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B cell antigen receptor expression and signalling

A dissertation submitted to the University of Cambridge
in candiditure for the degree of Doctor of Philosophy
1997

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Summary

The B cell antigen receptor (BCR), composed of surface immunoglobulin associated with the Ig-α/Ig-β heterodimer, mediates the antigen-dependent activation of B lymphocytes. Transmembrane signalling by the BCR occurs via the immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic domains of both Ig-α and Ig-β. The BCR of the IgM class, comprising two μ heavy chains and two light chains (κ or λ), plays a critical role not only in activation, but also in early development of the B cell.

In order to discriminate between the functions of the Ig-α and Ig-β polypeptides, transgenic mice expressing chimeric IgM BCR transgenes were analysed. Surface IgM molecules fused directly to the cytoplasmic domains of either Ig-α or Ig-β were sufficient to signal B cell activation in primary cells and in cell lines in response to antigen. In transgenic mice, they could also mediate the antigen-independent development of mature peripheral B cells and allelic exclusion of endogenous immunoglobulin heavy chains. However, when the Ig-β ITAM was mutated, signalling was abrogated. Thus, it appears that the individual Ig-α or Ig-β cytoplasmic domains can broadly perform the signalling activities of the intact BCR; a function for which an ITAM is essential.

The transcriptional activity of the Ig κ light chain enhancers was characterised in transgenic mice bearing different transgenes containing either the κ intron enhancer (Ei) or the κ 3′ enhancer (E3′), or both. In spleen, the expression of a rearranged κ transgene versus endogenous κ suggested that E3′, in the absence of Ei, is not only sufficient to drive transcription, but also becomes active early enough in B cell development to effect allelic exclusion. The activation of E3′ at this early stage is consistent with the finding that a rearranged μ transgene driven by the κ enhancers was able to rescue development blocked at the late pro-/pre-B cell stage in *μMT* B cell-deficient mice. Indeed, the expression of a human β-globin reporter transgene linked to E3′ in different bone marrow subsets indicates that E3′ does become transcriptionally active in pre-B cells, at a stage prior to Vκ rearrangement.

Declaration

This dissertation describes research carried out from January 1994 until March 1997 at the Medical Research Council Laboratory of Molecular Biology, Cambridge, UK. It is entirely my own work except David Gilmore operated the FACScan for flow cytometric analysis in chapters 3 and 6, and Andy Riddell operated the FACStar Plus for cell sorting in chapter 6. Sarah Davies performed the microinjection of zygotes for the transgenic mice in chapter 4, and as part of a collaborative project, Kerstin Meyer performed the RT-PCR analysis in chapter 6 .

I declare that my dissertation is not substantially the same as any that I have submitted for a degree of diploma or other qualification at any other University, and further, that no part has already been or is being concurrently submitted for any such degree, diploma or other qualification.

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April 1997

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Abbreviations

BCR	B cell antigen receptor
bp	basepair
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
C region	constant region of Ig
Con A	concanavalin A
cpm	counts per minute
D region	diversity region of Ig
DMEM	Dulbecco's minimum essential medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediamineglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Ei	immunglobulin κ light chain intron enhancer
E3'	immunglobulin κ light chain 3' enhancer
Eμ	immunoglobulin heavy chain enhancer
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	gram
GC	germinal centre
h	hour
H chain	heavy chain of Ig
HEL	hen egg lysozyme
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine phosphoribosyl transferase
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
ITAM	immunoreceptor tyrosine-based activation motif
J region	joining region of Ig
kb	kilobase
κ	immunoglobulin κ light chain
l	litre
L chain	light chain of Ig

LPS	lipopolysaccharide
m	milli
M	molar
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
min	minute
μ	immunoglobulin μ heavy chain, or micro
n	nano
n	number in study or group
NP-40	Nonidet P-40
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
PNA	peanut agglutinin
RBC	red blood cell
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
SA	streptavidin
SH domain	Src homology domain
SDS	sodium dodecyl sulphate
TdT	terminal deoxynucleotidyl transferase
TEL	turkey egg lysozyme
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling
U	unit
V	variable region of Ig

Acknowledgements

Many thanks to Michael Neuberger for his supervision and encouragement, and to Gareth Williams for his help and inexhaustible patience.

Thanks also to Sarah Davies for microinjection and sharing the frustrations of mice (ugh!!); Theresa Langford and Mike Norris and their staff for animal handling; David Gilmore and Andy Riddell for FACS operation; Facundo Batista, Anne Corcoran and Kerstin Meyer for comments on my draft attempts; and to all else in the lab and the LMB for their help, moral support and lots of fun! (at the LMB??)

Finally, I have greatly valued the love, support and prayers of my family.
Thank you Father God for Your guidance, grace and innumerable blessings.

Introduction

Chapter 1

Chapter 1: Introduction

1.1 B cell antigen receptor structure and function

1.1.1 Selection theories predicted an antigen receptor on B cells.

The immune system defends the body from disease and infection. Even before attack from bacteria, viruses and other pathogens, it is equipped to recognise and eliminate foreign antigens. Our understanding of the response mediated by antibodies (composed of immunoglobulin [Ig] molecules produced by B lymphocytes) has come a long way since the initial discovery of the *Antikörper* (antibody) in 1890 (von Behring and Kitasato, 1890). Immunity to diphtheria and tetanus was shown to be due to a large protein in the blood, a discrete entity that carried specificity against the toxins. A few years later, Ehrlich, studying the interactions between antigen and antibody, developed the side-chain theory and was the first to suggest the existence of a cell-surface receptor for toxin (antigen) (Ehrlich, 1897; Ehrlich, 1900). He proposed that certain cells having a natural affinity for toxin would bind toxin molecules via specific receptors on the surface, assimilate the toxin as a metabolite, and regenerate the receptors. Large amounts of toxin would cause the cell to over-compensate for the loss of the side-chain receptors and to regenerate excess side chains that were released into the body as anti-toxins (antibodies).

From the 1930s, the enormous range in antibody diversity (Landsteiner, 1933) favoured an instruction hypothesis where the antibody moulded its polypeptide sequence around the antigen template in a complementary conformation (Breinl and Haurowitz, 1930; Haurowitz, 1952; Pauling, 1940). However, the theory was later dismissed by the central dogma that information flowed in only one direction from DNA to RNA to protein (Crick, 1958), and that protein folding is determined by its polypeptide sequence, indicating that the specificity of an antibody must be encoded by its genes.

In the suggestion that the immune response was selective (rather than instructive) and that the production of pre-exisiting antibodies was enhanced after antigen recognition (Jerne, 1955), Ehrlich's original concept was revived in the late 1950s. These ideas developed into Burnet's clonal selection theory (Burnet, 1959): from the large pool of potential antibody-producing cells (each capable of making a different antibody), recognition of the antigenic determinant by a cell would trigger its clonal expansion. Daughter cells possessing the same properties for antigen recognition would be generated, or the clones would undergo cellular differentiation for

antibody secretion.

An important prediction to arise from the work of Ehrlich and Burnet was that a receptor must exist on the surface of the cell that would recognise the antigen in a similar way to the antibody itself.

Not long after publication of the clonal selection theory, rabbit lymphocytes were shown to proliferate *in vitro* when stimulated by anti-Ig antisera, indicating that a form of antibody was present on the B cell surface (Sell and Gell, 1965). Confirmation of surface Ig on lymphocytes came from direct fluorescent or radioactive detection (Pernis *et al.*, 1970; Raff *et al.*, 1970). These results also explained the background staining in earlier experiments when indirect immunofluorescence was used to detect histocompatibility antigens on living lymphocytes (Möller, 1961). Thus it was possible that antibody itself could act as the B cell antigen receptor (BCR). However, it was not clear until the development of molecular cloning, how the antibodies that were secreted and those that were expressed on the surface could share the same specificity for antigen.

1.1.2 Immunglobulin can be expressed in secretory and membrane forms.

Antibodies are made up of disulphide-linked Ig heavy (H) and light (L) chains containing amino-terminal variable (V) regions determining the antigen specificity and carboxy-terminal constant (C) regions (Fig. 1.1A). The five H chain isotypes (μ, δ, γ, α, ε) give rise to different classes (IgM, IgD, IgG, IgA and IgE, respectively) and can combine with either of the two L chain isotypes (κ and λ).

The μ H chain is the first class of BCR expressed during development. Its gene encodes two distinct forms which are generated by alternative splicing: a membrane form of μ, as well as that for secretory antibodies (Alt *et al.*, 1980; Early *et al.*, 1980; Rogers *et al.*, 1980). Both mRNAs encode the same V region and they differ only in their 3′ ends at Cμ4. Instead of the secretory tailpiece, the alternative splice of two 3′ exons (M1 and M2) generates the membrane form containing extracellular spacer, transmembrane and cytoplasmic regions appended to the Cμ4 exon. However, the short cytoplasmic tail thus determined only contains three amino acids, calling into question how surface IgM could transmit signals. It was suggested that IgM may be associated with other proteins in order to mediate the biological activities of the BCR.

1.1.3 Membrane Ig associates with the Ig-α/Ig-β heterodimer as the BCR complex. Plasmacytoma cell lines represent the terminal stage of development, when B cells have differentiated to secrete large amounts of antibody, and contain predominantly secretory transcripts. Transfection of the membrane-only form of μ resulted in its expression, but not its transport to the surface of the plasmacytoma, despite surface μ expression in pre-B and B cell lines (Hombach *et al.*, 1988; Sitia *et al.*, 1987). Instead, μ was retained in the endoplasmic reticulum and was subsequently degraded. However, the isolation of a plasmacytoma variant that did express the transfected μ on the cell surface (Hombach *et al.*, 1988) resulted in the discovery that the BCR is in fact a complex.

The BCR complex is composed of membrane Ig associated with a disulphide-linked heterodimer of Ig-α and Ig-β (Campbell and Cambier, 1990; Hombach *et al.*, 1990) (Fig. 1.1A). The genes encoding Ig-α and Ig-β (*mb-1* and *B29* respectively) had previously been isolated by subtractive hybridisation of B cell-specific mRNAs (Hermanson *et al.*, 1988; Sakaguchi *et al.*, 1988). Characterisation of their gene expression revealed that although Ig-β is expressed throughout B cell development, the expression of Ig-α is downregulated at the terminal plasma cell stage, consistent with the inability of membrane IgM to be transported to the surface in the plasmacytoma transfection experiments (Hombach *et al.*, 1988). Membrane μ requires the Ig-α/Ig-β heterodimer to sheath its relatively hydrophilic transmembrane region for stable insertion into the membrane, and this non-covalent association is affected by mutation of the μ transmembrane region (Williams *et al.*, 1990). Furthermore, the same Ig-α/Ig-β heterodimer associates with all five classes of membrane Ig (although the glycosylation patterns of the H chain isotypes and dependence for surface transport differ) and the BCR complex has been reconstituted in non-lymphoid cell lines (Venkitaraman *et al.*, 1991). Although it remains to be shown, one could speculate that the stoichiometry of the BCR is membrane IgM sheathed by two Ig-α/Ig-β heterodimers, since μ offers two identical surfaces for interaction with the Ig-α/Ig-β heterodimer (Reth, 1992) (Fig. 1.1A).

Whilst it is clear that membrane Ig is the central component of the BCR and that Ig-α and Ig-β are required for surface transport and biological activity, there have been reports of additional BCR constituents, such as the BCR-associated proteins (BAPs) that bind to the transmembrane regions of IgM and IgD (Kim *et al.*, 1994; Terashima *et al.*, 1994), and p40/42 which associates with the transmembrane or extracellular domains of the Ig-α/Ig-β heterodimer (Lee *et al.*, 1996). However, their functions are as yet unknown.

1.1.4 ITAMs are essential for transmembrane signalling by the BCR.

The Ig-α/Ig-β heterodimer not only facilitates surface transport of IgM, but provides the BCR with cytoplasmic domains for interacting with signalling molecules. The cytoplasmic tails of both Ig-α and Ig-β contain sequences bearing homology to several other cytoplasmic domains of receptors which are involved in the immune response, such as the T cell receptor (TCR) and the Fc receptor for IgE (FcεR) (Reth, 1989) (Fig. 1.1B). This conserved sequence has been designated the immunoreceptor tyrosine-based activation motif (ITAM) (Cambier, 1995). The ITAMs are essential for the initiation of transmembrane signalling by the BCR (Flaswinkel and Reth, 1994; Papavasiliou *et al.*, 1995; Sanchez *et al.*, 1993; Taddie *et al.*, 1994; Williams *et al.*, 1994).

Triggering of the BCR results in signal transduction via the Ig-α/Ig-β heterodimer. Protein tyrosine phosphorylations occur (Brunswick *et al.*, 1991; Campbell and Sefton, 1990; Gold *et al.*, 1990; Lane *et al.*, 1991), including phosphorylation of the two conserved tyrosines in the ITAM for recruitment of SH2 (Src homology 2) domain-containing molecules (Songyang *et al.*, 1993) such as protein tyrosine kinases, Syk and members of the Src family (Bolen, 1995). Subsequent signalling pathways diverge and include the activation of phospholipase C (PLC), Ras, phosphatidylinositol 3-kinase (PI3-K), or G proteins, ultimately regulating gene expression and determining cellular responses (Cambier *et al.*, 1994).

1.1.5 Are the signalling activities of Ig-α and Ig-β similar or different?

The evidence for the relative roles of Ig-α and Ig-β in BCR-mediated signalling are unclear. Several groups have attempted to discriminate between the activities of the individual polypeptides in cell line transfectants expressing chimeric receptors of extracellular and transmembrane domains of IgM, CD8 or FcγRII fused to either the Ig-α or the Ig-β cytoplasmic tail. Although some signalling assays suggest that the presence of the ITAM renders Ig-α and Ig-β functionally redundant, conflicting evidence suggests that the polypeptides perform distinct roles in signal transduction.

Some experiments in cell line transfectants expressing chimeric mouse IgM and human CD8 receptors suggest that both Ig-α and Ig-β can independently mediate signalling in several assays. The Ig-α and Ig-β cytoplasmic tails were shown to be sufficient to induce tyrosine phosphorylation of total cellular proteins or specific substrates (Vav, PI-3K, CD22), inositol phosphate production, calcium flux, and IL-2 production in 2PK3, A20, LK35.2 and DO-11.10 cell lines (Law *et al.*, 1993; Taddie *et al.*, 1994; Williams *et al.*, 1994).

Human IgM chimeras in A20 (Sanchez *et al.*, 1993) or mouse CD8 chimeras in K46 (Kim *et al.*, 1993) also exhibit no difference between Ig-α and Ig-β in mediating in the rise in intracellular calcium concentration in reponse to receptor crosslinking. However, the total tyrosine phosphorylation effected by Ig-β is impaired in comparison to the Ig-α response, both in the kinetics of the response and in the range of substrates phosphorylated. Further analysis of the calcium response by FcγRII chimeras in single cells suggests that the polypeptides differ, since Ig-α mediates a full calcium flux, whereas Ig-β exhibits oscillations of the calcium spikes (Choquet *et al.*, 1994). The dissection of the Ig-α and Ig-β ITAM sequences in single cell calcium analysis suggests that the four amino acid sequences that differ between the Ig-α (DSCM) and Ig-β (QTAT) ITAMs (see Fig. 1.1) can in part account for their differential signalling abilities (Cassard *et al.*, 1996). Mutation of the Ig-β QTAT sequence to DSCM rescued the calcium response mediated by Ig-β. However, the dissection is complicated by the apparent negative regulatory role of regions flanking the ITAM in Ig-β. Ig-α and Ig-β have also been proposed to bind different downstream effectors (Clark *et al.*, 1992). Indeed, the DSCM sequence of Ig-α appears to mediate the specific association of signalling molecules such as SHC (D'Ambrosio *et al.*, 1996).

Nevertheless, the discrimination of which cytoplasmic tail binds which effector may not be as important as the interplay between all the molecules recruited within the context of the intact BCR complex (Luisiri *et al.*, 1996).

1.1.6 BCR-mediated tyrosine phosphorylation activates multiple effectors.

BCR crosslinking leads to protein tyrosine phosphorylation of multiple substrates including several members of the Src family kinases, Lyn, Fyn, Blk, Lck (Burkhardt *et al.*, 1991; Campbell and Sefton, 1992; Clark *et al.*, 1992; Yamanashi *et al.*, 1991). The Src kinases can bind differentially to the downstream effectors PLCγ2, microtubule-associated protein (MAP) kinase, GTPase-activating protein (GAP), and PI3-K (Pleiman *et al.*, 1993). For example, BCR crosslinking results in the association of Lyn with the p85 subunit of PI 3-K and activation of both kinases (Yamanashi *et al.*, 1992). Recruitment of the Src kinases not only occurs to the activated BCR (Law *et al.*, 1993), but signalling molecules can associate with the resting BCR (Clark *et al.*, 1992). Lyn and Fyn can associate with both phosphorylated and non-phosphorylated forms of Ig-α (Pleiman *et al.*, 1994). Association of the Src kinases with the activated BCR occurs via binding of their SH2 domains to the phosphorylated Ig-α ITAM tyrosines essential for Src kinase activation (Flaswinkel and Reth, 1994). Association of the Src kinases with the resting BCR may occur via their SH4 domains which mediate

association both to the ITAM and with plasma membrane (Timson-Gauen *et al.*, 1996). The Src kinases may exhibit overlapping or redundant functions, however, gene-targetted Lyn-deficient mice exhibit impaired BCR-mediated signalling in the generation of peripheral B cells and also in the elimination of autoreactive B cells (Nishizumi *et al.*, 1995).

The Syk protein tyrosine kinase (Taniguchi *et al.*, 1991) plays a crucial role in BCR signalling and is tyrosine phosphorylated after crosslinking of membrane Ig (Hutchcroft *et al.*, 1992; Law *et al.*, 1993; Yamada *et al.*, 1993). The centrality of Syk to BCR signalling was revealed by mice rendered Syk-deficient by germline disruption (Cheng *et al.*, 1995; Turner *et al.*, 1995). The mice died perinatally due to severe haemorrhaging, but irradiated *RAG*$^{-/-}$ mice reconstituted by Syk-deficient foetal liver exhibited a block in B cell development from the pro-B to pre-B cell stage, similar to that observed in mice lacking membrane IgM or surrogate light chain (Kitamura *et al.*, 1992; Kitamura *et al.*, 1991). These findings indicate that Syk expression and function is essential for mediating the activities of the pre-BCR. Additionally, although a small number of immature B cells were detected, mature B cells were absent, suggesting that Syk is also required for the maintenance of the mature B cell pool (Turner *et al.*, 1995).

Syk bears homology to the TCR-associated ZAP-70 protein tyrosine kinase (Chan *et al.*, 1992) and both contain two SH2 domains (Pawson and Gish, 1992). Indeed, ZAP-70 reconstituted BCR signalling in Syk-deficient B cells demonstrating functional redundancy between the kinases (Kong *et al.*, 1995). Phosphorylation of both tyrosine residues in the ITAM is essential for high affinity binding of Syk and ZAP-70 via their dual SH2 domains (Bu *et al.*, 1995; Hatada *et al.*, 1995). Syk needs to bind to the doubly-phosphorylated ITAM before it can undergo tyrosine phosphorylation and activation (Kurosaki *et al.*, 1995). This can occur by autophosphorylation (Rowley *et al.*, 1995), although Syk can also be activated by the Src kinases (Kurosaki *et al.*, 1994).

It is not clear whether Syk or a Src family kinase initiates the tyrosine phosphorylation cascade (if that is a distinction that can be made). Different phosphorylated ITAMs vary in their preferences for binding Syk versus Lyn, although the Ig-α and Ig-β ITAMs bind Lyn in preference to Syk (Johnson *et al.*, 1995). Experiments using Lyn-deficient and Syk-deficient chicken B cell lymphomas (Takata *et al.*, 1994), suggest that Syk and Lyn may effect distinct signalling pathways or cooperate. Syk seems to be required for inositol phosphate turnover,

whereas Lyn mediates a calcium response independent of inositol phosphate generation.

The low molecular weight GTP-binding protein, Ras, is a key regulator of cellular activation and proliferation in a number of cell types (Lowy and Willumsen, 1993) and its activation state seems to be regulated by multiple mechanisms (Boguski and McCormick, 1993). Ras was found to co-cap with membrane Ig following BCR ligation (Graziadei *et al.*, 1990), and is activated in a PKC-independent manner (Harwood and Cambier, 1993). Furthermore, anti-Ig treatment of B cells leads to the activation of the Ras/Raf-1/MEK/MAP kinase pathway (Tordai *et al.*, 1994).

The Ras pathway was proposed to be regulated by the product of the *vav* proto-oncogene, Vav, which is a target for tyrosine phosphorylation after BCR crosslinking (Bustelo and Barbacid, 1992; Bustelo *et al.*, 1992; Margolis *et al.*, 1992). However, it was later suggested that Vav does not act as a GTP-exchange protein for Ras, but that Vav and Ras could mediate distinct, yet interactive, pathways. (Bustelo *et al.*, 1994). Nevertheless, the importance of Vav in BCR signalling was revealed in mice using the RAG-complementation approach, where embryonic stem cells bearing a germline disruption of the *vav* gene were injected into blastocysts from gene-targetted *RAG*$^{-/-}$ blastocysts (Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995). The B-1 lineage B cells were absent and the Vav-deficient conventional B cells were unable to undergo proliferation in response to anti-Ig, although the lipopolysaccharide (LPS) and CD40 pathways seemed unaffected.

The SH2 domain-containing protein, SHC (Pelicci *et al.*, 1992) may provide another means for Ras activation via the Grb2/Sos complex. BCR ligation results in tyrosine phosphorylation of SHC with recruitment of Grb2/Sos and membrane translocation of the SHC/Grb2 complex (Lankester *et al.*, 1994; Saxton *et al.*, 1994; Smit *et al.*, 1994). SHC not only binds to the phosphorylated ITAMs of both Ig-α and Ig-β via its SH2 domain (Baumann *et al.*, 1994), but also associates with the resting BCR via the four amino acid sequence DSCM in the non-phosphorylated ITAM of Ig-α (D'Ambrosio *et al.*, 1996).

Further downstream in BCR cell activation pathways, the Btk protein tyrosine kinase (Rawlings *et al.*, 1993) mediates binding via its pleckstrin homology domain (Musacchio *et al.*, 1993) to signalling molecules such as the β and γ subunits of heterotrimeric G proteins (Tsukada *et al.*, 1994), PKC (Yao *et al.*, 1994) and phosphatidylinositol-4,5-bisphosphate (Harlan *et al.*, 1994). A mutation in the

pleckstrin homology domain of mouse Btk results in the *xid* (X-linked immune deficiency) phenotype (Rawlings et al., 1993). The *xid* mouse exhibits defective BCR signalling: the B-1 cells are absent and the conventional B cells exhibited impaired responses to LPS or ligation of IgM or CD40 (Hasbold and Klaus, 1994; Scher, 1982; Wicker and Scher, 1986). Since the *xid* phenotype is more severe than the effect of disrupting *vav* (Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995), Btk may function downstream of Vav and may integrate the different B cell signalling pathways. It also may be regulated by the Src kinases (Saouaf *et al.*, 1994) since Fyn and Lyn bind to Btk via their SH3 domains (Cheng *et al.*, 1994).

1.1.7 Other cell surface receptors can modulate BCR signalling.

The physiological events in B cell development and activation are not mediated by the BCR in isolation, but result from a complex interplay of the BCR with other receptors that modulate its signals, such as CD19, FcγRIIb1 and CD22 (Doody *et al.*, 1996).

CD19, a surface glycoprotein expressed in the B lineage from early in development, is a target for tyrosine phosphorylation following BCR crosslinking (Fearon and Carter, 1995). When co-crosslinked with membrane Ig, it acts as a positive regulator and lowers the antigen threshold for triggering the BCR (Carter and Fearon, 1992). CD19 forms a complex with CD21 (complement receptor 2) and CD81 (TAPA-1) (Fearon and Carter, 1995). *In vivo*, coligation of the CD19 and BCR complexes via antigen-complement aggregates could provide a link between the innate response and T cell-dependent antibody responses by enhancing the potency of antigen for the BCR (Dempsey *et al.*, 1996). Indeed, gene-targetted mice lacking CD19 exhibit deficiencies in T cell-dependent responses and germinal centre formation (Engel *et al.*, 1995; Rickert *et al.*, 1995). The CD19-deficient mice also lack B-1 cells and this phenotype bears similarites to that of Vav-deficient mice (Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995). This is consistent with reports that CD19 activates Vav and MAP-K (Weng *et al.*, 1994), and that CD19 is necessary for self-renewal of the B-1 population (Krop *et al.*, 1996). The CD19 complex also associates with PI 3-K, and Src kinases Fyn and Lyn (Chalupny *et al.*, 1995; Tuveson *et al.*, 1993; van Noesel *et al.*, 1993).

