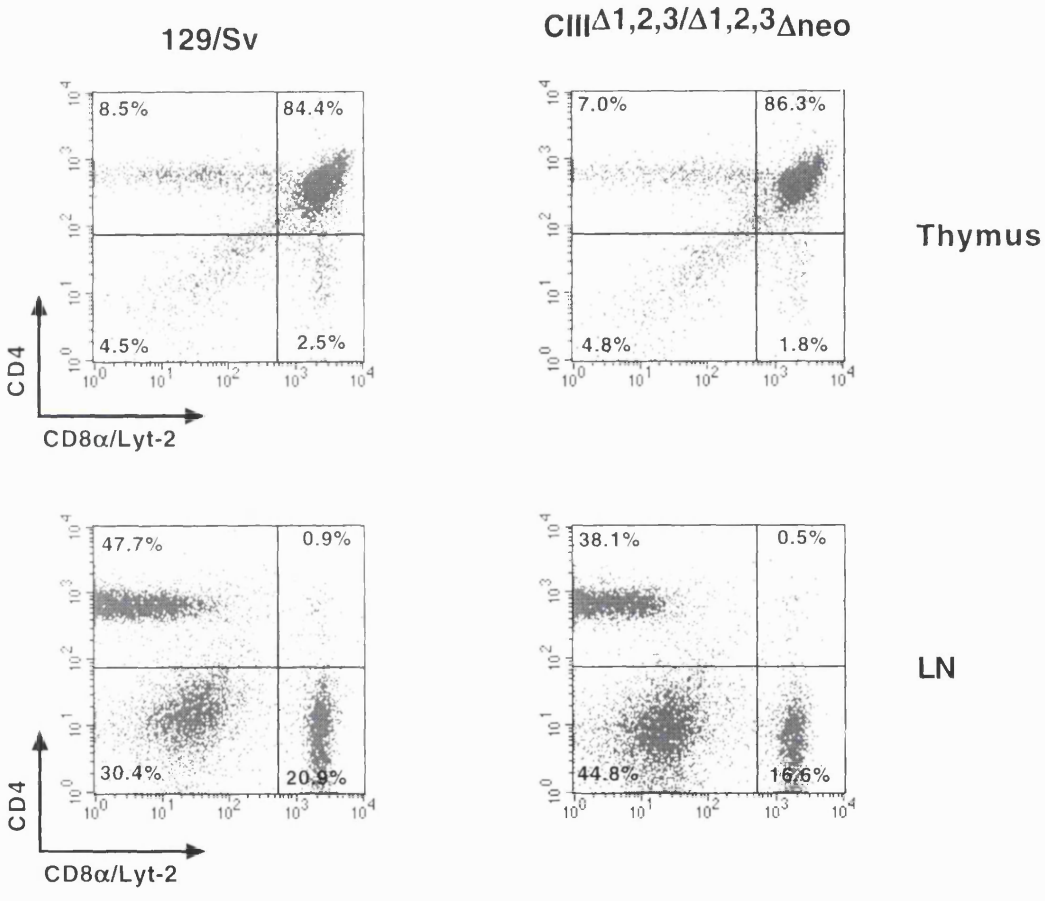
Thymocytes and peripheral T cell subsets are still not affected in $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ mice after removal of the neomycin gene

In order to examine the effect of $CIII^{\Delta 1,2.,3}$ deletion on CD8α expression in $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ mice and therefore exclude any influence that the presence of the neomycin gene might have had in the phenotype of the mutation, lymph node cells and thymocytes were isolated from wild type 129/Sv and $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ homozygous mutant mice and stained with antibodies against CD4 and CD8α/Lyt-2. Figure 15 shows the FACS profiles of lymph node cells (bottom panel) or thymocytes (top panel) isolated from 129/Sv wild type control mice (left-hand side) and $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ homozygous mutant mice (right-hand side) and stained with the above combination of antibodies. Cells were analysed by plotting CD8α/Lyt-2 against CD4 and electronically gating the different cell populations. The percentages of cells falling in each gate, as well as the mean fluorescence intensity (MFI) of the CD8α/Lyt-2 antibody are shown in the dot-plots. This analysis showed that FACS profiles of T cells and thymocyte subsets were similar for both wild type and mutant mice with no significant differences in either percentages of subsets or CD8α mean fluorescence intensity between the mice analysed.

Thus, analysis of $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ homozygous mutant mice showed that the deletion of all three sites of cluster III DNaseI-HSS had no effect on expression of the CD8α in the CD8αβ SP compartment, just like it was shown for the $CIII^{\Delta 1,2}$ deletion. The presence of the neomycin gene at this site in the mouse genome did not have any effect on the lack of phenotype and both $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ and $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ mice have very similar FACS profiles with the wild type controls. The most possible explanation for the lack of phenotype is that there are additional *cis*-acting regulatory elements that are involved in the regulation of CD8 expression in thymus derived T cells, such as elements in the CD8β gene, which are able to compensate for the loss of cluster III activity.

Figure 15: Thymocytes and peripheral αβTCR T cell subsets are still not affected CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ mice after deletion of the neomycin gene

FACS analysis of 129/Sv wild type and CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice. Thymocytes and lymph node cells were isolated and stained with anti-CD8α/Lyt-2 and anti-CD4 antibodies. T cell and thymocyte subsets were resolved by plotting CD4 against CD8α/Lyt-2 and analysed for CD8α expression. Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants. Both percentages of cells and levels of expression of CD8α are very similar between mutant and wild type control mice.


129/Sv
CIII Δ1,2,3/Δ1,2,3 Δneo
8.5%
84.4%
4.5%
2.5%
7.0%
86.3%
4.8%
1.8%
Thymus
47.7%
0.9%
30.4%
20.9%
38.1%
0.5%
44.8%
16.6%
LN
CD4
CD8α/Lyt-2

$CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ homozygous mutant mice lack CD8α gene expression on $\gamma\delta TCR^+$ CD8αα intraepithelial T cells

The deletion of all three DNaseI-HSS of cluster III had no effect on expression of the CD8α in the CD8αβ SP compartment. In order to assess whether additional *cis*-acting regulatory elements within the CD8 gene locus, such as elements in the CD8β gene, are able to compensate for the loss of cluster III, CD8α expression on IEL was investigated. These cells express CD8αα homodimers on both αβTCR or γδTCR lineage cells and the ones that express CD8αα homodimers have not previously expressed the CD8β gene (Hamerman et al., 1997).

In order to examine the effect of the $CIII^{\Delta 1,2,3}$ deletion on CD8α gene expression in IEL, these lymphocytes were isolated from $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ homozygous mutant mice, as well as from C57Bl/10 and 129/Sv wild type controls. IEL isolated from the above mice were stained with anti-γδTCR, anti-CD8α/Lyt-2 and anti-CD8β. As mentioned before γδTCR IEL express only CD8αα homodimers, so they all appear as $CD8\beta^-$ cells. Thus, $\gamma\delta TCR^+$ cells were gated and analysed by plotting CD8β against CD8α/Lyt-2. Subsequently, $CD8\beta^-$ cells were gated and analysed for CD8α/Lyt-2 expression in the histograms shown in figure 16. The percentages of positive cell populations are shown in the histograms. This analysis showed that only 35.2% of $\gamma\delta TCR^+$ CD8αα IEL isolated from $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ Δneo homozygous mutant mice were found to fall in the CD8α positive gate compared to 78% of positive cells in the C57Bl/10 and 86% in the 129/Sv controls. In addition, $\gamma\delta TCR^+$ CD8αα IEL isolated from $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ Δneo homozygous mutant mice expressed CD8α at 8-fold lower levels compared to the control mice.

IEL isolated from the same mice were also stained with anti-αβTCR, anti-CD8β and anti-CD8α/Lyt-2 antibodies. αβTCR IEL express CD8αα homodimers or CD8αβ heterodimers on their surface. In order to assess CD8α expression on IEL that have CD8αα homodimers on their surface (and are thought to be extrathymically derived), $\alpha\beta TCR^+$ cells were gated and were analysed by plotting CD8β against CD8α/Lyt-2. Subsequently, $CD8\beta^-$ cells were gated and analysed for CD8α/Lyt-2 expression in the histograms shown in figures 17 and 18. The percentages of positive cell populations are

shown in the histograms. In one experiment αβTCR$^+$ CD8αα IEL isolated from CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ Δneo homozygous mutant mice were found to express CD8α at much lower levels than the wild type controls (figure 17), similar to what was shown previously for the CIII$^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice Surprisingly, in later experiments, the percentages of αβTCR$^+$ CD8αα IEL isolated from the mutant mice were comparable to the controls. However, these cells were found to express CD8α at lower levels compared to the cells isolated from the C57Bl/10 and the 129/Sv controls (figure 18).

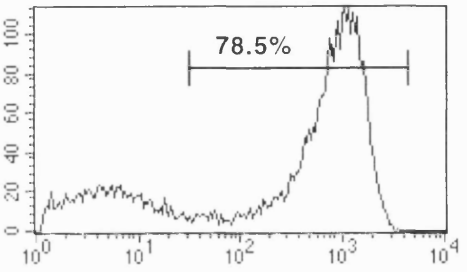
In order to assess CD8α expression on IEL that have CD8αβ heterodimers on their surface and are thought to have gone through normal development in the thymus, αβTCR$^+$ cells were gated and were analysed by plotting CD8β against CD8α/Lyt-2. Subsequently, CD8β$^+$ cells were gated and analysed for CD8α/Lyt-2 expression. This analysis showed that there is normal CD8α expression on αβTCR$^+$ CD8αβ IEL (data not shown), as was shown also in CIII$^{\Delta 1,2,3/\Delta 1,2,3}$Δneo homozygous mutant mice for T cells that migrated from the thymus to populate peripheral lymphoid organs.

In our CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice we observed a dramatic reduction of the CD8α gene expression in γδTCR$^+$ CD8αα IEL. This suggests, like in CIII$^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice described above, that in the presence of an active CD8β gene, cluster III activity is redundant in CD8αβ T cells. In IEL, however, which have not activated the CD8β gene, cluster III becomes important for CD8α gene expression.

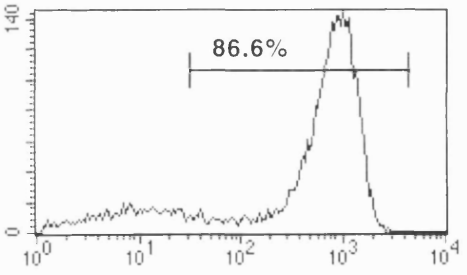
Figure 16: $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ mutant mice lack CD8α gene expression on $\gamma\delta TCR^{+}CD8\alpha\alpha$ intraepithelial T cells

Intraepithelial T cells were isolated from murine gut of wild type C57Bl/10 (top histogram), wild type 129/Sv (middle histogram) and $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous (bottom histogram) mice. IEL were stained with anti-γδTCR, anti-CD8α/Lyt-2 and anti-CD8β specific antibodies. $\gamma\delta TCR^{+}$ T cells were gated and analysed by plotting CD8β against CD8α/Lyt-2. Then, $CD8\beta^{-}$ cells were gated and analysed for CD8α/Lyt-2 expression. The percentages of positive cell populations are shown in the histograms. Mutant mice show a considerable reduction in the CD8α expression on $\gamma\delta TCR^{+}$ T cells compared to the wild type controls.

C57Bl/10
(CD8.2$^{+/+}$)



129/Sv
(CD8.2$^{+/+}$)



CIII$^{\Delta 1,2,3/\Delta 1,2,3\Delta neo}$
(CD8.2$^{\Delta 1,2,3/\Delta 1,2,3}$)

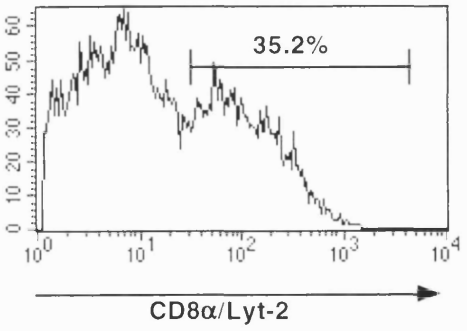
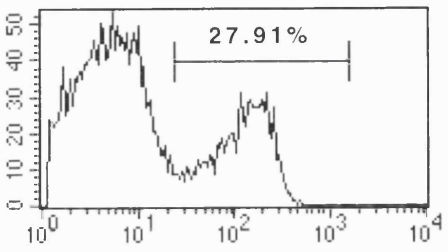


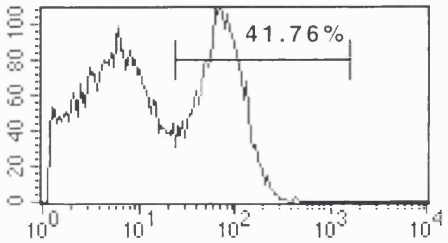
Figure 17: CIII$^{\Delta 1,2,3/\Delta 1,2,3}$Δneo mutant mice lack CD8α gene expression on αβTCR^{+}CD8αα intraepithelial T cells

Intraepithelial T cells were isolated from murine gut of wild type C57Bl/10 (top histogram), wild type 129/Sv (middle histogram) and CIII$^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous (bottom histogram) mice. IEL were stained with anti-αβTCR, anti-CD8α/Lyt-2 and anti-CD8β specific antibodies. αβTCR^{+} T cells were gated and analysed by plotting CD8β against CD8α/Lyt-2. Then, CD8β^{-} cells were gated and analysed for CD8α/Lyt-2 expression. The percentages of positive cell populations are shown in the histograms. Mutant mice show a considerable reduction in the CD8α expression on αβTCR^{+} IEL compared to the wild type controls.

C57Bl/10
(CD8.2$^{+/+}$)



129/Sv
(CD8.2$^{+/+}$)



CIIIΔ1,2,3/Δ1,2,3
(CD8.2Δ1,2,3/Δ1,2,3)

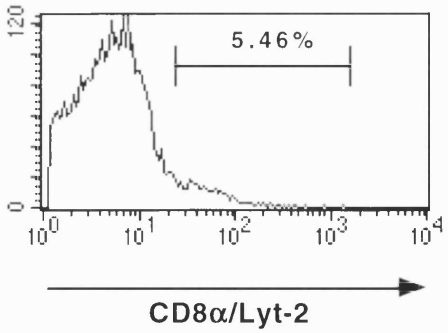
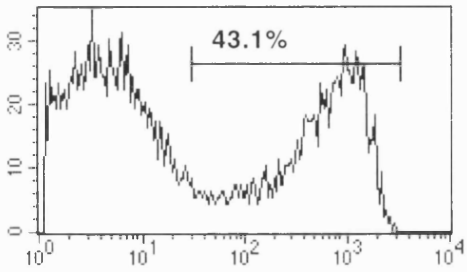


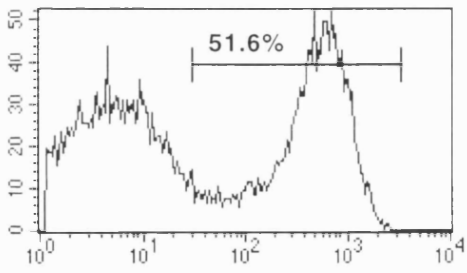
Figure 18: $CIII^{\Delta 1,2,3/\Delta 1,2,3}\Delta neo$ mutant mice have normal CD8α gene expression on αβTCR$^+$CD8αα intraepithelial T cells

Intraepithelial T cells were isolated from murine gut of wild type C57Bl/10 (top histogram), wild type 129/Sv (middle histogram) and $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous (bottom histogram) mice. IEL were stained with anti-αβTCR, anti-CD8α/Lyt-2 and anti-CD8β specific antibodies. αβTCR$^+$ T cells were gated and analysed by plotting CD8β against CD8α/Lyt-2. Then, CD8β$^-$ cells were gated and analysed for CD8α/Lyt-2 expression. The percentages of positive cell populations are shown in the histograms. Mutant mice show normal CD8α expression on αβTCR$^+$ T cells compared to the wild type controls.

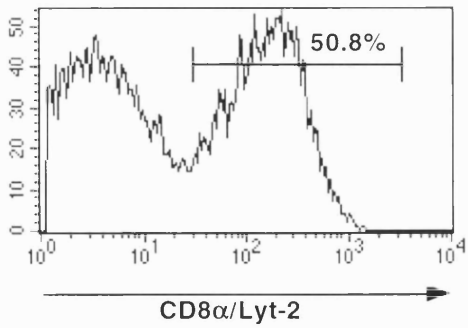
C57Bl/10
(CD8.2$^{+/+}$)



129/Sv
(CD8.2$^{+/+}$)



CIII$^{\Delta 1,2,3/\Delta 1,2,3\Delta neo}$
(CD8.2$^{\Delta 1,2,3/\Delta 1,2,3}$)



Deletion of cluster II DNaseI-HSS CII-2

The cluster II DNaseI-HSS located immediately upstream of the CD8α gene alone, was not sufficient to direct expression of the CD8α transgene in any thymocyte or T cell subset (Hostert et al., 1997), but in conjunction with CIII DNaseI-HSS it restored transgene expression in the immature DP thymocytes. Furthermore, CII-2 was absent in DNaseI treated splenocyte nuclei, suggesting that possibly an element responsible for CD8 expression in immature T cells lies in this region (Hostert et al., 1998). To address this question we generated mice that lack cluster II DNaseI-HSS CII-2.

Generation of CII $^{\Delta 2/\Delta 2}$ homozygous mutant mice

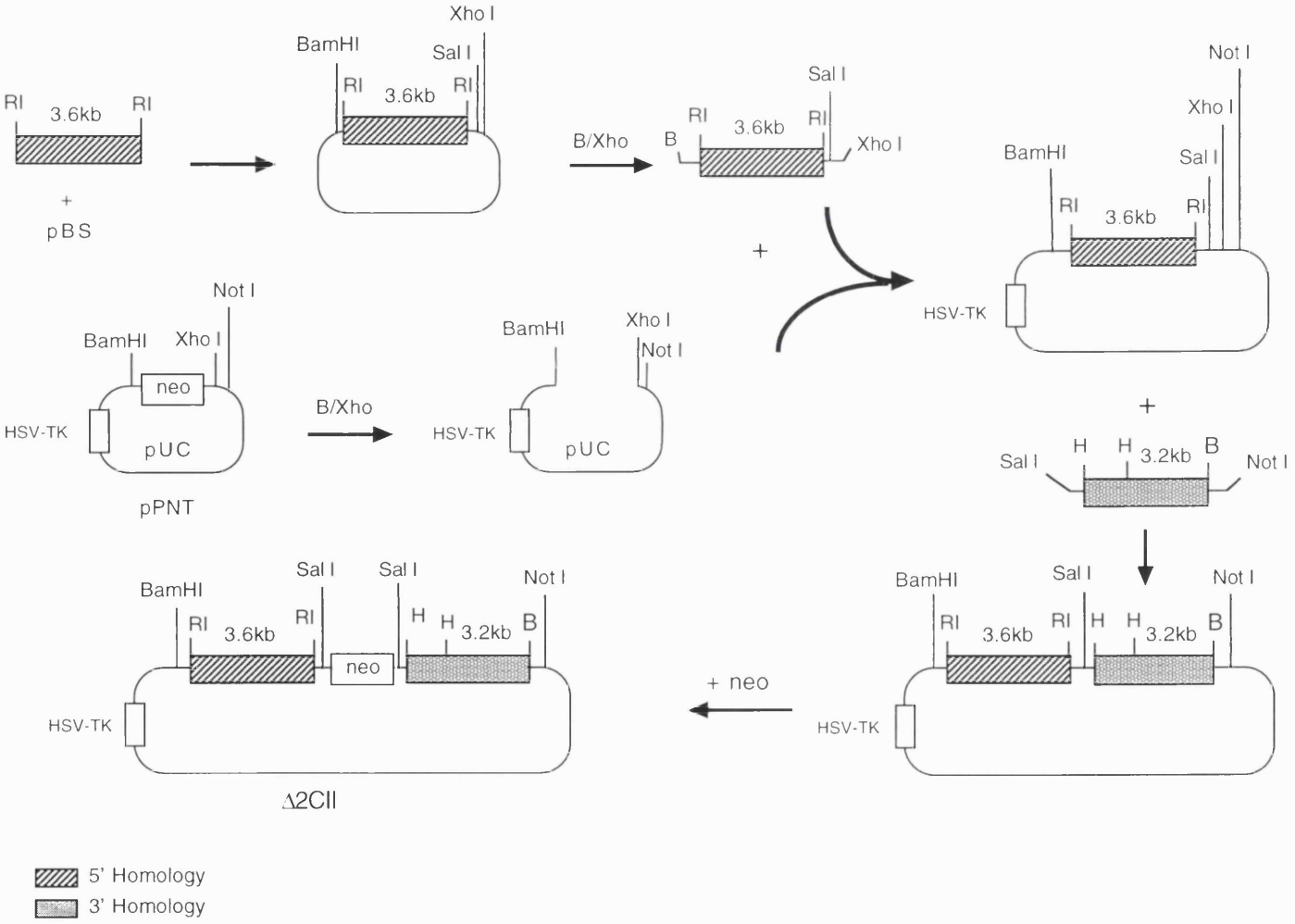
In order to delete DNaseI-HSS CII-2 of cluster II from the mouse genome, a targeting construct was introduced into embryonic stem cells by homologous recombination. For the generation of the targeting construct (figure 19) the following steps were taken: from the 129/Sv genomic λ phage library screening (see above), an 8.5kb BamHI genomic fragment containing the CD8α gene and 6.9kb upstream sequences was isolated and cloned into the pBluescript vector. A 3.6kb EcoRI genomic fragment was excised from this plasmid, it was ligated into the EcoRI site of pBluescript and then cut out as BamHI-XhoI fragment. This fragment was used as upstream homology for the targeting construct. For this purpose, it was ligated into the pPNT vector (Tybulewicz et al., 1991), that contains the HSV-tk gene.

From the same 8.5kb BamHI genomic fragment containing the CD8α gene and 6.9kb upstream sequences, a 3.2kb HindIII-BamHI genomic fragment was excised and cloned into pBluescript. Then, it was cut out as NotI-SalI fragment and used as downstream homology in the targeting vector. Finally, the 1.4kb fragment containing the neo gene flanked with loxP sites was ligated between upstream and downstream homology as SalI fragment to give the targeting construct, Δ2CII.

The construct was linearised with NotI and used to transfect D3 embryonic stem cells as described above. The concentration of the DNA was 50μg/ml and the selection used was 300μg/ml G418 and 2μM gancyclovir. The targeting event (figure 20A) replaces an 1.3kb genomic fragment containing the cluster II DNaseI-HSS CII-2 with the neomycin resistance gene. There is a SalI site artificially introduced in the 5' end of the neomycin gene, therefore the 8.0kb wild type PstI fragment is converted to a smaller 6.4kb mutant fragment in a PstI-SalI double digest as detected by the 0.6kb B/E probe.

Figure 19: Generation of the Δ2CII targeting construct

All the relevant restriction enzyme sites used for the cloning steps are shown. B, BamHI; RI, EcoRI; H, HindIII. The neomycin resistance gene (neo) and the herpes simplex virus thymidine kinase (HSV-tk) are indicated by open boxes and the upstream and downstream homologies of the targeting vector are shown by filled boxes. The generation of the Δ2CII targeting construct is described in detail in the text.


RI
3.6kb
RI
+
pBS
BamHI
Xho I
Sal I
RI
3.6kb
RI
B/Xho
B
RI
3.6kb
RI
Xho I
Sal I
+
Not I
Xho I
Sal I
BamHI
RI
3.6kb
RI
HSV-TK
+
Not I
BamHI
Xho I
neo
HSV-TK
pUC
pPNT
B/Xho
BamHI
Xho I
Not I
HSV-TK
pUC
Sal I
H
H
3.2kb
B
Not I
BamHI
Sal I
Not I
RI
3.6kb
RI
H
H
3.2kb
B
HSV-TK
+ neo
BamHI
Sal I
Sal I
Not I
RI
3.6kb
RI
neo
H
H
3.2kb
B
HSV-TK
Δ2CII
5' Homology
3' Homology

This probe is located upstream of the targeted region, 5' of the homology of the targeting vector (figure 20A). Figure 20B (middle) shows a Southern blot analysis of two positive clones (167 and 169) and 129/Sv genomic DNA digested with PstI-SalI and probed with the 0.6kb B/E probe.

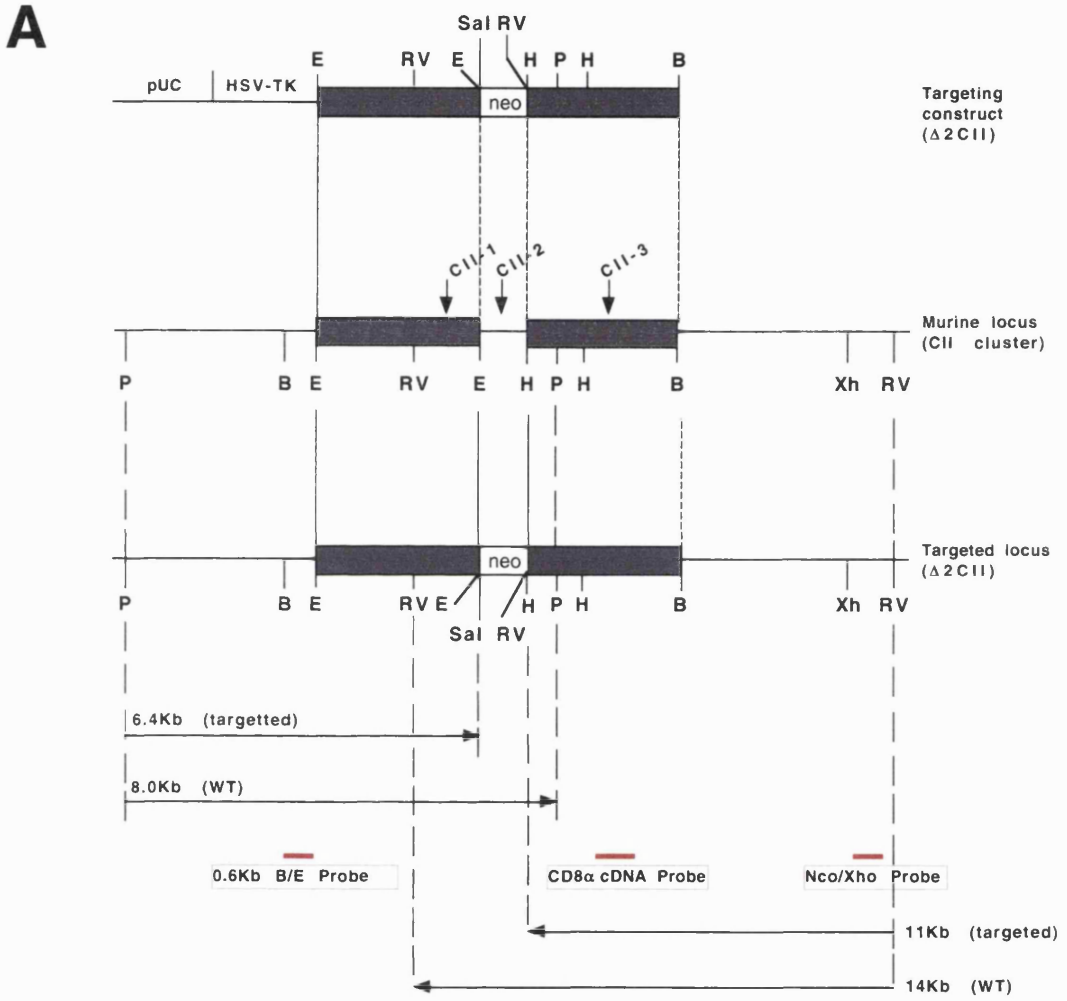
Of 576 resistant clones screened by Southern blot analysis and using the 0.6kb B/E probe, 15 were found to have undergone homologous recombination and the integrity of the locus was verified with additional probes located downstream of the targeted region and outside the homology (NcoI-XhoI probe) or within the region of homology (CD8α cDNA probe) and shown in figure 20A. Because of an EcoRV site introduced in the 3' end of the neomycin gene, the 14kb wild type EcoRV fragment is converted to a smaller 11kb mutant fragment, as detected with the NcoI-XhoI probe (figure 20B, left-hand side). In order to check further the integrity of the locus after the homologous recombination, an additional restriction enzyme was used and the Southern blot was hybridised with a probe that recognises a sequence inside the region of homology to ensure that there are no additional random integrations of the targeting construct elsewhere in the genome. For this purpose, a EcoRV-XhoI double digest was used and the Southern blot was hybridised with the CD8α cDNA probe. Figure 20B (right-hand side) shows the predicted conversion of the 8.5kb EcoRV-XhoI wild type genomic fragment to a smaller 5.2kb mutant fragment, as detected by the CD8α cDNA probe and no additional fragments could be detected, which indicated that there are no other random integrations of the targeting construct in the genome.