FcγRIIb1, which binds to the Fc regions of IgG (Ravetch, 1997), could act as a coreceptor with the BCR to mediate feedback of the concentration of serum antibodies. Its negative regulatory role in modulating BCR-mediated signals was first apparent in observations that anti-IgM-induced activation of B cells was

abrogated if intact antibody was used to crosslink the BCR (Phillips and Parker, 1984). Co-ligation of FcγRII1b with BCR results in the phosphorylation of a single tyrosine in an inhibitory motif contained in the FcγRIIb1 cytoplasmic domain. This phosphorylated tyrosine residue interacts with and activates the tyrosine phosphatase, SHP (PTP-1C) (D'Ambrosio *et al.*, 1995), and is essential for mediating the negative signal which inhibits the influx of extracellular calcium across the cell membrane (Choquet *et al.*, 1993; Muta *et al.*, 1994).

The SHP protein tyrosine phosphatase also interacts with CD22 (Campbell and Klinman, 1995; Doody *et al.*, 1995), a B cell-specific surface glycoprotein that associates with membrane Ig with low stoichiometry (Leprince *et al.*, 1993; Peaker and Neuberger, 1993). Triggering of the BCR leads to the rapid tyrosine phosphorylation of the cytoplasmic domain of CD22 (Schulte *et al.*, 1992); the phosphorylated tyrosines recruit SHP (Campbell and Klinman, 1995; Doody et al., 1995) and the tandem SH2 domains of Syk (Wienands *et al.*, 1995). The association with SHP suggests that CD22 is a negative regulator of BCR signalling and is consistent with the increase in B cell response resulting from ligating CD22 separately or preventing its association with the BCR (Doody *et al.*, 1995; Pezzutto *et al.*, 1988). Indeed, B cells from gene-targetted CD22-deficient mice are hyperresponsive to BCR-mediated signals, exhibiting not only enhanced B cell activation, but also advanced B cell maturation (O'Keefe *et al.*, 1996; Otipoby *et al.*, 1996; Sato *et al.*, 1996). CD22 is normally expressed at the mature B cell stage (Erickson *et al.*, 1996) and may act to increase the antigen threshold for BCR signalling after the stage when immature B cells are sensitive to self-antigens.

1.1.8 The BCR mediates antigen presentation to recruit help from T cells.

The signalling activities of the BCR are often not sufficient for B cell activation in response to T cell-dependent antigen, and in order to recruit T cell help (such as cytokine secretion), B cells must present antigen (Lanzavecchia, 1985). The BCR constitutively internalises bound antigen (Watts and Davidson, 1988) for processing and presentation as antigenic peptides complexed with MHC class II molecules (Pieters, 1997). The Ig-α/Ig-β heterodimer confers efficient antigen presentation (Patel and Neuberger, 1993) by driving the intracellular targetting of antigen (Aluvihare *et al.*, 1997). The relative contributions of Ig-α and Ig-β to the antigen presentation process are unclear; whilst chimeras bearing the individual Ig-α and Ig-β cytoplasmic tails have been shown to target antigen to different intracellular compartments (Bonnerot *et al.*, 1995), the relevance of these findings to BCR function *in vivo* remains to be shown.

Efficient antigen presentation by B cells for the stimulation of T cells also requires costimulation between the B and T cells, as suggested by the two-signal model of lymphocyte activation (Bretscher, 1992; Bretscher and Cohn, 1970; Schwartz, 1990). One such interaction results from the upregulation of B7 after BCR ligation and signalling. The B7 (CD80 and CD86) family of cell surface costimulatory molecules bind CD28 or CTLA4 expressed on the surface of T cells (June *et al.*, 1994), however, absence of this costimulatory signal leads to partial activation and anergy (Ho *et al.*, 1994). CD80 and CD86 are differentially regulated with respect to time and tissue-specificity, and have also been implicated in regulating the type of T-cell dependent response (Freeman *et al.*, 1995; Kuchroo *et al.*, 1995).

1.2 Immunoglobulin gene expression

1.2.1 Organisation of immunoglobulin genes

One limitation of the side chain theory (and later the clonal selection theory) became apparent after studies with artificial haptens indicating that the body contains antibodies with innumerable specifities and differing affinites (Landsteiner, 1933). This enormous diversity could not be explained by a simple model of pre-existing antibody side chains. Instead of each antibody being encoded by its own gene, it was proposed that immunoglobulin molecules may be generated by the recombination of two separate pools of genes: a very large pool for variable (V) regions and another for constant (C) regions (Dreyer and Bennett, 1965). The V regions would differ in antigen specificity but the functional properties of the antibody would be conferred by the C region. The prediction that lymphocyte DNA undergoes somatic rearrangement was borne out when λ L chain DNA was shown to hybridise to one fragment of myeloma DNA, but to two fragments from embryonic DNA (Hozumi and Tonegawa, 1976). Furthermore, the V regions are encoded by smaller gene segments that recombine (Tonegawa, 1983), providing diversity by combinatorial joining.

The mouse IgH locus is made up of exons encoding V and C regions (Fig. 1.2). The V region is composed of three families of gene segments, V_H, D_H and J_H; the choice within each family increases potential diversity. The C region exons encode for different isotypes of H chain, giving rise to the different classes of antibody. Mature B cells can co-express both IgM and IgD classes, which are generated by alternative splicing. B cell activation and differentiation can lead to the deletion of upstream C_H regions by class switching (Shimizu and Honjo, 1984), which is mediated by short tandem repeats known as switch recombination sequences (Radbruch *et al.*, 1986). Antibody class switching results in the expression of downstream H chain isotypes with different effector functions (Morgan and Weigle, 1987) but yet retain the same antigen specificity.

Mice can express either the κ or λ L chain isotype, although κ predominates and is expressed by 95% of B cells (Coleclough *et al.*, 1981). The mouse Igκ locus is made up of V and C region exons (Fig. 1.2), but it is less complex than the IgH locus. Only two families of V gene segments, Vκ and Jκ, rearrange to form the V region, and a single exon encodes the Cκ region.

1.2.2 Control and tissue-specificity of Ig gene expression

The expression of functionally rearranged Ig genes is regulated by the promoter and enhancer elements present at the loci (Staudt and Lenardo, 1991) (Fig. 1.2). The control elements ensure the cell type- and stage-specificity of expression. Most studies of the regulatory sequences have focussed on their transcriptional activities. However, the elements also play roles in such processes as demethylation and rearrangement of the gene segments.

All Ig promoters share two sequence motifs, a TATA box which is not cell-restricted, and an octamer motif (Staudt and Lenardo, 1991). The octamer present in the Ig promoter directed lymphoid-specific expression, and yet also activated transcription in other cell types, acting selectively with different enhancers (Parslow *et al.*, 1987). The conflict in specificity may be due to the differential expression and activity of the POU-domain family of transcription factors (Herr *et al.*, 1988). B cell specificity is driven primarily by Oct-2, which is restricted to the B cell lineage, but expressed throughout development (Staudt *et al.*, 1986). In contrast, Oct-1 is expressed by most cell types (Singh *et al.*, 1986; Sturm *et al.*, 1988).

Enhancer elements play an important role in directing events at the Ig loci. For example, two enhancers have been identified for the κ L chain, the κ intron enhancer (Ei) and the κ 3′ enhancer (E3′) (Meyer and Neuberger, 1989; Picard and Schaffner, 1984). Experiments in LPS-activated pre-B cell lines suggested that Ei is required at the pre-B cell stage for transcription (Atchison and Perry, 1987; Fulton and Van Ness, 1993; Lenardo *et al.*, 1987). Moreover, Ei plays a role in demethylation of the κ gene, possibly by directing locus accessibility prior to Vκ rearrangement (Lichtenstein *et al.*, 1994). The activity of Ei is dependent on binding of the transcription factor, NFκB (Atchison and Perry, 1987). In contrast, the second, stronger tissue-restricted enhancer, located 3′ of Cκ can drive the expression of κ in cell lines that lack NFκB and has been shown to be active from the B cell stage onwards in development (Meyer and Neuberger, 1989; Park and Atchison, 1991; Pongubala and Atchison, 1991).

1.2.3 V region rearrangement generates antibody diversity.

Early in B cell development, V region rearrangement occurs firstly at the IgH locus, in an ordered process with D_H-J_H joining followed by V_H-DJ_H (Alt *et al.*, 1984). Vκ-Jκ rearrangement generally occurs after V_H, but signals from the pre-BCR are not necessary for triggering this process (Ehlich *et al.*, 1993; Grawunder *et al.*, 1993). V gene segment rearrangement occurs at consensus recombination signal sequences

(RSSs) that flank the segment (Schatz *et al.*, 1992; Tonegawa, 1983). RSSs consist of a palindromic heptamer element (adjacent to the coding region) separated by a spacer region of 12 or 23 non-conserved nucleotides from a conserved nonamer element. Efficient joining of gene segments requires the interaction of a 12 bp spacer-containing RSS with a 23 bp spacer-containing RSS (the 12/23 rule); the presence of these two types of RSS is both necessary and sufficient (Aguilera *et al.*, 1987; Hesse *et al.*, 1989).

The recombination machinery involves the products of the recombination activating genes, *RAG-1* and *RAG-2* (Oettinger *et al.*, 1990; Schatz *et al.*, 1989), and the *scid* gene product, which is a DNA-dependent kinase (Kirchgessner *et al.*, 1995) also implicated double-strand break repair (Bosma *et al.*, 1983; Hendrickson *et al.*, 1991). Mice defective for these genes lack mature B and T cells due to impaired V rearrangement. However, the block in B cell development can be overcome by the introduction of functionally rearranged H and L chains (Chang *et al.*, 1995; Reichman-Fried *et al.*, 1990; Spanopoulou *et al.*, 1994; Young *et al.*, 1994). The expression of the *RAG* genes is biphasic; this pattern correlates with the two periods of V region rearrangement, firstly of the H chain and then of the L chain gene loci (Grawunder *et al.*, 1995).

The imprecise joining of gene segments, as well as the combinatorial mechanism, gives rise to diversity of the V regions. In the case of H chain rearrrangements, V_H regions can undergo the insertion of random "N" nucleotides by TdT (terminal deoxynucleotidyl transferase), creating additional diversity at the coding joints (Alt and Baltimore, 1982; Landau *et al.*, 1987). However, junctional diversity and N-region insertion can also give rise to non-functional genes due to out-of-frame joining or the introduction of stop codons.

1.3 B cell development

1.3.1 Expression of Ig and cell differentiation markers during B cell development

B cell development in the adult mouse bone marrow has been characterised by separating B lineage subpopulations according to the expression of Ig and different cell surface markers, status of V region rearrangement, and growth requirements. The system of dividing developmental stages varies between groups, but perhaps the most straightforward nomenclature is that of Hardy *et al.*, 1991, which I have adapted with additional data from reviewed in Rolink *et al.*, 1994 and Grawunder *et al.*, 1995, and summarised below (Fig. 1.3).

When a haematopoietic stem cell becomes committed to the B cell lineage, it may be identified by surface expression of B220 (CD45R). These pre-pro-B cells (Hardy fraction A) also express CD43, c-kit (CD117), but have not started to express any Ig. At this stage, the cells are dependent only on contact with stromal cells (Nishikawa *et al.*, 1988). CD19 is also expressed throughout B cell development, and may be the preferred marker for pan-B cell-specificity, since B220 is expressed on a minor subpopulation of natural killer cell progenitors that are negative for both heat stable antigen (HSA [CD24]) and BP-1, thus falling into fraction A (Rolink *et al.*, 1996).

Early pro-B cells begin to express HSA and IL-7 receptor (IL-7R), which correlates with their requirement for both stromal contact and the presence of IL-7. IL-7R is important for growth, but also induces H chain rearrangement (Corcoran *et al.*, 1996). The pro-B cells start D_H-J_H rearrangement with the expression of the recombinase activating genes *RAG-1* and *RAG-2*, and TdT (which mediates N region insertions). At later stages, pro-B cells also begin to express BP-1; the combination of CD43, HSA and BP-1 expression has been used to discriminate between the earliest stages in B cell development (Hardy fractions A to C).

Productive VDJ_H rearrangement leads to cytoplasmic μ expression at the large pre-B cell stage, and the cells no longer require stromal contact but still need IL-7. The association of functionally-rearranged μ with the surrogate light chains, V_{preB} and λ5, results in surface transport of the pre-BCR complex (with the Ig-α/Ig-β heterodimer (Brouns *et al.*, 1993; Gong and Nussenzweig, 1996; Matsuo *et al.*, 1993)). The pre-BCR signals a clonal expansion phase and the large majority of cells beginning to express μ or pre-BCR are in cell cycle (Karasuyama *et al.*, 1994). As the pre-B cells start to express CD25 (TAC, IL-2 receptor α chain) on the surface, they also downregulate the expression of *RAG-1*, *RAG-2* and TdT.

After the proliferative stage, the cells exit the cell cycle, becoming small pre-B cells. They lose expression of CD43 and surrogate light chains, but upregulate *RAG-1* and *RAG-2* expression again for Vκ-Jκ and Vλ-Jλ rearrangement. The productive rearrangement of L chain results in L chain association with μ and expression of the mature BCR. The immature B cells displaying surface IgM no longer need the recombination machinery and they also downregulate the expression of IL-7R and pre-B cell marker BP-1.

1.3.2 Role of the pre-BCR in developmental checkpoints

B cell development serves to generate antibodies recognising numerous antigens, however, the pre-BCR plays a crucial role even before the cell is exposed to antigen. Signals from the pre-BCR mediate several processes, including the indication of productive V_H rearrangement and expansion/survival of those cells, pro- to pre-B transition, allelic exclusion of IgH, and activation of V_L rearrangement, all of which may constitute distinct checkpoints in development.

The structure of the pre-BCR is not dissimilar to that of the BCR (Reth, 1992), since the Ig-α/Ig-β heterodimer is required for its surface expression and function (Brouns *et al.*, 1993; Gong and Nussenzweig, 1996; Matsuo *et al.*, 1993). μ H chains were detected on the surface of B cell progenitors without κ or λ L chains (Pillai and Baltimore, 1988), an observation that could be explained by the presence of two non-rearranging polypeptides, λ5 and Vpre-B, with homology to the C and V regions of the λL chain, respectively (Kudo and Melchers, 1987; Sakaguchi and Melchers, 1986). λ5 and VpreB act as surrogate light chains in the pre-BCR complex; λ5 binds covalently to μ whilst VpreB associates non-covalently (Karasuyama *et al.*, 1990; Tsubata and Reth, 1990).

Expression of a productively rearranged μ chain, surrogate light chains and the Ig-α/Ig-β heterodimer are essential for the function of the pre-BCR, since gene-targetted mice that cannot express membrane μ, λ5 , Ig-β or the cytoplasmic domain of Ig-α exhibit a block at the pro-B cell stage (Ehlich *et al.*, 1993; Gong and Nussenzweig, 1996; Kitamura *et al.*, 1992; Kitamura *et al.*, 1991; Torres *et al.*, 1996). Indeed, the pre-BCR complex seems to mediate its biological activities by transmembrane signalling. Although it is presumed to be an antigen-independent event, crosslinking μ, λ5 or Ig-α in pre-B cell lines results in downstream tyrosine phosphorylation and increases in intracellular calcium concentration (Matsuo *et al.*, 1993; Misener *et al.*, 1991; Takemori *et al.*, 1990).

Productive V_H rearrangement seems to act as a checkpoint whose completion is signalled via expression of the pre-BCR on the surface. Mice that are unable to rearrange VDJ_H due to germline disruption of the J_H locus (Chen *et al.*, 1993) or the *RAG-1*, *RAG-2* or *scid* genes involved in the recombination machinery (Bosma *et al.*, 1983; Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992) are blocked at development in pro-B cells. However, progression from the pro- to pre-B cell stage is rescued by the presence of a rearranged H chain or both H and L chain transgenes (Chang *et al.*, 1995; Reichman-Fried *et al.*, 1990; Spanopoulou *et al.*, 1994; Young *et al.*, 1994).

Most of the pre-B cells that express a productively-rearranged μ are in the S or G2 phase of the cell cycle (Karasuyama et al., 1994), a higher proportion than at any other stage in early B cell development, suggesting that the pre-BCR has signalled and licensed the functionally V_H-rearranged pre-B cell to undergo proliferative expansion (Rolink *et al.*, 1993).

Central to the clonal selection theory, is the prevention of expression of more than one antibody specificity. The mechanism that ensures monospecificity is known as allelic exclusion. Allelic exclusion of the IgH locus requires surface expression of the pre-BCR. Mice carrying μ transgenes encoding μ or membrane-only μ inhibited endogenous V_H rearrangements (Manz *et al.*, 1988; Nussenzweig *et al.*, 1987; Rusconi and Köhler, 1985; Weaver *et al.*, 1985). However, mice in which one allele of μ could only express the secretory form were not able to exclude the other IgH allele and generated B cells expressing both alleles (Kitamura and Rajewsky, 1992).

Several models have been proposed to account for allelic exclusion. Assuming that rearrangement proceeds independently on both IgH alleles, the stochastic model suggests that the process is intrinsically error-prone and that there is a low probability that two functional V_H rearrangements would be generated within a given time (Cohn and Langman, 1990; Coleclough *et al.*, 1981; Langman and Cohn, 1987; Walfield *et al.*, 1981). Alternatively, the expression of both alleles could be toxic to the B cells and cause the death of the double expressers (Wabl and Steinberg, 1982).

However, more recent evidence favours an ordered model, initally based on studies using Abelson-transformed pre-B cell lines (Alt *et al.*, 1981). Rearrangement is initiated at the IgH locus and μ chain expression from a productive V_H rearrangement prevents rearrangement of the other allele (Alt *et al.*, 1984). Allelic exclusion may be related to the downregulation of *RAG-1* and *RAG-2* gene

expression in pre-B cells, however, this offers only a partial explanation since *RAG* expression is upregulated again for V_L rearrangement (Grawunder *et al.*, 1995).

An additional form of regulation governing productive rearrangement may exist via a truncated form of membrane μ, Dμ, which lacks V_H segments and results from DJ_H rearrangements (Reth and Alt, 1984). The Dμ protein seems to be expressed in a similar complex to the pre-BCR (Tsubata *et al.*, 1991), and can also mediate allelic exclusion (Gu *et al.*, 1991; Löffert *et al.*, 1996). Dμ provides a possible mechanism for counterselecting rearrangement in reading frame (rf) 2, which is underselected in the repertoire compared to rf1 (Gu et al., 1991; Kaartinen and Mäkelä, 1985). Rf3 often contains stop codons which render those rearrangements non-productive.

Ig V region rearrangement in B cells usually begins at the IgH locus and it was proposed that the functionally-rearranged μ provided the signal to induce V_L rearrangement (Iglesias *et al.*, 1991; Reth *et al.*, 1987; Tsubata *et al.*, 1992). However, the detection of Vκ rearrangement in pre-B cell lines (Grawunder *et al.*, 1993; Kubagawa *et al.*, 1989; Schlissel and Baltimore, 1989) or pre-BCR-deficient mice (Blackwell *et al.*, 1989; Chen *et al.*, 1993; Ehlich *et al.*, 1993), indicated that the IgH and IgL loci rearrange independently. B cell development in λ5-deficient mice was rescued by a rearranged κ transgene, but not a rearranged κ targetted into the genomic κ locus (Pelanda *et al.*, 1996). However, since the transgene was expressed prematurely, it was suggested that the regulation of Vκ rearrangement operates at the level of gene expression.

Vκ rearrangement occurred only at a low frequency in the pro-B cells of pre-BCR-deficient mice (Chen *et al.*, 1993; Ehlich *et al.*, 1993). Although signals from the pre-BCR are not necessary for the initiation of V_L rearrangement, pre-BCR signalling may still be required for the upregulation of V_L rearrangement activity as suggested by Abelson-transformed pre-B cell line experiments (Iglesias *et al.*, 1991; Reth *et al.*, 1987; Tsubata *et al.*, 1992).

1.3.3 Selection and maturation of the peripheral B cell pool

The discrimination between self and non-self endures as one of the major immunological questions yet to be understood. However, transplantation experiments using dizygotic cows (Owen, 1945) or skin grafts on mice of different strains (Billingham *et al.*, 1956) demonstrated that self-recognition and tolerance were acquired during the development of lymphocytes, and indicated that the

potential antibody-producing B cells must be selected.

Tolerance induction seems to be correlated with the maturational status of the B cell rather than the form of the antigen (Klinman, 1996). Despite the daily generation of immature B cells estimated at 10-20 million (Osmond, 1991), few (3%) of these IgM+ B cells are selected into the long-lived peripheral B cell pool, in part reflecting negative selection processes that protect against autoreactive antibodies. Transgenic model systems have suggested that if B cells are strongly crosslinked by self-antigen, they may be eliminated in the bone marrow and prevented from migrating into the periphery (Chen *et al.*, 1995; Goodnow *et al.*, 1995; Hartley *et al.*, 1991; Nemazee and Bürki, 1989). Although immature B cells express IgM, they do not seem to respond to antigen in the same way as mature B cells; instead of undergoing activation and differentiation, apoptosis is induced in immature B cells (Monroe, 1996; Norvell *et al.*, 1995; Wechsler and Monroe, 1995; Yellen *et al.*, 1991). Levels of the *bcl-2* gene are downregulated in immature B cells (Li *et al.*, 1993; Merino *et al.*, 1994), suggesting that they are programmed to die unless they receive a signal (possibly mediated by the BCR) required for their survival and maturation (Torres *et al.*, 1996). Once rescued from apoptosis and selected into the long-lived peripheral B cell pool, levels of *bcl-2* expression increase again (Merino *et al.*, 1994).

Tolerance may also be induced when BCRs on immature B cells are inefficiently crosslinked by monovalent soluble antigen. B220 and surface IgM expression are downregulated, and the B cells are rendered anergic, as revealed by mice expressing an anti-HEL transgene (Goodnow *et al.*, 1988). Anergy can occur not only to B cells developing in the bone marrow, but also to mature B cells (Goodnow *et al.*, 1989). Furthermore, cell transfer experiments showed that these anergic cells are impaired when competing with wild type cells for populating the peripheral lymphoid organs, or when entering B cell follicles (Cyster *et al.*, 1994).

In response to autoantigen, immature B cells can undergo receptor editing (Gay *et al.*, 1993; Radic *et al.*, 1993; Tiegs *et al.*, 1993). At this stage in development they may still be expressing RAG-1 and RAG-2, allowing them to induce secondary L chain rearrangements and alter their antigen specificity (Feddersen and Van Ness, 1985; Harada and Yamagishi, 1991; Rolink *et al.*, 1993). However, immature B cells are short-lived, with a lifespan of only 3-4 days compared to that of the peripheral B cell pool, which may be months (Förster and Rajewsky, 1990; Rajewsky, 1993). If immature B cells do not receive a survival signal, they may die from apoptosis.

Survival of positive or negative selection events results in the co-expression of both IgM and IgD classes by the mature B cell. Maturation of the B cell also leads to the expression of other cell surface molecules such as CD22 (Erickson *et al.*, 1996), CD23 (Fcε receptor) (Conrad, 1991; Waldschmidt *et al.*, 1991), CD21/35 (complement receptors 2 and 1) (Ahearn and Fearon, 1989; Gelfand *et al.*, 1974) and varying levels of HSA expression, depending on the differentiation status of the B cell in the follicle or in the marginal zone (Allman *et al.*, 1993; Erickson *et al.*, 1996).

1.3.4 Affinity maturation and memory B cells

The germinal centre (GC) (Kelsoe, 1996; MacLennan, 1994) is a specialised microenvironment formed when B cells are activated by T cell-dependent antigen. The B cells migrate into and populate follicles, which also contain antigen-specific T cells (Fuller *et al.*, 1993) and follicular dendritic cells (FDCs) (Tew *et al.*, 1990). The FDCs retain antigen for long periods, bound to antibodies or in complexes with complement factors, and present antigen to drive the GC reaction for affinity maturation of serum antibodies. The GC B cells (identified by binding peanut agglutinin) within the polarised architecture of the GC (Liu *et al.*, 1991) are divided into the dark zone populated by rapidly proliferating centroblasts, and the light zone containing non-dividing centrocytes. It is believed that somatic hypermutation (Berek *et al.*, 1991; Jacob *et al.*, 1991) (when point mutations are accumulated by the V regions) occurs in the centroblasts, whereas the high-affinity mutant centrocytes are selected for their ability to bind antigen displayed by the FDC in the light zone.