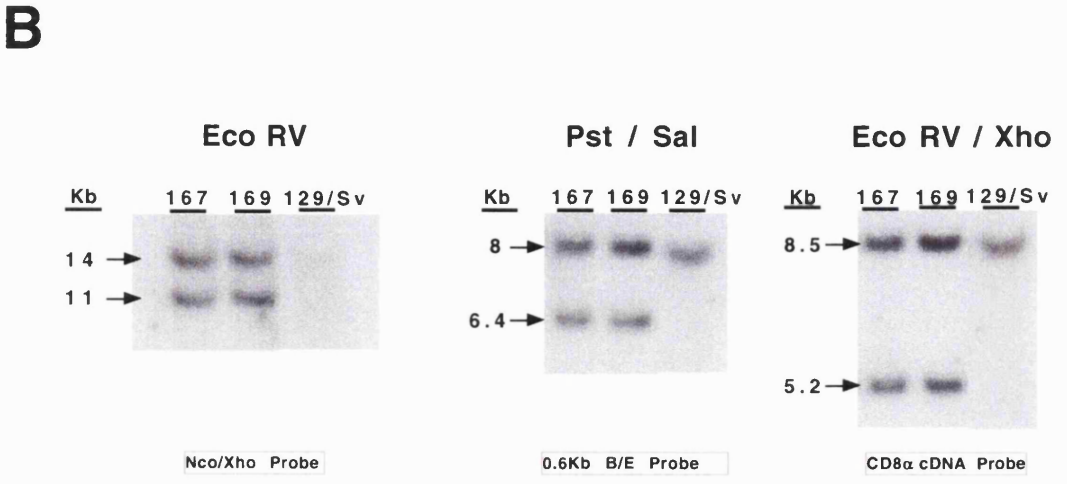
Eight of the clones that had a euploid karyotype (chromosome number of 40) were injected into 3.5-day blastocysts derived from C57Bl/6 females to generate chimeric mice. A total number of 770 blastocysts were injected and 85 mice were born. Most of these mice had a very low percentage of chimerism. This could have happened because the ES cells had differentiated and therefore they were not able to contribute to the generation of the mouse when they were injected into blastocysts. Only chimeric males coming from clone 169 had a high percentage of chimerism, they were bred to C57Bl/10 females and germline transmission was determined by agouti coat colour. Heterozygous mice that carry the $CII^{\Delta 2}$ deletion mutation were identified by Southern blot analysis and were bred to generate mice homozygous for the deletion. Mice heterozygous and homozygous for this mutation are designated $CII^{+/\Delta 2}$ and $CII^{\Delta 2/\Delta 2}$,

Figure 20: Deletion of DNaseI-HSS CII-2 by homologous recombination

(A) Diagram of the murine CD8 gene locus showing the targeting construct, DNaseI-HSS cluster CII and the targeted allele. The neomycin gene (open box, labeled neo) was flanked by 3.6kb 5' and 3.2kb 3' homologous DNA sequences (filled boxes). Restriction enzyme sites are shown by vertical bars and are labelled B, BamHI; RV, EcoRV; H, HindIII; P, PstI; E, EcoRI; Xh, XhoI; Sal, SalI. Vertical arrows indicate cluster CII DNaseI-HSS and the probes (0.6kb B/E, CD8α cDNA and NcoI/XhoI genomic fragments) used to detect homologous recombination events are shown as solid horizontal bars. Horizontal arrows illustrate the expected EcoRV fragments hybridising with the NcoI/XhoI probe (14kb wild type allele, 11kb targeted allele) and the expected PstI/SalI fragments hybridising with the 0.6kb B/E probe (8kb wild type allele, 6.4kb targeted allele).

(B) Southern blot analysis of two clones (167, 169) that had undergone homologous recombination, run along with wild type 129/Sv DNA. The sizes of wild type and mutant fragments, the restriction enzymes and the probes used are shown.


A
Sal RV
E
RV E
H P H
B
pUC
HSV-TK
neo
Targeting construct (Δ2CII)
CII-1
CII-2
CII-3
Murine locus (CII cluster)
P
B E
RV
E
H P H
B
Xh RV
Targeted locus (Δ2CII)
RV E
Sal RV
6.4Kb (targetted)
8.0Kb (WT)
0.6Kb B/E Probe
CD8α cDNA Probe
Nco/Xho Probe
11Kb (targeted)
14Kb (WT)


B
Eco RV
Kb
167 169 129/Sv
14
11
Nco/Xho Probe
Pst / Sal
Kb
167 169 129/Sv
8
6.4
0.6Kb B/E Probe
Eco RV / Xho
Kb
167 169 129/Sv
8.5
5.2
CD8α cDNA Probe

respectively. Figure 21A shows the Southern blot analysis of wild type 129/Sv, $CII^{+/\Delta 2}$ heterozygous and $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice and confirms the predicted conversion of the 14.0kb wild type EcoRV fragment to a smaller 11.0kb mutant fragment, as detected with the NcoI-XhoI probe shown in figure 20A.

Analysis of $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice

Expression of CD8 is affected in peripheral T cell subsets and to a lesser extent in thymocytes by deletion of CII-2 of cluster II

In order to examine the effect of $CII^{\Delta 2}$ deletion on CD8α expression, lymph node cells and thymocytes were isolated from wild type 129/Sv and $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice and stained with anti-CD4 and anti-CD8α/Lyt-2. Figure 21B shows the FACS profiles of lymph node cells (top panel) or thymocytes (bottom panel) isolated from 129/Sv wild type control mice (left-hand side) and $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice (right-hand side) stained with the above combination of antibodies. Cells were analysed by plotting CD8α/Lyt-2 against CD4 and electronically gating the different cell populations. The percentages of cells falling in each gate, as well as the mean fluorescence intensity (MFI) of the CD8α/Lyt-2 antibody are shown in the dot-plots. This analysis showed that 11.1% of peripheral T lymphocytes in $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice expressed both CD8α/Lyt-2 and CD4 on their surface and thus appeared as double positive cells. In addition, a small reduction in the percentage of CD4 SP cells was observed in $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice compared to the 129/Sv control mice. Thus, $CII^{\Delta 2/\Delta 2}$ mice contained cells which express CD8α/Lyt-2 on a proportion of the peripheral CD4 SP cells. However, these double positive cells expressed CD8α/Lyt-2 in lower levels than peripheral CD8 SP cells. Finally, no significant differences in CD8 SP cells were observed between $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice and 129/Sv controls, either in percentages of cells or in levels of CD8α/Lyt-2 expression.

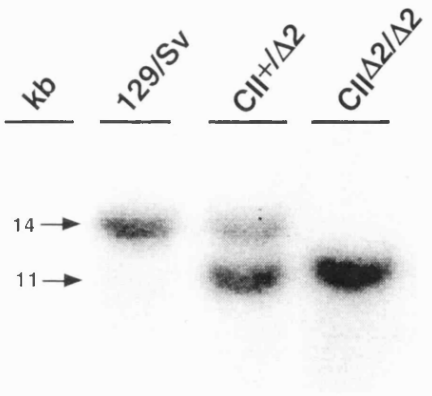
In thymocytes of $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice, there were no significant differences in percentages of double positive and CD8 SP cells compared to the 129/Sv control. However, it is possible that CD8α/Lyt-2 was expressed on a proportion of CD4 SP thymocytes, as it was seen in peripheral T cells, thus rendering some of these cells to

Figure 21: Expression of CD8 is affected in peripheral T cell subsets by deletion of CII-2 of cluster II

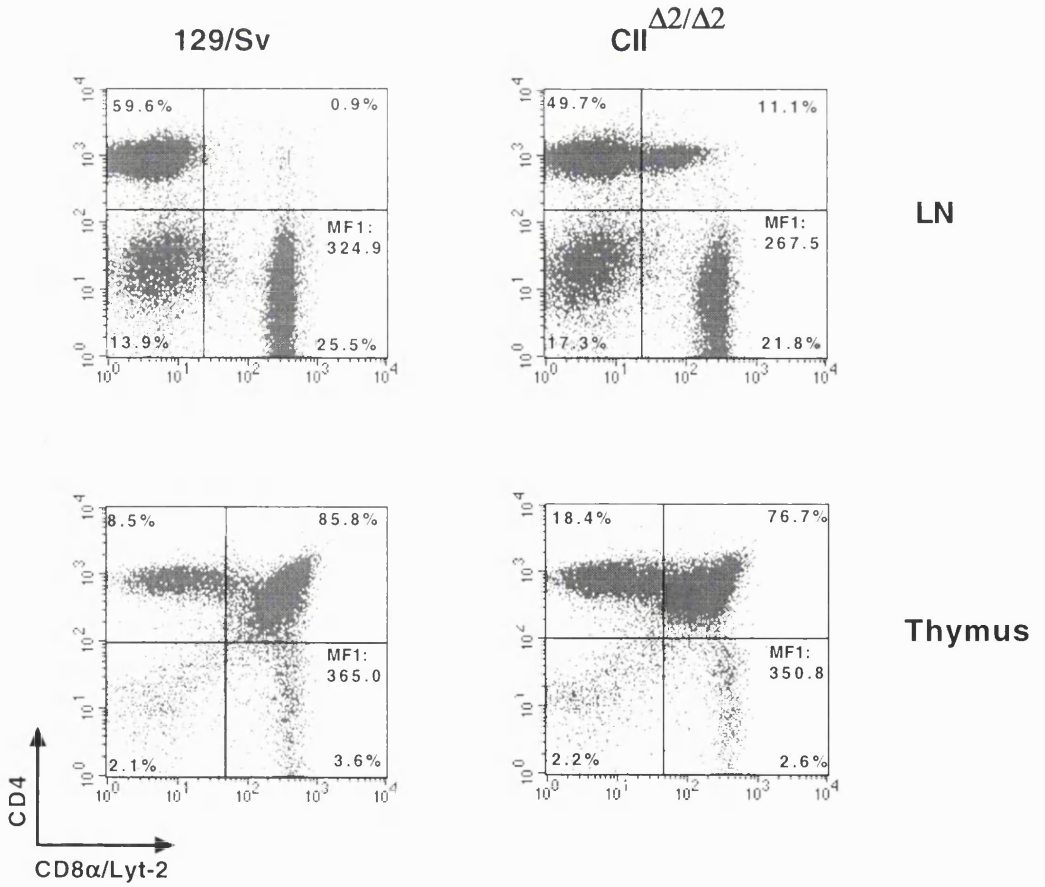
(A) Southern blot analysis of 129/Sv wild type, $CII^{+/\Delta 2}$ heterozygous and $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice. The DNA was digested with EcoRV and the Southern blot was hybridised with the NcoI/XhoI probe. When homologous recombination occurs, the wild type 14kb EcoRV fragment is converted to a mutant 11kb EcoRV fragment.

(B) FACS analysis of 129/Sv wild type and $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice. Thymocytes and lymph node cells were isolated and stained with anti-CD8α/Lyt-2 and anti-CD4 antibodies. T cell and thymocyte subsets were resolved by plotting CD4 against CD8α/Lyt-2 and analysed for CD8α expression. Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants. Deletion of CII-2 of cluster II from the mouse genome results in expression of CD8α on a proportion of the peripheral CD4 SP cells.

A


kb
129/Sv
CII+/Δ2
CIIΔ2/Δ2
14
11

B


129/Sv
CII Δ2/Δ2
59.6%
0.9%
MF1: 324.9
13.9%
25.5%
49.7%
11.1%
MF1: 267.5
17.3%
21.8%
LN
8.5%
85.8%
MF1: 365.0
2.1%
3.6%
18.4%
76.7%
MF1: 350.8
2.2%
2.6%
Thymus
CD4
CD8α/Lyt-2

double positive. This would be difficult to detect because of the presence of the double positive population. It was apparent, however, that the shape of the double positive population in $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice was different from that of 129/Sv control animals. As a result of that, the percentage of the cells falling in the CD4 SP gate was increased in mutant mice compared to the control.

Deletion of DNaseI-HSS CII-2 of cluster II caused expression of CD8α/Lyt-2 on a proportion of the peripheral CD4 SP cells. This means that the normal developmental expression of CD8 is disturbed in the $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice resulting in the appearance of double positive cells in peripheral lymphoid organs. This could happen if there is a silencing element lying in cluster II, which is responsible for absence of CD8 expression on CD4 SP cells. By deleting this element from the mouse genome, expression of CD8 is allowed, resulting in the appearance of the double positive peripheral T cells. However, it is not easy to explain the expression of CD8 only on a proportion of the CD4 cells while 49.7% of the peripheral lymph node cells appear to be CD4 SP cells. Alternatively this aberrant expression could be due to the presence of the neomycin gene in the place of cluster II DNaseI-HSS CII-2 left behind following the targeting event.

Deletion of the neomycin gene in $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice

In $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice, the 1.3kb genomic fragment containing the DNaseI-HSS CII-2 of cluster II was removed from the mouse genome and was replaced with the neomycin resistance gene that is 1.4kb long. This deletion had an effect on CD8 expression mainly on peripheral T cells. In the mutant mice described in the previous section, the neomycin gene is still present in the genome. It has been reported that in studies of complex regulatory elements *in vivo* the presence of the inserted selectable marker can influence the phenotype of the mutation. Therefore, the selectable marker must be removed from the mouse genome.

The neomycin gene used for the generation of the Δ2CII targeting vector was flanked by Cre recombinase target (loxP) sites in an orientation that would allow deletion of the neomycin gene upon Cre expression. This target site-specific reaction leaves a single loxP at the site of recombination. In order to delete the neomycin gene from the

genome of $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice and remove any influence that this might have in the phenotype of the mutation, the Cre recombinase was introduced into fertilised oocytes. For this purpose, a $CII^{\Delta 2/\Delta 2}$ homozygous mutant male mouse was mated with C57Bl/10 females. The oocytes were removed from the uteri of the females and were injected with a Cre expressing plasmid. Then, the oocytes were transferred into the uteri of foster mothers. Five mice were born that should all be heterozygous for the $CII^{\Delta 2}$ mutation. This was confirmed by Southern blot analysis, by using a PstI-SalI double digest and the 0.6kb B/E probe. Figure 22A shows a Southern blot analysis of the five heterozygous mice run next to 129/Sv genomic DNA, digested with PstI-SalI and probed with the 0.6kb B/E probe. It confirms the predicted conversion of the 8.0kb wild type PstI fragment to a smaller 6.4kb mutant fragment.

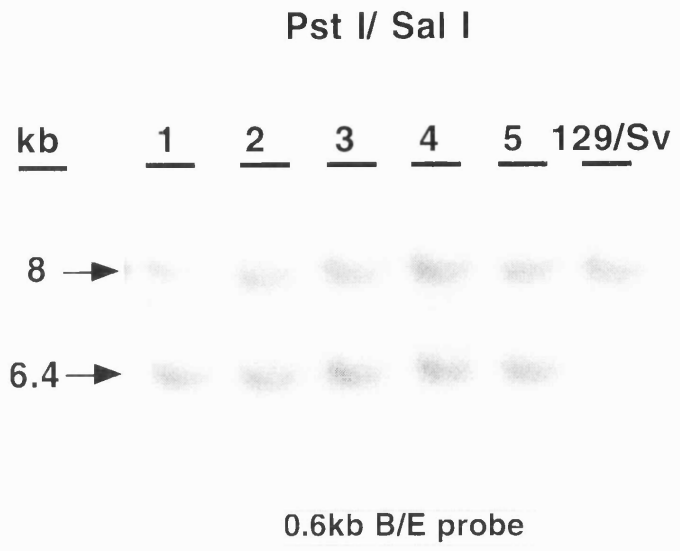
If deletion of the neomycin gene is achieved following Cre recombinase expression, the 8.5kb BamHI genomic fragment containing the DNaseI-HSS cluster II with the neomycin gene of the mutated allele will be converted to a smaller 7.2kb fragment, as detected by the 0.6kb B/E probe. However, the difference between the wild type BamHI fragment and the mutated fragment that comes from replacement of DNaseI-HSS CII-2 of cluster II with the neomycin gene, can not be detected because the two fragments are very similar in size. Thus, if there is no deletion of the neomycin gene, only one 8.5kb fragment will be detected by the 0.6kb B/E probe. Figure 22B shows the Southern blot analysis of the five heterozygous mice run next to 129/Sv genomic DNA, digested with BamHI and probed with the 0.6kb B/E probe. In three of the mice there is no deletion of the neomycin gene and therefore only an 8.5kb fragment is detected. In contrast, in two of these heterozygous mice, deletion of the neomycin gene has been mediated by Cre recombinase. The Southern blot confirms the predicted conversion of the 8.5kb mutant fragment with the neomycin gene to a smaller 7.2kb fragment after deletion of the neomycin gene. The heterozygous mice that no longer have the neomycin gene in their genome were bred to generate homozygous mice, that are designated $CII^{\Delta 2/\Delta 2}\Delta neo$.

Figure 22: Deletion of the neomycin gene in $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice

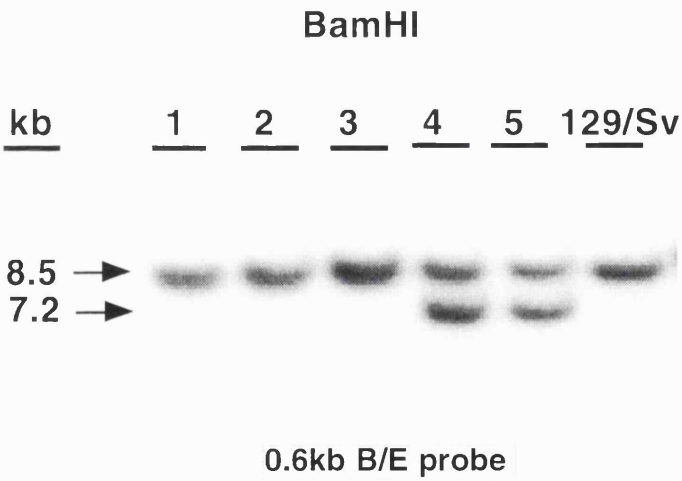
(A) Southern blot analysis of five heterozygous mice run next to 129/Sv genomic DNA, digested with PstI-SalI and hybridised with the 0.6kb B/E probe. After the homologous recombination event occurs, the wild type 8kb PstI fragment is converted to a mutant 6.4kb PstI/SalI fragment.

(B) Southern blot analysis of the same heterozygous mice described above run next to 129/Sv genomic DNA, digested with BamHI and hybridised with the 0.6kb B/E probe. All the mice have the 8.5kb fragment (both wild type fragment and mutated fragment with the neomycin gene). Two of the mice, that have deleted the neomycin gene, have the 7.2kb fragment.

A


Pst I/ Sal I
kb
1
2
3
4
5
129/Sv
8
6.4
0.6kb B/E probe

B


BamHI
kb
1
2
3
4
5
129/Sv
8.5
7.2
0.6kb B/E probe

Thymocytes and peripheral T cell subsets are not affected in $CII^{\Delta 2/\Delta 2}$Δneo mice after removal of the neomycin gene

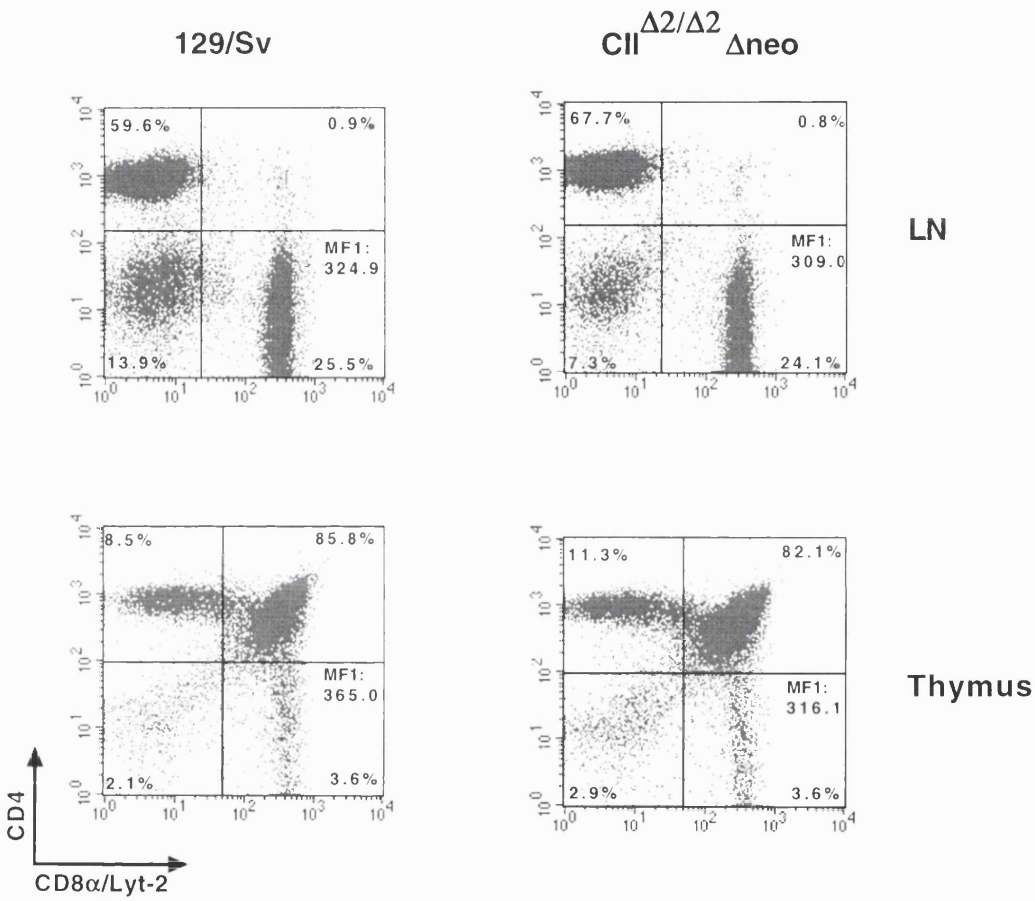
In order to examine whether the effect of the $CII^{\Delta 2}$ deletion on CD8α expression described above is still present in $CII^{\Delta 2/\Delta 2}$Δneo mice and therefore exclude any influence that the presence of the neomycin gene might have in the phenotype of the mutation, lymph node cells and thymocytes were isolated from wild type 129/Sv and $CII^{\Delta 2/\Delta 2}$Δneo homozygous mutant mice and stained with anti-CD4 and anti-CD8α/Lyt-2. Figure 23 shows the FACS profiles of lymph node cells (top panel) or thymocytes (bottom panel) isolated from 129/Sv wild type control mice (left-hand side) and $CII^{\Delta 2/\Delta 2}$Δneo homozygous mutant mice (right-hand side) and stained with the above combination of antibodies. Cells were analysed by plotting CD8α/Lyt-2 against CD4 and electronically gating the different cell populations. The percentages of cells falling in each gate, as well as the mean fluorescence intensity (MFI) of the CD8α/Lyt-2 antibody are shown in the dot-plots. This analysis showed that FACS profiles of T cells and thymocyte subsets were similar for both wild type and mutant mice with no significant differences in either percentages of subsets or CD8α mean fluorescence intensity between the mice analysed.

Thus, the deletion of DNaseI-HSS CII-2 of cluster II had no effect on expression of the CD8α in the CD8αβ SP compartment. If an element responsible for CD8α expression in double positive thymocytes lies in CII-2, as has been suggested from transgenic mice analysis, deletion of this region from the mouse genome would be expected to have an effect on CD8α expression on thymocytes. However, this did not happen and this could be due to the fact that in $CII^{\Delta 2/\Delta 2}$Δneo homozygous mutant mice, the first DNaseI-HSS of cluster II is still present and it is possible that alone it is sufficient to restore expression of the transgene in the double positive thymocytes, just as all three DNaseI-HSS of cluster III are individually sufficient to direct expression of a linked reporter gene to mature CD8 SP T cells.

It is apparent that the phenotype of the mutation observed in $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice which still had the neomycin gene in their genome, was an effect of the selectable marker. The promoter of the CD8α gene lies in the third DNaseI-HSS of cluster II. In $CII^{\Delta 2/\Delta 2}$ homozygous mutant mice the neomycin gene replaced the DNaseI-HSS CII-2 and thus is situated very close to the CD8α gene promoter. It is

Figure 23: Thymocytes and peripheral αβTCR T cell subsets are not affected in $CII^{\Delta 2/\Delta 2}$ Δneo mice after removal of the neomycin gene

(A) FACS analysis of 129/Sv wild type and $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ homozygous mutant mice. Thymocytes and lymph node cells were isolated and stained with anti-CD8α/Lyt-2 and anti-CD4 antibodies. T cell and thymocyte subsets were resolved by plotting CD4 against CD8α/Lyt-2 and analysed for CD8α expression. Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants. Both percentages of cells and levels of expression of CD8α are very similar between mutant and wild type control mice.