GCs are also necessary for memory B cell generation. GC B cells that survive repeated rounds of mutation and selection enter the memory B cell compartment and dominate later responses (Weiss and Rajewsky, 1990). These selected B cells leave the GC and populate the marginal zone (Liu *et al.*, 1988); their migration from the GC is suggested from the detection of mutated V regions (Dunn-Walters *et al.*, 1995). Memory B cells are generally resting and long-lived, and have undergone class-switching (Rajewsky, 1996). They can efficiently effect a secondary response to antigen, since re-activation induces the upregulation of costimulatory molecules more rapidly compared to naïve cells, thus mediating more efficient antigen presentation (Hayakawa *et al.*, 1987; Liu *et al.*, 1995).

1.3.5 B-1 cells: a separate lineage from conventional B cells

B-1 (Ly-1 B) cells (Hayakawa and Hardy, 1988) appear to constitute a separate B cell lineage from the conventional (or B-2; Kantor, 1991) B cells that primarily colonise the peripheral lymphoid organs. B-1 cells can be distinguished from conventional B

cells by expression of the cell surface molecules CD5, Mac-1 (Hardy *et al.*, 1994) and CD43 (Wells *et al.*, 1994), and they predominate in the B cell pool found in the peritoneum. They are generated early in ontogeny in the foetal liver and are self-renewing (Hardy et al., 1994; Hayakawa *et al.*, 1986), with a low turnover rate (Deenen and Kroese, 1993), unlike conventional bone marrow-derived B cells. Despite their relatively small numbers, B-1 cells produce at least 50% of serum IgM (Herzenberg *et al.*, 1986; Stall *et al.*, 1986). They are considered to be a major source of autoantibodies (Hayakawa and Hardy, 1988; Murakami *et al.*, 1992) and undergo "natural" antibody production without immunisation or exposure to environmental antigen (Bos *et al.*, 1989; Förster and Rajewsky, 1987).

1.4 Aims of this study

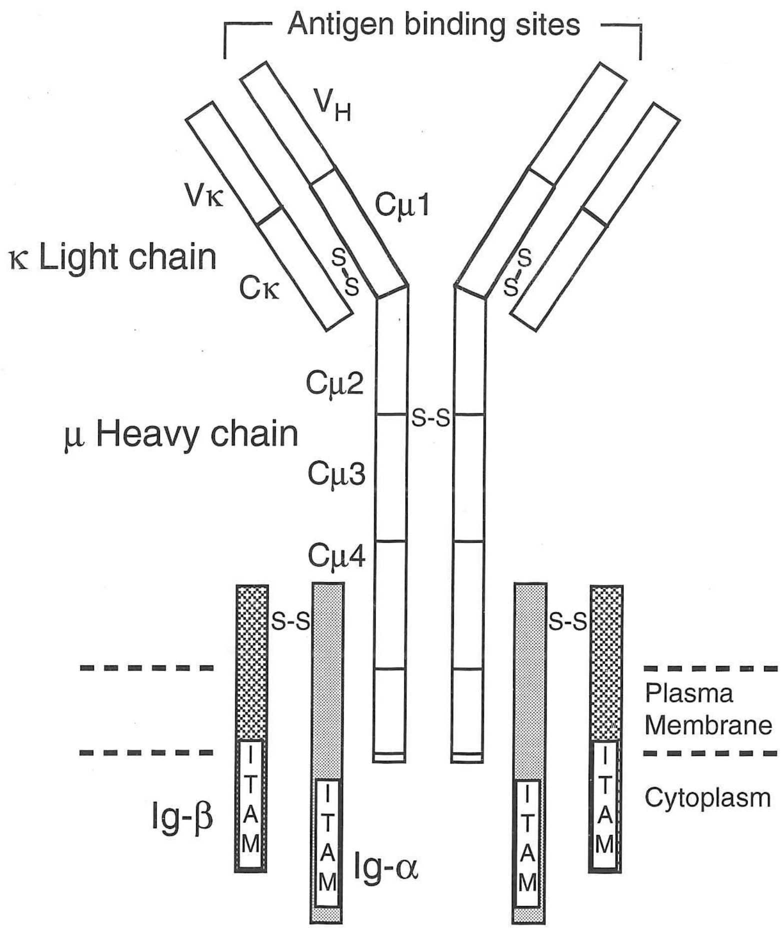
The analysis of B cell antigen receptor expression and signalling described in this study falls into two sections:

- The relative roles of Ig-α and Ig-β in transmembrane signalling mediated by the BCR complex (chapters 3 and 4)
- The transcriptional activity of the κ enhancer regulatory elements during B cell development (chapter 5 and 6).

The cytoplasmic domains of Ig-α and Ig-β are essential for the initiation of BCR-mediated signals. However the literature is unclear as to whether the two polypeptides are functionally redundant or if they have distinct roles to perform within the context of the intact BCR. To extend on the cell line transfection strategy used in experiments by others, I decided to use a transgenic approach to facilitate the discrimination of the signalling activities of Ig-α and Ig-β in primary cells and during B cell development.

Clearly, the effect of signals from the BCR is dependent on the timing of Ig expression. In the case of the Ig κ L chain gene, two enhancer elements, the κ intron enhancer and the κ 3′ enhancer, have been identified and are presumed to play distinct roles in regulating the expression of κ at different stages in B cell development. In order to characterise and discriminate the transcriptional activities of the two κ enhancers throughout differentiation of the B cell lineage *in vivo*, I took advantage of transgenic mice already available that expressed transgenes bearing either of the κ enhancers, or both.

A



B

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Consensus       D X X X - X X X X D X X Y X X L X X X X - - X X X Y X X L
                E                 E           I                         I

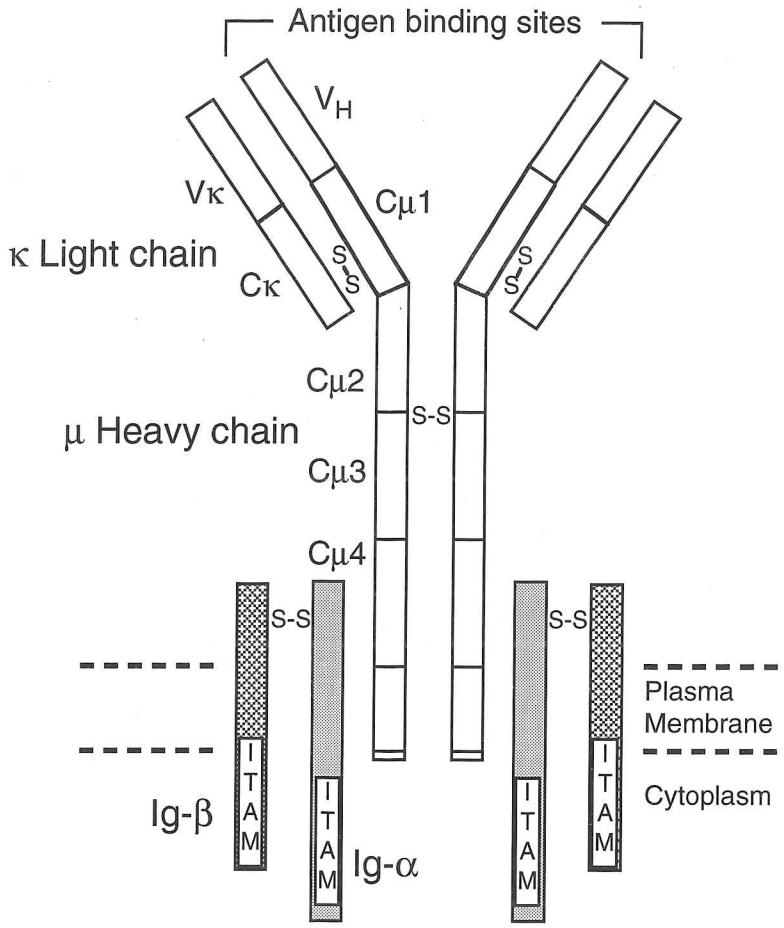
mouse Ig-α      D M P D - D Y E D E N L Y E G L N L D D - - C S M Y E D I
human Ig-α      D A G D - E Y E D E N L Y E G L N L D D - - C S M Y E D I
mouse Ig-β      D G K A - G M E E D H T Y E G L N I D Q - - T A T Y E D I
human Ig-β      D S K A - G M E E D H T Y E G L D I D Q - - T A T Y E D I

CD3-γ           D K Q T - L L Q N E Q L Y Q P L K D R E - - Y D Q Y S H L
CD3-δ           E V Q A - L L K N E Q L Y Q P L R D R E - - D T Q Y S R L
CD3-ε           N K E R P P P V P N P D Y E P I R K G Q - - R D L Y S G L
TCR-ζ/ηa        E T A A N L Q D P N Q L Y N E L N L G R - - R E E Y D V L
TCR-ζ/ηb        K Q Q R R R N P Q E G V Y N A L Q K D K M - A E A Y S E I
TCR-ζc          E R R R G K G H - D G L Y Q G L S T A T - - K D T Y D A L
TCR-ηc          E R R R G K G H - D G L Y D S H F Q A V Q F G N R R E R E