129/Sv
CII Δ2/Δ2 Δneo
59.6%
0.9%
MF1:
324.9
13.9%
25.5%
67.7%
0.8%
MF1:
309.0
7.3%
24.1%
LN
8.5%
85.8%
MF1:
365.0
2.1%
3.6%
11.3%
82.1%
MF1:
316.1
2.9%
3.6%
Thymus
CD4
CD8α/Lyt-2

possible that the presence of the neomycin gene promoter, although in the opposite transcriptional orientation, can cause some run through transcripts of the CD8α gene. It is also possible that the presence of the neomycin gene, that has a size similar to the deleted region, can allow binding of transcription factors that can lead to transcription of the CD8α gene in the CD4 lineage cells.

$CII^{\Delta 2/\Delta 2}\Delta neo$ homozygous mutant mice have normal CD8α gene expression on both $\gamma\delta TCR^+$ and $\alpha\beta TCR^+$ CD8αα intraepithelial T cells

The deletion of DNaseI-HSS CII-2 of cluster II had no effect on expression of the CD8α in the CD8αβ SP compartment. It is possible that other elements in the locus, like sequences in the CD8β gene, are able to compensate for the loss of CII-2, as it was shown for cluster III before. We sought to investigate the effect that this mutation had on intraepithelial lymphocytes that have never previously expressed the CD8β gene

In order to examine the effect of the $CII^{\Delta 2}$ deletion on CD8α gene expression in intraepithelial lymphocytes in the presence of the DNaseI-HSS cluster III, IEL were isolated from $CII^{\Delta 2/\Delta 2}\Delta neo$ homozygous mutant mice, as well as from C57Bl/10 and 129/Sv wild type controls. IEL isolated from the above mice were stained with anti-γδTCR, anti-CD8α/Lyt-2 and anti-CD8β. Subsequently, $\gamma\delta TCR^+CD8\beta^-$ cells were gated and analysed for CD8α/Lyt-2 expression in the histograms shown in figure 24. The percentages of positive cell populations are shown in the histograms. This analysis showed that in both the mutant mice and the wild type controls the percentages of $\gamma\delta TCR^+$ CD8αα cells that express CD8α were very similar. However, some variation in the levels of CD8α expression was observed due to mouse strain differences.

IEL isolated from the same mice were also stained with anti-αβTCR, anti-CD8β and anti-CD8α/Lyt-2. Subsequently, $\alpha\beta TCR^+CD8\beta^-$ cells were gated and analysed for CD8α/Lyt-2 expression in the histograms shown in figure 25. The percentages of positive cell populations are shown in the histograms. Similarly to $\gamma\delta TCR^+$ IEL described above, the percentage of $\alpha\beta TCR^+CD8\alpha\alpha$ IEL isolated from $CII^{\Delta 2/\Delta 2}\Delta neo$ homozygous mutant mice that express CD8α is comparable to the one from C57Bl/10 and the 129/Sv controls. In addition, these cells from all mice analysed express CD8α at comparable levels.

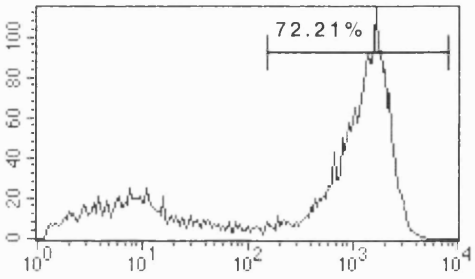
In order to assess CD8α expression on αβTCR^{+}CD8αβ IEL (that are thought to have gone through normal development in the thymus), αβTCR^{+}CD8β^{+} cells were gated and analysed for CD8α/Lyt-2 expression. This analysis showed that there is normal CD8α expression on αβTCR CD8αβ IEL (data not shown), as was shown also in CII$^{\Delta 2/\Delta 2}$Δneo homozygous mutant mice for peripheral T cells.

In conclusion, CII$^{\Delta 2}$ deletion did not have any effect on CD8α gene expression in intraepithelial lymphocytes. As shown in the previous sections, CD8α expression was dramatically affected in IEL when DNaseI-HSS cluster III was deleted from the mouse genome. In CII$^{\Delta 2/\Delta 2}$Δneo mice the DNaseI-HSS cluster III is still present and it could be responsible for CD8α expression in IEL.

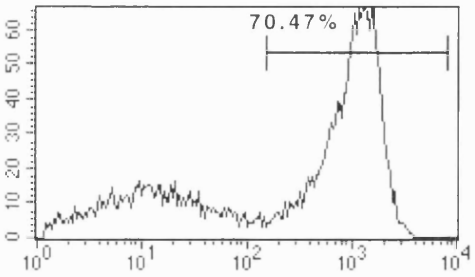
Figure 24: $CII^{\Delta 2/\Delta 2}$Δneo mutant mice have normal CD8α gene expression on γδTCR^{+}CD8αα intraepithelial T cells

Intraepithelial T cells were isolated from murine gut of wild type C57Bl/10 (top histogram), wild type 129/Sv (middle histogram) and $CII^{\Delta 2/\Delta 2}$ Δneo homozygous (bottom histogram) mice. IEL were stained with anti-γδTCR, anti-CD8α/Lyt-2 and anti-CD8β specific antibodies. γδTCR^{+} T cells were gated and analysed by plotting CD8β against CD8α/Lyt-2. Then, $CD8\beta^{-}$ cells were gated and analysed for CD8α/Lyt-2 expression. The percentages of positive cell populations are shown in the histograms. Mutant mice show normal CD8α expression on γδTCR^{+} T cells compared to the wild type controls.

C57Bl/10
(CD8.2$^{+/+}$)



129/Sv
(CD8.2$^{+/+}$)



CII$^{\Delta 2/\Delta 2}$
(CD8.2$^{\Delta 2/\Delta 2}$)

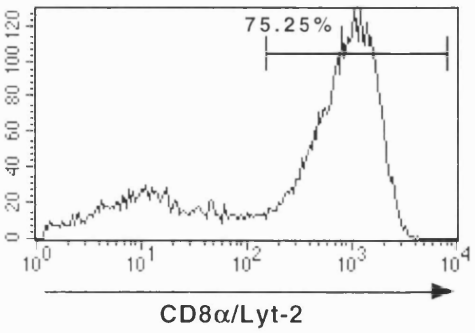
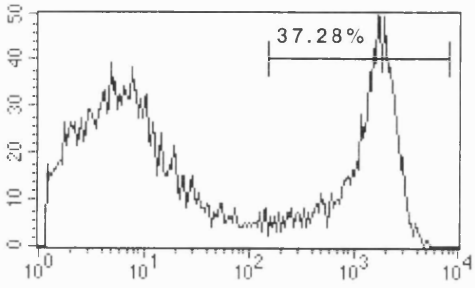


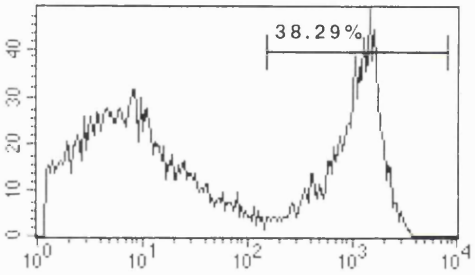
Figure 25: $CII^{\Delta 2/\Delta 2}\Delta neo$ mutant mice have normal CD8α gene expression on $\alpha\beta TCR^{+}CD8\alpha\alpha$ intraepithelial T cells

Intraepithelial T cells were isolated from murine gut of wild type C57Bl/10 (top histogram), wild type 129/Sv (middle histogram) and $CII^{\Delta 2/\Delta 2}\Delta neo$ homozygous (bottom histogram) mice. IEL were stained with anti-αβTCR, anti-CD8α/Lyt-2 and anti-CD8β specific antibodies. $\alpha\beta TCR^{+}$ T cells were gated and analysed by plotting CD8β against CD8α/Lyt-2. Then, $CD8\beta^{-}$ cells were gated and analysed for CD8α/Lyt-2 expression. The percentages of positive cell populations are shown in the histograms. Mutant mice show normal CD8α expression on $\alpha\beta TCR^{+}$ T cells compared to the wild type controls.

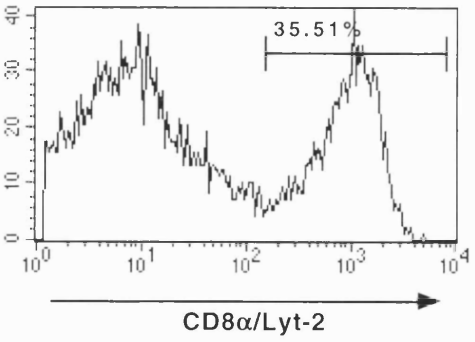
C57Bl/10
(CD8.2$^{+/+}$)



129/Sv
(CD8.2$^{+/+}$)



CII$^{\Delta 2/\Delta 2}$
(CD8.2$^{\Delta 2/\Delta 2}$)



Deletion of cluster II DNaseI-HSS CII-1 and CII-2

The cluster II DNaseI-HSS in conjunction with cluster III DNaseI-HSS was shown to restore transgene expression in the immature double positive thymocytes (Hostert et al., 1998). This, together with the absence of CII-2 from DNaseI treated splenocyte nuclei suggested that a possible immature element might lie in this region. However, the deletion of the second DNaseI-HSS of cluster II described above does not affect the expression of the CD8α gene in thymocytes or mature T cells of $CII^{\Delta 2/\Delta 2}\Delta neo$ homozygous mutant mice. In these mice, the first DNaseI-HSS of cluster II is still present and it is possible that alone it is sufficient to restore expression of the transgene in the double positive thymocytes, just as all three DNaseI-HSS of cluster III are individually sufficient to direct expression of a linked reporter gene to mature CD8 SP T cells. To further investigate the role of cluster II in the developmentally regulated expression of the CD8α gene in thymocytes and mature T cells we generated mice that lack cluster II DNaseI-HSS CII-1 and CII-2.

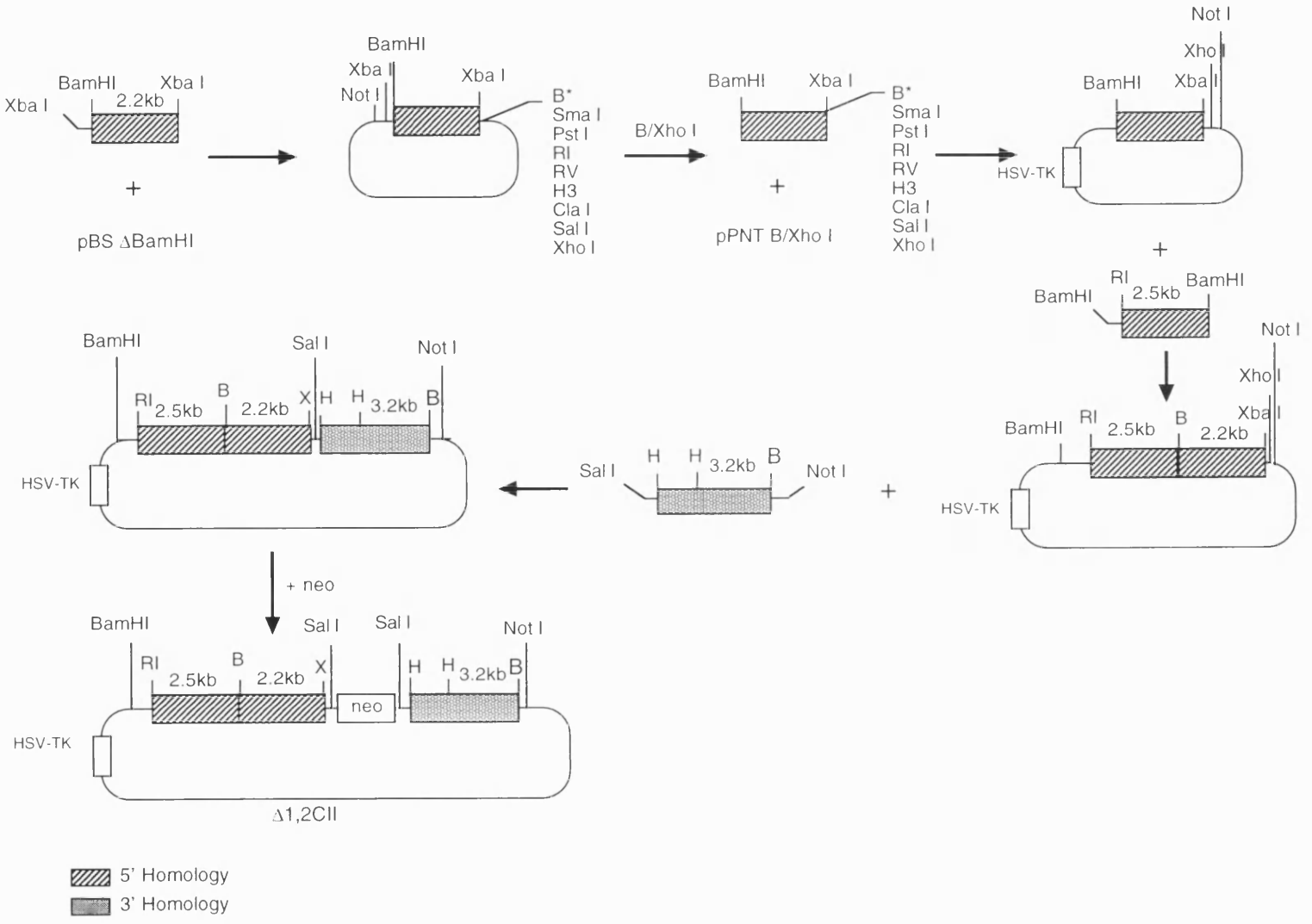
Generation of $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice

In order to remove DNaseI-HSS CII-1 and CII-2 of cluster II from the mouse genome, a targeting construct was introduced into embryonic stem cells by homologous recombination. From the 129/Sv genomic λ phage library screening (see above), an 8.5kb BamHI genomic fragment containing the CD8α gene and 6.9kb upstream sequences was isolated and cloned into the pBluescript vector. A 2.2kb BamHI-XbaI genomic fragment was excised from this plasmid as XbaI fragment, it was ligated into the XbaI site of pBluescript that had its BamHI site mutated and then cut out as BamHI-XhoI fragment. This fragment was used as upstream homology for the targeting construct. For this purpose, it was ligated into the pPNT vector, that contains the HSV-tk gene. A further 2.5kb EcoRI-BamHI fragment with additional upstream homology was excised from a plasmid that contained a 6kb BamHI genomic fragment located between cluster III and cluster II (isolated from λ phage library screening, see above) as EcoRI fragment and was cloned into pBluescript vector. It was then cut out as BamHI fragment and ligated into the targeting vector, in the 5' end of the 2.2kb BamHI-XbaI fragment, thus increasing the overall 5' homology to 4.7kb.

For downstream homology, a 3.2kb HindIII-BamHI genomic fragment was used (same as in the Δ2CII targeting vector described above). Finally, the 1.4kb fragment

Figure 26: Generation of the Δ1,2CII targeting construct

All the relevant restriction enzyme sites used for the cloning steps are shown. B, BamHI; RI, EcoRI; H, HindIII; X, XbaI; RV, EcoRV. The neomycin resistance gene (neo) and the herpes simplex virus thymidine kinase (HSV-tk) are indicated by open boxes and the upstream and downstream homologies of the targeting vector are shown by filled boxes. The asterisk indicated that the restriction site is destroyed. The generation of the Δ1,2CII targeting construct is described in detail in the text.


BamHI
Xba I
2.2kb
Xba I
+
pBS ΔBamHI
BamHI
Xba I
Not I
Xba I
B*
Sma I
Pst I
RI
RV
H3
Cla I
Sal I
Xho I
B/Xho I
BamHI
Xba I
B*
Sma I
Pst I
RI
RV
H3
Cla I
Sal I
Xho I
+
pPNT B/Xho I
Not I
Xho I
BamHI
Xba I
HSV-TK
+
BamHI
RI
2.5kb
BamHI
Not I
Xho I
Xba I
BamHI
RI
2.5kb
B
2.2kb
HSV-TK
Sal I
H
H
3.2kb
B
Not I
+
BamHI
Sal I
Not I
RI
2.5kb
B
2.2kb
X
H
H
3.2kb
B
HSV-TK
+ neo
BamHI
Sal I
Sal I
Not I
RI
2.5kb
B
2.2kb
X
neo
H
H
3.2kb
B
HSV-TK
Δ1,2CII
5' Homology
3' Homology

containing the neo gene flanked with loxP sites was ligated between upstream and downstream homology to give the targeting construct, Δ1,2CII (figure 26).

The construct was linearised with NotI and used to transfect D3 embryonic stem cells as described above. Out of 256 resistant clones that were screened by Southern blot analysis, 8 were found to have undergone homologous recombination. Two clones that had a euploid karyotype were used for injections into 3.5-day blastocysts. A total number of 197 blastocysts were injected and 21 mice were born that had very low percentage of chimerism. This could have happened because the ES cells had differentiated and therefore they were not able to contribute to the generation of the mouse when they were injected into blastocysts.

The targeting experiment was repeated with a different ES cell line. The same targeting construct was used to transfect PC3 embryonic stem cells (O'Gorman et al., 1997). This ES cell line was generated from mice transgenic for the Cre recombinase gene under the protamine 1 promoter, which is active in the terminal, haploid stages of spermatogenesis. The Cre recombinase is not expressed in the ES cells, but it is expressed in the male germ line and therefore it will lead to the deletion of the loxP flanked neomycin gene in the germ line of the male mice. The neomycin gene has been shown to affect gene expression, as described in the previous section, and it should be removed from the genome of the mutant mice. Using the PC3 ES cell line offers the advantage that the neomycin gene will be deleted from the mouse genome from the first generation. The PC3 embryonic stem cells are of 129/Sv origin and are maintained in the undifferentiated state by growth on a feeder layer of γ-irradiated primary embryonic fibroblasts like the D3 ES cell line and under the same culture conditions.

The Δ1,2CII targeting construct was used to transfect PC3 embryonic stem cells. The concentration of the DNA was 50μg/ml and the selection used was 300μg/ml G418 and 2μM gancyclovir. The targeting event (figure 27A) replaced the 3.4kb genomic fragment containing the cluster II DNaseI-HSS CII-1 and CII-2 with the neomycin resistance gene. Because of an EcoRV site introduced in the 3' end of the neomycin gene, the 14kb wild type EcoRV fragment is converted to a smaller 11kb mutant fragment, as detected with the NcoI-XhoI probe shown in figure 27A.

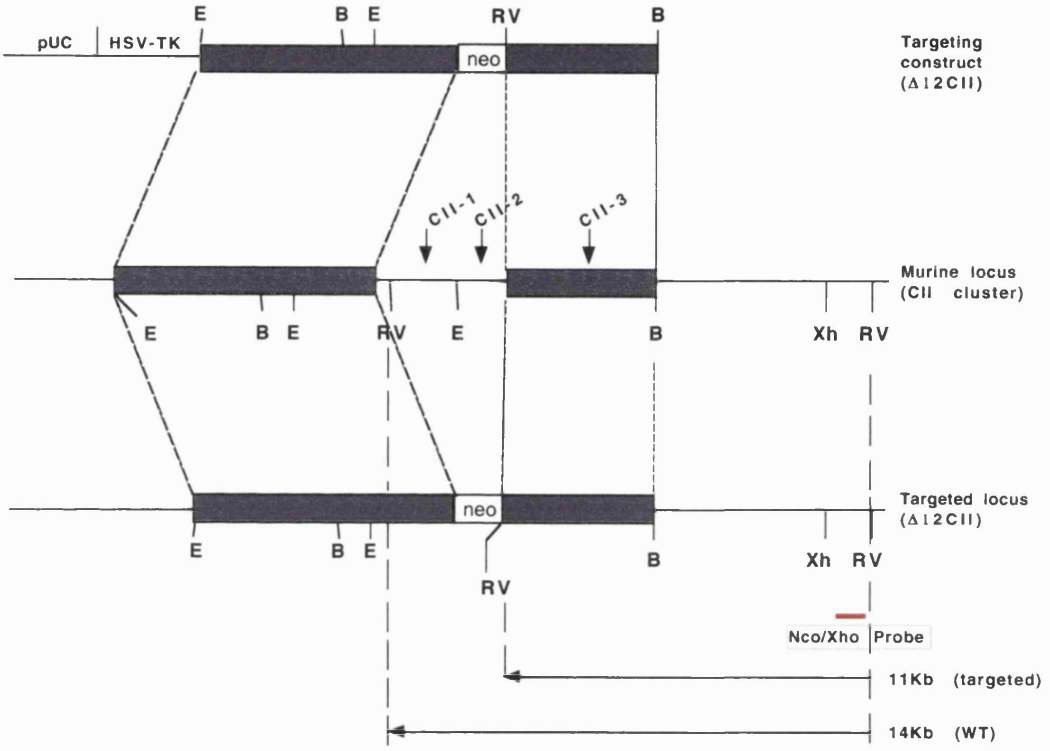
Out of 264 resistant clones that were screened by Southern blot analysis, 17 were found to have undergone homologous recombination. Figure 27B shows the

Figure 27: Deletion of DNaseI-HSS CII-1 and CII-2 by homologous recombination

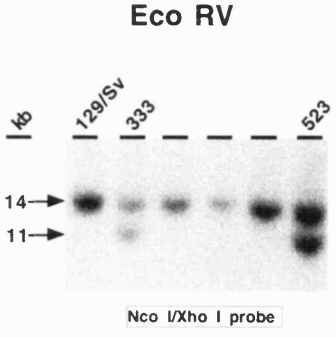
(A) Diagram of the murine CD8 gene locus showing the targeting construct, DNaseI-HSS cluster CII and the targeted allele. The neomycin gene (open box, labeled neo) was flanked by 4.7kb 5' and 3.2kb 3' homologous DNA sequences (filled boxes). Restriction enzyme sites are shown by vertical bars and are labelled B, BamHI; RV, EcoRV; H; E, EcoRI, Xh; XhoI Vertical arrows indicate cluster CII DNaseI-HSS and the probes (NcoI/XhoI genomic fragments) used to detect homologous recombination event is shown as solid horizontal bars. Horizontal arrows illustrate the expected EcoRV fragments hybridising with the NcoI/XhoI probe (14kb wild type allele, 11kb targeted allele).

(B) Southern blot analysis of two clones (333 and 523) that had undergone homologous recombination, run along with wild type 129/Sv DNA. The sizes of wild type and mutant fragments, the restriction enzyme and the probe used are shown.

A


E
B E
RV
B
pUC
HSV-TK
neo
Targeting construct (Δ12CII)
CII-1
CII-2
CII-3
Murine locus (CII cluster)
E
B E
RV
E
B
Xh RV
neo
Targeted locus (Δ12CII)
E
B E
RV
B
Xh RV
Nco/Xho Probe
11Kb (targeted)
14Kb (WT)

B


Eco RV
kb
129/Sv
333
523
14
11
Nco I/Xho I probe

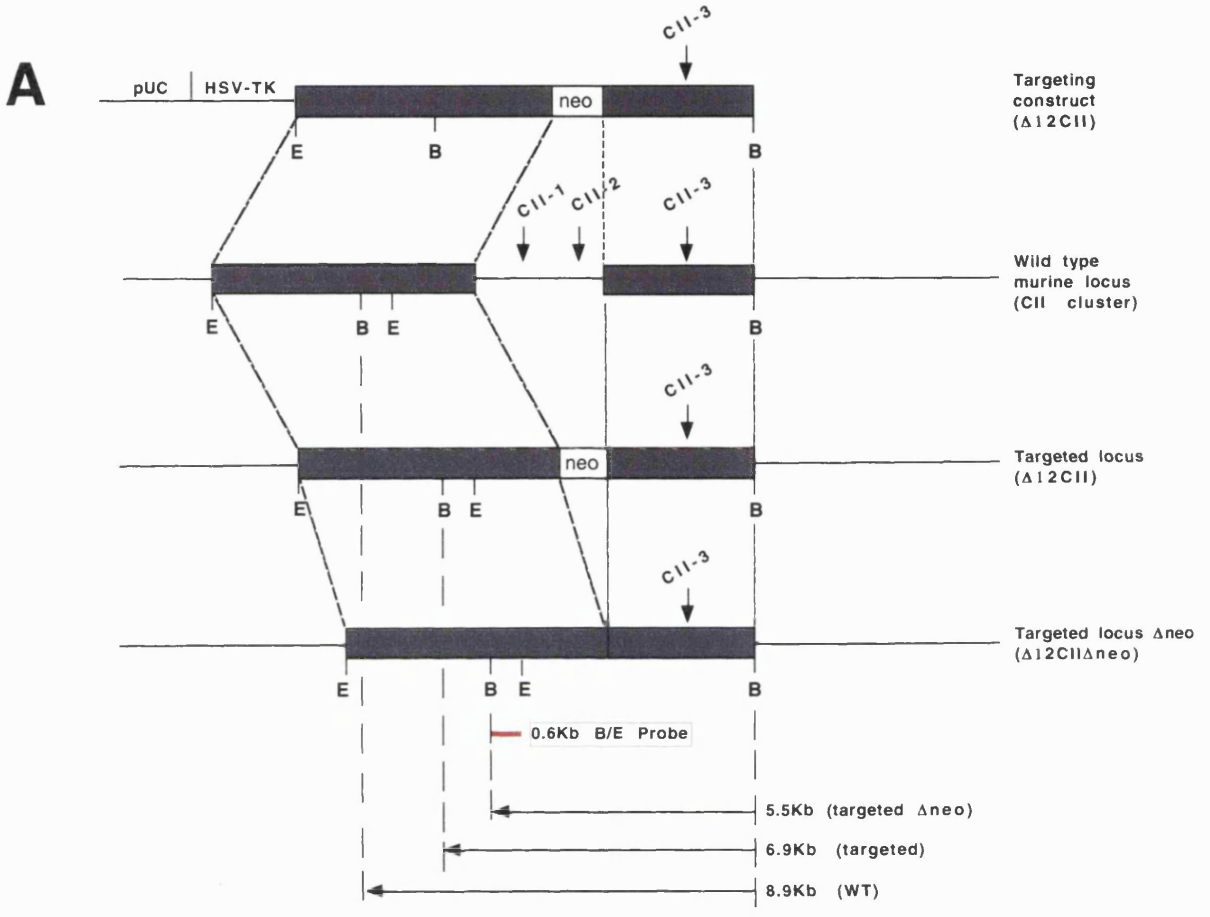
Southern blot analysis of two of the clones (number 333 and 523), run next to 129/Sv wild type DNA and confirms the predicted conversion of the 14kb wild type EcoRV fragment to a smaller 11kb mutant fragment, as detected by the NcoI-XhoI probe. Five of the positive clones were expanded and the integrity of the locus was verified with additional probes located upstream or within the targeted region (data not shown). Two of these clones (number 333 and 523) were checked for a euploid karyotype and were injected into 3.5-day blastocysts to generate chimeric mice. Chimeric males were bred to C57Bl/10 wild type females and germline transmission was determined by agouti coat colour of the offspring. In male chimeric mice, Cre recombinase is expressed in the germline, therefore the loxP flanked neomycin gene would be deleted from the genome of their offspring. Mutant heterozygous mice were bred to homozygocity for the mutated locus. Mice heterozygous and homozygous for the deletion mutation are designated $CII^{+/\Delta 1,2}\Delta neo$ and $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ respectively.