FcεRI-γ         A A I A S R E K A D A V Y T G L N T R N - - Q E T Y E T L
FcεRI-β         E L E S K K V P D D R L Y E E L N H V Y - - S P I Y S E L
```

Figure 1.1 The B cell antigen receptor complex contains ITAMs
(A) *Schematic diagram of the structure of the B cell antigen receptor.* The complex is composed of membrane immunglobulin associated with Ig-α and Ig-β, which contain ITAMs in their cytoplasmic domains. IgM, the first class expressed in ontogeny, is depicted, formed by μ heavy and κ light chain (V, variable region; C, constant region; S-S, disulphide bond).
(B) *Immunoreceptor tyrosine-based activation motif (ITAM).* Polypeptide sequences (Reth, 1989; Cambier *et al.*, 1994) from multichain immune recognition receptors are aligned with the conserved amino acids in bold type. Italics indicate residues proposed to account for differences in the signalling activities of Ig-α and Ig-β. Sequences are from mouse except where indicated and gaps (-) were inserted for optimal alignment.

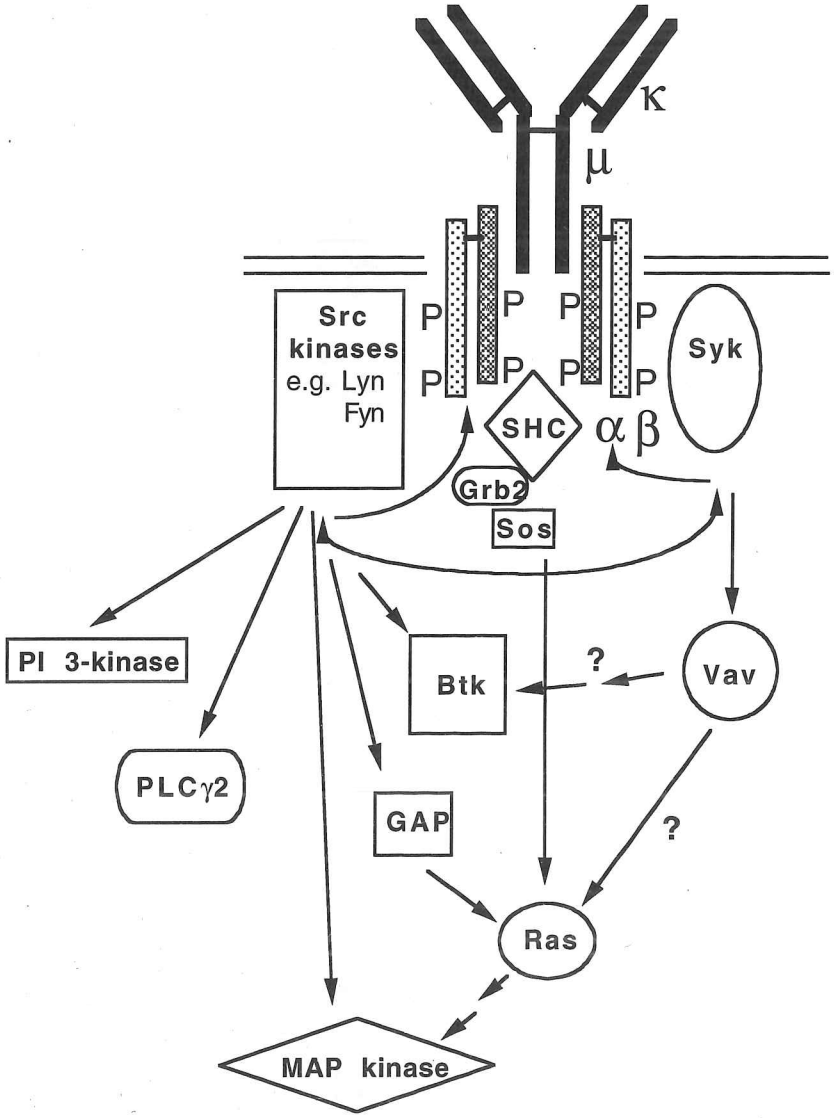
A



B

Consensus	**D**	X	X	X	-	X	X	X	X	**D**	X	X	**Y**	X	X	**L**	X	X	X	X	-	-	X	X	X	**Y**	X	X	**L**
	E									**E**						**I**														**I**
mouse Ig-α	**D**	M	P	D	-	D	Y	E	D	**E**	N	L	**Y**	E	G	**L**	N	L	D	*D*	-	-	*C*	*S*	*M*	**Y**	E	D	**I**
human Ig-α	**D**	A	G	D	-	E	Y	E	D	**E**	N	L	**Y**	E	G	**L**	N	L	D	*D*	-	-	*C*	*S*	*M*	**Y**	E	D	**I**
mouse Ig-β	**D**	G	K	A	-	G	M	E	E	**D**	H	T	**Y**	E	G	**L**	N	I	D	*Q*	-	-	*T*	*A*	*T*	**Y**	E	D	**I**
human Ig-β	**D**	S	K	A	-	G	M	E	E	**D**	H	T	**Y**	E	G	**L**	D	I	D	*Q*	-	-	*T*	*A*	*T*	**Y**	E	D	**I**
CD3-γ	**D**	K	Q	T	-	L	L	Q	N	**E**	Q	L	**Y**	Q	P	**L**	K	D	R	E	-	-	Y	D	Q	**Y**	S	H	**L**
CD3-δ	**E**	V	Q	A	-	L	L	K	N	**E**	Q	L	**Y**	Q	P	**L**	R	D	R	E	-	-	D	T	Q	**Y**	S	R	**L**
CD3-ε	N	K	E	R	P	P	P	V	P	N	P	D	**Y**	E	P	**I**	R	K	G	Q	-	-	R	D	L	**Y**	S	G	**L**
TCR-ζ/ηa	**E**	T	A	A	N	L	Q	D	P	N	Q	L	**Y**	N	E	**L**	N	L	G	R	-	-	R	E	E	**Y**	D	V	**L**
TCR-ζ/ηb	K	Q	Q	R	R	R	N	P	Q	**E**	G	V	**Y**	N	A	**L**	Q	K	D	K	M	-	A	E	A	**Y**	S	E	**I**
TCR-ζc	**E**	R	R	R	G	K	G	H	-	**D**	G	L	**Y**	Q	G	**L**	S	T	A	T	-	-	K	D	T	**Y**	D	A	**L**
TCR-ηc	**E**	R	R	R	G	K	G	H	-	**D**	G	L	**Y**	D	S	H	F	Q	A	V	Q	F	G	N	R	R	E	R	E
FcεRI-γ	A	A	I	A	S	R	E	K	A	**D**	A	V	**Y**	T	G	**L**	N	T	R	N	-	-	Q	E	T	**Y**	E	T	**L**
FcεRI-β	**E**	L	E	S	K	K	V	P	D	**D**	R	L	**Y**	E	E	**L**	N	H	V	Y	-	-	S	P	I	**Y**	S	E	**L**

Figure 1.1 The B cell antigen receptor complex contains ITAMs
(A) *Schematic diagram of the structure of the B cell antigen receptor.* The complex is composed of membrane immunglobulin associated with Ig-α and Ig-β, which contain ITAMs in their cytoplasmic domains. IgM, the first class expressed in ontogeny, is depicted, formed by μ heavy and κ light chain (V, variable region; C, constant region; S-S, disulphide bond).
(B) *Immunoreceptor tyrosine-based activation motif (ITAM).* Polypeptide sequences (Reth, 1989; Cambier *et al.*, 1994) from multichain immune recognition receptors are aligned with the conserved amino acids in bold type. Italics indicate residues proposed to account for differences in the signalling activities of Ig-α and Ig-β. Sequences are from mouse except where indicated and gaps (-) were inserted for optimal alignment.



BCR-mediated tyrosine phosphorylation
Summary of the interactions of signalling molecules recruited to the activated BCR (described in section 1.1.6). SH2 domain-containing proteins bind to the phosphorylated tyrosine residues (P) present in the ITAMs of Ig-α and Ig-β. The arrows represent the subsequent activation of donwstream targets by binding and/or tyrosine phosphorylation by the kinases.

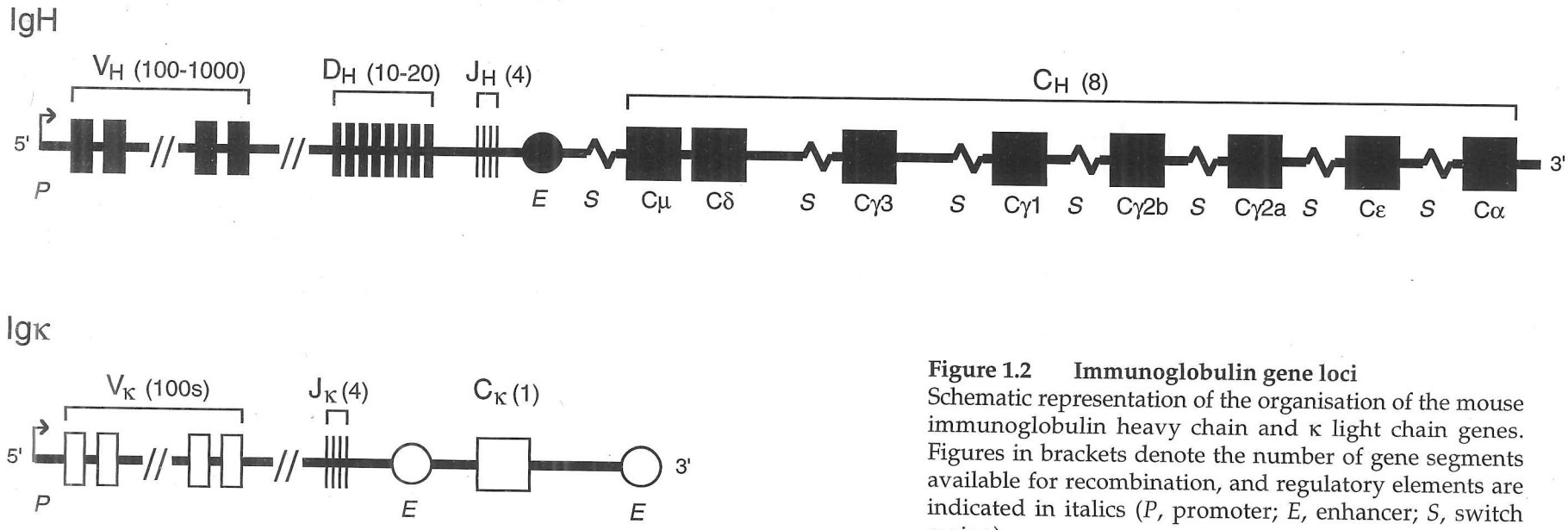


Figure 1.2 Immunoglobulin gene loci
Schematic representation of the organisation of the mouse immunoglobulin heavy chain and κ light chain genes. Figures in brackets denote the number of gene segments available for recombination, and regulatory elements are indicated in italics (*P*, promoter; *E*, enhancer; *S*, switch region).

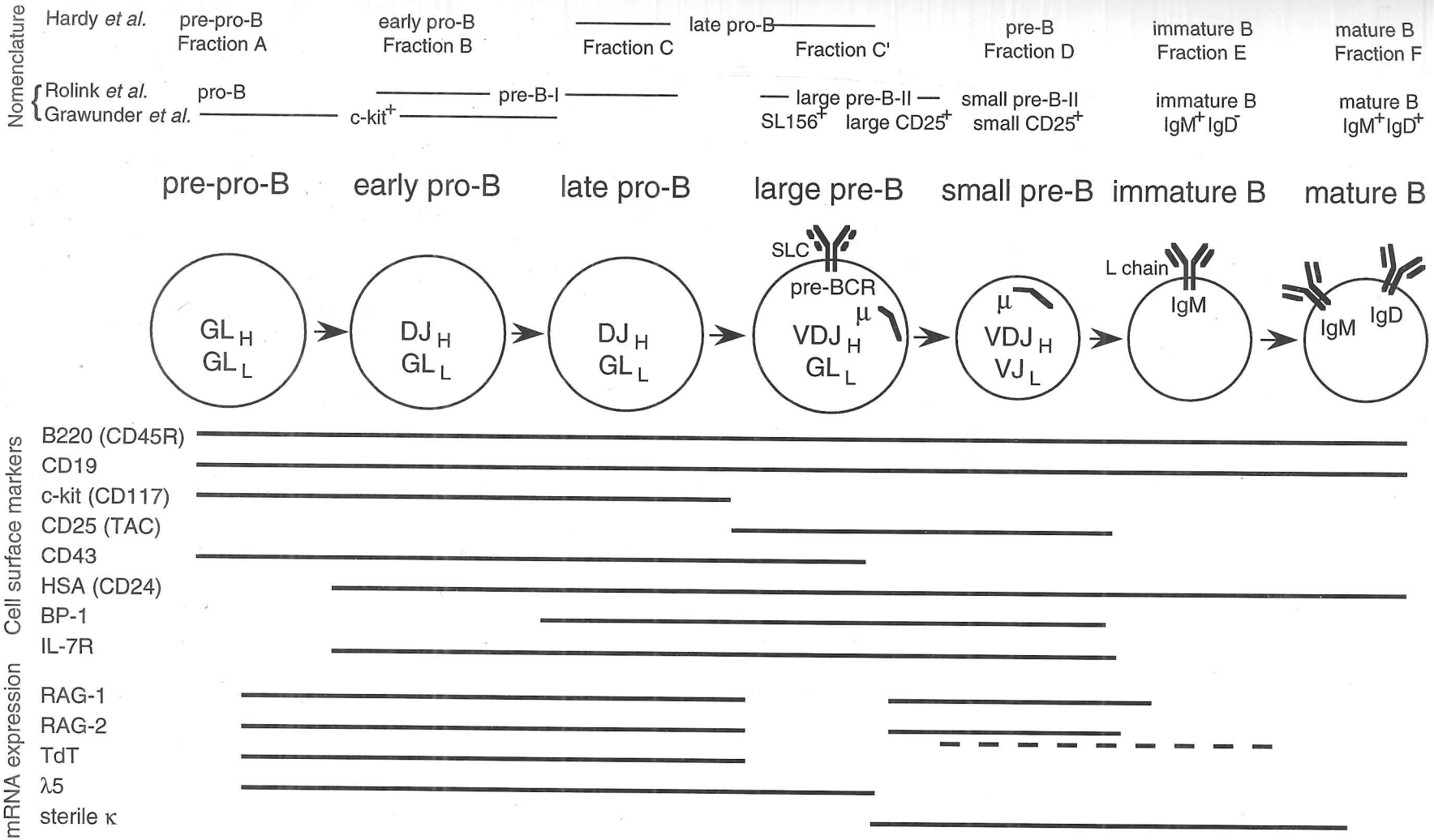
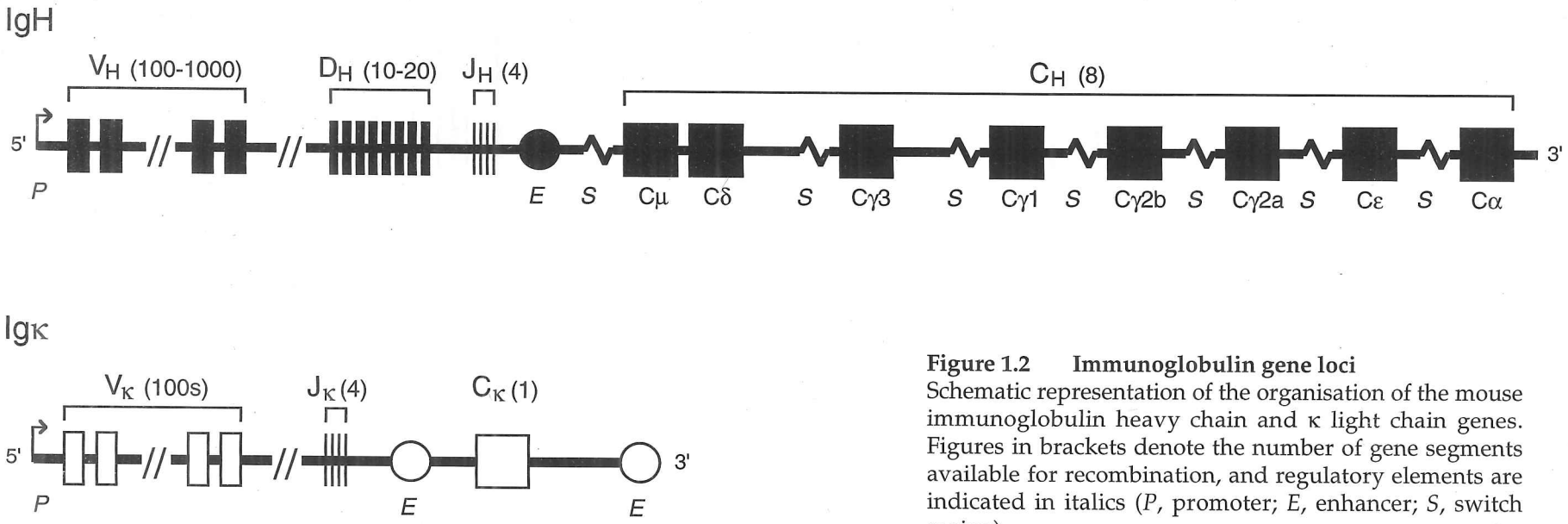


Figure 1.3 B cell development in mouse bone marrow.
Summary of stages defined by surface expression of immunoglobulin and cell differentiation markers, and status of V region rearrangement, based on Hardy *et al.* (1991), Rolink *et al.* (1994) and Grawunder *et al.* (1995). Large circles depict active cycling cells, whilst small circles depict resting cells (pre-BCR, pre-B cell antigen receptor; SLC, surrogate light chains). IgH and IgL rearrangment status of the cells are denoted by H and L subscripts respectively (GL, germline; V, D and J represent recombination of those gene segments). Bold lines indicate periods of expression of the protein or gene (dotted line denotes detection of RAG-2 protein).



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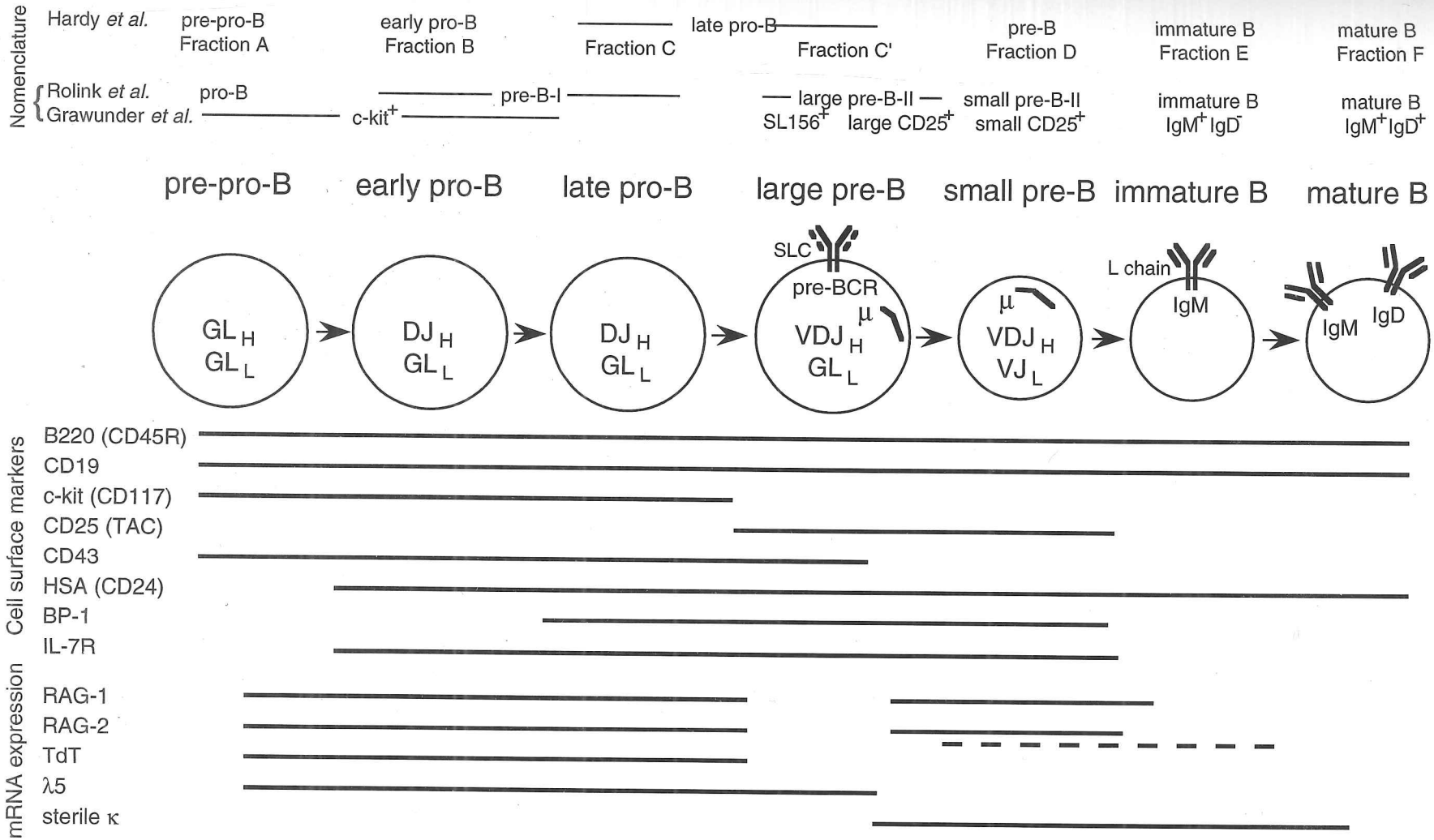

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Materials and Methods

Chapter 2

Chapter 2: Materials and Methods

Many of the procedures were followed as described in Sambrook *et al.*, 1989 or according to the maufacturer's instructions.

2.1 Materials

2.1.1 DNA constructs

Plasmids driving the expression of the *hen egg lysozyme (HEL)-specific receptors* were based on pSV2gpt and pSVneo (Fig. 3.1A). The κ transcription unit (Aluvihare *et al.*, 1997), in which the Vκ segment of the mouse D1.3 monoclonal antibody (Amit *et al.*, 1985; McCafferty *et al.*, 1990) is linked to rat Cκ, was assembled by exchanging the SacI-XhoI β-globin insert of Lκ-βG (Yelamos *et al.*, 1995) for an analogous PCR-generated SacI-XhoI fragment containing D1.3 Vκ. The H chain vectors derive from pSV-Vμ1 (Neuberger, 1983) (Fig. 5.1A) but with the V_{NP} V_H segment replaced by a PCR-generated PstI-BstEII D1.3 V_H segment (Fig. 3.1A). For the chimeric μ chains, the transmembrane and cytoplasmic domains of μ were replaced (using ApaI sites within Cμ4 and *gpt* selectable marker gene of the vector) by fusions of Cμ4 to the cytoplasmic domains of Ig-α alone (IgM/α) (Aluvihare *et al.*, 1997) or Ig-β (IgM/β) or mutant Ig-β with the ITAM tyrosines substituted by leucine ($IgM/\beta^{Y->L}$) (Patel and Neuberger, 1993) via a hydrophobic transmembrane region (derived from mouse H2-K^b) which facilitates surface transport without association of the Ig-α/Ig-β heterodimer (Williams et al., 1993; Patel and Neuberger, 1993).

The *V_{NP} Cμ[Ei,E3′]$^{\kappa}$ transgene* (provided by G. Williams) (Fig. 5.1A) also derives from plasmid pSV-Vμ1 (Neuberger, 1983) in which the region of DNA located between the most 5′ and 3′ XbaI sites in the J_H-Cμ intron was replaced by a 2.1 kb section of the mouse Jκ-Cκ intron spanning the κ intron enhancer/matrix attachment region (Ei/MAR) obtained by PCR using oligonucleotides MSN152 and MSN153. The XbaI-SacI 802 bp fragment containing the κ 3′ enhancer (E3′) was then introduced into the unique XhoI site located just 3′ of the CμM exons of pSV-Vμ1.

Construction of the *Lκ, Lκ[Ei/MAR] and Lκ[E3′]* (Fig. 6.1) have been described previously (Betz *et al.*, 1994).

The *βG[E3']* *transgene* (provided by G. Williams) (Fig. 6.5A) was assembled by inserting a 1 kb XbaI-SacI fragment including the mouse κ E3' into a Bluescript derivative in which a human β-globin gene had been inserted between the KpnI and XbaI sites. The KpnI-XbaI β-globin fragment was generated by PCR from human genomic DNA using oligonucleotides priming 105 nucleotides 5' of the transcription start site (introducing a 5' KpnI site) and 340 nucleotides 3' of the polyadenlyation signal (introducing a 3' XbaI site). The amplified β-globin gene (which extends from 5' of the promoter to 3' of the polyadenylation site) was assembled from two subfragments; the strategy involved a SpeI site in the second β-globin intron, 460 nucleotides downstream of the splice donor site.

2.1.2 Cell lines

Cell lines were usually cultured in DMEM/10% FCS/50 μM 2-ME and incubated at 37°C in 10% CO_2.

The *A20 transfectants expressing anti-HEL receptors* were established after plasmids encoding the anti-HEL-specific H chain constructs were linearised with ClaI and introduced by electroporation into a transfectant of the mouse A20 B cell lymphoma expressing the anti-HEL-specific κ L chain (provided by V. Aluvihare). After plating out to the density required to isolate single clones, stable transfectants were selected in DMEM/10% FCS/50 μM 2-ME containing 1 mg/ml G418 sulphate and 2 mg/ml mycophenolic acid, and screened by immunofluorescent microscopy for the expression of IgM and rat κ, discriminating the transfected Ig from the endogenous IgG_{2a} and mouse κ expressed by A20.

The *1E5.111 helper T cell hybridoma* (provided by L. Adorini) recognises HEL peptide (amino acids 108-116) in the context of MHC class II I-E^d molecules and is induced to secrete IL-2 (Adorini *et al.*, 1993).

2.1.3 Transgenic mice

Transgenic mice were established by microinjection of vector-free DNA fragments into the nuclei of (C57BL/6 x CBA/Ca) F1 zygotes (performed by S. Davies) and founder animals were bred with F1s, C57BL/6 or crossed with *μMT* B cell-deficient mice (Kitamura *et al.*, 1991) (provided by K. Rajewsky).

For the *anti-HEL-specific receptors*, an EcoRI fragment containing the L chain transcription unit was coinjected together with either a ClaI-XhoI (wild type IgM)

or ClaI-KpnI (chimeric IgM) fragment for the H chain (Fig. 3.1A). For all four constructs, multiple founders were identified that carried a H or L chain transgene. However, only one founder that had cointegrated both H and L transgenes was identified for each chimeric receptor construct; Southern analysis revealed that they were present in ten to twenty copies (S. Davies, unpublished observations). In the case of wild type HEL-specific IgM, two founders (lines A and B) that had cointegrated the H and L transcription units were studied, although only line B was bred into a *μMT* background. Litters were screened by PCR for presence of the H chain transgene and for endogenous background alleles; the κ transgene bearing a rat Cκ region was detected by ELISA. The presence of the IgM transgene in line B was detected using 5' oligonucleotide PCRD13 which primed within the D1.3 V_H region. The IgM ^{B} transgene could not, however, be detected using 5' oligonucleotide MSN146 which primed upstream of the TATA box, suggesting that integration may have resulted in the truncation of the 5' end of the IgM transgene bearing the promoter.

Although other founders expressing the *V_{NP} Cμ[Ei,E3']^κ transgene* had been generated, experiments were performed only on a line that had integrated a high number of copies (S. Davies, unpublished observations). Mice were screened by PCR.

Two founder lines each of the previously described *Lκ, Lκ[Ei/MAR] and Lκ[E3'] mice* (Betz et al., 1994) were analysed; the number of transgene copies is denoted by superscript and the mice were screened by ELISA. However, the Lκ[E3']^5 line used in Betz *et al.* (1994) was lost and Lκ[E3']^6 was used instead, revealing conflicting results. Confusion resulting from the difference in notation of the founder lines between Meyer *et al.* (1996), Betz *et al.* (1994), and the laboratory database may be clarified below.

Notation in Meyer *et al.* and in this study	**Notation in Betz *et al.* and Betz, PhD thesis**	**Notation in Neuberger group database**
Lκ[Ei/MAR]^4	Lκ[Ei/MAR] line B	DEL A
Lκ[Ei/MAR]^7	Lκ[Ei/MAR] line A	DEL C(I)
Lκ[E3']^2	Lκ[E3'] line A	EXO A
(Lκ[E3']^5)	Lκ[E3'] line B	EXO B
Lκ[E3']^6		EXO C

For the *βG[E3']* *transgene*, three founder lines were established, and although only line A, bearing 10-20 copies was analysed in this study, line B (line C on the lab database) bearing 5-10 copies was also used in Meyer *et al.* (1996). Litters were screened by PCR.

2.1.4 Oligonucleotides for PCR

For replacement of Eμ by Ei/MAR in V_{NP} Cμ[Ei,E3']$^{\kappa}$ transgene:

MSN152	GGACTAGTGATAGGAACAGAGCCACT
MSN153	GCTCTAGACAGAGATTCAGACCAGTTTA

For screening for anti-HEL D1.3 V region (0.3 kb):

PCRD13	CATCACATGCACCGTCTCAGG
MSN146	CGCGGATCCTTTTAAGGACTCACCTGAGG

For screening for the presence of a *μMT* allele (detects within *neo*, 192 bp):

TOKN30	CTTCTGAGGGGATCGGCA
OMN63	CGGCTCGAGGTTCAGAACAGGGTGACGGTGGTGCTGTAGAA

For screening to discriminate *μMT* alleles (1.3 kb) from wild type (0.2 kb)

MSN251	AGGGCACCCCACCCTTATGC
OMN63	CGGCTCGAGGTTCAGAACAGGGTGACGGTGGTGCTGTAGAA

For screening to discriminate IgH^a (209 bp) from IgH^b (231 bp) (detects γ2a intron):

MSN235	ATGATCCTCCTAGGATAGATGTCC
MSN236	GGAGTGGAGCTCTGGTCTAG

For screening for transgenic V_H (V_{NP} or D1.3 [except IgM^B]) (0.6 kb):

MSN145	CCGGGGAATTCTATGTATCCTGCTCATGAAT
MSN146	CGCGGATCCTTTTAAGGACTCACCTGAGG

For screening for βG[E3'] transgene (0.7 kb):

MSN212	CGCGAATTCTATTGCTTACATTTGCTTCTG
MSN213	CGCGGATCCCATTCTAAACTGTACCCTGT

For RT-PCR of β-globin transcripts (328 bp product):

CCTGAGGAGAAGTCTGCC

AGCACACAGACCAGCACG

For RT-PCR of HPRT transcripts (461 bp product):

TGCCGACCCGCAGTCCCAGCGTCG

GCTGTACTGCTTAACCAGGGAAAG

2.1.5 Immunoreagents (listed according to manufacturer)

Amersham International

streptavidin-FITC

Becton Dickinson

mouse anti-BrdU-FITC (clone: B44)

GIBCO BRL Life Technologies

rat anti-mouse B220(CD45R)-PE (clone: RA3-6B2)

streptavidin-RED670

Jackson ImmunoResearch Laboratories

goat anti-mouse IgM $F(ab')_2$ (115-006-020)

goat anti-mouse IgG $F(ab')_2$ (115-006-071)

goat anti-human IgG-FITC (109-095-008)

streptavidin-PE

Nordic

sheep anti-mouse IgD-FITC

PharMingen

mouse anti-mouse IgM^a-biotin (clone: DS-1)

rat anti-mouse CD4-PE (clone: RM4-5)

rat anti-mouse CD8a-FITC (clone: 53-6.7)

rat anti-mouse CD21/35(CR2/1) (clone: 7G6)

rat anti-mouse-CD23-PE (clone: B3B4)

rat anti-mouse HSA(CD24)-biotin (clone: M1/69)

rat anti-mouse CD25-FITC (clone: 7D4)

rat anti-mouse CD43-biotin (clone: S7)

rat anti-mouse CD44-PE (clone: 1M7)

rat anti-mouse B220(CD45R)-FITC (clone: RA3-6B2)

rat anti-mouse B220(CD45R)-PE (clone: RA3-6B2)

hamster anti-mouse CD69-FITC (clone: H1.2F3)

rat anti-mouse Thy1.2(CD90.2)-biotin (clone: 53-2.1)

Sigma

mouse anti-mouse Thy1.2(CD90.2)-FITC (clone: TS)

Southern Biotechnology Associates

goat anti-mouse IgM (1020-01)

goat anti-mouse IgM-FITC (1020-02)

goat anti-mouse IgM-biotin (1020-08)

rat anti-mouse IgD-PE (clone: ?)

goat anti-mouse IgG_1-FITC (1070-02)

goat anti-rat Ig-FITC (3010-02)

Non-commercial

mouse anti-mouse IgM^b-FITC (clone: MB86; Nishikawa *et al.*, 1986) (provided by M. Neuberger)

rat anti-mouse κ-biotin (clone: 187.1; Yelton *et al.*, 1981) (provided by R. Pannell)

mouse anti-rat κ-biotin (clone: RG7.1; Springer *et al.*, 1982) (provided by R. Pannell)

mouse anti-rat κ (clone: MRC OX12) (provided by R. Pannell)

mouse anti-rat κ-biotin (clone: MRC OX12) (provided by R. Pannell)

mouse anti-mouse BP-1-biotin (clone: BP-1; Cooper *et al.*, 1986)) (protein provided by G. Williams)

mouse anti-HEL (clone: HyHEL10; Smith Gill *et al.*, 1984) (protein gift from S. Smith-Gill)

mouse anti-D1.3 idiotype-FITC (clone: E5.2; Fields *et al.*, 1995) (cells gift from R. Poljak)

mouse anti-D1.3 idiotype-biotin (clone: E5.2)

mouse CTLA4-human IgG_1 fusion protein (Lane *et al.*, 1993) (cells gift from P. Lane)

2.1.6 Miscellaneous chemical and reagents

Standard chemical and reagents were obtained from BDH, Fischer Scientific, or Sigma, unless otherwise indicated.

Agarose (BioGene Limited)

Biotin-16-dUTP (Boehringer Mannheim)

Calf intestinal phosphatase (CIP) (Boehringer Mannheim)

Concanavalin A (Con A) (Sigma C-5275)

Fluorescein isothiocyanate (FITC) isomer I (Sigma F5720)

Foetal calf serum (FCS) (Hyclone Laboratories Inc.)

Gelase enzyme (Epicentre Technologies)

Gentamicin (GIBCO BRL)

G418 sulphate, Geneticin (GIBCO BRL)

Horse radish peroxidase (HRP), avidin conjugate (Dako)

Interleukin-2 (IL-2), human (Boehringer Mannheim 1204 700)

Interleukin-4 (IL-4), recombinant mouse (Genzyme)

Lipopolysaccharide (LPS) from *E. coli* 0111:B4 (Sigma L-4391)

Mycophenolic acid (GIBCO BRL)

Phenol, Tris-equilibrated (United States Biochemical)

Protein A-sepharose (Pharmacia)

Protein G-sepharose, pre-packed 1 ml HiTrap columns (Pharmacia)
Proteinase K (Boehringer Mannheim)
Restriction endonucleases (New England Biolabs)
Sephadex G-25 M, pre-packed PD10 columns (Pharmacia)
Sucrose (Sigma S-1888) (added to BrdU water)
Sulfo-NHS-biotin (Pierce)
Taq polymerase (Promega)
Terminal deoxynucleotidyl transferase (TdT) (Promega)
[^{3}H]Thymidine (Amerham)
T4 DNA ligase (New England Biolabs)

2.1.7 Buffers and solutions

Restriction enzyme buffer: 0.1 volume 10X TMN (1 ml 10X TMN:
1 mM DTT 150 µl 1M Tris pH 7.4
1 mM spermidine 150 µl $MgCl_2$
750 µl NaCl)

10X TBE: 0.89 M Tris HCl
0.89 M boric acid
25 mM EDTA pH 8.3

Agarase buffer: 10mM bis Tris-HCl pH 6.5
1 mM EDTA
100 mM NaCl

TB buffer pH 7.5: 50 mM Tris-HCl
100 mM EDTA pH 8.0
100 mM NaCl
1% SDS

2 X TY medium: 1.6% tryptone
1% yeast extract
0.5% NaCl pH 7.4

Phosphate-buffered saline (PBS): for 1 litre: 7.25 g NaCl
3.96 g $Na_2HPO_4.2H_20$
1.31 g $NaH_2PO_4.H_2O$

RNA lysis buffer: 0.15 M NaCl (for 50 ml: 7.5 ml 1M
10 mM Tris pH 7.4 0.5 ml 1M
0.5 % NP-40 0.25 ml
1 mM $MgCl_2$ 50 µl 1M)
in dpc H_20 (prepared according to (Sambrook et al., 1989))

FACS buffer: PBS
1% FCS
0.05% azide

ABTS-peroxide substrate: dissolve 10 mg tablet in: 7.7 ml 0.1M trisodium citrate
9 ml 0.1M ctric acid
add 3.5 µl H_2O_2 just before use

Red blood cell (RBC) lysis buffer: 1 mM NH_4HCO_3 (for 1 litre: 79 mg)
114 mM NH_4Cl (for 1 litre: 6 g)

1% paraformaldehyde: for 10 ml: 0.1 g paraformaldehyde
1.5 ml H_20
1 drop 1M NaOH
warm tube under hot tap to dissolve
then 1 ml 10X PBS
6.9 ml H_20
check pH 6.8-7.0

DNase I reaction mix: 150 mM NaCl
5mM MgCl2
10 µM HCl
300 µg/ml DNase I (about 500 Kunitz U/ml)

TdT reaction buffer: 0.5 M cacodylic acid, sodium salt, pH 6.8
1 mM $CoCl_2$
0.5 mM DTT
0.5 ml/ml BSA
0.15 NaCl

2.2 *Molecular techniques*

2.2.1 Plasmid construction

To prepare DNA fragments for cloning, 4-8 µl of plasmid DNA (from miniprep) was digested at 37°C for at least 1 h in a total volume of 40 µl containing 2 µl of each restriction enzyme and 4 µl of the 10X commercial enzyme buffer, and the enzymes were often heat inactivated by incubation for 20 min at 65°C.
Restriction fragments containing the plasmid vector were 5′ dephosphorylated by adding 2 µl of calf intestinal phosphatase (CIP) and 5 µl 10X CIP buffer in a total volume of 50 µl, incubating for 30 min at 37°C.
The DNA fragments were separated by agarose gel electrophoresis, depending on their size (often 0.7% agarose in 1X TBE buffer) and purified by running the band into 3MM filter paper backed with dialysis membrane slotted into the lane of the gel. The DNA was eluted by microcentrifugation using 1X TBE buffer to wash.
The DNA was ethanol-precipitated by adding 0.1 volumes of 3 M sodium acetate pH 5.5, followed by 3 volumes of 95% ethanol and placing on dry ice until frozen. After spinning in a microfuge for 5 min, the pellet was aspirated and resuspended in an appropriate volume of H_20 (often 25 µl).

DNA ligations were carried out in a total volume of 10 µl containing 1 µl of vector fragment to about 3 µl of each insert fragment, 1 µl of T4 DNA ligase and 1 µl 10X ligase buffer enzyme. Reactions (including controls for vector and inserts alone) were left at room temperature (or at 16°C) overnight.

Competent TG1 E. coli for transfection were prepared by the method of (Hanahan, 1983) and were prepared freshly prior to use.
Each ligation reaction was transfected into 200 µl TG1, by leaving on ice for 10-20 min before heat shocking at 42°C for 2 min and immediately placing back on ice. After addition of 400 µl of 2 X TY broth, the cells are allowed to recover for 1 h at 37°C with shaking before plating out on three 2 X TY + ampicillin (100 µg/ml) agar plates and incubating at 37°C overnight inverted.