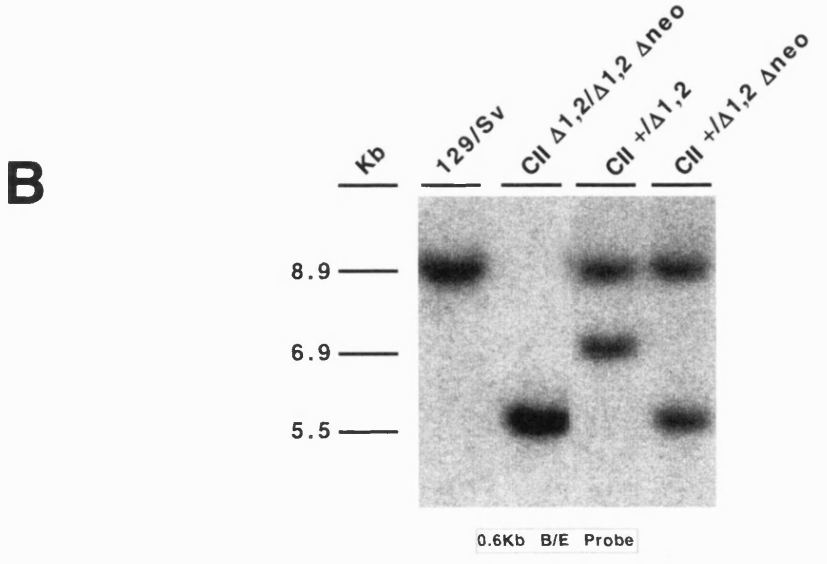
Because of the replacement of a 3.4kb genomic region with the 1.4kb neomycin gene, the 8.9kb wild type BamHI fragment is converted to a smaller 6.9kb mutant fragment, as detected with the 0.6kb B/E probe shown in figure 28A. After deletion of the neomycin gene, the 6.9kb mutant fragment is converted to an even smaller 5.5kb fragment, as detected with the same probe. Out of 22 heterozygous mice born from the chimeras, 20 were found to have deleted the neomycin gene, however 2 mice still contained the neomycin gene in their genome. Figure 28B shows the Southern blot analysis of wild type 129/Sv, $CII^{+/\Delta 1,2}\Delta neo$ and $CII^{+/\Delta 1,2}$ heterozygous and $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice and confirms the predicted conversion of the 8.9kb wild type BamHI fragment to a smaller 6.9kb mutant fragment before deletion of the neomycin gene and a 5.5kb mutant fragment after deletion of the neomycin gene, as detected with the 0.6kb B/E probe shown in figure 28A.

Figure 28: Deletion of DNaseI-HSS CII-1 and CII-2 by homologous recombination

(A) Diagram of the murine CD8 gene locus showing the Δ1,2CII targeting construct, DNaseI-HSS cluster CII, the targeted allele and the targeted allele after deletion of the neomycin gene. The neomycin gene (open box, labelled neo) was flanked by 4.7kb 5' and 3.2kb 3' homologous DNA sequences (filled boxes). Restriction enzyme sites are shown by vertical bars and are labelled B, BamHI; E, EcoRI. Vertical arrows indicate cluster II DNaseI-HSS and the probe (0.6kb B/E genomic fragment) used to detect homologous recombination events and deletion of the neomycin gene is shown as a solid horizontal bar. Horizontal arrows illustrate the expected BamHI fragments hybridising with the probe (8.9kb wild type allele, 6.9kb targeted allele, 5.5kb targeted allele after deletion of neomycin gene).

(B) Southern blot analysis of 129/Sv wild type, $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous, $CII^{+/\Delta 1,2}$ heterozygous and $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice. The replacement of 3.4kb genomic region with the neomycin gene results in the reduction of a wild type 8.9kb BamHI fragment to a mutant 6.9kb BamHI fragment. After deletion of the neomycin gene, the 6.9kb mutant fragment is converted to a smaller 5.5kb fragment.


A
pUC
HSV-TK
neo
CII-3
CII-1
CII-2
E
B
Targeting construct (Δ12CII)
Wild type murine locus (CII cluster)
Targeted locus (Δ12CII)
Targeted locus Δneo (Δ12CIIΔneo)
0.6Kb B/E Probe
5.5Kb (targeted Δneo)
6.9Kb (targeted)
8.9Kb (WT)


B
Kb
129/Sv
CII Δ1,2/Δ1,2 Δneo
CII +/Δ1,2
CII +/Δ1,2 Δneo
8.9
6.9
5.5
0.6Kb B/E Probe

Analysis of CII$^{\Delta 1,2/\Delta 1,2}$Δneo homozygous mutant mice

Expression of CD8 is affected in thymocytes and peripheral T cell subsets by deletion of CII-1 and CII-2 of cluster II

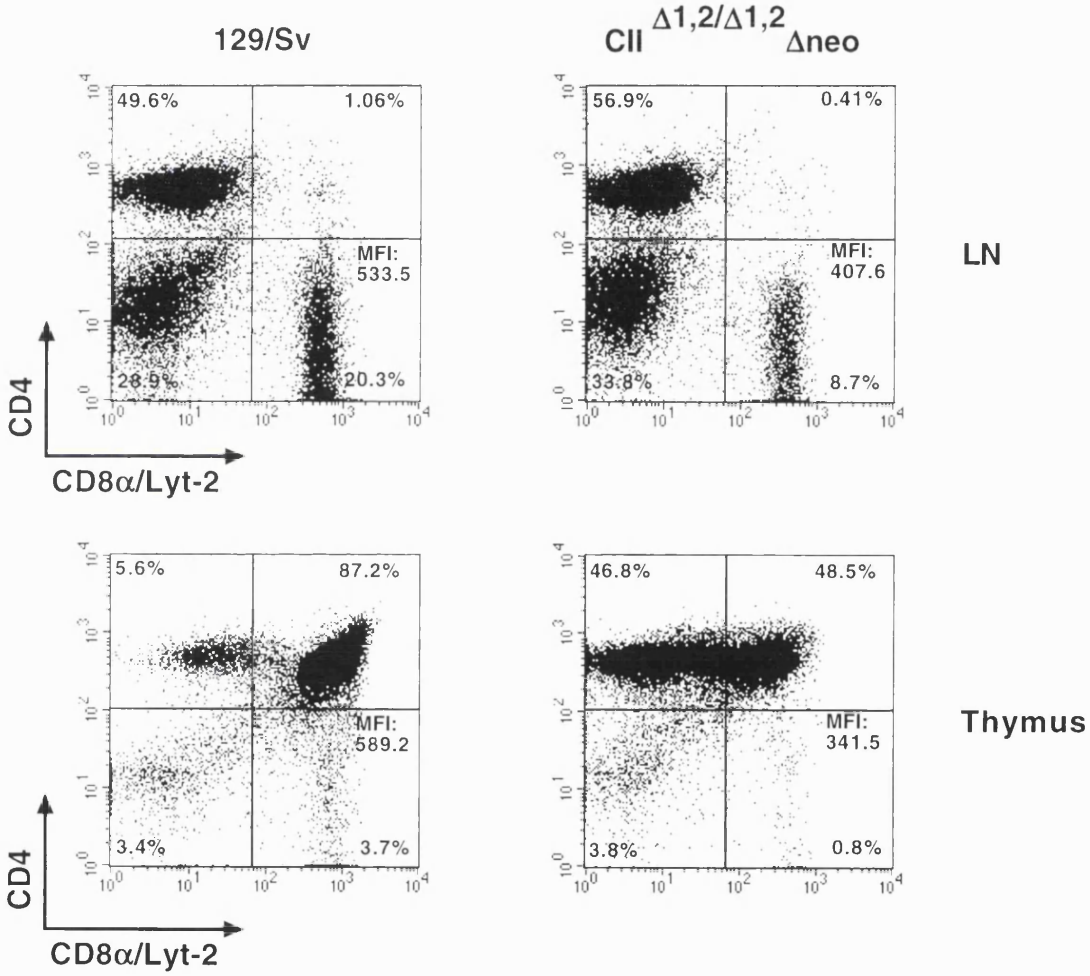
In order to examine the effect of CII$^{\Delta 1,2}$ deletion on CD8α expression, lymph node cells and thymocytes were isolated from wild type 129/Sv and CII$^{\Delta 1,2/\Delta 1,2}$Δneo homozygous mutant mice and stained with anti-CD4 and anti-CD8α/Lyt-2. Figure 29 shows the FACS profiles of lymph node cells (top panel) or thymocytes (bottom panel) isolated from 129/Sv wild type control mice (left-hand side) and CII$^{\Delta 1,2/\Delta 1,2}$Δneo homozygous mutant mice (right-hand side) stained with the above combination of antibodies. Cells were analysed by plotting CD8α/Lyt-2 against CD4 and electronically gating the different cell populations. The percentages of cells falling in each gate are shown in the dot-plots. This analysis showed that in peripheral T lymphocytes there was a substantial reduction in the percentage of cells that expressed CD8α in CII$^{\Delta 1,2/\Delta 1,2}$Δneo mice compared to the wild type control. Furthermore, the CD4:CD8 ratio was increased from 2-3:1 in the wild type control animals to 6:1 in CII$^{\Delta 1,2/\Delta 1,2}$Δneo mutant mice, while the total number of CD4$^+$ cells remained unaltered. In addition, the CD8α mean fluorescence intensity (MFI) in CII$^{\Delta 1,2/\Delta 1,2}$Δneo mice was decreased compared to the 129/Sv control mice.

In thymocytes of CII$^{\Delta 1,2/\Delta 1,2}$Δneo mutant mice, the level of CD8α expression was lower both in DP and in CD8 SP cells compared to the wild type controls. In addition, in the CII$^{\Delta 1,2/\Delta 1,2}$Δneo mutant mice, only 48.2% of the thymocytes appeared to be DP, compared to 83.7% in the wild type control and there was also a reduction in the number of CD8 SP compared to the 129/Sv controls. In contrast, there was an increase in the number of cells that fall into the CD4 compartment in CII$^{\Delta 1,2/\Delta 1,2}$Δneo mutant mice (47.9%) compared to the wild type control (8.8%).

CII$^{\Delta 1,2/\Delta 1,2}$Δneo homozygous mutant mice have a regulatory region for expression of the CD8α gene deleted from their genome. It is, therefore, possible that the majority of the cells in the CD4 gate are immature cells that appear like CD4 SP cells in this analysis due to the lack of CD8 on their surface.

Figure 29: Expression of CD8 is affected in thymocytes and peripheral T cell subsets by deletion of DNaseI-HSS CII-1 and CII-2

FACS analysis of 129/Sv wild type and $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice. Lymph node cells and thymocytes were isolated and stained with anti-CD8α/Lyt-2 and anti-CD4 antibodies. T cell and thymocyte subsets were resolved by plotting CD4 against CD8α and analysed for CD8α expression. Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants and mean fluorescence intensities (MFI) for CD8α are also shown. Deletion of Cluster II causes an abnormal subset distribution in the thymus with fewer DP and CD8 SP cells and an increase in cells with a CD4 SP phenotype.


129/Sv
CII Δ1,2/Δ1,2 Δneo
49.6%
1.06%
MFI:
533.5
28.9%
20.3%
56.9%
0.41%
MFI:
407.6
33.8%
8.7%
LN
5.6%
87.2%
MFI:
589.2
3.4%
3.7%
46.8%
48.5%
MFI:
341.5
3.8%
0.8%
Thymus
CD4
CD8α/Lyt-2

CD4$^+$ cells in thymocytes of CII$^{\Delta1,2/\Delta1,2}$Δneo mice have an immature phenotype

As described in the previous section, thymocytes from CII$^{\Delta1,2/\Delta1,2}$Δneo mutant mice showed an increased number of cells falling in the CD4 gate. These cells could arise because of a diversion of the lineage commitment process towards CD4 cells caused by the deletion of cluster II sequences. Alternatively, they may represent thymocytes in the process of differentiation that failed to express the CD8 gene. In order to distinguish between the two possibilities, we examined the pattern of expression on these cells of several markers that are modulated in different stages of development. Thymocytes were isolated from 129/Sv wild type control mice and CII$^{\Delta1,2/\Delta1,2}$Δneo homozygous mutant mice and stained with antibodies against CD4, CD8α and a number of maturation or activation markers, such as αβTCR, CD69, CD5 and HSA. The first three are surface molecules that are upregulated during the late stages of maturation following positive selection and HSA is a molecule that is downregulated during this process.

Figure 30 shows the FACS profiles of thymocytes isolated from 129/Sv wild type mice (left-hand side) and CII$^{\Delta1,2/\Delta1,2}$Δneo mutant mice (right-hand side) stained with anti-CD4, anti-CD8α/Lyt-2 and anti-αβTCR. Cells were analysed by plotting CD8α/Lyt-2 against CD4 and electronically gating the individual thymocyte subpopulations. Gated cell populations were then analysed for the expression of αβTCR in the histograms underneath the corresponding FACS dot-plots. In wild type mice, DN (A) and DP (B) thymocytes express low levels of TCR (TCR^{lo}). During their development in the thymus T cells upregulate TCR, they go through positive and negative selection and turn off either CD4 or CD8 coreceptor to become CD8 SP or CD4 SP cells respectively, expressing high levels of αβTCR (TCR^{hi}) (C and D). In CII$^{\Delta1,2/\Delta1,2}$Δneo mice, the pattern of expression of αβTCR in DN (A) and DP (B) thymocytes was very similar to that observed in the corresponding thymocyte subsets of 129/Sv wild type mice. In the CD4 gate (C), however, only 15.2% of the cells falling in the gate had upregulated αβTCR. In contrast, the majority of these cells were αβTCR^{lo} and thus appeared to have an immature phenotype. In CII$^{\Delta1,2/\Delta1,2}$Δneo mutant mice, there was a small number of thymocytes that completed selection and became CD8 SP cells, but those cells had normal levels of αβTCR on their surface (D). A small peak of αβTCR^{lo} cells was

observed in the $CD8^+$ cell compartment in both $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mutant mice and 129/Sv control mice, which probably corresponds to the $CD4^-CD8^+$ intermediate immature population between the DN and $CD4^+CD8^+$ DP cells (MacDonald et al., 1988).

To further investigate the characteristics of the cells falling in the CD4 gate, thymocytes were stained with more maturation markers. CD69 is an early activation marker whose onset of expression is closely correlated with positive selection (Bendelac et al., 1992, Chan et al., 1993). Figure 31 shows the FACS profiles of thymocytes isolated from 129/Sv wild type mice (left-hand side) and $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mutant mice (right-hand side) stained with anti-CD4, anti-CD8α/Lyt-2 and anti-CD69. Cells were analysed by plotting CD8α/Lyt-2 against CD4 and electronically gating the individual thymocyte subpopulations. Gated cell populations were then analysed for the expression of CD69 in the histograms underneath the corresponding FACS dot-plots. In wild type mice, DN (A) and DP (B) thymocytes express low levels of CD69. This activation marker is then upregulated upon selection, so CD4 and CD8 SP cells express intermediate to high levels of CD69 on their surface (C and D). In $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mice, the pattern of expression of CD69 in DN (A) and DP (B) thymocytes was very similar to that observed in the corresponding thymocyte subsets of 129/Sv wild type mice. In the CD4 gate (C), however, only 13.05% of the cells falling in the gate have upregulated CD69. In contrast, the majority of these cells expressed low levels of CD69 and thus appeared to have an immature preselection phenotype. In $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mutant mice, the small number of thymocytes that completed selection and became CD8 SP cells had normal levels of CD69 on their surface (D).

Figure 30: Thymocytes appearing within the CD4 SP gate have an immature phenotype (αβTCR)

FACS analysis of 129/Sv wild type and $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice. Thymocytes were isolated and stained with anti-CD8α/Lyt-2, anti-CD4 and anti-αβTCR antibodies. Cell populations were identified by plotting CD4 against CD8α/Lyt-2. Different cell populations (A, double negative; B, double positive; C, CD4 SP; D, CD8 SP) were electronically gated and analysed for αβTCR expression in the histograms shown underneath the corresponding dot-plot. The percentages of cells positive for each gated population are shown in the histograms. Large numbers of $CD4^{+}CD8^{-}$ cells found in the thymus of $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mice show levels of expression of αβTCR consistent with an immature phenotype of these cells.

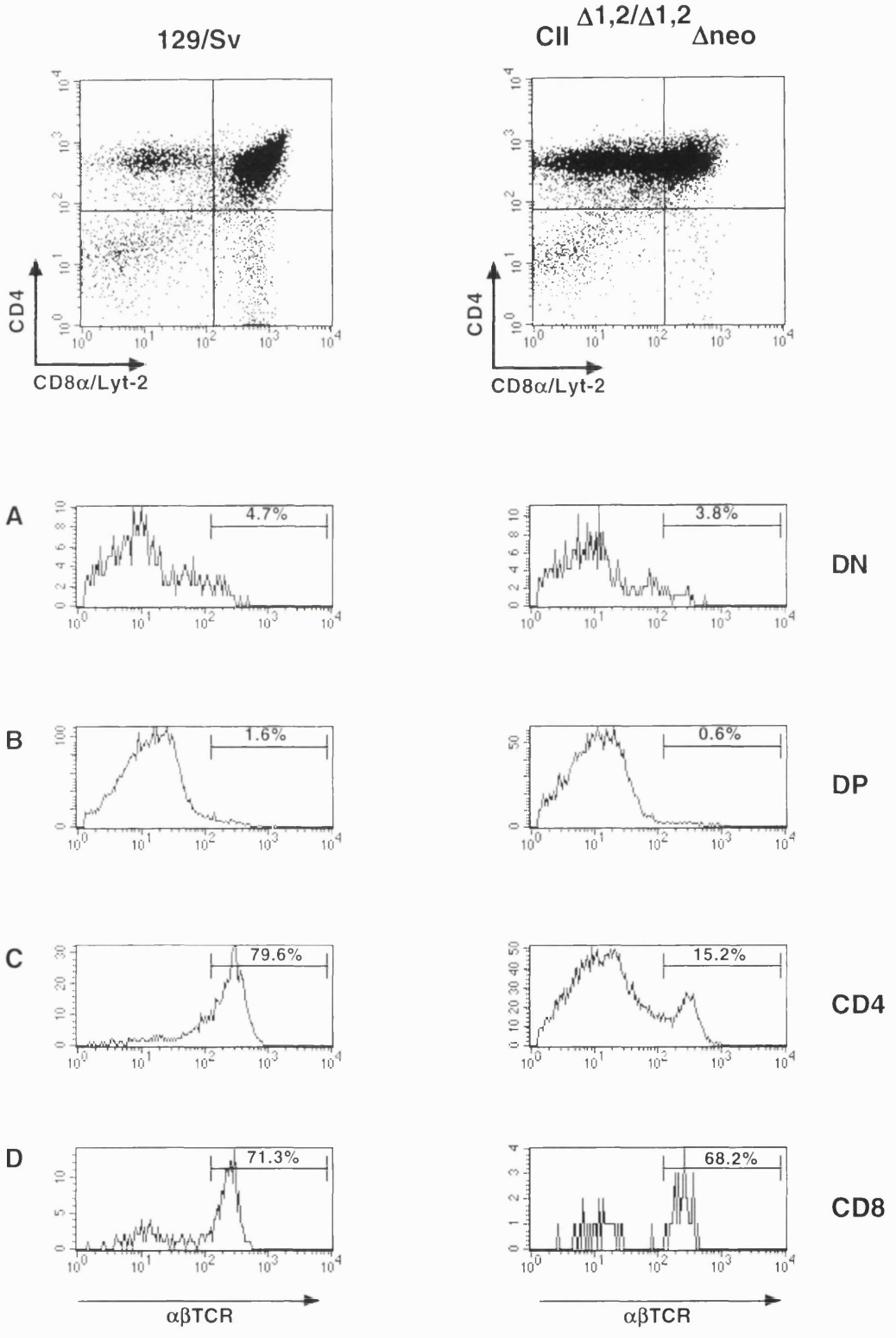

129/Sv
CII Δ1,2/Δ1,2 Δneo
CD4
CD8α/Lyt-2
A
4.7%
3.8%
DN
B
1.6%
0.6%
DP
C
79.6%
15.2%
CD4
D
71.3%
68.2%
CD8
αβTCR

Figure 31: Thymocytes appearing within the CD4 SP gate have an immature phenotype (CD69)

FACS analysis of 129/Sv wild type and $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice. Thymocytes were isolated and stained with anti-CD8α/Lyt-2, anti-CD4 and anti-CD69 antibodies. Cell populations were identified by plotting CD4 against CD8α/Lyt-2. Different cell populations (A, double negative; B, double positive; C, CD4 SP; D, CD8 SP) were electronically gated and analysed for CD69 expression in the histograms shown underneath the corresponding dot-plot. The percentages of cells positive for each gated population are shown in the histograms. Large numbers of $CD4^{+}CD8^{-}$ cells found in the thymus of $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mice show levels of expression of CD69 consistent with an immature phenotype of these cells.

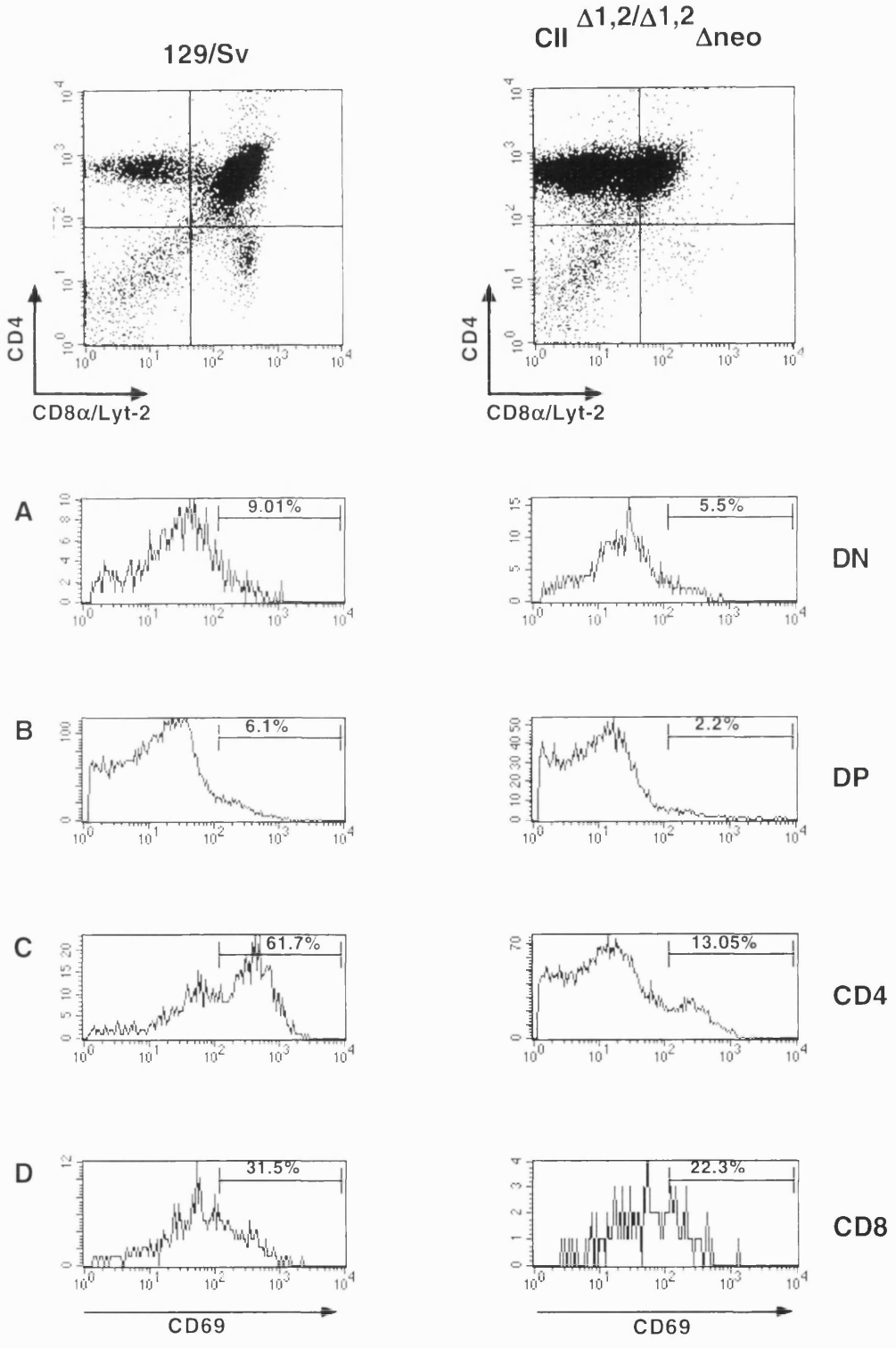
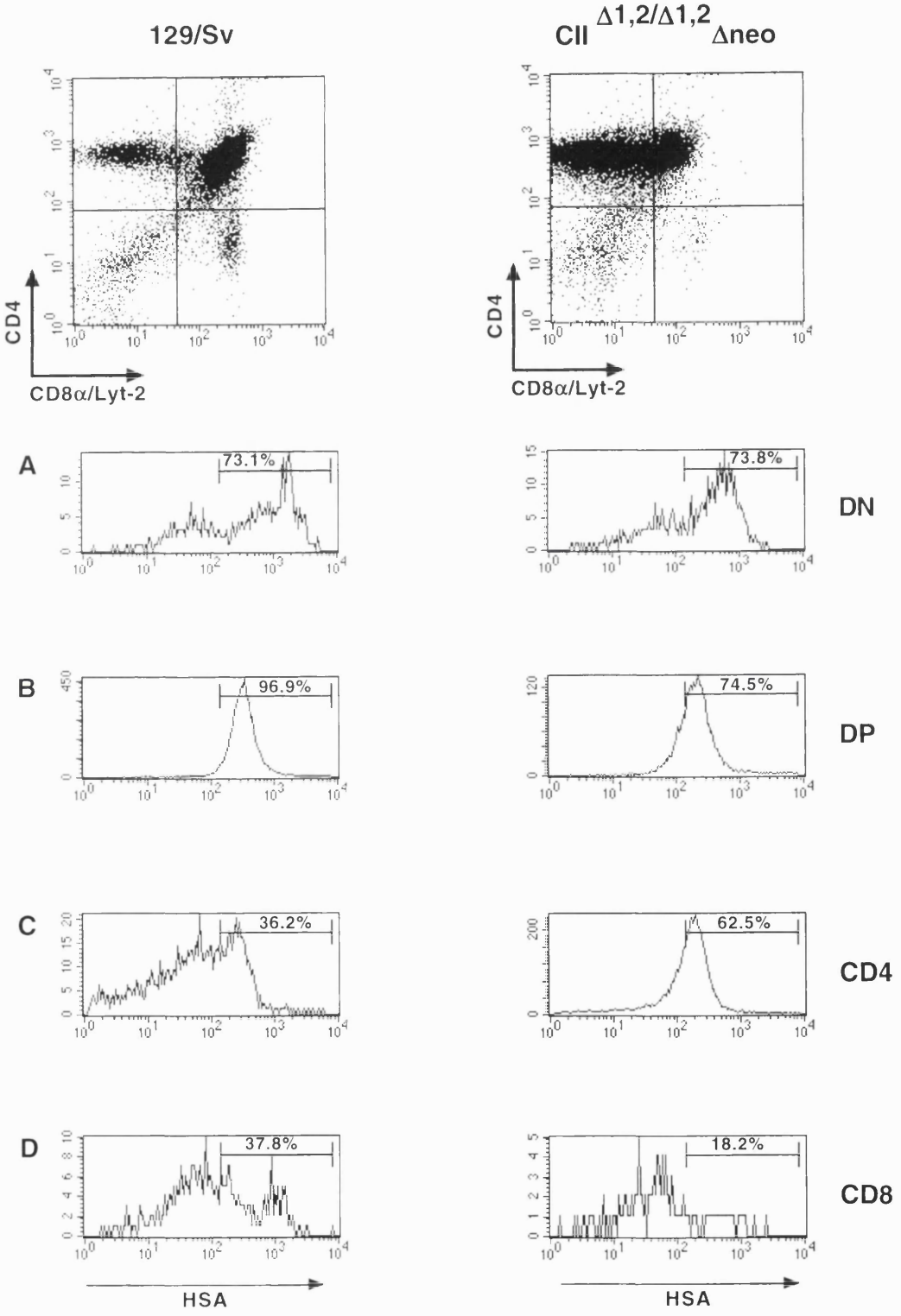

129/Sv
CII Δ1,2/Δ1,2 Δneo
CD4
CD8α/Lyt-2
CD4
CD8α/Lyt-2
A
9.01%
5.5%
DN
B
6.1%
2.2%
DP
C
61.7%
13.05%
CD4
D
31.5%
22.3%
CD8
CD69
CD69

Figure 33: Thymocytes appearing within the CD4 SP gate have an immature phenotype (HSA)

FACS analysis of 129/Sv wild type and $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice. Thymocytes were isolated and stained with anti-CD8α/Lyt-2, anti-CD4 and anti-HSA antibodies. Cell populations were identified by plotting CD4 against CD8α/Lyt-2. Different cell populations (A, double negative; B, double positive; C, CD4 SP; D, CD8 SP) were electronically gated and analysed for HSA expression in the histograms shown underneath the corresponding dot-plot. The percentages of cells positive for each gated population are shown in the histograms. Large numbers of $CD4^{+}CD8^{-}$ cells found in the thymus of $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mice show levels of expression of HSA consistent with an immature phenotype of these cells.