If colony numbers suggested the ligations had worked, about ten colonies were picked and *minipreps* of plasmid DNA were prepared from 5 ml overnight cultures of 2 X TY + ampicillin broth by alkaline lysis using the Promega Wizard Miniprep Kit. Larger quantities of plasmid from 100-200 ml cultures were prepared using the Promega Wizard Maxiprep Kit and by adding 20 µl DNA to 380 µl H_20, an estimate of mg/ml was equivalent to optical density at 260 nm.

For *analytical digestion of plasmid DNA*, 1 µl of miniprep DNA using 1 µl of each restriction enzyme was digested in a total volume of 15 µl restriction enzyme buffer for 1 h at 37°C. The fragment bands were resolved by agarose gel electrophoresis.

2.2.2 Preparation of DNA fragments for microinjection

Choosing restriction sites to remove as much of the vector as possible, the plasmids were digested as described for cloning. The fragments were resolved by electrophoresis of a 0.7% low melting point agarose gel and the bands were removed as gel slices which were equilibrated in 5 ml 1X agarase buffer overnight at 4°C. The slices were weighed (usually about 200 mg), melted at 70°C for 10 min, and pre-incubated at 40°C for 5 min before adding 1 µl of gelase enzyme per 100 mg and incubating for 2 h. After digestion, the DNA solution was spun in a microfuge for 20 min. The supernatant containing the DNA was suitable for microinjection without additional purification and the concentration (often used undiluted at about 2-5 ng/µl) was estimated by comparing aliquots with known quantities of phage λ HindIII digest markers on agarose gels.

2.2.3 PCR screening of transgenic mice

Genomic mouse DNA was prepared by digesting 1-2 cm tail biopsies in 750 µl of TB buffer supplemented with 450 µg of proteinase K (22.5 µl of 20 mg/ml in H_20) at 55°C overnight, with occasional mixing. The DNA was purified by successive extractions with 750 µl Tris-equilibrated phenol, 750 µl phenol/chloroform/isoamyl alcohol (25:24:1) and 750 µl chloroform, microfuging for 5 min to separate the aqueous and organic phases. The DNA was precipitated by adding to 1.3 ml 95% ethanol, drawn out using a disposable inoculating loop, and washed in 50 µl of 70% ethanol before the pellet was left to dry out. 100 µl H_20 was added to the pellet and the DNA was often left to dissolve at 4°C overnight.

PCR reactions were carried out in a total volume of 50 µl containing 1 µl genomic DNA from tail, 5 µl 10X Taq buffer, 2 µl 10 mM dNTP, 2.5 µl of each oligo diluted to 10 pmol/µl and ≤1µl Taq polymerase. One drop of mineral oil was added to each tube and the reactions were incubated according to a "touchdown" programme (succcessive steps have decreased annealing temperatures) with a 5 min polymerase extension time. PCR products were analysed by 2.0% agarose gel electrophoresis.

2.2.4 Cytoplasmic RNA preparation

Lymphocytes were pelleted (1,100 rpm, 4°C, 5 min) and washed in cold PBS, removing all supernatant. The cells were resuspended in 0.4 ml of cold RNA lysis buffer, and were microfuged at 4°C for 5 min. The supernatant lysate was transferred into 200 µl phenol + 50 µl 10% SDS, vortexed hard and microfuged again. The supernatant was re-extracted with 200 µl phenol, then 40 µl 3M sodium acetate pH 5.5 was added with 1 ml RNase-free 95% ethanol to precipitate at -20°C overnight. RNA preps were often stored at -20°C or -70°C until further analysis.

2.2.5 RT-PCR analysis (performed by Kerstin Meyer)

0.1-1.0 µg RNA was reverse transcribed with MMLV reverse transcriptase using 100 ng $(dT)_{15}$ primer for 1 h at 37°C. Aliquots of the reaction were then used in PCR annealed at 60°C for 1 min, extended at 72°C for 1 min and denatured at 92°C for 1 min for a total of 30 cycles.

2.3 *Antibody-mediated techniques*

2.3.1 Preparation of non-commercial immunoreagents

Cells expressing the *monoclonal antibody* or transfected fusion protein were grown to a density of about $5x10^5$ cells/ml in DMEM/5-10% FCS and were transferred (70-100 ml per tube of dialysis membrane) to an OBC ("oscillating bubble chamber" - designed by Milstein and Pannell). The OBC was filled with about 1.5 L of serumless DMEM which was changed every 2-3 days for 14 days. The culture supernatants were harvested for purification.

The desired protein was purifed by *affinity chromatography*. Where possible, a protein A-sepharose column were used to recover protein of interest: a 1 ml column was equilibrated with 100 mM Tris pH 8.0, the antibody supernant (equilibrated by adding 0.1 volume of 1M Tris pH 8.0) was loaded, washed with 100 mM Tris, then 10mM Tris, and eluted with 100 mM glycine pH 3.0 in 500 µl fraction, neutralising with 50 µl of 1M Tris pH 8.0. However, 1 ml pre-packed Hi-Trap Protein G columns were used for purifying mouse IgG_1 monoclonal antibodies (FCS did not contribute much to recovered protein) equilibrating 20 mM sodium phosphate buffer pH 7.0, eluting with 100 mM glycine pH 2.7 and neutralising with 0.1 volume 1 M Tris pH 9.0.
Protein concentration of the recovered material was estimated by optical density at 280 nm (where OD 1.0 is approximately equivalent to about 1.4 mg IgG) and purity was checked by SDS-PAGE.

Biotinylation was performed by dialysing the antibody prep (about 1 mg/ml) against 50 mM sodium bicarbonate buffer pH 8.5 at 4°C and after addition of the sulfo-NHS-biotin, the reaction was incubated with inversion at room temperature of 2-3 h, before dialysis against PBS at 4°C.

For *FITC conjugation*, the antibody prep (about 1 mg/ml) was dialysed against FITC conjugation buffer pH 9.3 at 4°C. FITC (isomer I, Sigma) was dissolved in buffer at 1 mg/ml and 0.3 volumes was added to the antibody solution and incubated in the dark with inversion at room temperature for 3 h. The free FITC molecules were removed by gel filtration using prepacked PD10 columns containing Sephadex G-25 M, equilibrating and eluting with PBS.

2.3.2 Immunofluorescent microscopy

15-well multi-test microscope slides were coated with one drop per well of 1 mg/ml poly-L-lysine, left at room temperature for 10-15 min, rinsed in distilled water and left to dry vertically.

A cell suspension in PBS was spotted onto the wells and the slides were placed into a humid box for 20-60 min at 37°C. The cells were fixed by placing the slides in methanol at -20°C for only 20 min and left to dry at -20°C for 10 min (or stored indefinitely).

To stain, the slides were dipped three times into PBS and not allowed to dry out during subsequent steps. Using filter paper, the wells were wiped around and after the slide was placed in a humid box, 4 µl of diluted FITC-conjugated immunoreagent in PBS/1% FCS/0.05% azide was pipetted per well and left for 20 min at room temperature. Rinsing in PBS and staining was repeated for each layer of reagent. Finally, the slide was dipped a three times in PBS and wiped, before drops of glycerol/PBS/0.05% azide and the coverslip were applied, sealed with nail varnish for analysis under the fluorescence microscope.

2.3.3 Flow cytometry

For surface staining, 0.5-2x10^6 cells per 6 ml round-bottomed tube (Falcon 2058) were pelleted (1,100rpm, 4°C, 5 min), the medium aspirated, resuspended in 60 µl diluted immunoreagent(s) in PBS/1% FCS/0.05% azide (FACS buffer) and left for at least 20 min on ice. After filling the tube with cold FACS buffer to wash, the procedure was repeated for each layer of reagent (e.g. subsequent staining with streptavidin [SA]-conjugates or secondary antisera). Immediately before filling the tube for the final wash, propidium iodide (PI; to indicate viability) was added if desired.

For cytoplasmic staining, cells were washed in cold PBS, pelleted, the medium aspirated and fixed in 0.5 ml cold PBS/3.7% formaldehyde for 10 min on ice. 0.5 ml PBS/0.1% NP-40 was added to permeabilise, the tube inverted to mix, and left on ice for 5 min. After the cells were pelleted and the medium aspirated, they were resuspended in FACS buffer for staining as above.

For *flow cytometric analysis*, the cells were resuspended in 0.5-1.0 ml FACS buffer. Becton Dickinson FACScan or FACSCalibur instruments with LYSYS II or CELLQuest software were used. In addition to experimental samples, unstained and single fluorochrome (FITC, PE, RED670, PI) controls for each reagent were for included for setting gains and compensation. Viable cells were gated by forward and side scatter and for PI exclusion where possible.

To enrich subpopulations, cells were surface stained as above under sterile conditions, and the cells were sorted on Becton Dickinson FACStar Plus (operated by D. Gilmore and A. Riddell). They were collected for immediate cytoplasmic RNA isolation or cultured in DMEM/10% FCS/50 μM 2-Me/50 μg/ml gentamicin.

2.3.4 ELISA screening of transgenic mice

96-well flat-bottomed plates were coated with 1 μl/ml mouse anti-rat κ (MRC OX12) or 5 μg/ml goat anti-mouse IgM in PBS (50 μl per well) overnight at 4°C or for 2 h at 37°C. Plates were blocked by addition of 150 μl/well of PBS/ 5% FCS/ 0.05% azide for 30 min at 37° (and could be stored at 4°C for months).

After washing three times with PBS, 50 μl diluted mouse serum was added to each well and incubated for 1.5 h at 37°C. Serum was diluted in PBS/5% FCS and for each mouse, usually two dilutions were analysed in duplicate (for rat κ in the range 1/10 - 1/500; for μ, 1/400 - 1/2,000). After careful washing with PBS three times, mouse serum antibodies were detected using 50 μl per well of 1 μg/ml mouse anti-rat κ-biotin (MRC OX12) or goat anti-mouse IgM-biotin in PBS/5% FCS, incubating for 45 min at 37°C. 50 μl/well avidin-conjugated horse radish peroxidase (1:2,000 in PBS/ 5% FCS) was added for 15 min at 37°C after washing with PBS three times. The final washes consisted of three with PBS, followed by three with distilled water. 50 μl/well of the ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid)-peroxide substrate was added and left to develop for a few minutes before the reaction was stopped with 50μl/well of 0.1M citric acid/ 0.05% axide. If desired the OD at 405 nm was read on a Molecular Devices plate reader using Softmax software.

2.4 Cellular assays

2.4.1 Preparation of lymphoid tissues from mice

Single cell suspensions were prepared from mouse lymphoid tissues. Spleen and thymus were dissected, bone marrow was washed from within the femurs, and Peyer's patches were pinched off the gut. Tissues were disrupted by gentle teasing with forceps and by pushing gently through a cell strainer into PBS or culture medium.

If desired, *erythrocytes were depleted* by resuspending the cells (after pelleting at 1,100 rpm at 4°C for 5 min) in 1 ml PBS or medium and adding 4 ml RBC lysis buffer. After gentle inversion to mix, the cells were left for 10 min on ice or room temperature before washing with PBS or medium.

2.4.2 Antigen presentation

In a flat-bottomed 96-well plate, triplicate wells each containing 10^5 A20 B cells and 10^5 1E5.111 T cell hybridomas were cultured in 200 µl complete medium (DMEM/10% FCS/50 µM 2-ME) supplemented with 10-fold serial dilutions of antigen hen egg lysozyme (HEL) or turkey egg lysozyme (TEL). After incubation for 24 h at 37°C/10% CO_2, the plates were spun down (1,100 rpm, 4°C, 5 min) and the supernatants (50 µl per well) were transferred to a fresh plate and to each well was added $2.5x10^5$ HT2 (IL-2 dependent) T cells in 50 µl complete medium. After 20-24 h at 37°C/10% CO2, each well was pulsed with 10 µl of 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in PBS for 4-6 h, then the crystals were dissolved by vigorous pipetting after the addition of 110 µl of 5% formic acid in isopropanol. The optical density at end point 570-650 nm was measured on Molecular Devices plate reader using Softmax software and units of IL-2 produced were calibrated against recombinant IL-2 standards.

2.4.3 B cell activation

The production of IL-2 by A20 cells was measured by adapting the antigen presentation assay without T cells. 10^5 B cells were incubated with 10-fold serial dilutions of HEL or TEL antigen or $F(ab')_2$ goat anti-mouse IgM or anti-IgG antiserum and subsequent stages proceed as for the antigen presentation assay.

To monitor proliferative responses, splenocytes that had been depleted of erythrocytes were cultured in triplicate aliquots ($2x10^5$ in 200 µl) in RPMI/10% FCS/10 µM 2-ME in the presence of 1 µg/ml LPS or 10 µg/ml $F(ab')_2$ goat anti-mouse IgM + IL-4 (100 U per 200 µl). After 48 h, the cells were pulsed with 0.5 µCi [^{3}H]thymidine for 15 h before scintillation counting.

To monitor CTLA4 binding, spleen cells depleted of erythrocytes were cultured (10^6 cells/ml) in medium in the presence or absence of 50 µg/ml LPS, 10 µg/ml $F(ab')_2$ goat anti-mouse IgM, 1.0 µg/ml HEL or 10 µg/ml monoclonal E5.2 anti-D1.3 idiotype antibody for 24 h prior to staining with mCTLA4-Hγ1 fusion protein, goat anti-human IgG-FITC and propidium iodide.

κ L chain expression in LPS-activated B cells was analysed after incubation of splenic B cells in medium supplemented with 50 µg/ml LPS for 48 h. Cells were harvested, fixed and permeabilised before cytoplasmic staining with mouse anti-rat κ-biotin or rat anti-mouse κ-biotin followed by SA-PE.

2.4.4 T cell activation

Splenocytes depleted of erythrocytes were cultured ($5x10^5$/ml) in the presence or absence of 2 µg/ml concanavalin A for 24 h before staining for the surface expression of Thy 1.2 (CD90.2), CD4, CD44, CD25, CD69 and IgM and analysis by flow cytometry.

2.4.5 BrdU uptake

Mice were given two intraperitoneal injections 4 h apart of 1 mg BrdU (5-bromo-2-deoxyuridine) in PBS and the drinking water was supplemented with 1 mg/ml BrdU and 1% sucrose for 72 h before analysis of bone marrow and spleen. Cells were surface stained with rat anti-B220-PE and either goat anti-IgM-biotin or anti-D1.3 idiotype-biotin (both revealed by SA-RED670) prior to fixation. The cells were washed (pelleted at 1,1000 rpm, 4°C, 5 min) and resuspended in 200 µl PBS, and fixed by injecting into 1 ml ice-cold 70% ethanol to prevent cell clumping. After 30 min on ice, the fixed cells were pelleted at 1,500 rpm (and at subsequent spins) and resuspended in 500 µl PBS to which was added 500 µl of 2% paraformaldehyde/ 0.01% Tween 20 in PBS, and left overnight at 4°C. After washing once in PBS, the cells were resuspended in 1 ml of DNase I reaction mix for 10 min at room temperature. The cells were washed once with FACS buffer, stained with anti-BrdU-FITC for 20 min at room temperature, then washed and resuspended for analysis.

2.4.6 TUNEL analysis

Splenocytes that had been depleted of erythrocytes were cultured (10^6 per 1 ml) in the presence and absence of 10 μg/ml $F(ab')_2$ goat anti-mouse IgM for 0, 4, 8 or 24 h. Cells were harvested and surface stained with rat anti-B220-PE before fixation for TUNEL (terminal dexoynucleotidyl transferase [TdT]-mediated dUTP-biotin nick end labelling). After washing in PBS (pelleted at 1,100 rpm, 4°C, 5 min) the cells were resuspended in 250 μl PBS and fixed by adding 750 μl of ice-cold 95% ethanol dropwise over a period of 5-10 sec while gently vortexing to prevent cell clumping. After incubation for 20 min on ice, the cells were washed on ice and pelleted at 1,500 rpm (fixation causes shrinkage). The cells were fixed with 1 ml of 1% paraformaldehyde in PBS by adding dropwise and gently vortexing, and incubated at room temperature for 30 min. Cells were washed in PBS (when they could be stored for a few days at 4°C in the dark), followed by TdT reaction buffer prior to resuspension of the cells in 50 μl TdT reaction buffer containing 5U TdT and 10 μM biotin-dUTP. The TdT reaction was incubated for 45 min at 37°C. After washing the cells were stained with SA-FITC for analysis by flow cytometry.

Results

Expression and function of chimeric B cell antigen receptors in cell line transfectants

Chapter 3

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The Ig-α/Ig-β heterodimer plays a critical role in B cell antigen receptor (BCR) function, not only for surface transport of membrane IgM (Williams *et al.*, 1990), but also because the ITAMs (immunoreceptor tyrosine-based activation motifs) (Reth, 1989) present in the cytoplasmic domains of both polypeptides are essential for the initiation of transmembrane signalling (Flaswinkel and Reth, 1994; Papavasiliou *et al.*, 1995; Sanchez *et al.*, 1993; Taddie *et al.*, 1994; Williams *et al.*, 1994). Aside from the ITAMs, Ig-α and Ig-β differ significantly and are well conserved in evolution (Hermanson *et al.*, 1988; Müller *et al.*, 1992; Sakaguchi *et al.*, 1988; Yu and Chang, 1992). Thus, it has been disputed whether Ig-α and Ig-β mediate essentially similar activities or if they perform distinct roles. The abilities of the individual cytoplasmic domains have been determined in cell lines transfected with chimeric receptors bearing either the Ig-α or Ig-β tail alone. Some groups found no differences in the signalling activities of either the Ig-α or Ig-β chimeras compared to wild type BCR (Law *et al.*, 1993; Taddie *et al.*, 1994; Williams *et al.*, 1994). Others, however, found that whilst the Ig-α chimeras could signal, signalling by the Ig-β chimeras was impaired to variable extents, depending on the nature of the chimeric constructs and the assay conditions (Choquet *et al.*, 1994; Kim *et al.*, 1993; Sanchez *et al.*, 1993). Furthermore, it has been suggested that the Ig-α and Ig-β cytoplasmic domains bind different sets of downstream effectors (Cassard *et al.*, 1996; Clark *et al.*, 1992).

The conflicting evidence regarding the relative roles of Ig-α and Ig-β may be due to the use of cell lines. A cell line provides a convenient model system of a homogeneous population of cells that is relatively easy to maintain. However, different cell lines may express limiting amounts of signalling molecules and they are subject to the effects of long-term culture. Thus, they may not accurately reflect events in primary cells.

An alternative approach to cell line transfectants is the introduction of transgenes the germline of mice in order to generate a good source of primary B cells expressing antigen-specific receptors. Transgenic mice made it possible to extend the study of Ig-α and Ig-β in BCR signalling to primary cells. In preparation for the transgenic experiments, it was first necessary to prepare and check the transgene constructs. This chapter describes the BCR transgenes and

their expression and function in cell line transfectants. Additional findings to result from these studies were that the cytoplasmic tails of Ig-α or Ig-β could independently mediate antigen-induced signals in this cell line system, but that mutation of the Ig-β ITAM tyrosines abrogated signalling.

3.1 Anti-HEL receptor transgene constructs contain genomic regulatory elements.
In order to discriminate between the independent functions of Ig-α and Ig-β, a set of plasmids was constructed encoding BCRs of μ H chains and κ L chains bearing the V_H and V_L regions of the D1.3 monoclonal antibody (Amit *et al.*, 1985) that specifically binds the antigen hen egg lysozyme (HEL) (Fig. 3.1).

The wild type μ construct derived from pSV-Vμ1 (Neuberger, 1983) (which includes the appropriate genomic elements of the V_H promoter and IgH enhancer [Eμ] for efficient tissue-specific expression in mice), but with the V_{NP} V_H replaced by the D1.3 V_H (Fig. 3.1A). For the μ chimeras, ApaI fragments extending from Cμ4 into the pSV2gpt plasmid vector were exchanged to encode for the extracellular μ domains fused via a hydrophobic transmembrane region to the cytoplasmic domains of Ig-α alone (IgM/α) (Aluvihare *et al.*, 1997) or Ig-β (IgM/β) or mutant Ig-β with the ITAM tyrosines substituted by leucine (IgM/$\beta^{Y->L}$) (Patel and Neuberger, 1993) (Fig. 3.1A and B). The transmembrane segment (which derives from the H2-K^b gene) confers surface transport without detectable association with the Ig-α/Ig-β heterodimer (Patel and Neuberger, 1993; Williams *et al.*, 1993).