129/Sv
CII Δ1,2/Δ1,2 Δneo
CD4
CD8α/Lyt-2
A
73.1%
73.8%
DN
B
96.9%
74.5%
DP
C
36.2%
62.5%
CD4
D
37.8%
18.2%
CD8
HSA
HSA

CD8 SP compartments (C and D, left-hand side). In $CII^{\Delta1,2/\Delta1,2}$Δneo mice, the pattern of expression of HSA in DN (A) and DP (B) thymocytes was very similar to that observed in the corresponding thymocyte subsets of 129/Sv wild type mice. In the CD4 gate (C), however, the majority of the cells (62.5%) falling in the gate had not down-regulated HSA. These cells still expressed high levels of HSA and thus appeared to have an immature phenotype. A small peak of cells expressing high levels of HSA was observed in the $CD8^+$ cell compartment in 129/Sv control mice, which corresponded to the $CD4^-$ $CD8^+$ intermediate immature population between the DN and $CD4^+CD8^+$ DP cells (Shortman et al., 1988). In $CII^{\Delta1,2/\Delta1,2}$Δneo mutant mice, the small number of thymocytes that completed selection and became CD8 SP cells had down-regulated HSA on their surface (D).

Thymocytes from $CII^{\Delta1,2/\Delta1,2}$Δneo mutant mice showed an increased number of cells falling in the CD4 gate. The results obtained from staining those cells with different maturation markers indicate that the majority of those cells have an immature phenotype and thus are cells that would be DP, but appear like CD4 SP cells due to the lack of CD8 on their surface.

In $CII^{+/\Delta1,2}$ thymuses the two alleles are regulated independently

In the $CII^{\Delta1,2/\Delta1,2}$Δneo homozygous mutant mice a reduction in the percentage of DP thymocytes was observed compared to the wild type controls. In addition, there was an increased number of cells with immature phenotype that appeared in the CD4 gate when thymocytes were stained with antibodies against CD4 and CD8. Furthermore, the number of cells that passed selection and became CD8 SP was reduced in the $CII^{\Delta1,2/\Delta1,2}$Δneo homozygous mutant mice. It is possible that deletion of cluster II DNaseI-HSS CII-1 and CII-2 leads to a developmental arrest and the cells can not reach the DP stage where they receive the appropriate maturation signals to activate the CD8 locus in SP thymocytes. In order to assess whether deletion of cluster II from the mouse genome affects maturation and selection of thymocytes or just transcription of the CD8α gene, $CII^{+/\Delta1,2}$Δneo heterozygous mutant mice were analysed, in which the products of the two CD8α alleles could be distinguished. These mice were generated by crossing $CII^{\Delta1,2/\Delta1,2}$Δneo homozygous mutant mice (carrying the CD8α/Lyt-2.2 allele) to CBA/Ca

wild type mice (carrying the CD8α/Lyt-2.1 allele). The CD8α/Lyt-2 allelic difference is detectable at the protein level using specific monoclonal antibodies. This allows expression of the $CII^{\Delta 1,2}$ mutated allele to be distinguished from the wild type CBA/Ca allele. (CBA/Ca x 129/Sv) F1 mice were used as non-transgenic controls since they carry both the CD8α/Lyt-2.1 (CBA/Ca encoded) and CD8α/Lyt-2.2 (129/Sv encoded) allele. In $CII^{+/\Delta 1,2}$Δneo heterozygous mutant mice the presence of one fully functional CD8α allele allows the cells to reach the DP stage, where the appropriate maturation signals would be available and the expression of the $CII^{\Delta 1,2}$ mutated allele can be determined on these DP cells.

Thymocytes isolated from (CBA/Ca x 129/Sv) F1 (figure 34, left-hand side) and $CII^{+/\Delta 1,2}$Δneo heterozygous mutant mice (figure 34, right-hand side) were stained with anti-CD8α/Lyt-2.1, anti-CD4 and anti-CD8α/Lyt-2.2. Individual cell populations were identified by plotting CD8α/Lyt-2.1 against CD4 (top panel) or CD8α/Lyt-2.2 against CD4 (bottom panel) and electronically gating the different cell populations. The percentages of cells falling in each gate are shown in the dot-plots. This analysis showed that FACS profiles of thymocyte subsets identified by plotting CD8α/Lyt-2.1 against CD4 (top panel) were similar for both (CBA/Ca x 129/Sv) F1 wild type and $CII^{+/\Delta 1,2}$Δneo heterozygous mutant mice with no significant differences in percentages of cell subsets between the mice analysed. This indicates that in the thymuses of $CII^{+/\Delta 1,2}$Δneo heterozygous mutant mice developmental progression is not perturbed.

In addition, the FACS profiles of thymocyte subsets identified by plotting CD8α/Lyt-2.2 against CD4 in (CBA/Ca x 129/Sv) F1 wild type mice (bottom panel, left-hand side) were found to be very similar to the analysis obtained with the previous set of antibodies. In contrast, FACS profiles of $CII^{+/\Delta 1,2}$Δneo thymocytes obtained by plotting CD4 against CD8α/Lyt-2.2 (bottom panel, right-hand side) were similar to those obtained from $CII^{\Delta 1,2/\Delta 1,2}$Δneo homozygous mutant mice as described in previous section. Thus, there was a reduction in the DP and CD8 SP thymocytes as defined by the CD8α/Lyt-2.2 allele and an increase in the number of cells falling in the CD4 gate.

This experiment suggests that the two alleles are regulated independently and that the presence of one wild type allele is enough to get the cells through selection. Staining thymocytes of $CII^{+/\Delta 1,2}$Δneo heterozygous mutant mice with an antibody that

recognises the wild type allele gives FACS profiles similar to the wild type control, which indicates that cells mature normally in heterozygous mice. However, in the presence of a functional CD8α allele (CD8α/Lyt-2.1), the expression of the mutated allele (CD8α/Lyt-2.2) is still perturbed. In $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice there is an increase in the percentage of DN cells as defined by the mutated allele (CD8α/Lyt-2.2) compared to the DN cells as defined by the wild type allele (CD8α/Lyt-2.1). This must account for the cells that express the CD8α/Lyt-2.1 wild type allele and thus are CD8 SP, but do not express the mutated allele and therefore appear like DN when stained for the CD8α/Lyt-2.2 allele.

These data suggest that the regulatory region that is deleted in $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice does not lead to a developmental arrest of thymocytes. In $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice, the cells are allowed to reach the DP stage because of the presence of one wild type CD8α allele, but the expression of the mutated allele remains affected. Therefore the effect observed following the deletion of cluster II must be in *cis*.

Variegated expression of CD8 ($CII^{\Delta 1,2}$) allele on DP thymocytes of heterozygous mice (as defined by the wild type allele)

As described in the previous section, $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice carrying one CD8α/Lyt-2.1 wild type allele and one CD8α/Lyt-2.2 mutated allele showed normal expression of the wild type allele, while the expression of the mutated allele was perturbed. In order to assess the pattern of expression of the CD8α/Lyt-2.2 mutated allele on DP thymocytes (as defined by the CD8α/Lyt-2.1 wild type allele), thymocytes were isolated from (CBA/Ca x 129/Sv) F1 control mice (figure 35, left-hand side) and $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice (figure 35, right-hand side) and were stained with anti-CD8α/Lyt-2.1, anti-CD4 and anti-CD8α/Lyt-2.2. Cells were analysed by plotting CD8α/Lyt-2.1 against CD4 and electronically gating the individual thymocyte subpopulations. Gated cell populations were then analysed for the expression of CD8α/Lyt-2.2 in the histograms underneath the corresponding FACS dot-plots. The anti-CD8α/Lyt-2.2 antibody stained the CD8α/Lyt-2.1 positive cells in the (CBA/Ca x 129/Sv) F1 control mice whilst staining could not be observed on the $CD4^-CD8^-$ DN cell

subset. However, a small peak of positive cells was observed in the CD4 subset of (CBA/Ca x 129/Sv) F1 control mice. We believe that these are cells that have turned off the CD8α/Lyt-2.1 allele and thus appear as CD4 SP cells as defined by the CD8α/Lyt-2.1 antibody, but they still have the CD8α/Lyt-2.2 allele on their surface. As discussed in the previous section, this is further indication that the two CD8α/Lyt-2 alleles appear to be independently regulated and they could be turned on and off at different times.

In $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice, only 44.1% of the DP and 39.2% of the CD8 SP thymocytes, as defined by the CD8α/Lyt-2.1 wild type allele, also expressed the CD8α/Lyt-2.2 mutated allele on their surface. However, the majority of these cell subsets expressed low levels or no CD8α/Lyt-2.2 allele at all on their surface. This is consistent with a variegated expression of the CD8α/Lyt-2.2 allele on DP and CD8 SP thymocytes (as defined by the wild type allele) in $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice.

Figure 36 shows the FACS profiles of lymph node T cells isolated from (CBA/Ca x 129/Sv) F1 wild type mice (left-hand side) and $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice (right-hand side) stained with CD8α/Lyt-2.1, CD4 and CD8α/Lyt-2.2 antibodies. Cells were analysed by plotting CD8α/Lyt-2.1 against CD4 and electronically gating the individual thymocyte subpopulations. Gated cell populations were then analysed for the expression of CD8α/Lyt-2.2 in the histograms underneath the corresponding FACS dot-plots. The anti-CD8α/Lyt-2.2 antibody stained only CD8α/Lyt-2.1 positive cells in both the (CBA/Ca x 129/Sv) F1 control mice and the $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice whilst staining was absent on the CD4 single positive and the $CD4^{-}CD8^{-}$ DN cell subsets. In the $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice a very small proportion of the CD8 SP cells as defined by the CD8α/Lyt-2.1 wild type allele were found not to express the CD8α/Lyt-2.2 allele on their surface. Thus, variegation in the expression of the CD8α/Lyt-2.2 mutated allele was observed also in peripheral T cells, but to a lesser extend than in thymocytes. This could happen if only cells that expressed both alleles migrated from the thymus to peripheral lymphoid organs or if CD8 SP thymocytes in $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice managed to activate the CD8α/Lyt-2.2 locus as maturation progressed and therefore, express both alleles when they reached peripheral lymphoid organs.

Figure 34: In ΔCII thymuses the two allelic CD8 loci are regulated independently

FACS analysis of (129/Sv x CBA/Ca) F1 wild type and $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice. The wild type mice express a wt-CD8.1 allele and a wt-CD8.2 allele, whereas the mutant mice express a $CII^{\Delta 1,2}$-CD8.2 and a wt-CD8.1 alleles. Thymocytes were isolated and stained with anti-CD8α/Lyt-2.1, anti-CD4 and anti-CD8α/Lyt2.2 antibodies. FACS profiles were identified by plotting CD4 against CD8α/Lyt-2.1 (top dot-plot panels) or CD4 against CD8α/Lyt2.2 (bottom dot-plot panels). Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants. FACS profiles of thymocytes identified by plotting CD4 against CD8α/Lyt2.1 were similar for both control F1 mice and for $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mice. In contrast, FACS profiles from $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mice obtained by plotting CD4 against CD8α/Lyt2.2 were similar to those obtained from $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mice.

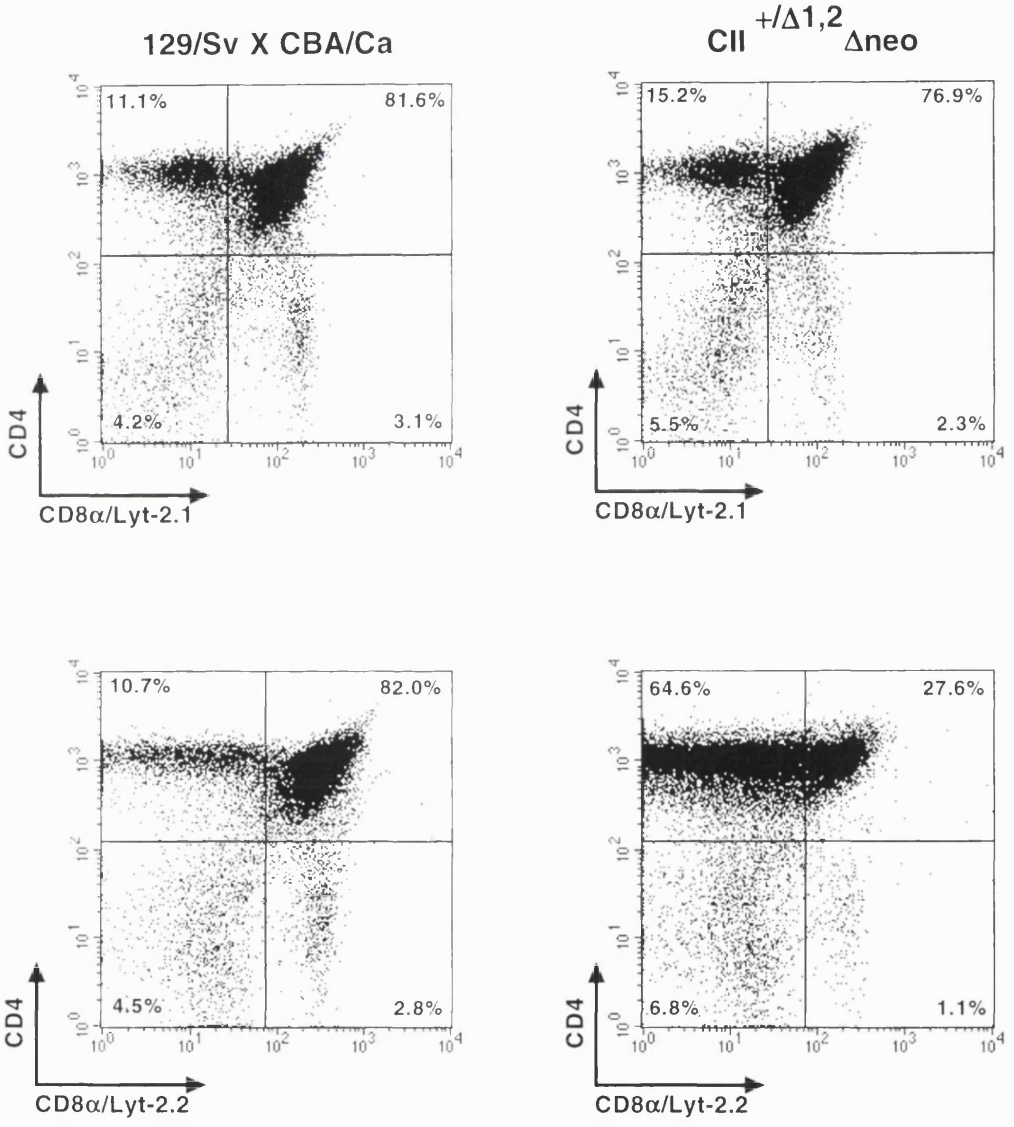

129/Sv X CBA/Ca
CII +/Δ1,2 Δneo
11.1%
81.6%
4.2%
3.1%
15.2%
76.9%
5.5%
2.3%
10.7%
82.0%
4.5%
2.8%
64.6%
27.6%
6.8%
1.1%
CD4
CD8α/Lyt-2.1
CD8α/Lyt-2.2

Figure 35: Variegated expression of CD8 ($CII^{\Delta 1,2}$) allele on DP thymocytes of heterozygous $CII^{+/\Delta 1,2}\Delta neo$ mice

(A) FACS analysis of thymocytes isolated from (129/Sv x CBA/Ca) F1 wild type and $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice. The wild type mice express a wt-CD8.1 allele and a wt-CD8.2 allele, whereas the mutant mice express a $CII^{\Delta 1,2}$-CD8.2 and a wt-CD8.1 alleles. Thymocytes were isolated and stained with anti-CD8α/Lyt2.1, anti-CD4 and anti-CD8α/Lyt2.2 antibodies. FACS profiles were identified by plotting CD4 against CD8α/Lyt2.1. Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants. Different cell populations (DN, double negative; DP, double positive; CD4 and CD8) were electronically gated and analysed for CD8α/Lyt2.2 expression in the histograms shown underneath the corresponding dot-plot. The percentages of cells positive for CD8α/Lyt2.2 are shown in the histograms. In the wild type mice almost all the DP and CD8 SP cells as defined by CD8α/Lyt2.1 and CD4, also express CD8α/Lyt2.2 on their surface. In $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice, in contrast, only a proportion of DP and CD8 SP cells as defined by CD8α/Lyt2.1 and CD4 were found to express also the mutant CD8α/Lyt2.2 allele. Deletion of the CII regulatory region caused variegation of expression of the CD8 locus in the thymus particularly among the DP population.

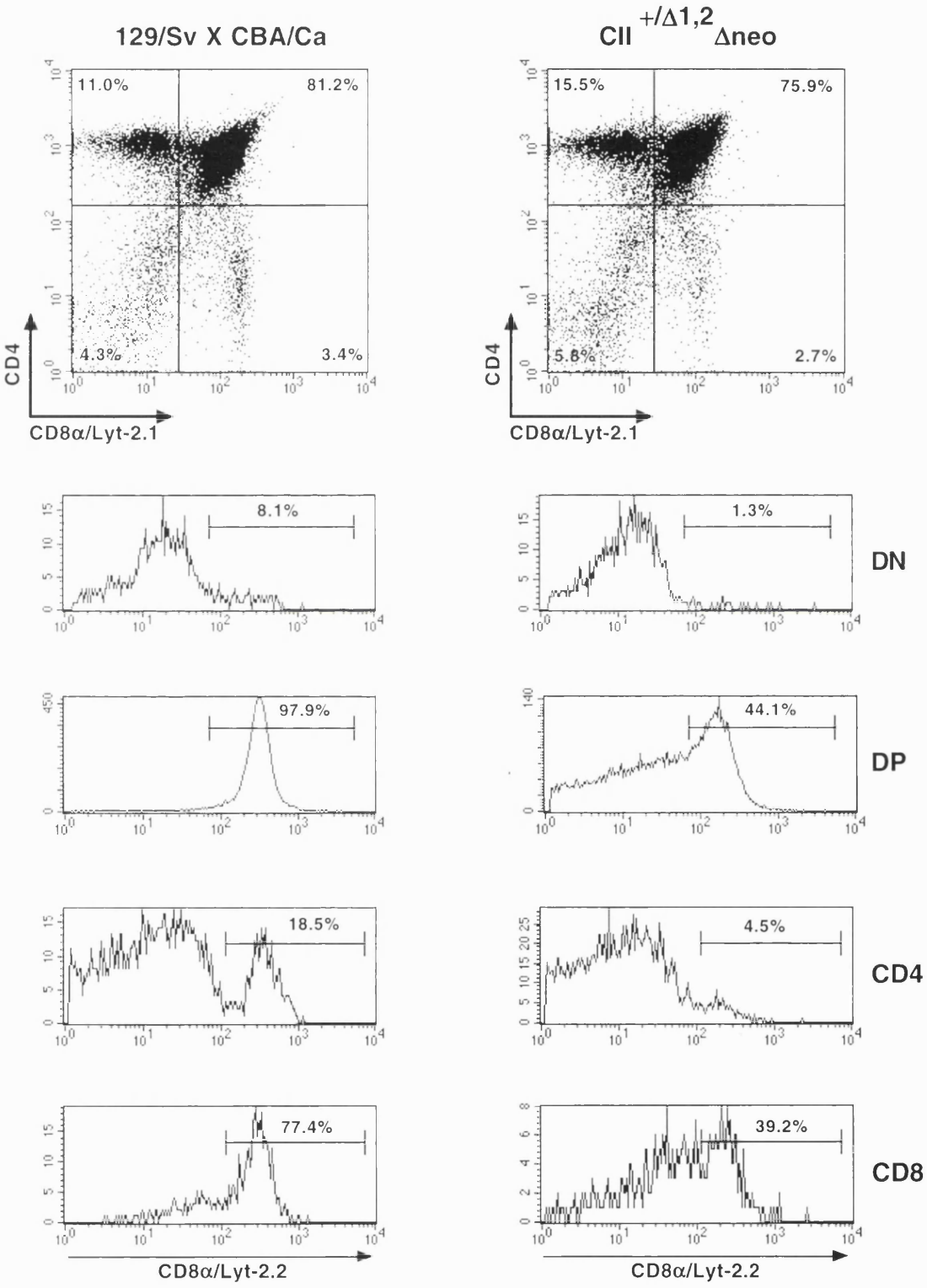
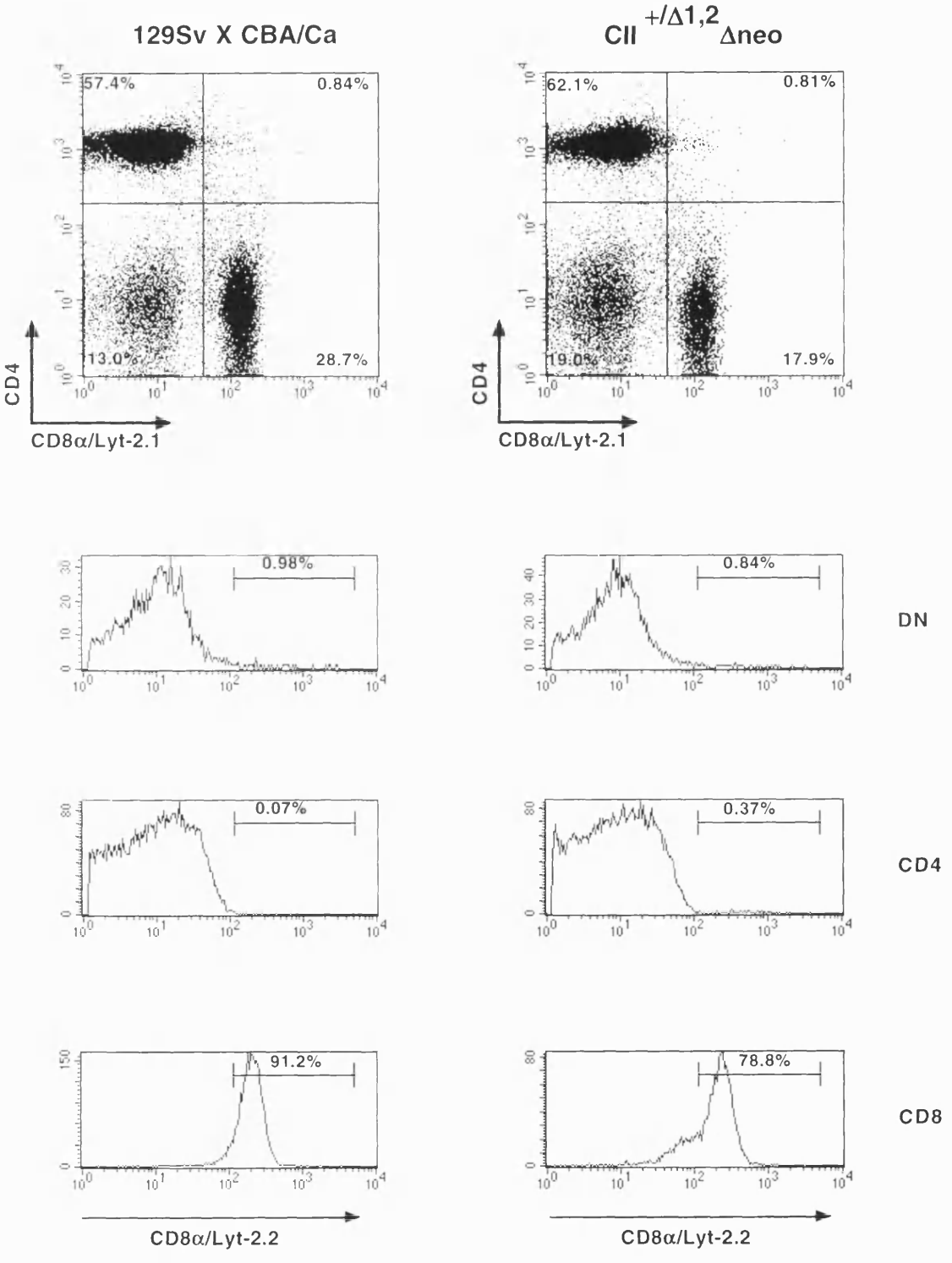

129/Sv X CBA/Ca
CII +/Δ1,2 Δneo
11.0%
81.2%
4.3%
3.4%
15.5%
75.9%
5.8%
2.7%
CD4
CD8α/Lyt-2.1
8.1%
1.3%
DN
97.9%
44.1%
DP
18.5%
4.5%
CD4
77.4%
39.2%
CD8
CD8α/Lyt-2.2

Figure 36: Variegated expression of CD8 ($CII^{\Delta 1,2}$) allele on peripheral lymphocytes of heterozygous $CII^{+/\Delta 1,2}\Delta neo$ mice

FACS analysis of lymph node cells isolated from (129/Sv x CBA/Ca) F1 wild type and $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice. The wild type mice express a wt-CD8.1 allele and a wt-CD8.2 allele, whereas the mutant mice express a $CII^{\Delta 1,2}$-CD8.2 and a wt-CD8.1 alleles. Lymph node cells were isolated and stained with anti-CD8α/Lyt2.1, anti-CD4 and anti-CD8α/Lyt2.2 antibodies. FACS profiles were identified by plotting CD4 against CD8α/Lyt2.1. Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants. Different cell populations (DN, double negative; CD4 and CD8) were electronically gated and analysed for CD8α/Lyt2.2 expression in the histograms shown underneath the corresponding dot-plot. The percentages of cells positive for CD8α/Lyt2.2 are shown in the histograms. In the wild type mice almost all the CD8 SP cells as defined by CD8α/Lyt2.1 and CD4, also express CD8α/Lyt2.2 on their surface. In $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice, in contrast, only a proportion of CD8 SP cells as defined by CD8α/Lyt2.1 and CD4 were found to express also the mutant CD8α/Lyt2.2 allele. In peripheral lymphocytes, there is variegation of expression of the mutant allele, but it is reduced compared to the thymus.