The κ L chain construct (Aluvihare *et al.*, 1997) bore a rat Cκ region for discrimination from endogenous κ in mice or in mouse B cell lines (Fig. 3.1A). It also contained the required regulatory elements of Vκ promoter, intron enhancer (Ei) and 3′ enhancer (E3′).

3.2 The transgenic receptors are expressed on the surface of A20.
A20 is a mouse B cell lymphoma that expresses endogenous BCRs composed of IgG_{2a} and κ. The set of μ H chain plasmids was transfected into an A20 transfectant that already expressed the anti-HEL rat κ L chain as a means for checking that the DNA constructs were functional and could be expressed properly.

After initial screening by immunofluorescent microscopy, clones of each transfection were selected for analysis by flow cytometry (Fig. 3.2). All of the

transfected receptor constructs were detected by staining for IgM and rat κ, indicating that the plasmids could indeed encode the H and L chains for functional expression as surface receptors on B cells. Although each transfectant clone varied in expression level and homogeneity, the positive populations were enriched by cell sorting if necessary.

3.3 The anti-HEL V region mediates specific antigen presentation.

Having shown that the transfected H and L chain C regions were detectable with μ- and rat κ-specific antisera, it remained to be shown that the D1.3 V region was also expressed correctly and could bind antigen specifically.

One of the functions of the BCR is to internalise and target antigen intracellularly for processing and presentation to specific T cells. Antigen presentation by B cell lines can be monitored by the levels of IL-2 secreted by the antigen-specific helper T cell hybridoma in a colorimetric assay. The antigen presentation assay provided a convenient system not only for verifying antigen binding, but also for assessing receptor function. Indeed, it has been shown that an IgM/β chimeric receptor could mediate antigen presentation with similar efficiency to the wild type IgM receptor (Patel and Neuberger, 1993).

For the HEL-specific antigen presentation assay, the A20 transfectants expressing the D1.3 receptors were incubated with HEL, and the presentation of antigen was detected by the 1E5.111 T cell hybridoma which recognises HEL peptide amino acids 108-116 in the context of I-E^d (Adorini *et al.*, 1993). The sequence of this peptide is identical in HEL and the related turkey egg lysoyme (TEL) (Canfield, 1963; LaRue and Speck, 1970), thus HEL and TEL share the same epitope recognised by 1E5.111. However, the epitope recognised by D1.3 is specific to HEL and not TEL (Harper *et al.*, 1987). Therefore, the specific internalisation and presentation HEL via the D1.3 receptors could be monitored using the uptake and presentation of TEL as a control for non-specific fluid phase-mediated internalisation.

The receptor constructs all contained the D1.3 V region since they shared the same L chain and the H chains had been derived by "tail swapping" of the same H chain plasmid clone bearing the D1.3 V_H. A representative IgM/β transfectant (clone-14) was assessed for its ability to present HEL but not TEL, alongside a wild type IgM transfectant (AK1158-7) which has already been shown to mediate specific HEL presentation (Aluvihare *et al.*, 1997). From the levels of IL-2 secreted

by the T cell hybridoma, it is clear that both IgM and IgM/β receptors mediated the specific presentation of HEL (Fig. 3.3B). HEL presentation was evident from 0.01 μg/ml, at 100-fold lower concentrations than that of the non-specific presentation of TEL which was detectable from 1.0 μg/ml (Fig. 3.3B). Thus the expression of the D1.3 V region was intact and functional, also confirming that the Ig-β cytoplasmic tail alone can mediate antigen presentation.

Surprisingly, IL-2 was detected in the "no T" control containing B cells and antigen, but no T cells. Only the antigen-induced T cell hybridoma was expected to secrete IL-2. Since the "no T" cultures contained A20 transfectant cells incubated with 0.1 μg/ml HEL, the IL-2 production indicated that A20, as well as IE5.111 could be induced to secrete IL-2.

The A20 transfectants were made available to others in the group who have continued the analysis of antigen presentation by comparing the presenting activities of IgM/α versus IgM/β (Aluvihare *et al.*, 1997). Both chimeric receptors mediated the efficient presentation of HEL, suggesting that the cytoplasmic domains of either Ig-α or Ig-β are sufficient to drive intracellular targetting of antigen for presentation and revealing no major differences in their contribution to this BCR function.

3.4 HEL stimulates greater IL-2 production in A20 transfectants than anti-Ig.

Antibodies are routinely used to mimic antigen crosslinking of BCRs in biochemical signalling assays. For example, after ligation with anti-IgM, IgM/β chimeras have been shown to mediate downstream tyrosine phosphorylation within five minutes (Law *et al.*, 1993; Williams *et al.*, 1994). The finding that A20 can be stimulated to secrete IL-2 suggested that IL-2 production could be exploited as a signalling assay in response to anti-Ig. Since cytokine secretion occurs further downstream than tyrosine phosphorylation events, it might provide a more sensitive readout for signalling.

When the IgM/β transfectant was incubated with increasing concentrations of anti-IgM, IL-2 secretion was induced from 1.0 μg/ml anti-IgM (Fig. 3.4A). Anti-IgM did not induce untransfected A20 to signal, but IL-2 production was detectable when the endogenous BCRs were crosslinked by 100 μg/ml anti-IgG. These results confirmed that the cytoplasmic tail of Ig-β alone is sufficient to mediate signalling in response to anti-IgM. However, the levels of IL-2 were rather low, making detection difficult and limiting the sensitivity range of the

assay.

Recalling the signal clearly apparent in the antigen presentation assay (Fig. 3.3B), a greater response might be conferred using the natural antigen HEL, rather than anti-Ig. Indeed, IgM/β did produce higher levels of IL-2 in response to HEL compared to anti-IgM (Fig. 3.4B). IL-2 production in IgM/β was triggered from 0.1 μg/ml HEL, but untransfected A20 did not respond to HEL. Thus, the signalling ability of the Ig-β cytoplasmic tail was further demonstrated in mediating the response to the receptor's natural antigen. Furthermore, monitoring IL-2 production in A20 transfectants seemed to be a promising signalling assay in terms of using antigen, rather than anti-Ig, and sensitivity.

The evidence that 0.1 μg/ml HEL induced the IgM/β transfectant to secrete IL-2 does not invalidate the earlier conclusions that IgM and IgM/β can mediate specific antigen presentation since IL-2 production (by the T cell hybridoma) was clearly evident after incubation with 0.01 μg/ml HEL.

3.5 BCR signalling in A20 may be induced by multivalent forms of HEL.

It is curious that HEL should induce the BCR to signal, since HEL is a monovalent antigen and effective signal transduction requires receptor aggregation. One explanation might be that HEL exists in a higher valency form than a monomer. For example, HEL may form dimers on an opposite face from the D1.3 epitope that facilitates crosslinking of the receptors.

In order to test whether multimeric forms of HEL existed, a 1 mg/ml solution of HEL in PBS was analysed by HPLC (high-performance liquid chromatography) at 4°C (Fig. 3.5). Although the large majority of the protein was present in the monomer peak, two other peaks were detected and they may correspond to HEL dimers and trimers. Thus it may be possible that HEL aggregates crosslink the D1.3 receptors and induce BCR signalling, even if present in such small quantities.

3.6 HEL-induced signalling is specific over a range of concentrations.

The IL-2 assay system was further characterised by determining the concentration range for which signalling in response to HEL was specific. The dose response of IgM/β to HEL shows that IgM/β secreted IL-2 when incubated with 0.1 to 100 μg/ml HEL (Fig. 3.6). Untransfected A20 was not induced by HEL and IgM/β did not respond to TEL, indicating that the HEL-induced response was specific to the

presence of D1.3 receptors as well as the recognition of HEL by D1.3. Interestingly, higher concentrations of HEL seemed to inhibit the IL-2 signal.

3.7 IgM/α and IgM/β but not IgM/$\beta^{Y->L}$ can signal in A20.

The development of an antigen-dependent signalling assay facilitated the comparison of the different receptors by monitoring their abilities to induce IL-2 secretion in response to HEL.

The IgM/β transfectant has already been shown to secrete IL-2 after incubation with HEL, therefore it was not surprising that the wild-type IgM receptor mediated signalling (Fig. 3.7A). Additionally, IgM/α was induced to produce IL-2. Thus the IgM/α and IgM/β chimeras, as well as the wild type IgM receptor mediated responses to antigen, indicating that the individual cytoplasmic domains of Ig-α or Ig-β are sufficient for signalling in A20. The differences in the levels of IL-2 secreted by IgM, IgM/α and IgM/β may simply reflect the expression levels of the A20 transfectants (Fig. 3.7B).

In contrast to the response of IgM/β, the IgM/β^{Y-L} transfectant did not secrete IL-2 (Fig. 3.7A). Mutation of the tyrosine residues prevented signalling, revealing that the ITAM tyrosines are essential for the ability of the Ig-β cytoplasmic domain to signal.

3.8 Discussion

Resulting from the original intention to examine the roles of Ig-α and Ig-β in transgenic mice, plasmids encoding wild type and chimeric BCRs were constructed to confer anti-HEL specificity and to include genomic regulatory elements for their appropriate expression in mice. In order to check the receptor constructs for expression and function, they were transfected into the A20 cell line. The results in this chapter have not only shown that they were correctly expressed and assembled as anti-HEL receptors on the cell surface, but that a transfected BCR could also mediate the functions of antigen presentation and antigen-induced transmembrane signalling. Thus, the constructs proved satisfactory for generating transgenic mice.

In addition, the cell line experiments resulted in the development of an antigen-induced signalling assay. The production of IL-2 was detected as a readout for the response of the anti-HEL A20 transfectants to the natural antigen HEL, extending experiments that have used antibodies to crosslink and induce A20 to secrete IL-2

(Beaufils *et al.*, 1993; Justement *et al.*, 1989).

It was intriguing that HEL induced BCR signalling since it was expected to behave as a monovalent antigen. HPLC analysis suggested that small quantities of HEL may be present in dimeric or trimer forms which could mediate crosslinking of the receptors. Indeed, it has been possible in ELISAs to capture HEL with the same anti-HEL mAb as used to subsequently detect HEL (G. Williams, unpublished observations). The observation that the same epitope of HEL can be recognised on two faces of the protein is consistent with the presence of the antigen as a dimer. Whilst it remains to be shown whether these results can be extrapolated to other assay conditions, the dimerisation of HEL offers an explanation that could be pursued.

The basis of the possible dimerisation of HEL is unclear. If a distinct surface of HEL mediates association, then only BCRs that recognise epitopes on the outer faces of the dimer could be crosslinked. However, B cells expressing receptors bearing HyHEL10 V regions also undergo HEL-induced activation (Cooke *et al.*, 1994), even though HyHEL10 binds to an epitope on a different face of the antigen from D1.3 (Davies *et al.*, 1990).

It could be argued that the crosslinking of anti-HEL receptors resulted from the aggregation of HEL with serum proteins over the 24 h-culture period in the IL-2 assay. However, anti-HEL receptors have been shown to mediate increases in intracellular calcium levels within 1 min of the addition of HEL, and tyrosine phosphorylated substrates were detected after 5 min incubation with HEL in serum-free medium (Cooke *et al.*, 1994).

Other mechanisms to explain the triggering of the BCR by HEL may be that HEL mediates co-crosslinking of the BCR with other receptors, or that HEL does bind as a monovalent antigen, but effects a conformational change in the BCR resulting in transmembrane signalling.

IL-2 production was stimulated over the range of 0.1 to 100 μg/ml HEL, but was inhibited at higher concentrations. It is possible that the BCRs become saturated at high HEL concentrations and are ligated separately, preventing agregation and signalling. Alternatively, at high concentrations HEL may bind to other receptors, such as Fc receptors, that negatively regulate BCR signalling, or the presence of excess HEL may result in receptor downmodulation over the 24 h-

assay period without generating detectable IL-2 levels.

Anti-Ig is standardly used for activation at 1-20 μg/ml, both in biochemical assays over a few minutes and physiological assays over days. In the IL-2 assays shown here, $F(ab')_2$ fragments were used instead of whole Ig to eliminate inhibitory effects resulting from Fc receptor coligation. It was thus surprising when anti-Ig stimulation of A20 did not induce much IL-2 production. Perhaps, anti-Ig over-crosslinked the BCRs. Membrane IgM that is highly crosslinked by anti-Ig patches and caps by association with the cytoskeleton (Williams et al., 1994), and IgM has been shown to be partitioned in the detergent-insoluble phase after cell lysis (Braun *et al.*, 1982). Thus the receptors may be rendered inaccessible to downstream signalling molecules by association with the cytoskeleton. It will be interesting to see whether HEL, like anti-Ig, causes BCRs to patch and cap.

Application of the IL-2 assay to discriminate between the functions of Ig-α and Ig-β, indicated that whereas the Ig-β ITAM tyrosines are essential, the Ig-α and Ig-β cytoplasmic domains can independently mediate signalling, with no significant differences in their abilities. The results are in agreement with previous findings in cell lines (Law *et al.*, 1993; Sanchez *et al.*, 1993; Taddie *et al.*, 1994; Williams *et al.*, 1994), although in conflict with others (Choquet *et al.*, 1994; Kim *et al.*, 1993; Sanchez *et al.*, 1993). The limitations of cell line systems discussed at the beginning of this chapter and the original decision to generate transgenic mice were thus reinforced.

Nevertheless, HEL-induced IL-2 production by A20 provides a convenient signalling assay, and opens up the possibilities of testing further mutant chimeric receptors to assess the roles of BCR components. Other parameters important to signalling such as affinity for antigen could also be monitored.

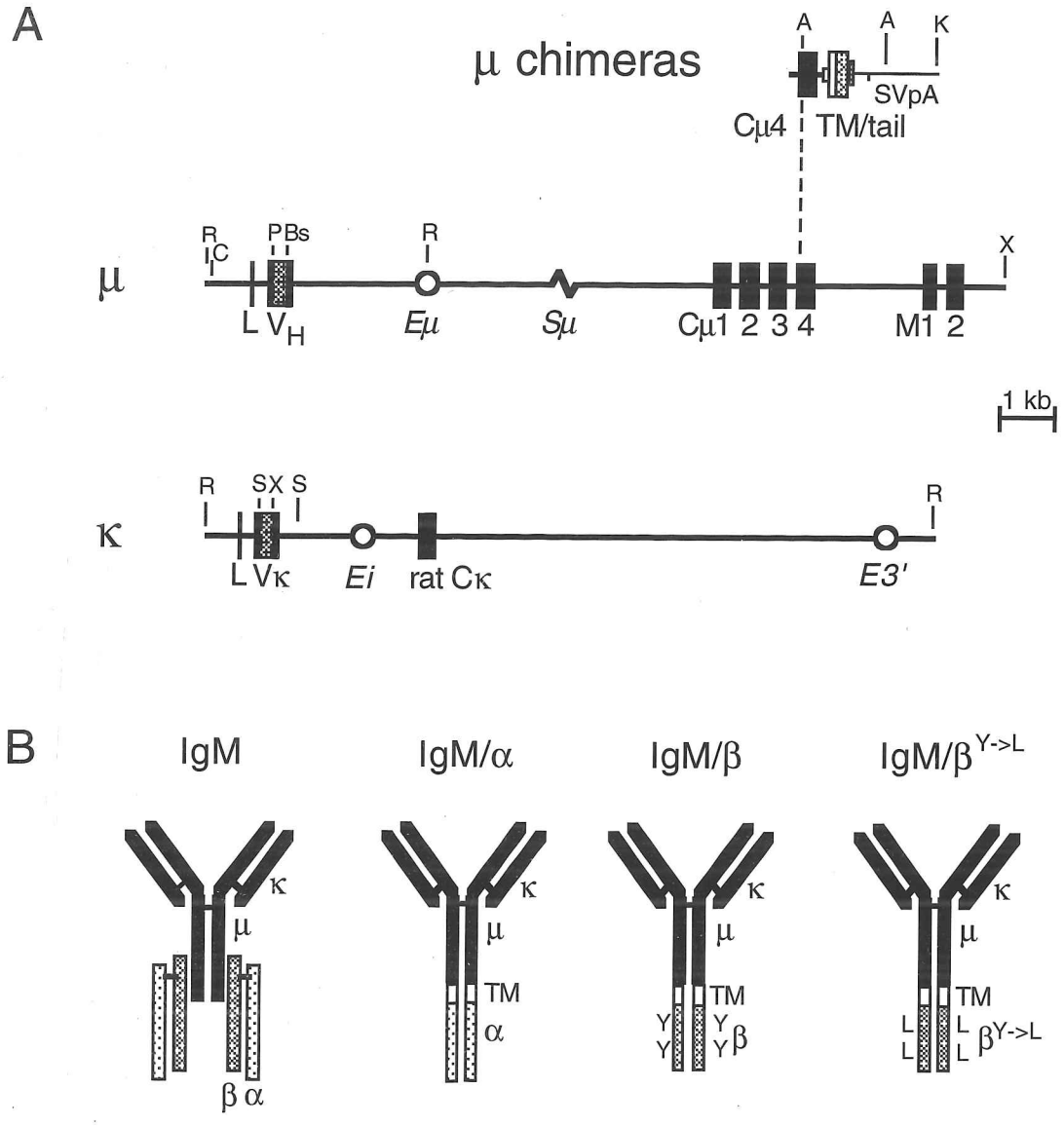

Figure 3.1 Chimeric receptor transgenes

(A) *Schematic representation of the DNA constructs.* Coding regions are shown in rectangles with regulatory regions indicated in italics.The μ constructs contained the anti-hen egg lysozyme (HEL) D1.3 V_H region inserted as a PstI-BstEII fragment. The 3' exons of μ were exchanged for ApaI fragments extending from within Cμ4 and bearing the chimeric exons of a transmembrane region (TM) derived from H2-K^b fused to the cytoplasmic domains of Ig-α or Ig-β. The κ construct contained the D1.3 Vκ region inserted as a SacI-XhoI fragment. Vector-free fragments were generated for microinjection: wild type μ (ClaI-XhoI), μ chimeras (ClaI-KpnI), κ (EcoRI). (R, EcorI; C, ClaI; P, PstI; Bs, BstEII; A, ApaI; K, KpnI; S, SacI; X, XhoI)

(B) *Predicted structures of the chimeric receptors.* The extracellular IgM (μ and κ) domains were fused via a hydrophobic transmembrane region (TM) to the cytoplasmic tail of Ig-α, Ig-β or a mutated Ig-β with the ITAM tyrosines mutated to leucine (Ig-$\beta^{Y->L}$).

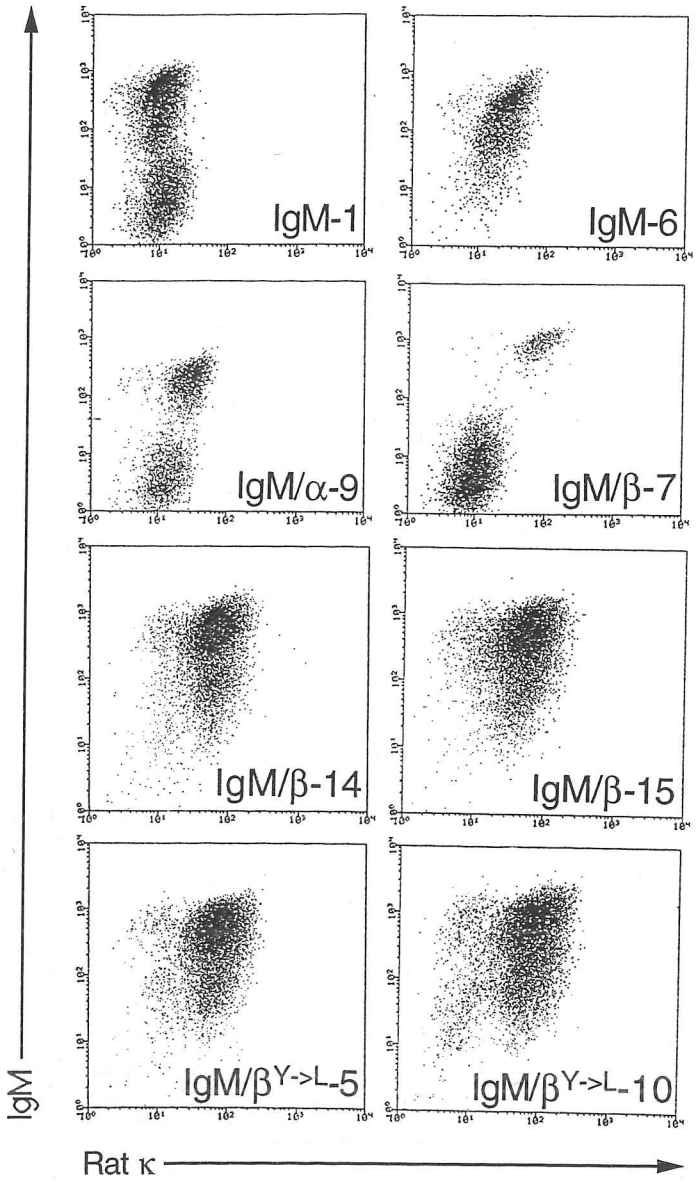

Figure 3.2 Expression of A20 transfectants
The transfected receptors were identified from the endogenous IgG2a, κ of A20 by staining with goat anti-IgM-biotin (revealed by SA-PE) and goat anti-rat Ig-FITC (for rat Cκ). Figures after transfectant denote clone number.

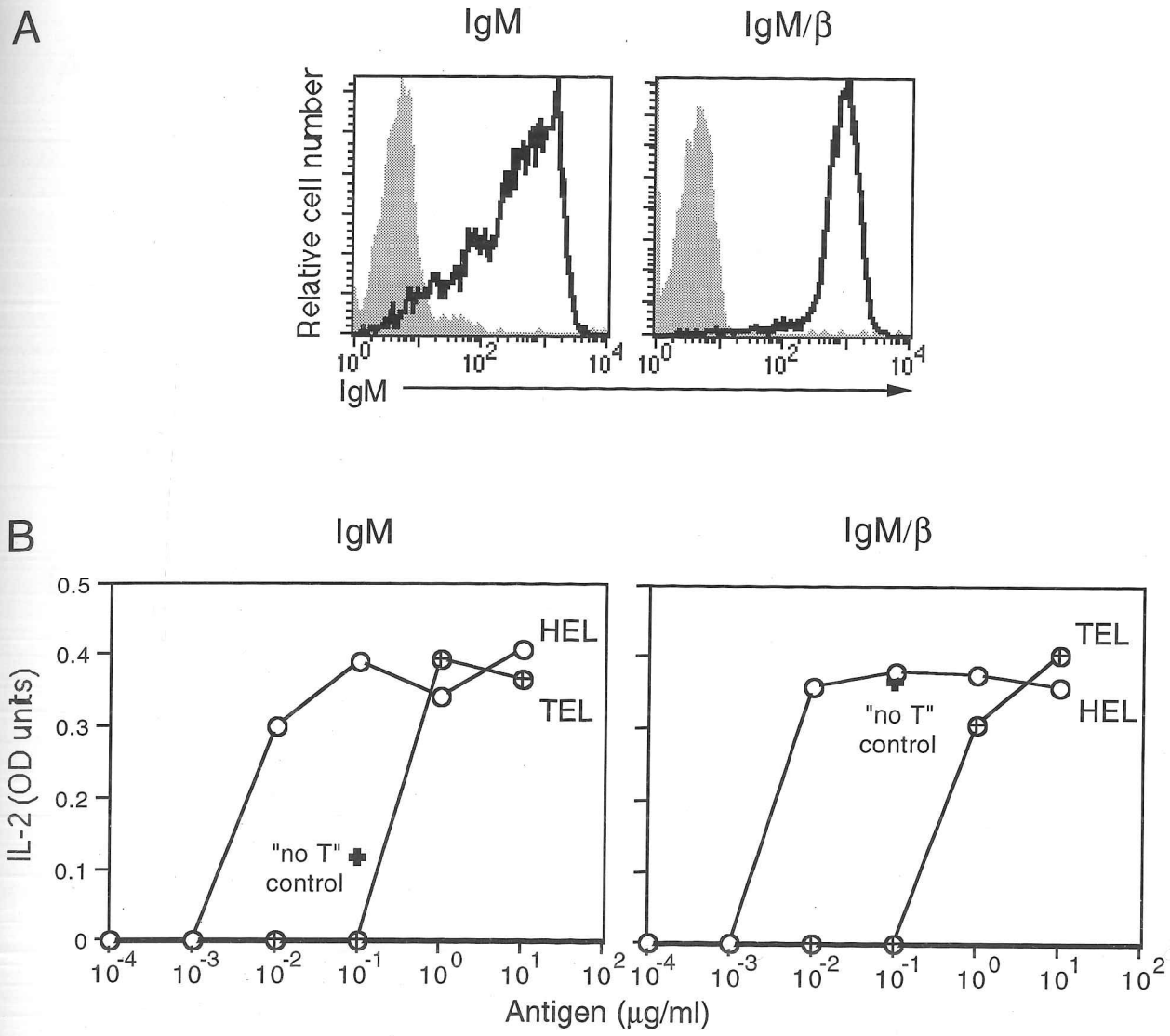

Figure 3.3 Antigen presentation assay

(A) *Expression levels of the A20 transfectants used in the assay.* Flow cytometric analysis of IgM (AK1158 clone-7) and IgM/β (clone-14) after staining with goat anti-IgM-FITC (unstained controls depicted by shaded histograms).

(B) *IgM/β, as well as IgM, mediates specific antigen presentation of HEL, but not TEL.* Levels of IL-2 (expressed as OD units) produced after antigen presentation of hen egg lysozyme (HEL; open circles) or turkey egg lysozyme (TEL; crossed circles) by A20 to T cell hybridoma 1E5.111 for 24 h. The "no T" control (filled cross) value resulted from culture of A20 and 0.1 μg/ml HEL in the absence of T cells.

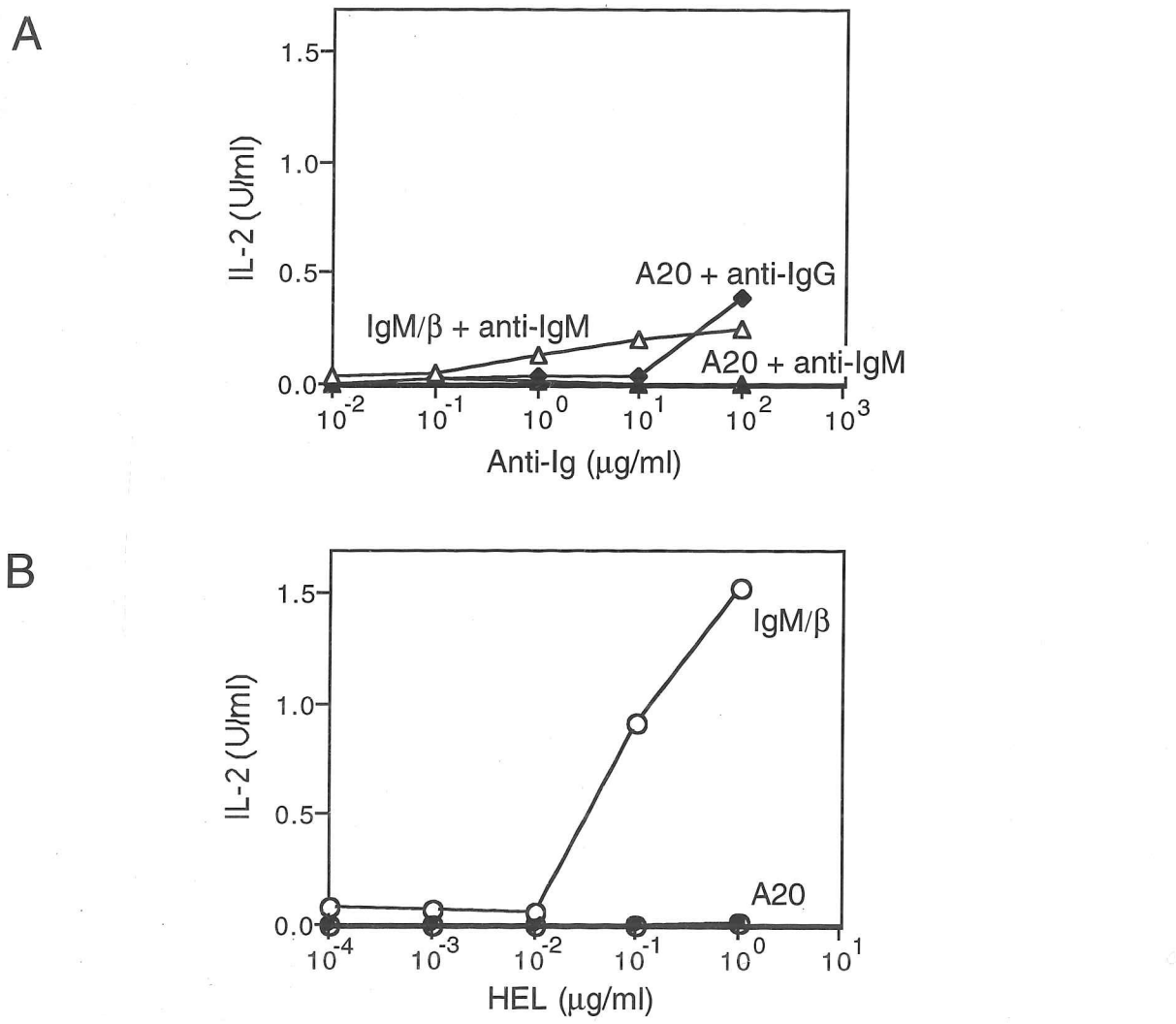

Figure 3.4 Activation of A20 by HEL versus anti-Ig

(A) *Anti-IgM triggers IgM/β but the signal is weak.* IL-2 production after 24 h incubation of IgM/β (clone-14) transfectant (open symbols) and untransfected A20 (filled symbols) with goat anti-IgM $F(ab')_2$ (triangles) or goat anti-IgG $F(ab')_2$ (diamonds).

(B) *HEL induces a greater IL-2 response than anti-Ig.* Levels of IL-2 secreted after incubation of untransfected (A20; filled circles) and transfected (IgM/β, open circles) A20 for 24 h with HEL.

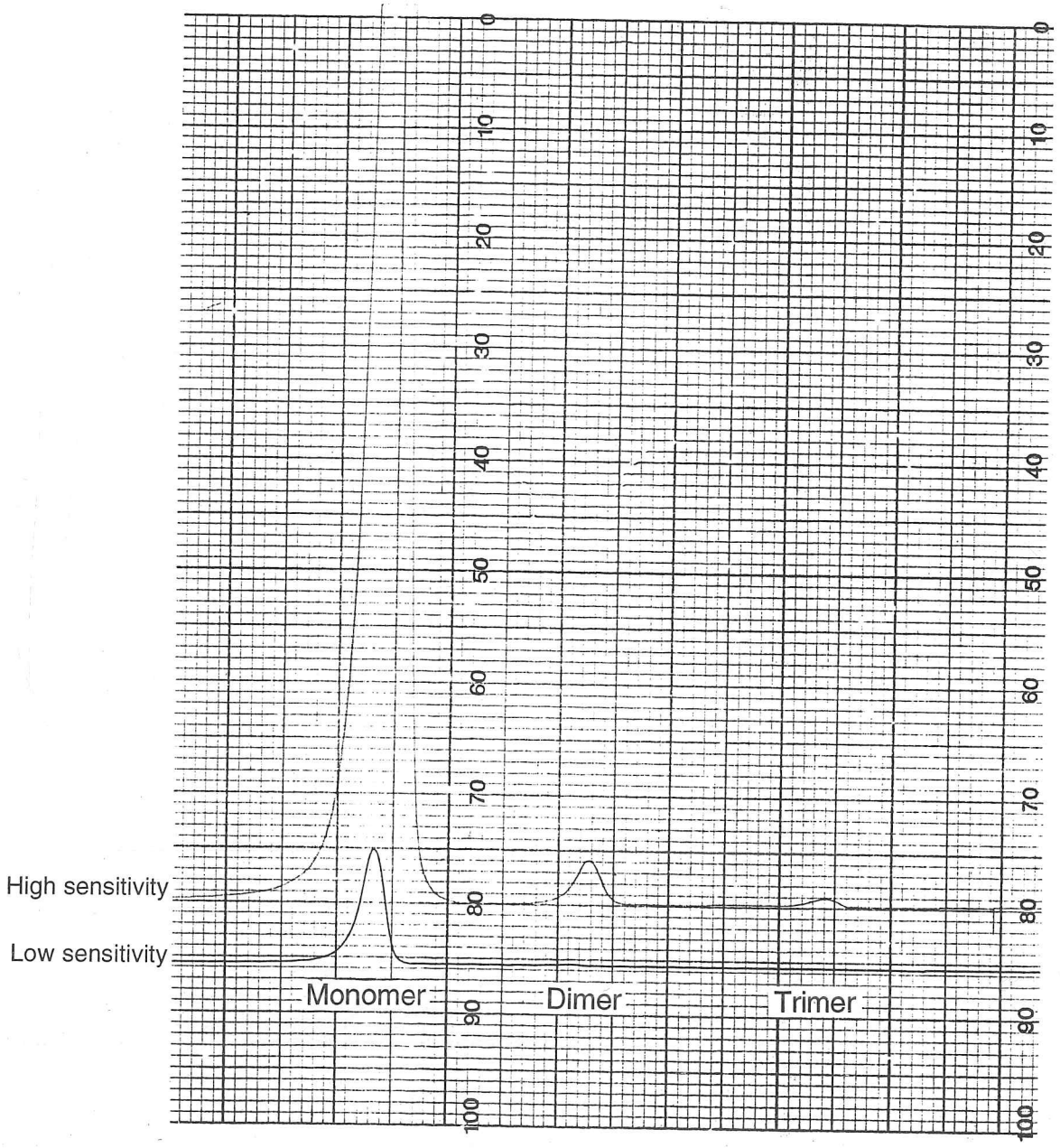

Figure 3.5 HPLC analysis of HEL
A solution of 1 mg/ml HEL (Sigma L-6876) in PBS was analysed by HPLC (high-performance liquid chromatography) at 4°C and the OD at 280nm was detected at high and low sensitivity, revealing possible dimer and trimer forms in addition to the predominant monomer peak.

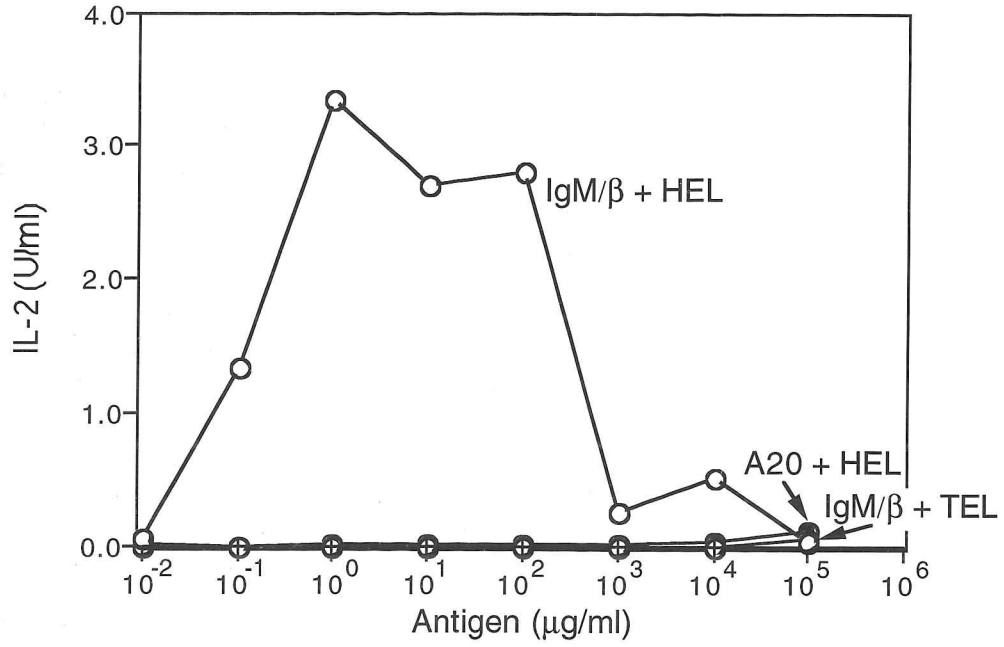

Figure 3.6 Dose response curve for HEL-specific signalling
IL-2 was measured after 24 h culture of IgM/β (clone-14) transfectant with HEL (open circles) or TEL (crossed circles) and untransfected A20 with HEL (filled circles).

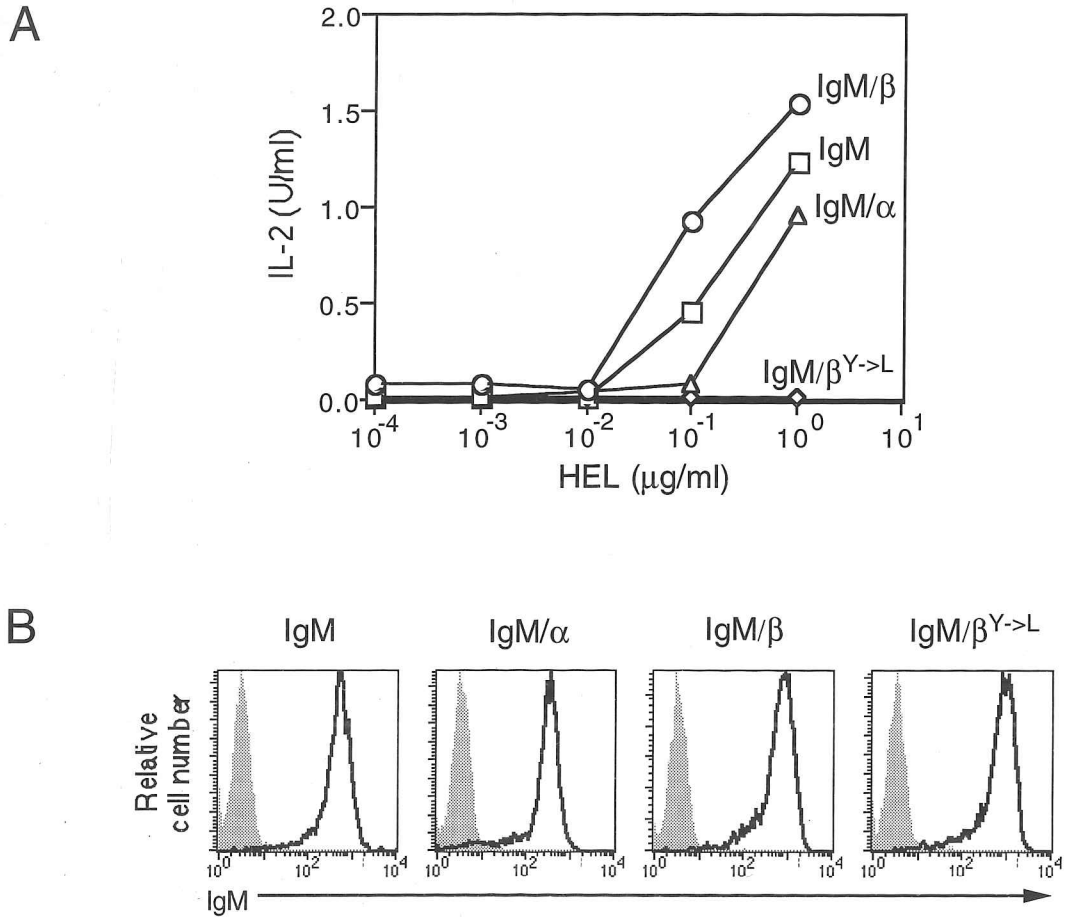

Figure 3.7 IgM/α or IgM/β, but not IgM/$\beta^{Y->L}$, can signal in A20.
(A) *Signalling by the A20 transfectants in response to HEL.* Levels of IL-2 produced after culturing IgM (clone-6) (squares), IgM/α (-9) (triangles), IgM/β (-14) (circles) and IgM/$\beta^{Y->L}$ (-5) (diamonds) transfectants with HEL for 24 h.
(B) *Expression of the transfectants used in the assay.* For flow cytometric analysis, cells were stained with goat anti-IgM-FITC (shaded histograms denote unlabelled controls).

Results

B cell antigen receptor signalling in transgenic mice

Chapter 4

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B cell antigen receptor signalling in transgenic mice

It has not been clear whether the Ig-α and Ig-β polypeptides perform similar or different activities within the context of the BCR complex. In the previous chapter, both IgM/α and IgM/β chimeric receptors mediated signalling, suggesting that the cytoplasmic domains of Ig-α and Ig-β are functionally redundant. These results are consistent with some previous findings, but disagree with others. Thus, persevering with chimeric receptors in cell lines seemed unlikely to clarify the situation.

Primary B cells might provide a more sensitive and physiological assay system, so transgenic mice were generated expressing the anti-HEL BCRs. An additional advantage of the transgenic system is that the effect of the transgenes could be monitored at different stages in B cell development. Indeed, the BCR plays a critical role in developmental processes that occur independent of antigen, as well as in B cell activation and other antigen-induced differentiation events.

4.1 The transgenes are expressed in splenic B cells.