129Sv X CBA/Ca
CII +/Δ1,2 Δneo
57.4%
0.84%
13.0%
28.7%
62.1%
0.81%
19.0%
17.9%
CD4
CD8α/Lyt-2.1
0.98%
0.84%
DN
0.07%
0.37%
CD4
91.2%
78.8%
CD8
CD8α/Lyt-2.2

$CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice have normal CD8α gene expression on both $\gamma\delta TCR^+$ and $\alpha\beta TCR^+$ CD8αα intraepithelial T cells

In order to examine the effect of the $CII^{\Delta 1,2}$ deletion on CD8α gene expression in intraepithelial lymphocytes, IEL were isolated from $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice (carrying the CD8α/Lyt-2.2 allele) and $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mice (carrying one wild type CD8α/Lyt-2.1 allele and one mutatedCD8α/Lyt-2.2 allele). The heterozygous mice were generated by crossing $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mice (carrying the CD8α/Lyt-2.2 allele) to CBA/Ca wild type mice (carrying the CD8α/Lyt-2.1 allele). 129/Sv wild type mice were used as non-transgenic controls, as well as (CBA/Ca x 129/Sv) F1 mice since they carry both the CD8α/Lyt-2.1 (CBA/Ca encoded) and CD8α/Lyt-2.2 (129/Sv encoded) allele.

IEL isolated from the above mice were stained with anti-γδTCR, anti-CD8α/Lyt-2.2 and anti-CD8α/Lyt-2. Subsequently, $\gamma\delta TCR^+$ cells were gated and analysed for CD8α/Lyt-2 and CD8α/Lyt-2.2 expression. Results were very similar and only CD8α/Lyt-2.2 expression is shown in the histograms in figure 37. The percentages of positive cell populations are shown in the histograms. This analysis showed that the percentages of $\gamma\delta TCR^+$ CD8αα IEL isolated from $CII^{\Delta 1,2/\Delta 1,2}$ homozygous and $CII^{+/\Delta 1,2}$ heterozygous mutant mice that expressed CD8α were comparable to those found in 129/Sv and (CBA/Ca x 129/Sv) F1 control mice. In addition, $\gamma\delta TCR^+$ CD8αα IEL isolated from $CII^{\Delta 1,2/\Delta 1,2}$ homozygous mice and 129/Sv wild type controls expressed CD8α at similar levels.

IEL isolated from the same mice were also stained with anti-αβTCR, anti-CD8β and anti-CD8α/Lyt-2 or with anti-αβTCR, anti-CD8β and anti-CD8α/Lyt-2.2. In order to assess CD8α expression on $\alpha\beta TCR^+CD8\alpha\alpha$ IEL (that are thought to be extrathymically derived), $\alpha\beta TCR^+CD8\beta^-$ cells were gated and analysed for CD8α/Lyt-2 and CD8α/Lyt-2.2 expression. Results were again very similar and only CD8α/Lyt-2.2 expression is shown in the histograms in figure 38. The percentages of positive cell populations are shown in the histograms. As it was shown for the $\gamma\delta TCR^+$ IEL, the percentages of $\alpha\beta TCR^+CD8\alpha\alpha$ IEL that expressed CD8α were found to be similar in $CII^{+/\Delta 1,2}$ heterozygous and $CII^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mice compared to the wild type

controls. However, some variation in the levels of CD8α expression was observed between the mice analysed. Further investigation is required to elucidate the effect of the mutation on levels of CD8α expression.

Thus, deletion of DNaseI-HSS CII-1 and CII-2 of cluster II had an effect on CD8α expression on thymocytes and peripheral T cells of $CII^{\Delta 1,2/\Delta 1,2}$Δneo mice, but it did not affect IEL that are thought to be extrathymically derived. This suggests that the cluster II sequences that have been deleted in the mutant mice are responsible for expression of the CD8α in T cells, which have passed through development in the thymus.

Expression of CD8 is affected in αβTCR$^+$ CD8αβ intraepithelial T cells by deletion of CII-1 and CII-2 of cluster II

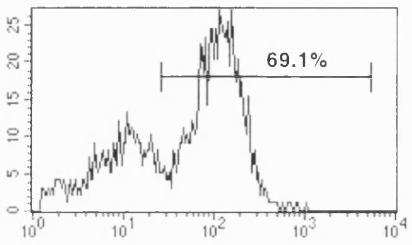
As described above, the $CII^{\Delta 1,2}$ deletion had no effect on intraepithelial lymphocytes that express CD8αα homodimers, maybe because cluster III sequences are still present. In contrast, the deletion was found to cause variegation in the expression of the CD8α/Lyt-2.2 mutated allele in thymocytes and to a lesser extend in peripheral T cells. We sought to investigate whether the same effect was evident in αβTCR$^+$CD8αβ IEL that have gone through normal development in the thymus.

For this purpose, IEL were isolated from $CII^{\Delta 1,2/\Delta 1,2}$Δneo homozygous (carrying the CD8α/Lyt-2.2 allele), $CII^{+/\Delta 1,2}$Δneo heterozygous mice (carrying one wild type CD8α/Lyt-2.1 allele and one mutated CD8α/Lyt-2.2 allele), as well as from (CBA/Ca x 129/Sv) F1 and 129/Sv controls. αβTCR$^+$ cells were gated and were analysed by plotting CD8β against CD8α/Lyt-2 or CD8α/Lyt-2.2. Figure 39 shows the FACS profiles of IEL from the above mice stained with anti-CD8β and anti-CD8α/Lyt-2 (left-hand side) or anti-CD8β and anti-CD8α/Lyt-2.2 (right-hand side). The percentages of cells falling in each gate are shown in the dot plots. This analysis showed that there is a small reduction in the percentage of αβTCR CD8αβ IEL in $CII^{\Delta 1,2/\Delta 1,2}$Δneo homozygous compared to 129/Sv wild type controls. This is consistent with the expression of CD8α in lymph node cells described in a previous section.

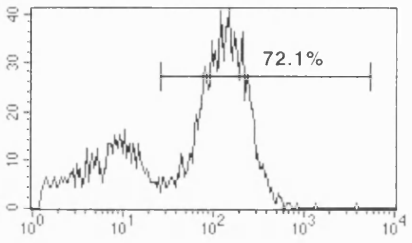
Figure 37: $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mutant mice have normal CD8α gene expression on γδTCR$^+$CD8αα intraepithelial T cells

Intraepithelial T cells were isolated from murine gut of wild type 129/Sv (top histogram), wild type (CBA/Ca x 129/Sv) F1 (second line histograms), $CII^{+/\Delta 1,2}$ heterozygous (third line histograms) and $CII^{\Delta 1,2/\Delta 1,2}$ homozygous (bottom histograms) mice. IEL were stained with anti-γδTCR, anti-CD8α/Lyt-2 and anti-CD8α/Lyt-2.2 specific antibodies. γδTCR$^+$ T cells were gated and analysed for CD8α/Lyt-2.2 expression. The percentages of positive cell populations are shown in the histograms. Mutant mice show normal CD8α expression on γδTCR$^+$ T cells compared to the wild type controls.

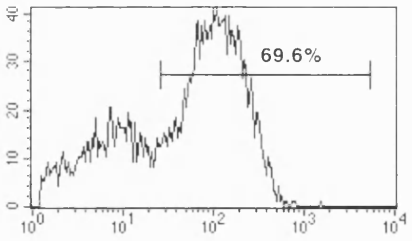
129/Sv
(CD8.2$^{+/+}$)



CBA/Ca X 129/Sv
(CD8.1^{+}/CD8.2^{+})



CII$^{+/\Delta 1,2}$
(CD8.1^{+}/CD8.2$^{\Delta 1,2}$)



CII$^{\Delta 1,2/\Delta 1,2}$
(CD8.2$^{\Delta 1,2/\Delta 1,2}$)

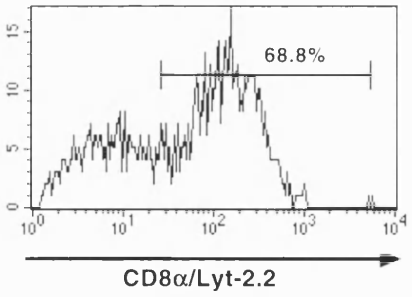
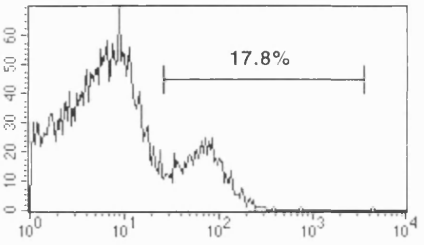


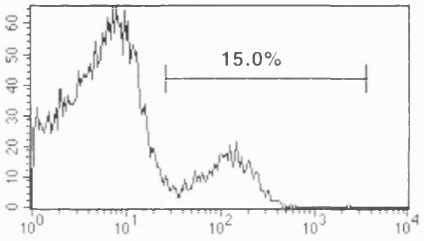
Figure 38: $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mutant mice have normal CD8α gene expression on $\alpha\beta TCR^{+}CD8\alpha\alpha$ intraepithelial T cells

Intraepithelial T cells were isolated from murine gut of wild type 129/Sv (top histogram), wild type (CBA/Ca x 129/Sv) F1 (second line histograms), $CII^{+/\Delta 1,2}$ heterozygous (third line histograms) and $CII^{\Delta 1,2/\Delta 1,2}$ homozygous (bottom histograms) mice. IEL were stained with anti-αβTCR, anti-CD8β and anti-CD8α/Lyt-2.2 specific antibodies. $\alpha\beta TCR^{+}$ T cells were gated and analysed by plotting CD8β against CD8α/Lyt-2.2. Then, $CD8\beta^{-}$ cells were gated and analysed for CD8α/Lyt-2.2 expression. The percentages of positive cell populations are shown in the histograms. Mutant mice show normal CD8α expression on $\alpha\beta TCR^{+}CD8\alpha\alpha$ T cells compared to the wild type controls.

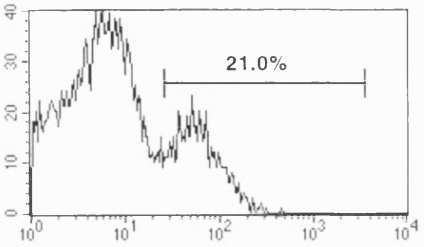
129/Sv
(CD8.2$^{+/+}$)



CBA/Ca X 129/Sv
(CD8.1^{+}/CD8.2^{+})



CII$^{+/\Delta 1,2}$
(CD8.1^{+}/CD8.2$^{\Delta 1,2}$)



CII$^{\Delta 1,2/\Delta 1,2}$
(CD8.2$^{\Delta 1,2/\Delta 1,2}$)

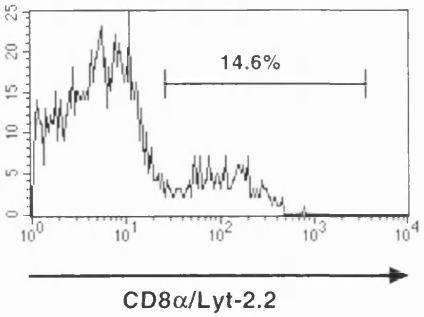
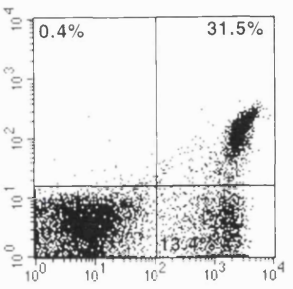
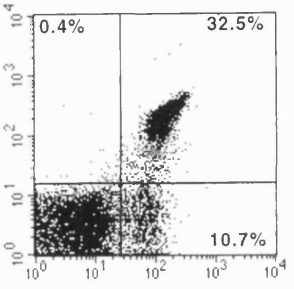


Figure 39: Expression of CD8 is affected in αβTCR$^+$CD8αβ intraepithelial T cells by deletion of CII-1 and CII-2 of cluster II

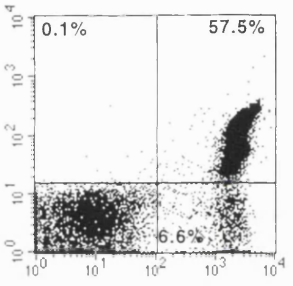
Intraepithelial T cells were isolated from murine gut of wild type 129/Sv (top plots), wild type (CBA/Ca x 129/Sv) F1 (second line plots), CII$^{+/\Delta 1,2}$ heterozygous (third line plots) and CII$^{\Delta 1,2/\Delta 1,2}$ homozygous (bottom plots) mice. IEL were stained with anti-αβTCR, anti-CD8β and anti-CD8α/Lyt-2.2 or anti-CD8α/Lyt-2 specific antibodies. αβTCR$^+$ T cells were gated and analysed by plotting CD8β against CD8α/Lyt-2 (left-hand side) or CD8α/Lyt-2.2 (right-hand side). The percentages of positive cell populations are shown in the dot plots.

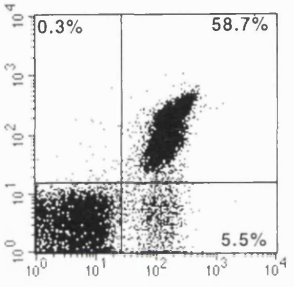
129/Sv
(CD8.2$^{+/+}$)



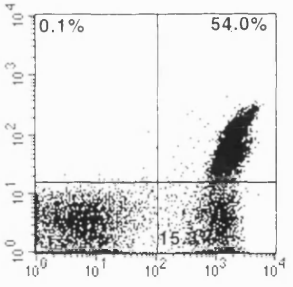


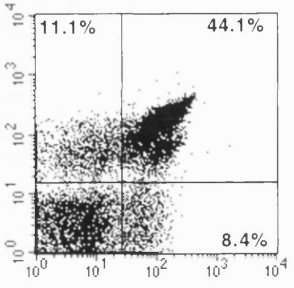
CBA/Ca X 129/Sv
(CD8.1^{+}/CD8.2^{+})



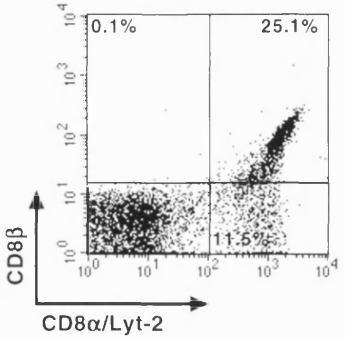


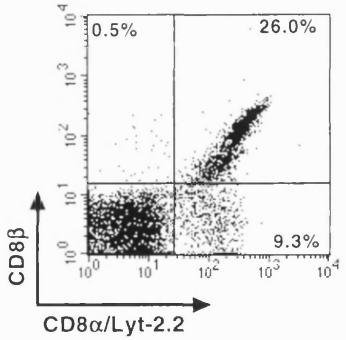
CII$^{+/\Delta 1,2}$
(CD8.1^{+}/CD8.2$^{\Delta 1,2}$)





CII$^{\Delta 1,2/\Delta 1,2}$
(CD8.2$^{\Delta 1,2/\Delta 1,2}$)





Staining of IEL from $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mice with anti-CD8β and anti-CD8α/Lyt-2.2 showed that 11.1% of the $CD8\beta^+$ cells appeared to be $CD8\alpha^-$ and 44.1% were $CD8\alpha^+$. This was not the case when the cells were stained with anti-CD8β and anti-CD8α/Lyt-2, where all the $CD8\beta^+$ cells are also $CD8\alpha^+$. It is known that there are no CD8ββ homodimers and that the β chain can not come to the cell surface without the α chain. Therefore, we assume that the cells that appear to be $CD8\beta^+CD8\alpha$/Lyt-2.2^- express the CD8α/Lyt-2.1 wild type allele on their surface, but not the CD8α/Lyt-2.2 mutated allele. Thus, 78% of the CD8αβ cells as defined by the wild type allele also express the mutated allele on their surface. This is consistent with the variegation in the expression of the CD8α/Lyt-2.2 mutated allele that was observed in peripheral T cells found in lymph nodes (see figure 36 above).

Expression of CD8 is dramatically affected in thymocytes and peripheral T cells by deletion of CII-1 and CII-2 of cluster II in the absence of a second CD8α allele

Deletion of DNaseI-HSS CII-1 and CII-2 of cluster II caused a substantial reduction in the percentage of thymocytes and peripheral T cells that express CD8α in $CII^{\Delta 1.2/\Delta 1.2}\Delta neo$ mice compared to the wild type control. In order to examine the expression of one mutated allele alone in the absence of second CD8α allele, $CII^{-/\Delta 1,2}$ mice were generated. For this purpose, a $CII^{+/\Delta 1,2}\Delta neo$ ($CD8^{+/\Delta 1,2}$) heterozygous mutant mouse was crossed to a CD8α heterozygous null ($CD8^{+/-}$) mouse. The offspring of this cross would have the following genotypes in terms of CD8 expression: $CD8^{+/+}$ (expressing two wild type alleles), $CD8^{+/-}$ (expressing one wild type allele), $CD8^{+/\Delta 1,2}$ (expressing one mutated allele and one wild type allele) and $CD8^{-/\Delta 1,2}$ (expressing only one mutated allele). A $CD8^{\Delta 1,2/\Delta 1,2}$ homozygous mouse (expressing two mutated alleles) was also used for the experiment. Thymocytes and lymph node cells from these mice were isolated and stained with anti-CD4 and anti-CD8α/Lyt-2. Cells were analysed by plotting CD4 against CD8α/Lyt-2 and gating the different cell populations. The percentages of cells falling in each gate are shown in the dot plots.

Figure 40 (left-hand side) shows the FACS profiles of thymocytes isolated from the mice described above and stained with anti-CD4 and anti-CD8α/Lyt-2. The

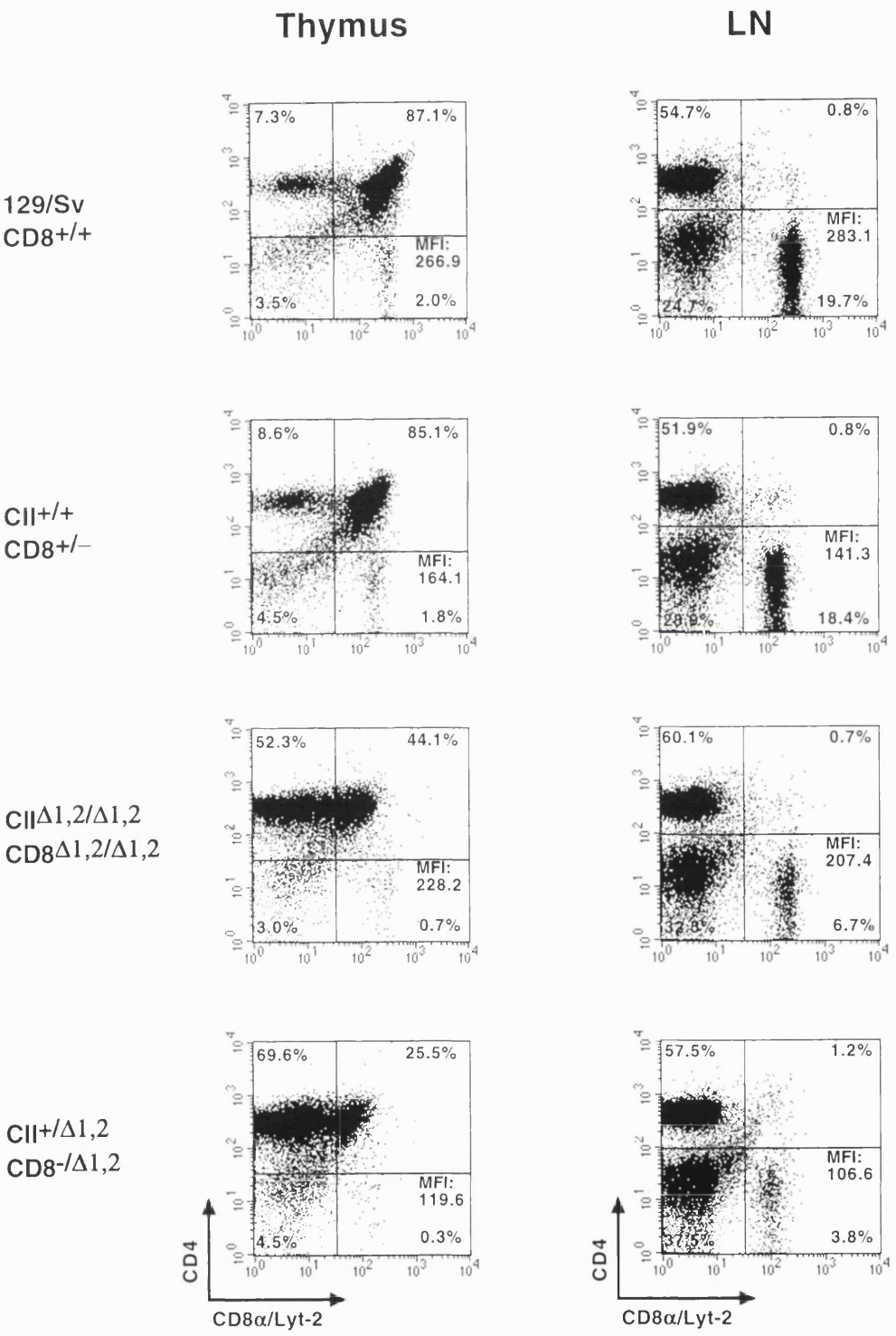
heterozygous null mouse, that has only one functional allele, expresses CD8α at lower levels that the wild type control, but the percentages of cells falling in each gate are the same as in the wild type control mouse. The $CD8^{\Delta 1,2/\Delta 1,2}$ homozygous mutant mouse, that has two mutated alleles, expresses CD8α at lower levels than the wild type mouse. In addition, in this mouse the percentages of DP and CD8 SP cells are reduced, as a result of the deletion mutation. In the $CD8^{-/\Delta 1,2}$ mouse, that expresses only one mutated allele, the effect is even more dramatic. The percentages of DP and CD8 SP thymocytes in this mouse are reduced compared to the $CD8^{\Delta 1,2/\Delta 1,2}$ homozygous mutant control mouse and those cells express CD8 at even lower levels compared to the control. Furthermore, the percentage of cells falling in the CD4 gate is increased, showing a further block in the DN to DP transition of thymocytes.

Similarly, in the peripheral T lymphocytes of $CD8^{-/\Delta 1,2}$ mice (figure 40, right-hand side) the percentage of CD8 SP cells that leave the thymus and populate the lymph nodes is reduced compared to the $CD8^{\Delta 1,2/\Delta 1,2}$ homozygous control. Those cells express CD8α at even lower levels than the control mice.

Thus, in $CD8^{-/\Delta 1,2}$ mice (in the absence of a second CD8α allele), there are very few cells that get to the DP stage and even fewer cells manage to pass through selection in the thymus and migrate to populate peripheral lymphoid organs.

Figure 40: Expression of CD8 is dramatically affected in thymocytes and peripheral T cells by deletion of CII-1 and CII-2 of cluster II in the absence of a second CD8α gene

Thymocytes (left-hand side) or lymph node cells (right-hand side) were isolated from mice carrying two wild type CD8α alleles ($CD8^{+/+}$) (top plots), or one wild type allele ($CD8^{+/-}$) (second line plots), or two mutated alleles ($CII^{\Delta 1,2/\Delta 1,2}$) (second line plots), or one mutated allele ($CII^{-/\Delta 1,2}$) (bottom plots). Cells were stained with anti-CD4 and anti-CD8α/Lyt-2 specific antibodies. T cell and thymocyte subsets were resolved by plotting CD4 against CD8α and analysed for CD8α expression. Percentages of $CD4^+$ SP, $CD8^+$ SP, $CD4^+CD8^+$ DP and $CD4^-CD8^-$ DN cell subsets are indicated in the quadrants and mean fluorescence intensities (MFI) for CD8α are also shown. Expression of CD8 is dramatically affected in the mutant mice in the absence of a second CD8α gene.


Thymus
LN
129/Sv
CD8+/+
7.3%
87.1%
MFI:
266.9
3.5%
2.0%
54.7%
0.8%
MFI:
283.1
24.7%
19.7%
CII+/+
CD8+/–
8.6%
85.1%
MFI:
164.1
4.5%
1.8%
51.9%
0.8%
MFI:
141.3
28.9%
18.4%
CIIΔ1,2/Δ1,2
CD8Δ1,2/Δ1,2
52.3%
44.1%
MFI:
228.2
3.0%
0.7%
60.1%
0.7%
MFI:
207.4
32.8%
6.7%
CII+/Δ1,2
CD8-/Δ1,2
69.6%
25.5%
MFI:
119.6
4.5%
0.3%
57.5%
1.2%
MFI:
106.6
37.5%
3.8%
CD4
CD8α/Lyt-2
CD4
CD8α/Lyt-2

Kinetics of thymocyte maturation in $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice

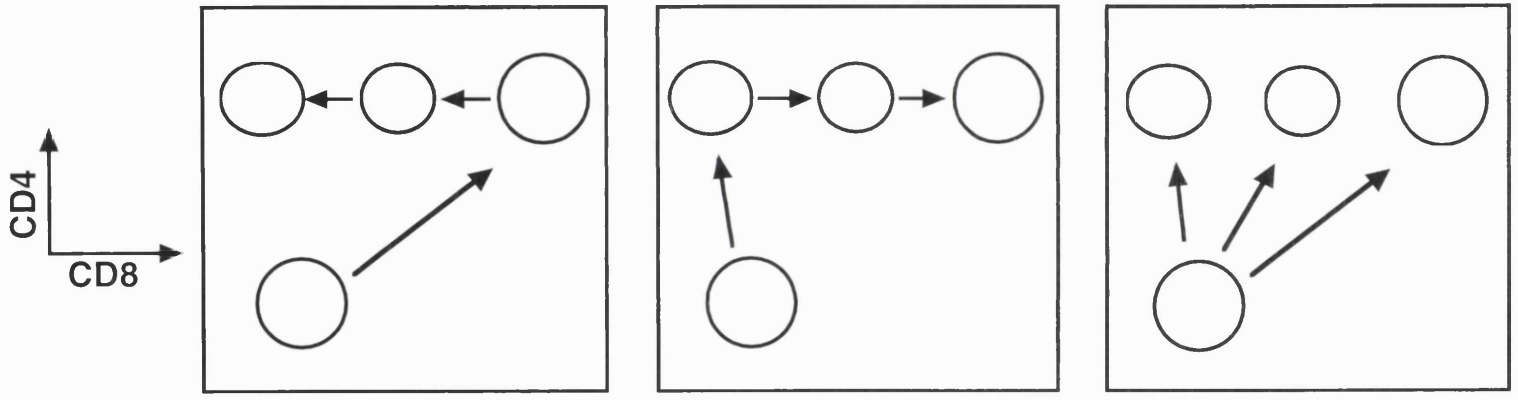
Cluster II DNaseI-HSS was shown to play a role in the regulation of expression of the CD8α gene in transgenic animal studies (Hostert et al, 1998). Deletion of DNaseI-HSS CII-1 and CII-2 of cluster II from the mouse genome resulted in different FACS profiles of thymocytes of $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice compared to wild type controls when they were stained with antibodies against CD4 and CD8. In this analysis, an increased number of immature cells was observed in the CD4 gate and it is not clear whether these cells differentiate directly from immature $CD4^-CD8^-$ DN cells or they were $CD4^+CD8^+$ DP cells that lost or have not acquired yet expression of CD8 on their surface. Thus, there are three main possibilities for the kinetics of thymocyte maturation in $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice, that are shown in figure 41. The first possibility is that the CD8α is turned on on all cells and there is normal progression from the DN to the DP stage, but they do not maintain the CD8 on the surface because of the lack of Cluster II. The second possibility is that the transcriptional machinery in the mutant mice is slower than in wild type animals, therefore, thymocytes aquire CD8 slowly and become DP. However, most of them do not reach the DP stage within their 3 day life span. Finally, there is the possibility that all subpopulations of immature thymocytes expressing normal, low levels or no CD8 at all on their surface, appear directly from the DN population simultaneously. The latter possibility is highly reminiscent of the phenomenon of Position Effect Variegation (PEV)

One means of evaluating the precursor/product relationship between the DP and the CD4 SP thymocytes in the $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mutant mice, is to chart the flow of 5-bromo-2-deoxyuridine (BrdU) into the different thymocyte populations. BrdU is incorporated into the DNA of cycling cells and has been employed to study the dynamics of thymocyte populations in normal animals (Lucas et al, 1993). The BrdU labelling experiments (data not shown) suggested that the two populations incorporate BrdU at the same rate. However, from this experiment it is not clear whether the cells in the CD4 intermediate gate differentiate from cells in the DN or the DP compartment. Furthermore, the presence of cells with immature phenotype within the CD4 gate that were proliferating (and thus incorporated BrdU), complicated the interpretation of the result.

Figure 41: Kinetics of generation of the different immature subpopulations in $CII^{\Delta 1,2/\Delta 1,2}$ mutant mice

Diagram of three probable ways of thymocyte maturation in $CII^{\Delta 1,2/\Delta 1,2}$ mutant mice. Circles indicate cell populations and arrows show transition of cells from one population to the other during maturation. Cells could activate the CD8 locus, but gradually loose CD8 gene expression (left-hand side), they could aquire CD8 expression slowly (middle) or there could be variegation of CD8 expression with some cells activating the CD8 locus and some cells remaining negative (right-hand side).

Hypothesis



Loss of CD8 expression

Slow aquisition of CD8 expression

Variegated CD8 expression

In order to demonstrate that there are proliferating cells in the CD4 intermediate and CD4 gates, thymocytes were stained with anti-CD4, anti-CD8α/Lyt-2 and 7AAD. figure 42 shows 7AAD labeled thymocytes from C57Bl/10 wild type (left-hand side) and $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice (right-hand side). This experiment showed that in C57Bl/10 control mice there were dividing cells in the DN (A) and DP (B) compartments as well as in the CD8 (E). In contrast, in the CD4 intermediate (C) and the CD4 (D) gates, there were no proliferating cells. In the $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice, however, there was 7AAD labeling in all the compartments, which indicated that there were dividing cells in all the gates.

An alternative means of monitoring thymocyte maturation is to follow their generation through ontogeny. The first precursor T cells derived from haemopoietic stem cells in the foetal liver or the bone marrow in the adult, migrate to populate the thymus at day eleven of embryonic development (Owen and Ritter, 1969), where they develop into mature T cells. Until day 15 of foetal development all thymocytes are double negative and it is at day 16 of gestation that double positive cells first appear.

In order to follow the generation of thymocytes through ontogeny in the mutant mice, $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice of 129/Sv background (carrying the CD8α/Lyt-2.2 allele) were crossed to CBA/Ca wild type mice (carrying the CD8α/Lyt-2.1 allele). The offspring of this breeding are $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice that carry one CD8α/Lyt-2.1 wild type allele (CBA/Ca encoded) and one CD8α/Lyt-2.2 mutated allele (129/Sv encoded). (CBA/Ca x 129/Sv) F1 mice that carry both the CD8α/Lyt-2.1 and CD8α/Lyt-2.2 wild type alleles were used as non-transgenic controls. At day 15 of gestation the thymuses were removed from the embryos and were used for foetal thymus organ cultures (FTOC).

Thymocytes were stained immediately after the thymuses were removed from the embryos and after one day and three days of culture with anti-CD4, anti-CD8α/Lyt-2.1 and anti-CD8α/Lyt-2.2. Figure 43 shows the FACS profiles of thymocytes from (CBA/Ca x 129/Sv) F1 wild type mice (left-hand side) and $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant mice (right-hand side) stained with the above combination of antibodies at day 15 of embryonic development (top panels) or after one day in culture. Cells were analysed by plotting CD8α/Lyt-2.2 against CD4. Percentages of cells in each subpopulation are

shown in the FACS dot plots. In the figure it is apparent that at day 15 of embryonic development, all thymocytes were DN. After one day in culture, the first DP thymocytes started to appear in the wild type control (bottom panel, left-hand side). However, in the $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mutant embryonic thymus there were cells in both the DP and the CD4 compartments. This result suggested that there is a variegation in CD8 expression in the absence of cluster II from the mouse genome. After only one day in culture, there were already immature thymocytes expressing normal, low levels or no CD8 at all on their surface and there was not enough time for cell movement from one compartment to the other. Therefore, it seams that all those subpopulations appeared at the same time.

The thymic development was followed in vitro until day three of culture. Figure 44 summarises the FTOC experiment. The thymocytes of $CII^{+/\Delta 1,2}\Delta neo$ embryonic thymuses were stained with anti-CD4, anti-CD8α/Lyt-2.1 and anti-CD8α/Lyt-2.2. Cells were analysed by plotting CD8α/Lyt-2.1 against CD4 and electronically gating the DP population (gate A). Gated cells were then analysed for the expression of CD8α/Lyt-2.2 in the histogram next to the FACS dot plot. Percentages of negative (gate B) or positive (gate C) cells for CD8α/Lyt-2.2 expression are shown in the table underneath the two plots. The variability between the samples in each population is indicated by the standard deviation, also shown in the table. Seven thymuses were analysed the first day and 3 the third day of the FTOC experiment. The percentages of both $CD4^+CD8\alpha/Lyt\text{-}2.1^+/Lyt\text{-}2.2^-$ and $CD4^+CD8\alpha/Lyt\text{-}2.1^+/Lyt\text{-}2.2^+$ subpopulations were very similar between day 1 and day 3 of culture. This suggests that cells expressing high or low levels of CD8α/Lyt-2.2 appeared at the same rate. This result supports the hypothesis that all cells appear simultaneously and directly from the DN population. It is possible that this reflects a form of variegation of expression of the CD8 mutant allele, resulting from the removal of DNaseI-HSS CII-1 and CII-2 of cluster II. In the mutant mice, some thymocytes early in their development decide to open up the CD8 locus and become $CD4^+CD8^+$ double positive cells, while some others do not activate the locus and give rise to immature thymocytes that appear as single positive CD4 cells due to the lack of CD8.

Figure 42: Thymocytes appearing within the CD4 SP gate include a large number of cycling cells

FACS analysis of 129/Sv wild type and $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ homozygous mutant mice. Thymocytes were isolated and stained with anti-CD8α/Lyt-2, anti-CD4 and 7AAD. Cell populations were identified by plotting CD4 against CD8α/Lyt-2. Different cell populations were electronically gated as indicated in the dot-plots (gates A, B, C, D and E) and analysed for 7AAD labeling in the histograms shown underneath the corresponding dot-plot. The percentages of cells labeled with 7AAD are shown in the histograms. Next to the histograms are shown the different gates (left-hand side) and the gated cell populations (right-hand side) (DN, double negative; DP, double positive; CD4 INT, CD4 intermediate; CD4 and CD8). In $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ mice in gates (C) and (D) there is an increased number of 7AAD labeled cells compared to the wild type control, which indicates that there is a large proportion of cycling cells within these populations.

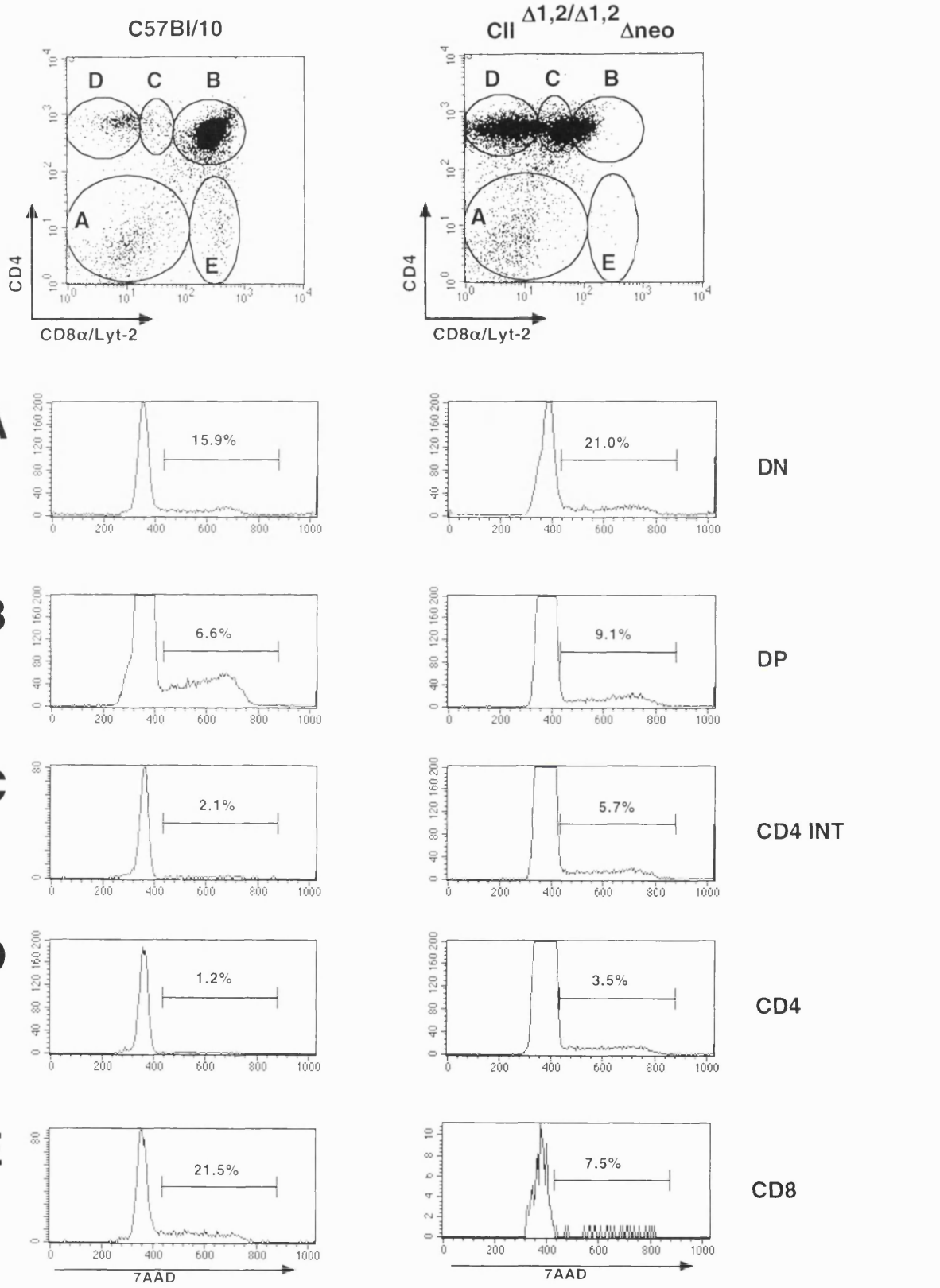

C57Bl/10
CII Δ1,2/Δ1,2 Δneo
D C B
A E
CD4
CD8α/Lyt-2
A
15.9%
21.0%
DN
B
6.6%
9.1%
DP
C
2.1%
5.7%
CD4 INT
D
1.2%
3.5%
CD4
E
21.5%
7.5%
CD8
7AAD
7AAD

Figure 43: In $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mice both CD4 SP and DP cells appear within 24 hours

Day E15 foetal lobes from $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ heterozygous mice and (129/Sv x CBA/Ca) F1 wild type control mice were placed in culture and allowed to develop for further one or three days. The mutant mice express a $CII^{\Delta 1,2}$-CD8.2 and a wt-CD8.1 alleles. Thymocytes were isolated from the foetal lobes and stained with anti-CD8α/Lyt-2.1, anti-CD4 and anti-CD8α/Lyt-2.2 antibodies. FACS analysis of thymocytes isolated from foetal lobes of $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mice and (129/Sv x CBA/Ca) F1 controls at day E15 (top panels) or after one day in culture (bottom panels). Cells were analysed by plotting CD4 against CD8α/Lyt-2.2. Percentages of $CD4^+$ SP, $CD8^+$ SP and $CD4^+CD8^+$ DP cell subsets are indicated in the quadrants. In (129/Sv x CBA/Ca) F1 wild type mice, mostly DP thymocytes appear within 24 hours. In contrast, in $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mice within 24 hours both CD4 SP and DP cells are evident.

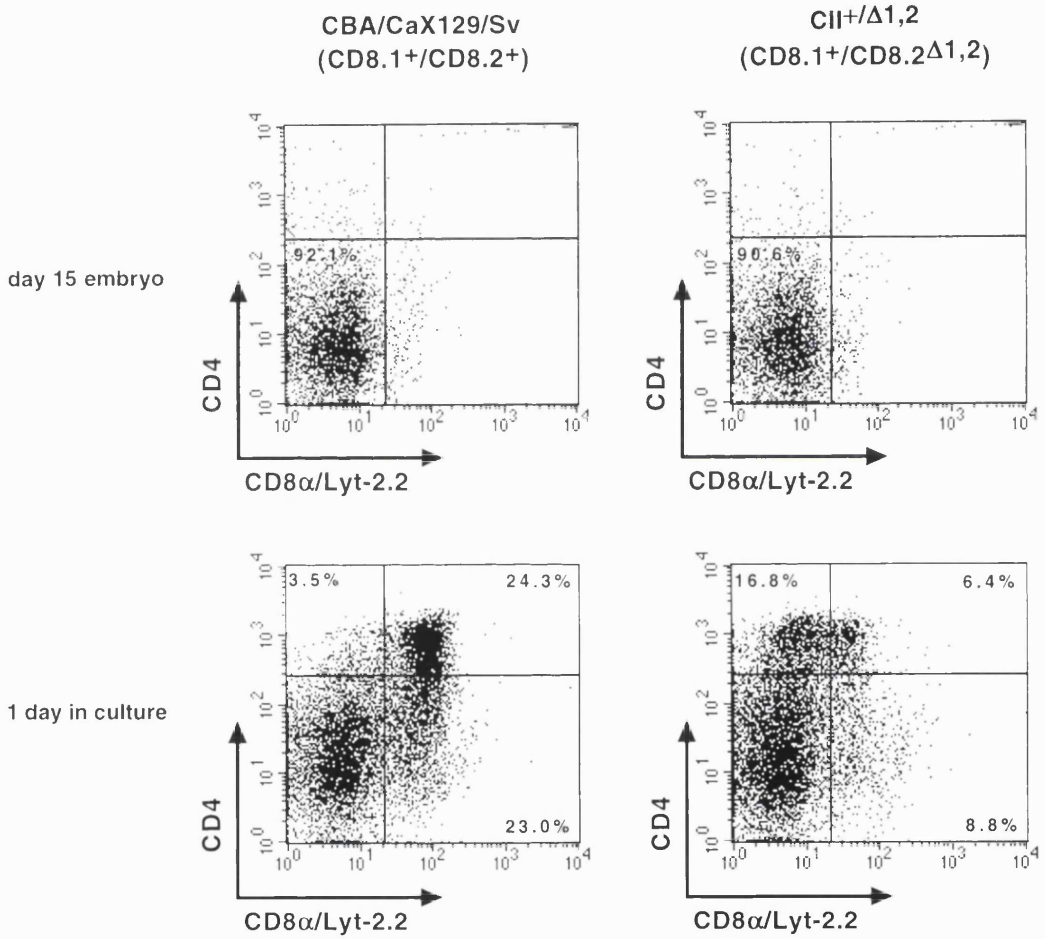
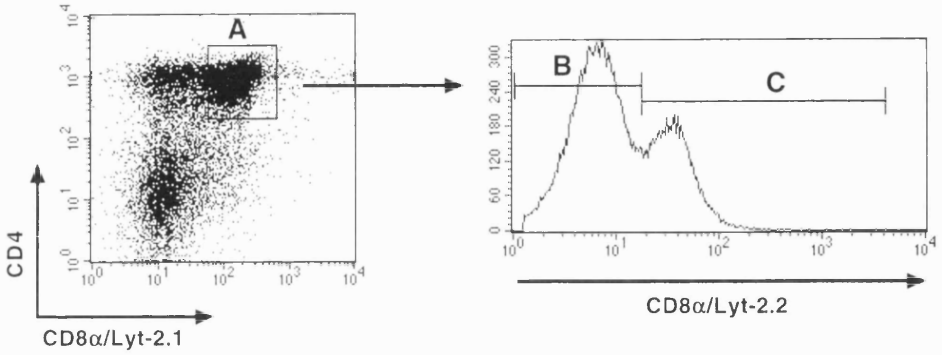

CBA/CaX129/Sv
(CD8.1+/CD8.2+)
CII+/Δ1,2
(CD8.1+/CD8.2Δ1,2)
day 15 embryo
1 day in culture
92.1%
90.6%
3.5%
24.3%
23.0%
16.8%
6.4%
8.8%
CD4
CD8α/Lyt-2.2

Figure 44: In $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mice both CD4 SP and DP cells appear at the same rate through ontogeny

Day E15 foetal lobes from $CII^{\Delta 1,2/\Delta 1,2}\Delta neo$ heterozygous mice and (129/Sv x CBA/Ca) F1 wild type control mice were placed in culture and allowed to develop for further one or three days. The mutant mice express a $CII^{\Delta 1,2}$-CD8.2 and a wt-CD8.1 alleles. Thymocytes were isolated from the foetal lobes and stained with anti-CD8α/Lyt-2.1, anti-CD4 and anti-CD8α/Lyt-2.2 antibodies. Thymocytes isolated from foetal lobes of $CII^{+/\Delta 1,2}\Delta neo$ heterozygous mice were analysed by plotting CD4 against CD8α/Lyt-2.1. DP thymocytes were electronically gated as indicated in the dot-plot (gate A) and analysed for CD8α/Lyt-2.2 expression in the histogram shown next to the dot-plot. Cells negative (gate B) or positive (gate C) for CD8α/Lyt-2.2 were gated. Percentages of cells within the $CD4^+CD8.1^+CD8.2^+$ (gate C) or the $CD4^+CD8.1^+CD8.2^-$ (gate B) population in day 1 and day 3 of culture are shown. The number of foetal lobes analysed each day is shown (n=7 for day 1 and n=3 for day 3) The standard deviation of the samples for each population and each day is also shown. The rate at which the DP and the CD4 SP populations appear is the same between these two time points. Those two populations arise simultaneously and independently from each other.

A



B

Gate	Phenotype	% of each subset Day 1	% of each subset Day 3
B	CD4+CD8α/Lyt-2.1+/Lyt-2.2-	70.1±5.6 (n=7)	75.7±5.4 (n=3)
C	CD4+CD8α/Lyt-2.1+/Lyt-2.2+	30.1±5.6 (n=7)	24.4±5.4 (n=3)

DISCUSSION

Subset specific and developmentally regulated expression of CD8 has been achieved by the generation of transgenic mice carrying a 80kb genomic fragment from the murine CD8 locus (Hostert et al., 1997). This suggested that the major regulatory elements for expression of the CD8α and β genes are localised within this genomic fragment. This result is consistent with the finding that a 95kb genomic fragment spanning the human CD8β gene could direct expression of CD8β on DP thymocytes and mature CD8 SP T cells in transgenic mice (Kieffer et al., 1997). DNaseI-HSS analysis over the CD8 gene locus revealed four clusters of DNaseI-HSS, three of which (clusters II, II and IV) were found to be specific for T cells. Further deletion analysis using a combination of HSS or clusters to prepare reporter constructs and analysing them for enhancer activity in transgenic mice was very important to characterise the role of individual DNaseI-HSS within the 80kb murine CD8 locus (Hostert et al., 1997, Hostert et al., 1998, Ellmeier et al., 1997, Ellmeier et al., 1998, Zhang et al., 1998). In order to determine the role of different DNaseI-HSS clusters in CD8 gene regulation in the context of the endogenous chromosomal location, we deleted these regions from the mouse genome by homologous recombination.

Gene targeting

The 'targeted mutagenesis' technology to derive mice for the study of gene function has been used extensively. In the vast majority of these targeting experiments, embryonic stem (ES) cells derived from 129 mouse substrains have been an essential component. For reasons that are not completely understood, 129 genetic background is particularly permissive for the derivation of ES cell lines that can be manipulated in culture while remaining competent to repopulate the mouse germline. As a result, new mutations are usually derived within the 129 mouse genome. The two different ES cell lines (D3 and PC3) used in this work are both of 129 origin.

Maintaining a mutation on a standard inbred background has numerous advantages, such as reproducible results, reduced variability between samples and availability of genetically defined controls. In the case that a different genetic background is required for the analysis of the mutation, the mice carrying the desired mutation can be backcrossed to a mouse strain different than the 129. However, it is easier and faster to breed the chimera to 129 wild type mice and, thus, keep the mutation

in the 129 background. Usually, the chimera is bred to C57Bl/10 or C57Bl/6 mice (black coat) in order to take advantage of the coat colour to determine germline transmission (agouti). Chimeras with the higher percentages of germline transmission are bred to 129 wild type mice in order to derive the mutation in the 129 background. The latter was done in the work described in this thesis and all the homozygous mutant mice analysed are in the 129 background, with the exception of the $CIII^{\Delta 1,2,3}$ deletion. In this case, the chimera gave germline transmission only once, when crossed to a C57/Bl/10 wild type mouse. The heterozygous mouse that resulted from this breeding was used to generate the colony of $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ mutant mice and therefore this mutation is in a mixed background.

The genetic purity of the resulting 129 mouse depends on two main factors. One of them is the match between the ES cell line and the DNA used for the generation of the targeting construct. The use of isogenic DNA constructs has been proven very important, for both keeping the genetic purity of the line generated, as well as obtaining higher efficiency in the gene targeting (te Riele et al., 1992). Base-pair mismatches may strongly affect the efficiency of homologous recombination. The second factor is the match between the ES cell line and the mouse strain to which the chimera is bred. An effort is made to use wild type mice of 129 origin for breeding with chimeras in order to have the new mutations in a genetically pure background. However, it has been shown that there are numerous 129 substrains, that vary extensively among each other (Simpson et al., 1997). The history of 129 substrains is very complex and confusing and 129 is not considered an inbred line today.

The two ES cell lines (D3 and PC3) used to generate the knockout mice described in this thesis are both of 129/Sv origin. However, they belong to different substrains, the D3 being of 129/SvPas origin and the PC3 of 129/SvJae origin. For reasons mentioned above, all the DNA fragments used to generate the targeting constructs were of 129/Sv origin and either existed in the laboratory, or were obtained from the library screening described in the results section. In addition, the chimeras were bred to 129/SvEv wild type mice kept in the animal colony, which is a different substrain than the ES cells. Therefore, some variation in the levels of CD8α expression in our mutant mice could be due to genetic variability between the different 129 substrains.