Once the anti-HEL BCR transgene constructs had been checked for correct expression and function (see Chapter 3), vector-free DNA fragments were prepared for microinjection into (CBA/Ca x C57Bl/6) F1 zygotes. Each H chain fragment of the wild type IgM (ClaI-XhoI) or the chimeric IgM/α, IgM/β or IgM/$\beta^{Y->L}$ (ClaI-KpnI) constructs was co-injected with the κ L chain EcoRI fragment bearing a rat Cκ region (see Fig. 3.1B).

Putative founder mice were screened by ELISA for the expression of transgenic (rat) κ and genomic integration of the H chain transgenes was detected by PCR. Mutiple founders were identified carrying either the H or L chain transgenes, however, only one founder for each chimeric receptor was found to have cointegrated both H and L chain constructs. Two founders (lines A and B) for the wild type IgM receptor had cointegrated the H and L chains. Depending on the experiment, mice were bred with (CBA/Ca x C57Bl/6) F1, C57Bl/6 or *μMT* B-cell deficient mice (Kitamura *et al.*, 1991). Analyses were performed on multiple individuals from each transgenic line, although only line B of the IgM transgenic mice was crossed into a *μMT* background.

With the generation of founder lines that had integrated the transgenes into the

genome, the expression of the transgenic receptors was analysed by flow cytometry (Fig. 4.1). Transgenic κ could be discriminated from the endogenous κ since the L chain transgene bore a rat Cκ region. The expression of secretory transgenic κ had already been detected by ELISA in the serum of the mice during screening (not shown), and staining for rat κ revealed that the transgenic κ chains were expressed on the cell surface in spleen (Fig. 4.1A).

The detection of transgenic μ H chain was not as straightforward as that of transgenic κ. The antigen binding site of the BCRs is formed by both V_H and V_L regions, thus specific binding of the antigen HEL would give an indication of the expression of both H and L chain transgenes. Attempts to obtain good staining with HEL conjugates (to biotin or FITC) proved unsuccessful. However, since numerous anti-HEL monoclonal antibodies have been generated and characterised, it was possible to visualise HEL binding using a second anti-HEL antibody, HyHEL10 (Smith Gill *et al.*, 1984). HyHEL10 recognises a complementary epitope to D1.3 and binds to a different face of HEL (Davies *et al.*, 1990). When the splenic B cells were identified by the B cell marker B220 (CD45R), HEL binding revealed that both H and L chain transgenes were expressed as receptors on the B cell surface (Fig. 4.1B).

Binding of the D1.3 idiotype to HEL has been characterised and compared to the binding of D1.3 to an anti-D1.3 idiotype, E5.2 (Fields *et al.*, 1995). The monoclonal antibody E5.2 mimics the binding of HEL by forming similar structural contacts within the antigen binding site of D1.3. Thus, the transgenic receptors could also be identified by staining for D1.3 with the anti-D1.3 idiotype. The pattern of B cell staining with the E5.2 antibody seemed similar to that of HEL itself, and it is evident that all the transgenic lines do express their transgenes (Fig. 4.1C), although the levels of expression and proportion of transgenic B cells ($B220^+D1.3$ $idiotype^+$) varied (Table 4.1), with IgM/α least efficiently expressed.

These results demonstrate that splenic B cells of all the transgenic mice express their H and L chain transgenes, forming a functional D1.3 idiotype that binds its antigen, HEL.

Unexpectedly, it is apparent that the D1.3 transgenes were also expressed on a proportion $B220^-$ cells in the spleens of mice bearing the chimeric transgenes (Fig. 4.1C).

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Table 4.1 B cell populations in transgenic mice.
Cells from spleen and bone marrow of non-transgenic (Non-tg) and transgenic mice were analysed by flow cytometry as in Fig. 4.1C. Mean values are shown with standard deviations in brackets for *n* individuals.

	Spleen			Bone marrow		
	Total number of cells	%B220$^+$	%B220$^+$ D1.3$^+$	Total number of cells	%B220$^+$	%B220$^+$ D1.3$^+$
Non-tg	9.4×10^7 (2.4×10^7) *n*=18	57 (7.7) *n*=18	2 (0.9) *n*=11	1.7×10^7 (8.6×10^6) *n*=7	23 (3.7) *n*=7	0 (0.0) *n*=2
IgM^A	4.2×10^7 (1.5×10^7) *n*=6	15 (5.2) *n*=6	9 (5.8) *n*=6	1.9×10^7 (8.5×10^6) *n*=2	13 *n*=1	6 *n*=1
IgM^B	5.2×10^7 (2.5×10^7) *n*=2	26 (1.4) *n*=2	20 (0.7) *n*=2	2.2×10^7 *n*=1		
IgM/α	5.1×10^7 (2.1×10^7) *n*=9	18 (7.4) *n*=9	4 (1.5) *n*=9	1.9×10^7 (7.0×10^6) *n*=3	15 (0.0) *n*=2	6 (0.0) *n*=2
IgM/β	2.6×10^7 (8.9×10^6) *n*=4	21 (1.5) *n*=3	12 (2.1) *n*=3	1.1×10^7 (3.7×10^6) *n*=2	14 *n*=1	8 *n*=1
IgM/β$^{Y->L}$	1.8×10^8 (1.1×10^8) *n*=5	46 (10) *n*=5	38 (7.9) *n*=5	1.4×10^7 (7.1×10^5) *n*=2	9 *n*=1	8 *n*=1

4.2 Splenic T cells also express the chimeric transgenes.
Expression of the chimeric transgenes was most obvious in the spleens of IgM/β and IgM/β$^{Y->L}$ mice (Fig. 4.1C). In order to identify the cells that aberrantly expressed the transgenic receptors, IgM/β$^{Y->L}$ mice were analysed further, since transgene expression might be least perturbed by any signalling ability.

When IgM/β$^{Y->L}$ splenocytes were stained for different cell lineage markers, IgM was detected on the surface of cells stained for Thy1.2 (CD90.2) (Fig. 4.2A), but not Mac-1 or Gr-1 (not shown), indicating that the B220$^-$ cells expressing surface IgM were a subset of T cells.

The splenic T cells from IgM/β$^{Y->L}$ mice that expressed surface IgM seemed to reflect the activity of the IgH enhancer (Eμ) in the T cell lineage (Cook *et al.*, 1995), and μ transgenic mice often express μ intracellularly in thymus and peripheral T cells (Lamers *et al.*, 1989; Storb *et al.*, 1986). When IgM/β$^{Y->L}$ thymus was analysed, cytoplasmic μ (IgM) was indeed expressed in most thymocytes, although it was not transported to the surface (Fig. 4.2B).

The inability of the IgM/$\beta^{Y->L}$ receptor to be detected on the surface of the thymocytes suggested that the receptor complex may be incomplete. The chimeric μ chains contain a hydrophobic transmembrane region deriving from H2-K^b which facilitates surface transport of the chimeras in the absence of Ig-α and Ig-β (Williams *et al.*, 1993). Thus, unlike wild type μ, the chimeric receptors do not require association with the Ig-α/Ig-β heterodimer for surface transport, and indeed no such complex has been detected (Patel and Neuberger, 1993). However, L chain association is still required. The surface expression of transgenic κ was detected in a proportion of IgM/$\beta^{Y->L}$ splenic T cells similar to the subpopulation that expressed surface IgM (Fig. 4.2A). Furthermore, the intact transgenic D1.3 idiotype formed by both V_H and Vκ regions can be identified on $CD4^+$ and $CD8^+$ subsets of splenic T cells. However, the κ transgene was not detected in thymus (not shown). Thus, the pattern of transgenic κ expression suggests that the κ transgene acts as the limiting factor for surface transport of the chimeric μ H chain.

The expression of transgenic κ in peripheral T cells may be regulated by binding of the transcription factor NFκB to the κ intron enhancer (Staudt and Lenardo, 1991). Following activation of the T cell, NFκB activity increases (Baeuerle and Henkel, 1994) and may induce transcription of the rearrranged κ transgene. Association of the transgenic κ chain with the chimeric μ chain renders the chimeric receptor competent for surface expression. To test this hypothesis, splenic T cells were assessed for increases in surface expression of the IgM/$\beta^{Y->L}$ receptors after activation for 24 h. Splenocytes from IgM/$\beta^{Y->L}$ mice were cultured in the presence and absence of the lectin concanavalin A (Con A), which crosslinks CD3 receptors, inducing T cell activation. After 24 h, the surface expression of activation markers CD44, CD25 and CD69 was upregulated on the splenic T cells (gated $Thy1.2^+$ or $CD4^+$) (Fig. 4.2 C), indicating that the cells were in an activated state. However, the expression of IgM/$\beta^{Y->L}$ did not increase and in fact, incubation of the splenic T cells reduced the number of IgM^+ cells detected. Thus, the conditions of the 24 h Con A culture were not sufficient to induce further expression, but it is possible that *in vivo* activation of the T cells had been induced by other mechanisms.

Nevertheless, it is clear that the non-$B220^+$ cells expressing the chimeric transgenes are peripheral T cells of both $CD4^+$ and $CD8^+$ subpopulations.

4.3 IgM/α and IgM/β, but not IgM/$\beta^{Y->L}$, generate a peripheral B cell pool.

BCR expression and signalling is essential for differentiation of the B cell lineage, as illustrated by the *μMT* mouse which bears a germline disruption in the membrane exons of the μ H chain (Kitamura *et al.*, 1991). Inability to express membrane μ prevents assembly of the pre-BCR and results in a block at the pro-B cell stage (fraction C, according to (Hardy *et al.*, 1991)) (Ehlich *et al.*, 1993). Thus, no $B220^+IgM^+$ cells could be detected in the bone marrow or the spleen of *μMT* mice, in contrast to non-transgenic wild type mice (Fig. 4.3).

Introduction of all the anti-HEL transgenes into a *μMT* background resulted in the detection of $B220^+IgM^+$ bone marrow cells (Fig. 4.3 and Table 4.2). In non-transgenic mice, the expression of surface IgM can be used as a differentiation marker for immature and mature B cells. However, in transgenic mice bearing rearranged IgM transgenes, the expression of surface IgM may be misleading since the H and L chain transgenes do not need to undergo V region rearrangement. By-passing the rearrangement stages in differentiation may cause premature expression of transgenic IgM and apparent acceleration of the B cell precursor through early development.

One indication of a functional BCR is its ability to drive the generation of $B220^+IgM^+$ cells in the peripheral B cell pool. When cells from the peripheral lymphoid organ, spleen, were analysed, it was clear that the IgM transgene overcame the defect in the *μMT* mouse (Fig. 4.3 and Table 4.2), indicating that the wild type anti-HEL receptor can mediate B cell development. In addition, both IgM/α and IgM/β chimeras effected reconstitution of a splenic B cell population in *μMT* mice.

In contrast, the IgM/$\beta^{Y->L}$ transgene did not rescue the *μMT* phenotype (Fig. 4.3 and Table 4.2). No $B220^+IgM^+$ cells were identified in the spleen, although they were detected in the bone marrow. These results indicated that although IgM/$\beta^{Y->L}$ could be expressed on the surface of B cell precursors in the bone marrow, it proved inadequate to reconstitute a peripheral B cell pool. The only IgM^+ cells detected in the spleen were the aberrant T cells previously identified (see Fig. 4.2).

The results from the *μMT* reconstitution assay indicate that the Ig-β ITAM tyrosines are essential for signalling, but suggest that the cytoplasmic domains of either Ig-α or Ig-β are sufficient mediate the generation of peripheral B cells in *μMT* B cell-deficient mice.

Table 4.2 B cell populations in transgenc mice crossed into a *μMT* background.
Spleen and bone marrow cells from transgenic mice in a *μMT* background or non-transgenic (Non-tg) controls were analysed by flow cytometry as in Fig. 4.3. Numbers indicate mean values with standard deviations in brackets for *n* individuals.

		Spleen		Bone Marrow		
Transgene	Background	Total number of cells	%B220$^+$ IgM$^+$	Total number of cells	%B220$^+$	%B220$^+$ IgM$^+$
Non-tg	Wild type	9.4×10^7 (2.4×10^7) *n*=18	57 (6.4) *n*=8	1.7×10^7 (8.6×10^6) *n*=7	23 (3.7) *n*=7	9 (2.3) *n*=6
Non-tg	μMT	6.4×10^7 (2.5×10^7) *n*=4	0 (0.5) *n*=4	2.2×10^7 (1.1×10^7) *n*=3	6 (0.6) *n*=3	0 (0.0) *n*=3
IgM^B	μMT	5.6×10^7 (2.3×10^7) *n*=4	26 (19) *n*=4	2.0×10^7 (9.0×10^6) *n*=4	15 (10) *n*=4	7 (5.7) *n*=4
IgM/α	μMT	4.6×10^7 (2.4×10^7) *n*=8	15 (6.6) *n*=8	1.9×10^7 (6.4×10^6) *n*=6	10 (3.5) *n*=6	6 (1.9) *n*=6
IgM/β	μMT	3.9×10^7 (1.0×10^7) *n*=5	15 (6.0) *n*=5	2.1×10^7 (7.4×10^6) *n*=4	7 (3.4) *n*=4	5 (2.0) *n*=4
IgM/β$^{Y->L}$	μMT	3.3×10^7 (1.8×10^7) *n*=2	1 (0.7) *n*=2	1.3×10^7 *n*=1	4 *n*=1	3 *n*=1

4.4 The transgenic B cells turnover more rapidly *in vivo*.

The splenic B cell populations generated by the IgM, IgM/α and IgM/β transgenes in *μMT* mice was reduced compared to that of non-transgenic wild type mice (Fig. 4.3 and Table 4.2). Fewer B cells seemed to be generated in the bone marrow. However, an alternative explanation for the reduced B cell numbers could be an increased turnover or shorter lifespan of the B cells *in vivo*.

Cell turnover can be measured by the uptake of BrdU (5-bromo-2-deoxyuridine) in proliferating cells. BrdU is an analogue of thymidine that can be incorporated during DNA replication and can be detected using a specific anti-BrdU antibody. For the *in vivo* production of cells in mice, BrdU can be administered as an injection or added to the drinking water. Depending on the period of BrdU uptake, the proportions of cells that are labelled with BrdU indicate the relative lifespans of different cell populations.

After 72 h of BrdU incorporation, most of the $B220^+$ bone marrow cells of the non-transgenic mice had taken up BrdU (Fig. 4.4), but the majority did not express surface IgM, indicating that most of the newly-generated $B220^+$ cells in the bone marrow are early B cell progenitors that have yet to express a functional BCR.

BrdU uptake was also detected in most of the $B220^+$ cells in the bone marrow of the transgenic mice (Fig. 4.4). However, the effects of the transgenes on the bone marrow cells were difficult to determine. Although transgenic receptors (stained for D1.3 idiotype) were expressed on a large proportion of the transgenic $B220^+BrdU^+$ cells, the transgenes are not reliable markers of differentiation status. Nevertheless, these results indicate that in the bone marrow, B cell turnover is rapid in all the transgenic mice as well as in the non-transgenic mice.

B cells in the periphery are long-lived compared to those with a rapid turnover in the bone marrow. Thus, in non-transgenic mice, a small proportion of splenic $B220^+$ cells had incorporated BrdU (Fig. 4.4). By comparison, BrdU uptake by the transgenic ($D1.3^+$) B cells was significantly increased in the spleens of IgM and IgM/β, but not IgM/$\beta^{Y->L}$ mice. The relative proportions of $BrdU^+$ cells suggest that the splenic B cells are shorter-lived in the transgenic mice than in non-transgenic mice. Since the expression of a functional transgenic receptor, whether wild type or chimeric IgM, increased B cell turnover, it is possible that the shorter lifespan of the transgenic B cells is due to the D1.3 V region specificity.

The IgM/α transgene was expressed at consistently lower levels in the spleen (Fig. 4.4; also Fig. 4.1 and 4.3), making identification of the IgM/α transgenic B cells less straightforward than for the other transgenic mice. Therefore the results for BrdU uptake by the IgM/α splenic B cells were difficult to interpret.

However, despite its poor expression in the spleen, IgM/α was clearly detected in the bone marrow (Fig. 4.4). Indeed, BCR expression was lower on the $BrdU^-B220^+$ cells compared to the $BrdU^+B220^+$ cells in the IgM, IgM/α and IgM/β transgenic mice, but not in the non-transgenic controls. The reduced levels of transgene expression in both the longer-lived $BrdU^-$ B cells and the more mature splenic B cells may indicate that expression of the anti-HEL receptor transgenes is downregulated over the lifetime and maturation of the transgenic B cells (possibly another effect of the D1.3 specificity).

These results suggest that the D1.3 idiotype confers a common effect of increased turnover of the transgenic B cells. Thus, the reduced splenic B cell pools reconstituted in *μMT* mice may be explained by the shorter lifespan of peripheral B cells as well as the decreased generation of B cell precursors in the bone marrow of the transgenic mice.

4.5 The reconstituted B cells reveal altered expression of mature B cell markers.
The IgM, IgM/α and IgM/β transgenes rescued the development of B cells in *μMT* B cell-deficient mice. Such transgenic *μMT* mice and their B cells will be referred to as *reconstituted* mice and *reconstituted* B cells for the remainder of this chapter since *transgenic* may be ambiguous since it also describes the IgM/$\beta^{Y->L}$ transgene which does not effect *μMT* reconstitution. The reconstituted mice provided a source of transgenic B cells in the absence of contaminating B cells that expressed endogenous BCRs. For example, the reconstituted splenic B cell population could be analysed without enriching for transgenic B cells or anti-IgM could be used for detecting or stimulating the transgenic B cells, since the only IgM expressed on the surface was transgenic IgM and not endogenous membrane IgM.

In order to determine any differences in the extent of maturation effected by the chimeric IgM/α and IgM/β receptors compared to the wild type IgM BCR, the reconstituted B cells were characterised by the expression of cell surface markers (Fig. 4.5).

B-1 cells constitute a separate B cell lineage in the peritoneum that can be discriminated from conventional mature B cells by the expression of the cell surface marker, CD43. B-1 cells are self-renewing (not bone marrow-derived), but have a low turnover rate. The anti-HEL transgenes seemed to increase the turnover of transgenic B cells and therefore may reduce the size of the long-lived B-1 pool. Indeed, the IgM, IgM/α and IgM/β mice in a *μMT* background had fewer peritoneal B cells (Fig. 4.5A). However, the reconstituted mice contained reduced numbers of total $B220^+$, and as a proportion of the total peritoneal B cells, the $CD43^+B220^+$ B-1 cell pools were of similar size to those in non-transgenic wild type mice.

During the development of conventional B cells in the bone marrow of adult mice, CD43 is expressed on pro-B cells before the expression of the BCR. Indeed, surface IgM is not yet expressed on the $CD43^+$ pro-B cells in the non-transgenic

bone marrow (Fig. 4.5B). However, IgM/α and IgM/β chimeras, but not the wild type IgM transgene, are detected on $CD43^+$ pro-B cells, suggesting that the chimeric transgenes are expressed prematurely.

At later stages in B cell development, peripheral B cells express the mature B cell markers CD22, CD21/35 (complement receptors 2 and 1) and CD23 (Fc receptor for IgE), as observed in the splenic B cells from non-transgenic mice (Fig. 4.5C). CD22 expression appeared relatively unaffected in the IgM, IgM/α and IgM/β splenic B cells, although a few IgM^+CD22^- B cells were detected in the IgM/α and IgM/β spleens.

The splenic B cell populations from IgM/α and IgM/β exhibited a shift to lower expression of CD21/35 and CD23, compared to non-transgenic mice (Fig. 4.5C). The reduced proportions of B cells expressing high levels of CD21/35 and CD23 suggest that the chimeric transgenes do not effect the same degree of maturation as for non-transgenic peripheral B cells. However, analysis of these mature B cell markers on splenic B cells from wild type IgM transgenic mice also revealed CD21/35 and CD23 expression altered from the non-transgenic controls.

Characterisation of the different B cell populations by cell surface markers suggests that although total peripheral B cell numbers are reduced, the B-1 pool seems unaffected. However, the IgM/α and IgM/β chimeric transgenes appear to be expressed prematurely in pro-B cells in the bone marrow. Furthermore, the phenotype conferred by the IgM, IgM/α and IgM/β transgenes in spleen suggests that the anti-HEL receptors do not drive the same distribution of maturational development of peripheral B cells as that observed in non-transgenic mice.

4.6 The transgenic B cells proliferate poorly *in vitro*.

In chapter 3, A20 transfectants expressing the IgM/α and IgM/β receptors transfected mediated signalling in response to the antigen HEL, but it remains to be shown that the chimeras can mediate antigen-dependent activation in primary cells.