The targeting efficiency for different genes can vary considerably. This can be due to different sizes of targeting vectors, the transcriptional activity of the targeted gene or the chromosomal location of the gene. A lot of variation in the targeting efficiency was observed in the experiments described here, although they all involve the same gene locus. This could be due to different length of upstream and downstream homologies used in each targeting vector, as well as different length of deleted regions in each case.

In order to exclude any possibility that the phenotype observed in the mutant mice is due to a mutation acquired by ES cells as a result of the targeting process, but not due to the deletion introduced with the targeting vector, two individual clones were used to generate mutant mice whenever possible. Unfortunately, this was not possible for the $CIII^{\Delta 1,2,3}$ deletion mutation, because only one chimera gave germline transmission, and for the $CII^{\Delta 2}$ mutation, because only chimeras coming from a single clone gave germline transmission. However, the phenotype of the $CIII^{\Delta 1,2,3}$ deletion was the same as the $CIII^{\Delta 1,2}$ deletion and the $CII^{\Delta 2}$ mutation did not have any significant phenotype.

Cre/loxP System

The Cre/loxP system has been used extensively for conditional mutations in order to achieve gene deletion in a time- and/or tissue specific manner or for deletion of selectable markers that are left in the genome after gene targeting experiments (Jiang and Gridley, 1997). In the work described here, different regulatory regions of the CD8α gene are deleted from the genome in an effort to investigate their function in their normal chromosomal location and are replaced by the neomycin gene. The selectable marker is flanked by loxP sites in order to be able to delete it and exclude any influence it might have in the phenotype of the mutation (also see below).

In three of the mutant mouse lines described here the neomycin gene was deleted after introducing Cre. This was done either by injecting a Cre expressing plasmid into oocytes ($CIII^{\Delta 1,2,3}$ and $CII^{\Delta 2}$ deletion) or by using the PC3 ES cell lines for the targeting experiment ($CII^{\Delta 1,2}$ deletion), which expresses Cre under the protamine 1 promoter and induces deletion in the male germline (O'Gorman et al., 1997). In the $CIII^{\Delta 1,2,3}$ deletion there was no change in the phenotype of the mutation before and after deletion of the neomycin gene, whereas in the $CII^{\Delta 2}$ deletion there was a difference that is discussed in a

later section. In contrast, in the $CIII^{\Delta 1,2}$ deletion the neomycin gene was not deleted and the phenotype described here refers to a mutant mouse that has the neo integrated in the genome. However, the neomycin gene was deleted by Ellmeier et al (1998) in a very similar deletion mutation. That experiment showed that there was no difference in the phenotype as a result of the presence of neo.

The Cre recombinase catalyses the recombination between loxP sites with high efficiency. However, chronic high-level expression of Cre recombinase in developing spermatids has been reported to lead to male sterility (Schmidt et al., 2000). The cause of sterility is likely because of Cre-mediated genomic rearrangements, perhaps at pseudo-loxP sites within the mouse genome. Such sites have been identified within the mouse and the human genomes and have been shown to function in *in vitro* assays (Thyagarajan et al., 2000). In order to avoid any unwanted rearrangement caused by Cre, it is very important to remove or inactivate it after deletion is achieved. In our mutant mice the expression of Cre was transient for the $CIII^{\Delta 1,2,3}$ and $CII^{\Delta 2}$ deletions and the Cre DNA does not integrate in the oocyte genome with high efficiency and therefore it should not cause any problem. In the $CII^{\Delta 1,2}$ deletion, however, the gene encoding Cre recombinase is integrated in the mouse genome and is expressed in the late stages of spermatogenesis. In this case, after the desired deletion was achieved, the mice were screened for the presence of Cre (data not shown) and the ones that did not carry it in their genome were used for further breeding.

Deletion of cluster III DNaseI-HSS CIII-1 and CIII-2

From transgenic mice analysis, a subregion was identified that contains one cluster of tissue specific DNaseI-HSS, cluster III. This cluster was found to be sufficient to direct reporter transgene expression to the mature CD8 SP T cells and CD8αα intraepithelial lymphocytes. In contrast, expression of the transgene reporter was absent from the immature DP thymocytes in those transgenic mice (Ellmeier et al., 1997, Hostert et al., 1997).

The role of CIII$^{\Delta 1,2}$ deletion in thymus-derived T cells

In order to examine the role of CIII DNaseI-HSS we generated mice that lack two of the three DNaseI-HSS of this cluster. Staining of T cells from spleen, lymph nodes and thymus with CD4 and CD8 antibodies showed no significant difference both in percentages of cells that express CD8 on their surface and in levels of CD8 expression, between 129/Sv control mice and our mutant mice. Thus, the deletion of two DNaseI-HSS of cluster III had no effect on expression of the CD8α in the CD8αβ SP compartment. This could be due to the fact that this mutation deletes only two of the three DNaseI-HSS of cluster III and, thus, it leaves behind the third HSS of this cluster. From transgenic mice analysis (Hostert et al., 1997) it is known that DNaseI-HSS CIII-1 and CIII-2 alone or in combination are sufficient to direct expression of a linked reporter transgene in CD8 SP T cells, as the whole of cluster III. Therefore it is possible that the third site alone can also direct expression of the CD8α gene on T cells of thymic origin. Another possible explanation is that additional *cis*-acting regulatory elements are involved in the regulation of CD8 expression in thymus derived T cells, such as elements in the CD8β gene or within cluster II, which are able to compensate for loss of cluster III activity.

The role of CIII$^{\Delta 1,2}$ deletion in intestinal IEL

Intraepithelial lymphocytes (IEL) are either of αβTCR or γδTCR lineage. The ones that are of αβTCR lineage express CD8αα homodimers or CD8αβ heterodimers on their surface, whereas the ones that are of γδTCR lineage express only CD8αα homodimers. The CD8αα$^+$ IEL (of both the αβTCR and γδTCR lineage) are primarily of extrathymic origin, whereas the CD8αβ$^+$ IEL require the thymus for development (Poussier and Julius, 1994, Rocha et al., 1995). Recent studies on the methylation state of cytosines of an approximately 1kb portion of the CD8β gene 5' regulatory region suggested that CD8αα$^+$ IEL have never previously expressed CD8β (Hamerman et al., 1997).

In our mutant mice we observed a dramatic reduction of the CD8α gene expression in CD8αα$^+$ IEL of both the αβTCR and γδTCR lineage. In contrast, the CD8αβ$^+$ IEL showed normal CD8α expression, as it was shown for other thymus derived

T cells. This suggests that in the presence of an active CD8β gene, cluster III activity is redundant in CD8αβ T cells. In IEL, however, which have not activated the CD8β gene, cluster III becomes important for CD8α gene expression. Alternatively, the regulatory element lying in the cluster III region might be singularly responsible for CD8α expression in the extrathymically derived lymphocyte population and other regulatory elements exist within the CD8 locus that direct CD8 expression in immature thymocytes and peripheral CD8 T cells.

The reason for loss of CD8αα expression on most, but not all $CIII^{\Delta 1,2}$-null IEL is not known. If cluster III is responsible for CD8α expression in extrathymically derived IEL, it is possible that CD8α expression is not completely lost because of the third DNaseI-HSS of cluster III that is still present in the mutant mice. It is also possible that there are sublineages of γδTCR and αβTCR IEL that differ in their ability to employ one or more enhancer elements other than the DNaseI-HSS CIII-1 and CIII-2. These other enhancers may be able to partially compensate in the $CD8\alpha\alpha^{low}$ αβTCR or γδTCR lineage.

Cluster III expression is restricted to the *cis* allele

It is possible that regulatory elements can act *in trans* via a diffusible product (RNA or protein) and therefore a mutation in one allele may disturb the expression of the other allele. In order to address whether the influence of the deletion of cluster III DNaseI-HSS is restricted to the *cis* CD8α allele, we analysed heterozygous mice that had one wild type CD8α/Lyt-2.1 allele and one mutant CD8α/Lyt-2.2 allele. This analysis showed that CD8α/Lyt-2.2 expression was absent from $CD8\alpha\alpha^{+}$ IEL of both the αβTCR and γδTCR lineage, whereas CD8α/Lyt-2.1 expression from the other allele was unaffected. We conclude, therefore, that the cluster III DNaseI-HSS are unable to stimulate CD8α gene transcription *in trans*.

Deletion of cluster III DNaseI-HSS CIII-1, CIII-2 and CIII-3

The deletion of two of the three DNaseI-HSS of cluster III did not affect the expression of the CD8α gene in thymocytes or mature T cells of the mutant mice. This could be due to the presence of the third DNaseI-HSS of this cluster in the genome of the mutant mice. In order to investigate further the function of cluster III, mice that lack all three DNaseI-HSS of cluster III were generated.

Analysis of $CIII^{\Delta 1,2,3}$-null mice showed that FACS profiles of T cells and thymocyte subsets were similar for both wild type and mutant mice with no significant differences in either percentages of subsets or levels of CD8α expression. Thus, deletion of all three sites of cluster III DNaseI-HSS had no effect on expression of the CD8α gene in the CD8αβ SP compartment. The most possible explanation (like it was suggested above) is that there are additional *cis*-acting regulatory elements that are involved in the regulation of CD8 expression in thymus derived T cells, such as elements in the CD8β gene, which are able to compensate for the loss of cluster III activity.

The role of $CIII^{\Delta 1,2,3}$ deletion in intestinal IEL

The effect of $CIII^{\Delta 1,2,3}$ deletion on CD8α gene expression in thymus-independent IEL was examined. The expression of CD8αα homodimers of the γδTCR lineage was severely compromised in $CIII^{\Delta 1,2,3}$-null mice, similarly to what was shown for the $CIII^{\Delta 1,2}$ deletion. Surprisingly, this was not true for the $\alpha\beta TCR^{+}$ CD8αα IEL isolated from these mice. In this cell subset, the percentage of cells expressing CD8α was sometimes much lower than the wild type controls, while some other times the percentage in the mutant mice was comparable to the controls. However, in the latter cases the levels of CD8α expression were lower in the mutant mice compared to the wild type controls. This difference in the results could be explained with the difference in the age of mice used for the experiments. It is known that IEL appear late in life and increase in numbers with aging (Rocha et al., 1992, Klein, 1996). It is possible that, for unknown reasons, IEL of αβTCR lineage are accumulated in mice faster than γδTCR IEL. Therefore, when older $CIII^{\Delta 1,2,3}$-null mice are used for an experiment, they would show percentages of CD8α expressing cells comparable to the controls in αβTCR IEL, whereas in γδTCR lineage IEL the CD8α expressing cells would be lower in the mutant mice

compared to the controls. In contrast, when younger mice are analysed, the percentages of cells expressing CD8α within both αβTCR and γδTCR IEL would be lower in $CIII^{\Delta 1,2,3}$-null compared to the wild type controls.

Alternatively, the difference in the percentage of $\alpha\beta TCR^+$ CD8αα IEL and levels of CD8α expression in $CIII^{\Delta 1,2,3}$-null mice between experiments could be due to genetic variability in the line. As it was mentioned above, the $CIII^{\Delta 1,2,3/\Delta 1,2,3}$ line is in a mixed background (129/Sv and C57Bl/10) and it is possible that this gives rise to genetic differences between mice of the same line.

The result of the $CIII^{\Delta 1,2,3}$ deletion in the IEL is not conclusive and it must be further investigated. However, it supports the conclusion from the previous knockout, that in the presence of an active CD8β gene cluster III activity is redundant in CD8αβ T cells. In IEL, however, which have never activated the CD8β gene, cluster III becomes important for CD8α gene expression. The possibility that cluster III is responsible for CD8α expression in extrathymically derived lymphocytes (of γδTCR lineage) and not in thymus dependent T cells can not be excluded.

Deletion of cluster II DNaseI-HSS CII-2

The cluster II DNaseI-HSS is located immediately upstream of the CD8α gene. This cluster alone was not sufficient to direct expression of a CD8α transgene in any thymocyte or T cell subset (Hostert et al., 1997), but in conjunction with cluster III it restored transgene expression in the immature DP thymocytes. In addition, CII-2 was absent in DNaseI treated splenocyte nuclei. This suggested that an element responsible for expression in immature thymocytes lies in this region (Hostert et al., 1998). In order to identify regulatory elements responsible for CD8 expression in DP thymocytes, we generated mice that lack CII-2 from their genome.

The effect of the neomycin gene in the phenotype of the $CII^{\Delta 2}$ deletion

The $CII^{\Delta 2}$ deletion caused a disturbance in the normal developmental expression of CD8, resulting in the appearance of double positive cells in peripheral lymphoid organs. One explanation for this could be that there is a silencing element lying in the cluster II region which is responsible for absence of CD8 expression on CD4 SP cells. It

is known that a silencing element is involved in the regulation of CD4 (Sawada et al., 1994, Siu et al., 1994), but there is no indication so far about the presence of such an element in the regulation of CD8 expression. However, if this is true, it is not easy to explain the expression of CD8 only on a proportion of the CD4 cells. Alternatively, this aberrant expression could be due to the presence of the neomycin gene in the place of cluster II DNaseI-HSS CII-2 left behind following the targeting event.

It has been reported that in studies of complex regulatory elements *in vivo* (Fiering et al., 1995, Mei et al., 2000) or studies of gene function, the presence of the inserted selectable marker can influence the phenotype of the mutation or the expression of neighbouring genes (Rijli et al., 1994). Therefore, it is very important that the selectable marker is removed from the mouse genome. For this reason, the neomycin gene was flanked with loxP sites in all the targeting experiments described here and it was deleted from the mouse genome by introducing Cre recombinase.

After deletion of the neomycin gene, the $CII^{\Delta 2}$ deletion did not show any effect in the expression of the CD8α gene. Thus, the phenotype of the mutation was an effect of the selectable marker. In previous reports about the effects of selectable markers in the phenotypes of mutations, these effects were negative. Thus, the presence of the neomycin gene in the regulatory region of a gene either decreased the expression level of the relevant gene (Mei et al., 2000) or affected the expression of the gene causing a lethal phenotype that was not real (Fiering et al., 1995).

In contrast, in the $CII^{\Delta 2}$ deletion, the presence of the neomycin gene had a positive effect, as it caused expression of CD8α on some peripheral CD4 cells that would not normally express CD8 on their surface. One possible explanation for this is the following: the promoter of the CD8α gene lies in third DNaseI-HSS of cluster II. This means that in the mutant mice the neomycin gene is situated very close to the CD8α gene promoter. It is possible that the presence of the neomycin gene promoter, although in the opposite transcriptional orientation, can cause some run through transcripts of the CD8α gene. It is also possible that the presence of the neomycin gene, can allow binding of transcription factors that can mediate transcription of the CD8α gene in the CD4 cells. However, the reason for the effect being present in some and not all the CD4 cells is not clear.

The role of $CII^{\Delta 2}$ deletion in thymus-derived T cells and intestinal IEL

Analysis of $CII^{\Delta 2/\Delta 2}$ mice after deletion of the neomycin gene showed that these mice had normal CD8α gene expression in CD8αβ SP cells and in $CD8\alpha\alpha^{+}$ IEL of both γδTCR or αβTCR lineage. Transgenic mice analysis results suggested that an element responsible for CD8α expression in immature DP cells lies in CII-2. However, deletion of this region did not have any effect on CD8α expression in thymocytes. This could be due to the fact that in the $CII^{\Delta 2}$ deletion, the first DNaseI-HSS of cluster II is still present in the mutant mice. There is now evidence in the laboratory, that this alone is sufficient to restore expression of a transgene in the double positive thymocytes, just as all three sites of cluster III are individually sufficient to direct expression of a linked reporter gene to mature CD8 SP T cells. There seems to be a lot of genetic redundancy in the regulatory elements controlling CD8α gene expression, with some of them having apparently the same function with others and being able to compensate for the loss of some.

Deletion of cluster II DNaseI-HSS CII-1 and CII-2

The cluster II consists of three DNaseI-HSS, as mentioned above. It contains the promoter of the CD8α gene, which is believed to coincide with the third HSS of the cluster, and two possible upstream regulatory elements (Hostert et al., 1997). From transgenic mice analysis there is indication that these regulatory elements are responsible for expression of a CD8α reporter gene in immature thymocytes (Hostert et al., 1998, Zhang et al., 1998). In order to investigate the role of cluster II in the regulated expression of the CD8α gene in thymocytes and mature T cells we generated mice that lack cluster II DNaseI-HSS CII-1 and CII-2.

The role $CII^{\Delta 1,2}$ deletion in thymus-derived T cells and intestinal IEL

Analysis of $CII^{\Delta 1,2}$-null mice showed that the $CII^{\Delta 1,2}$ deletion had two main effects: it affected the levels of expression of the CD8 gene and caused an abnormal subset distribution in the thymus with fewer DP and CD8 SP cells and an increase in cells with a CD4 SP phenotype. Staining with several markers that are modulated in different stages of thymocyte development, showed that the increased numbers of

thymocytes falling within the CD4 SP gate are immature cells that would normally appear in the DP gate, but fail to do so due to the lack of CD8 expression on their surface. Thus, the $CII^{\Delta 1,2}$ deletion did not affect the lineage commitment decisions towards CD4 cells. Rather, it disturbed the proper developmental expression of CD8 on a proportion of thymocytes.

In order to examine the effect of the $CII^{\Delta 1,2}$ deletion in intraepithelial lymphocytes, IEL were isolated and stained with the appropriate antibodies for both γδTCR and αβTCR cells. This analysis showed that the $CII^{\Delta 1,2}$ deletion did not have any effect on IEL that are extrathymically derived. This suggested that the cluster II sequences that were deleted in the mutant mice are responsible for expression of the CD8α in T cells, which have passed through development in the thymus. The normal CD8α expression on IEL of mutant mice also indicated that the promoter of the CD8α gene is functional and, thus, the phenotype of the mutation in thymocytes and peripheral T cells is not due to a defect in the promoter caused by the deletion mutation. In contrast, the CD8αβ expressing IEL of $CII^{\Delta 1,2}$-null mice showed a similar phenotype to the rest of the peripheral T cells.

As was mentioned before, in mice lacking the cluster II regulatory sequences only a proportion of thymocytes manage to activate the CD8 locus, whereas a large number of the cells fail to do so. One explanation for this phenomenon is that there are two distinct populations amongst the DP cells, one which is dependent on the presence of cluster II in order to activate the locus and one which is able to open the locus by using some other regulatory region in the locus (eg cluster IV) that has also been implicated in the early activation of the locus (Zhang et al., 1998, Ellmeier et al., 1998, Hostert et al., 1998). It is possible, that the cells that depend on cluster II for activation of the locus do not manage to do so in the absence of this cluster, while the cells that use cluster IV do open the locus and give rise to the DP cells that exist in the mutant mice.

In $CII^{\Delta 1,2}$-null thymuses the two allelic CD8 loci are regulated independently

In $CII^{\Delta 1,2}$-null mice the increase of immature, phenotypically CD4 SP cells and their inability to express the CD8 gene could be a direct *cis* effect on the locus due to the removal of cluster II sequences. Alternatively, this could be the result of an effect *in*

trans that results in a developmental disturbance at the DN to DP transition. In order to distinguish between these two possibilities, advantage was taken of the existence of two allelic forms of the CD8α gene (Lyt-2.1 and Lyt-2.2), for which specific monoclonal antibodies are available. Analysis of heterozygous mice indicated that deletion in one of the alleles did not affect the developmental progression to the DP stage. The two alleles seemed to be regulated independently and in the presence of an intact CD8α locus that allows normal maturation of thymocytes, the expression of the mutated allele is still perturbed. Thus, the phenotype observed in $CII^{\Delta 1,2}$-null mice is due to a direct *cis* effect on the activation of expression of the CD8 locus, caused by the deletion of the cluster II regulatory sequences. Therefore, the influence of the deletion of cluster II DNaseI-HSS is restricted to the *cis* CD8α allele, as it was shown for cluster III in a previous section.

Another indication that the two CD8 alleles are regulated independently and they can be turned on and off at different times came from analysis of (CBA/Ca x 129/Sv)F1 wild type mice that carry both CD8α alleles. Thymocytes from these mice were stained anti-CD8α/Lyt-2.1, anti-CD8α/Lyt-2.2 and anti-CD4. Subsequently, CD4 SP cells (as defined by CD4 and CD8α/Lyt-2.1 alleles) were gated and analysed for CD8α/Lyt-2.2 expression. This analysis showed that a small proportion of these cells still had the CD8α/Lyt-2.2 allele on their surface, although they had switched off the CD8α/Lyt-2.1 allele and looked CD4 SP in that respect.

A result of this independent regulation of the two CD8α alleles is that in heterozygous mutant mice the cells are allowed to reach the DP stage because the wild type allele is expressed normally, but the mutation has affected the normal developmental expression of the mutated allele. Therefore, only a proportion of the DP cells (as defined by the wild type allele) were also found to express the mutated allele. This is consistent with a hypothesis that deletion of the cluster II regulatory region caused variegation of the expression of the CD8 locus in the thymus particularly among the DP population. Similar analysis of peripheral lymphocytes showed that variegation of the mutant allele was reduced in the periphery. This could be possible if the cells that express both alleles had an advantage in maturation, migration and survival processes. Alternatively, some of the cells that were positively selected while expressing only the wild type allele in the thymus, gradually managed to activate the CD8 gene on the mutated locus with time.

Kinetics of CD8 locus activation during thymocyte maturation in $CII^{\Delta 1,2}$-null mice

Deletion of cluster II regulatory sequences from the CD8 locus resulted in a *cis* effect that led to its impaired activation. Thymocytes from $CII^{\Delta 1,2}$-null mice contained a large number of immature 'DP' cells that do not express the CD8 gene. However, it is not clear whether a) these cells differentiated from cells that had activated the CD8 locus and gradually lost expression of the CD8α gene, or b) they represent cells that are slow in activating the CD8 locus, or finally, c) whether these cells arise simultaneously and directly from the DN population bearing a CD8 locus that is either activated or silenced. The latter possibility is highly reminiscent of the phenomenon of Position Effect Variegation (PEV).

Initial experiments trying to delineate the precursor/product relationship between the DP and CD4 SP thymocytes in the $CII^{\Delta 1,2}$-null mice using BrdU labelling showed that the two populations incorporated BrdU at the same rate suggesting simultaneous generation of these populations from the DN precursors. However, the presence of cycling cells within these populations as indicated by 7AAD staining complicated the interpretation of the result.

Subsequently, the appearance of the different thymocyte populations of $CII^{\Delta 1,2}$-null mice during ontogeny was examined. This experiment showed that CD4 SP and DP thymocytes (within the genuine DP population) accumulated at the same rate. This suggested that these two populations do not have a precursor/product relationship, but they arise simultaneously directly from the DN population. If this is true, it is possible that it reflects a form of variegation of expression of the CD8 mutant locus, resulting from the removal of HSS CII-1 and CII-2 of cluster II, with some cells activating the CD8 locus while in some other cells the CD8 locus remains silent. However, we have not excluded formally the possibility that there is a precursor/product relationship between the different populations.

Are there immature CD8 SP cells CII$^{\Delta 1,2}$-null mice?

It is known that during T cell development in the thymus, in the DN to DP transition the cells pass through an intermediate CD8 SP stage (Paterson and Williams, 1987). These cells are immature and express only the CD8 coreceptor on their surface. Subsequently, they turn on also the CD4 molecule and they become DP cells. In the CII$^{\Delta 1,2}$-null mice the immature CD8 cells are affected in numbers, but still exist. Staining thymocytes with antibodies against different maturation markers showed the same pattern of expression between the mutant mice and the wild type controls. Thus, there was a small proportion of cells with immature characteristics within the CD8 SP thymocytes. This result was further confirmed from the 7AAD labelling experiment, where a higher percentage of CD8 SP than CD4 SP cells were labeled. 7AAD labelling is indicative of cycling and, thus, immature cells. However, the percentage of labeled cells in the CII$^{\Delta 1,2}$-null mice is much lower than the control, which could mean that the mutant mice have a decreased CD8 SP population. The presence of a reduced immature CD8 SP population in the CII$^{\Delta 1,2}$-null mice can explain the existence of the DP cells in these mice. It also explains the existence of fewer DP thymocytes in the mutant mice and which arise from the DN population through the immature CD8 SP cells.

Possible involvement of chromatin structure in the phenotype of the CII$^{\Delta 1,2}$ deletion

There is a possibility that the regulation of chromatin structure of the CD8 locus is perturbed in CII$^{\Delta 1,2}$-null mice. The ability to express or not a gene depends on the state of chromatin. Co-operation of all the regulatory elements that are acting within the locus ensures the timely and tissue specific expression of the gene. Removal of a gene from its normal chromosomal location, such as in translocations or transgenesis, can result in different patterns of expression. This phenomenon is known as Position Effect Variegation (PEV) (Festenstein et al., 1996, Kioussis and Festenstein, 1997). In such cases, within a population of cells belonging to the same lineage and containing identical genetic information, some cells open the locus and express the genes, whereas some cells silence them. The silencing is usually associated with heterochromatinisation of the locus (Karpen, 1994).

In the work described here the locus has not been removed from its natural location, but a crucial element for its function has been removed. However, the phenotype of the mutation bears many similarities to PEV: cells from the same lineage that have identical genetic information either decide to open up the CD8 locus and become DP thymocytes or decide to close down the locus and give rise to immature cells that appear like CD4 SP cells. There has been one other report that describes similar disturbances in expression due to deletion of regulatory elements. In that case, it was shown that deletion of the Ig enhancer results in variegating expression of the immunoglobulin locus (Ronai et al., 1999).

A possible hypothesis in how chromatin is involved in the phenotype of the $CII^{\Delta 1,2}$ deletion is the following: during the transition from DN to DP a process is initiated which results in opening the CD8 locus in all thymocytes. These initiation events involve chromatin remodelling which results in the establishment of an active/open chromatin domain (Paro, 1995). However, maintenance of an active chromatin state may be the responsibility of other regulatory elements within the locus. It is possible that cluster II is important for these initiation events and deletion of cluster II sequences makes the cells vulnerable to suppressive effects and results in random activation of the locus. In the cells in which activation of the CD8 locus is achieved other regulatory regions take over the maintenance, such as cluster III sequences, which have been shown to direct expression of reporter genes only in mature CD8 SP cells in transgenic studies. Such a specialised role in initiating a specific chromatin state has been recently reported for the CD4 silencer. In that case it was shown that the silencer element was necessary to establish an inactive state of the CD4 gene in some cells, but it was not needed for the maintenance of that inactive state (Zou et al., 2001).

Another possible explanation for the partial activation of the CD8 locus in $CII^{\Delta 1,2}$-null mice, is the deletion of sequences with matrix attachment characteristics that have been mapped in the cluster II region (Lee et al., 1994, Banan et al., 1997). Matrix attachment regions (MARs) can anchor a locus in specific sites within the nucleus of cells. Removal of such sequences from the CD8 locus of developing thymocytes may result in inaccessibility of the locus to necessary factors for its activation.

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