The proliferation of cells following stimulation can be judged by the incorporation of $[^3H]$thymidine during DNA replication. After incubation with anti-IgM + IL-4 for three days, splenic B cells from non-transgenic mice showed a strong proliferative response (Fig. 4.6A). In contrast, when reconstituted B cells from the IgM/α and IgM/β chimeras crossed into a *μMT* background were

cultured with anti-IgM + IL-4, they accumulated few counts over the IL-4 alone controls. Surprisingly, even the wild type IgM transgenic B cells proliferated poorly.

The poor proliferative response of the transgenic B cells to anti-IgM + IL-4 might be due to defective BCR-mediated signalling. Alternatively, the reconstituted B cells might exhibit an inability to undergo proliferation to any stimulant. In order to discriminate between the two possibilities, the B cells were incubated with lipopolysaccharide (LPS) which induces B cell proliferation by a different mechanism from that mediated by the BCR. When incubated with LPS, the proliferation of the IgM, IgM/α and IgM/β reconstituted B cells was again greatly diminished compared to the non-transgenic controls (Fig. 4.6B).

The poor proliferation of the IgM, IgM/α and IgM/β splenic B cells in response to both anti-IgM + IL-4 and LPS therefore seemed to reflect an intrinsic defect of the reconstituted B cells expressing anti-HEL transgenes, regardless of the wild type or chimeric nature of receptors.

4.7 The transgenic B cells die more quickly *in vitro*.

The reduced proliferative response of the transgenic B cells revealed an inability to induce proliferation, possibly due to a defect required for the proliferative process. Another explanation, in view of their increased turnover *in vivo* (Fig. 4.4), might be that the transgenic B cells die more quickly than non-transgenic B cells when cultured *in vitro* .

Preliminary experiments, using propidium iodide to stain dead cells as a crude estimate of B cell viability, showed an overall trend. The IgM, IgM/α and IgM/β reconstituted B cells all revealed a decrease in viability compared to B cells from non-transgenic mice after 24 h. For example, cultures of B cells with anti-IgM resulted in only 20-30% viability of the transgenic B cells versus 65% of the non-transgenic controls. The reduced viability of the IgM, IgM/α and IgM/β B cells suggests that they may behave like immature B cells, which are susceptible to deletion and are induced to die by apoptosis following BCR crosslinking.

Dying cells may be identified by the characteristic feature of DNA fragmentation which occurs during the early stages of apoptosis. Biotin-conjugated dUTP can be added to the broken ends of the double-stranded DNA using the enzyme terminal deoxynucleotidyl transferase (TdT). The cells thus labelled can be

visualised by flow cytometry. Such TUNEL (TdT-mediated dUTP-biotin nick end-labelling) analysis revealed that almost all the splenic B cells from transgenic and non-transgenic mice had been labelled by 24 h incubation in the presence of anti-IgM (Fig. 4.7). However, a large proportion of the IgM/α and IgM/β B cells were $dUTP^+$ after incubation for only 4 h. By 8 h, the majority of B cells expressing the wild type IgM transgene, as well as the IgM/α and IgM/β chimeras had incorporated dUTP. These results suggest that the B cells expressing the chimeric receptors were already dying from as early as 4 h, with almost all the reconstituted B cells induced to die within 8 h. The increased rate of dUTP labelling in the transgenic versus non-transgenic B cells suggests that the B cells expressing the anti-HEL receptors are more susceptible to cell death.

Little difference was observed in the rate of dUTP incorporation between B cells cultured in the presence, and absence, of anti-IgM (Fig. 4.7), suggesting that the cell death detected was not induced by BCR crosslinking. Thus, it is possible that the B cells are undergoing spontaneous apoptosis. Cell death is a dynamic process, however, and TUNEL may also detect necrotic cells, if analysis is performed at later stages.

The phenotypic analysis of the transgenic B cells included faster cell turnover *in vivo* (Fig. 4.4), lower expression of mature B cell markers (Fig. 4.5), poor proliferation (Fig. 4.6) and increased cell death (Fig, 4.7). Taken together, these results suggest that the D1.3 V region specificity may confer a (possibly more immature) phenotype that is common to all the anti-HEL transgenic receptors and is not due to their specific cytoplasmic domains.

4.8 IgM/α and IgM/β can signal to upregulate B7.

BCR-mediated signalling results in the upregulation of B7 costimulatory molecules on the B cell surface, which can be detected by the binding of its natural ligand, CTLA4. An increase in binding of a CTLA4 fusion protein was detected within a few hours and was clearly evident after 24 h stimulation (Fig. 4.8A).

Although the reconstituted transgenic B cells exhibited increased cell death, it was still possible to monitor the B7 response of those cells that remained viable over the 24 h period. Indeed, CTLA4 binding increased on reconstituted splenic B cells from the wild type IgM transgenic in a *μMT* background in response to anti-IgM (Fig. 4.8B). Similarly, B7 upregulation was also mediated by crosslinking the

IgM/α and IgM/β chimeras.

The upregulation of B7 was also monitored in B cells from the transgenic mice in a wild type background. The antigen HEL and anti-D1.3-idiotype antibody induced B7 upregulation in the IgM, IgM/α and IgM/β transgenics, but not in the non-transgenic controls (Fig. 4.8C). The variations in levels of CTLA4 binding may be due to the presence of contaminating non-transgenic B cells from the non-*μMT* wild type background, since the cells were gated by expression of B220. These experiments also facilitated the analysis of IgM/$\beta^{Y->L}$, which did not mediate B7 upregulation in response to HEL or anti-idiotype.

Thus, IgM/α and IgM/β, but not IgM/$\beta^{Y->L}$ receptor, were able to signal B cell activation, indicating that the cytoplasmic tails of both Ig-α and Ig-β can independently mediate a response to antigen in primary cells, but that the Ig-β ITAM tyrosines are essential.

4.9 IgM/α and IgM/β mediate allelic exclusion although less efficiently than IgM.
The B cell pool recognises diverse antigens, but it is essential that each B cell expresses antibodies of a monoclonal specificity. Early in B cell development, a functionally-rearranged Ig chain is expressed as a (pre-)BCR, which mediates allelic exclusion by signalling to inhibit rearrangement and prevent expression of the other allele.

In order to analyse the abilities of the transgenes to mediate allelic exclusion, it was necessary to discriminate transgenic from endogenous Ig. One approach was to exploit H chain isotype expression, since the IgD expressed in a transgenic mouse would derive only from the endogenous alleles. Whilst the transgenes encode only the IgM class, normal (non-transgenic) mature B cells can co-express both IgM and IgD classes. In a non-transgenic mouse, most of the splenic B cells expressed high levels of IgD, but not the transgenic D1.3 idiotype (Fig. 4.9A). In striking contrast, the wild type IgM transgene suppressed endogenous IgD expression and the B cells expressed only the transgene. In addition, both IgM/α and IgM/β were also able to mediate allelic exclusion. However, some IgD-expressing B cells leaked through, suggesting that the chimeras were less efficient than the wild type IgM transgene. Nevertheless they were more effective than the IgM/$\beta^{Y->L}$ receptor, which could not exclude co-expression of the transgene and IgD on most cells.

An alternative way of discriminating the transgenes from the endogenous alleles was to take advantage of the different mouse strain allotypes, since the transgenes encoded the IgM^a allotype. The transgenic receptors (identified by D1.3 or IgM^a) were bred into a background that expressed only the IgH^b allotype. Thus, the expression of transgenic versus endogenous Ig could be stained for either D1.3 versus IgM^b, or IgM^a versus IgM^b (Fig. 4.9B). The IgM^a versus IgM^b staining also facilitated the detection of allelic exclusion exhibited by a non-transgenic (IgH^a x IgH^b) F1 mouse. The patterns of allelic exclusion seen for each mouse with either set of reagents are consistent with the above results, although the combination of weaker staining by the anti-IgM^a reagent and consistently lower expression of the transgene makes exclusion less obvious in IgM/α.

The patterns of expression of transgenic versus endogenous Ig show that IgM efficiently excludes, IgM/α and IgM/β effect less complete exclusion, and IgM/$\beta^{Y->L}$ does not inhibit endogenous Ig expression. Although an intact ITAM is necessary, both Ig-α and Ig-β seem able, yet inefficient, to mediate allelic exclusion, suggesting that they have broadly similar signalling abilities.

4.10 The combination of IgM/α and IgM/β enhances allelic exclusion.

Since the BCR complex contains both Ig-α and Ig-β polypeptides, the IgM/α transgenic was crossed with IgM/β in order to determine whether the expression of both IgM/α and IgM/β together might enhance the ability of the individual chimeras to effect complete allelic exclusion.

Characterisation of the (IgM/α)x(IgM/β) double transgenic showed that the transgenic receptors were expressed at equivalent levels to those of the IgM/β parent in the spleen (Fig. 4.10A). The single IgM/α and IgM/β chimeras have been shown to mediate antigen-induced activation, therefore it was not surprising that splenic B cells expressing both chimeras were also able to upregulate B7 in response to HEL and anti-idiotype (Fig. 4.10B). In contrast to the IgM/α or IgM/β parents, however, (IgM/α)x(IgM/β) did exhibit more efficient allelic exclusion, inhibiting endogenous IgD expression similarly to the wild type IgM transgene (Fig. 4.10C).

The experiments comparing the activities of wild type IgM with those of chimeric IgM/α or IgM/β, or both together, suggest that whilst the cytoplasmic domains of either Ig-α or Ig-β are largely able to mediate the signalling activites of the BCR, the cooperation of both polypeptides appears to be required for

optimal function.

4.11 Discussion

The generation of transgenic mice expressing chimeric BCRs has facilitated the study of the roles of Ig-α and Ig-β in B cell development and activation in primary cells. Although the Ig-β ITAM tyrosines are necessary for signalling, the cytoplasmic domains of either Ig-α or Ig-β alone can broadly perform the functions of an intact BCR in the antigen-induced upregulation of B7, and in B cell maturation and allelic exclusion during development. These results do not highlight any major differences between the roles of Ig-α and Ig-β, yet do suggest that the combination of the two polypeptides may be necessary for optimal signalling potential of the BCR.

The abilities of IgM/α and IgM/β to mediate events in antigen-independent B cell development are consistent with reports published during the course of this work. Using transgenic mice expressing analogous IgM chimeras, the individual cytoplasmic domains of Ig-α and Ig-β were shown to have sufficient and equivalent functions in two checkpoints: pro- to pre-B cell transition and alleic exclusion (Papavasiliou *et al.*, 1995; Papavasiliou *et al.*, 1995). The progression of pro- to pre-B cells (judged by the loss of CD43 expression) was effected by the chimeric receptors in the bone marrow of *RAG-1*$^{-/-}$ mice that are rendered both B and T cell-deficient by germline disruption of a gene essential for V region recombination. The chimeras also inhibited endogenous V_H rearrangement, detected by PCR, in a wild type background. However, Ig-β ITAM signalling activity was abolished by mutation of the essential tyrosine residues to phenylalanine. The experiments in this chapter not only confirm these conclusions but extend the study to later stages of B cell differentiation: the generation of peripheral B cells in *μMT* mice and antigen-dependent activation of the reconstituted mature B cells.

Allelic exclusion was also exhibited by mice expressing compromised BCRs due to gene-targetting of the *mb-1* gene, resulting in truncation of the Ig-α cytoplasmic domain. Bone marrow analysis of such *mb-1*$^{\Delta c/\Delta c}$ mice suggests that early B cell development proceeds relatively normally in the absence of signals from Ig-α, presumably via Ig-β alone (Torres *et al.*, 1996). However, B cell maturation is severely impaired and the long-lived peripheral B cell pool was reduced to 1% of controls, possibly due to a signalling defect in a selection or survival checkpoint which is required for maturation and migration from the

bone marrow into the periphery. In view of the striking *mb-1*$^{\Delta c/\Delta c}$ phenotype, there was surprisingly little difference between the effects of the wild type IgM transgene and the IgM/α and IgM/β chimeras (Table 4.2). Indeed, compared to normal mice, all three anti-HEL transgenes generated reduced splenic B cell numbers in the reconstitution of *μMT* B cell-deficient mice. The apparent conflict in peripheral B cell numbers may be attributable to differences in the receptor complexes. It is possible that truncation of endogenous Ig-α confers a defect in surface transport of the BCR (and therefore in BCR function), although the few peripheral B cells that were detected in *mb-1*$^{\Delta c/\Delta c}$ mice did not show decreased surface IgM expression. In contrast, the chimeric IgM transgenes can be transported to the cell surface in the absence of an attendant Ig-α/Ig-β heterodimer due to the hydrophobic H2-K^{b} transmembrane segment (Williams *et al.*, 1990), yet do not show detectable association with endogenous Ig-α or Ig-β (Patel and Neuberger, 1993).

A possible indication of Ig-α and Ig-β function in selection of immature B cells to the long-lived mature B cell pool, is that expression of IgM/α (and to a less obvious extent IgM/β) seemed to be downregulated during cell lifespan or maturation. The newly-generated B cells in bone marrow expressed high levels of transgenic BCRs, and yet the longer-lived BrdU^{-} cells (Fig. 4.4), or the mature splenic B cells, expressed fewer receptors (Fig. 4.3). The apparent decrease in transgene expression may be due to elimination of the high expressers or selection and exit from the bone marrow of the low expressers. Although highly speculative, if transgenic BCR is used as a (rather unreliable) indicator of B cell maturity, then perhaps signalling via Ig-α alone advances the differentiation of the B cell, causing a shift from IgM^{hi} to IgM^{lo} expresssion as exhibited by B cells from gene-targetted CD22-deficient mice (O'Keefe *et al.*, 1996; Otipoby *et al.*, 1996; Sato *et al.*, 1996). Indeed, whilst the transgenes bearing D1.3 specificity exhibited a common phenotype, IgM/α often deviated less from normal mice, whereas IgM/β was more extreme, in displaying the altered phenotype of increased cell turnover *in vivo* and cell death *in vitro*, and lower expression of mature B cell markers.

Caution must be exercised when deriving conclusions from the small differences between IgM, IgM/α and IgM/β because founder effects cannot be excluded and the site of transgene integration may influence its function. Regrettably, although other founder lines were produced, they either did not express both the H and L chain transgenes or subsequent breeding proved difficult to establish. It

was difficult to detect whether the IgM/α or IgM/β chimeras revealed compromised signalling abilities because of the predominant phenotype common to the anti-HEL transgenes. Although a difference is seen between the wild type IgM and chimeric receptors in the *μMT* reconstitution assay, its significance is doubtful in the absence of multiple founder lines. The fact that even the wild type IgM BCR drives B cell maturation altered from normal suggests that the transgene specificity is not neutral and illustrates the limitations of monoclonal transgenic mice. Whilst some rearranged μ transgenes behave as hoped and can mediate allelic exclusion (Nussenzweig *et al.*, 1987), others are selected against (Forni, 1990; Grandien *et al.*, 1990) and even prove to be deleterious to B cells (Herzenberg *et al.*, 1987; Müller *et al.*, 1989) without known auto-antibody specificity. It appears that specificity may be important in determining the signalling potential of the transgene as a BCR since μ transgenes that strongly inhibit V_H rearrangment also allow B cell precursor development (Chang *et al.*, 1995). Nevertheless, it is clear in this chapter that the cytoplasmic domains of Ig-α and Ig-β are independently sufficient to generate a peripheral B cell pool.

Despite IgM/α and IgM/β behaving similarly to the IgM transgene in B cell maturation and activation, allelic exclusion of endogenous IgH was the only readout to show a significant difference in the signalling abilities of the chimeric versus wild type BCRs. IgM/α and IgM/β bore rearranged V regions and thus could be expressed to inhibit the endogenous V_H regions before they start to rearrange, but their signalling was inefficient and some endogenous alleles escaped exclusion. However, expressing both IgM/α and IgM/β together exhibited an enhanced activity and rescued the deficiency. Although the synergy demonstrated by (IgM/α)x(IgM/β) seems to indicate that the chimeras form a heterodimeric structure, the biochemical basis has yet to be proven (by, for example, immunoprecipitation). Nevertheless, these results are consistent with reconstitution studies revealing cooperativity between the two polypeptides (Luisiri *et al.*, 1996). Chimeras composed of the extracellular domains of the PDGF receptor fused to the Ig-α or Ig-β cytoplasmic tails were expressed in cell line transfectants. Although the chimeric homodimers could transmit signals, only the chimeric heterodimer (generated by specific ligand-induced dimerisation) could mediate downstream tyrosine phosphorylation similar in range and intensity to the endogenous BCR. Furthermore, Ig-β seemed to lower the stimulation threshold and enhance the activity of Ig-α by transferring its signalling potential since its own phosphorylation was quenched when

complexed with Ig-α. Therefore the assumption that the roles of Ig-α and Ig-β within an intact BCR could be elucidated by discerning their independent functions may be misleading and it seems more appropriate to examine how they co-operate. The cytoplasmic domains of Ig-α and Ig-β need to recruit downstream effectors such as protein tyrosine kinase Syk and the Src family kinases. It thus seems reasonable that the structure for binding these signalling molecules is optimal only in the context of the heterodimer. Indeed "cross-talk" between Ig-α and Ig-β and co-modulation of the each other's activities may occur within the intact BCR.

No major differences were observed between the signalling activities of IgM/α and IgM/β. However, B cell maturation and activation are complex, involving several discrete stages throughout differentiation: progression through development in the bone marrow, allelic exclusion of both H and L chains, selection or deletion, migration into the periphery, activation, clonal expansion, antibody secretion, memory generation, affinity maturation. The roles of Ig-α and Ig-β may vary in their contribution to BCR function at these stages, either by by mediating qualitatively different signals, or by signalling in different cellular contexts as the expression of possible downstream molecules for recruitment changes. It will be interesting to discover the biochemical mechanisms involved in each of these developmental checkpoints.

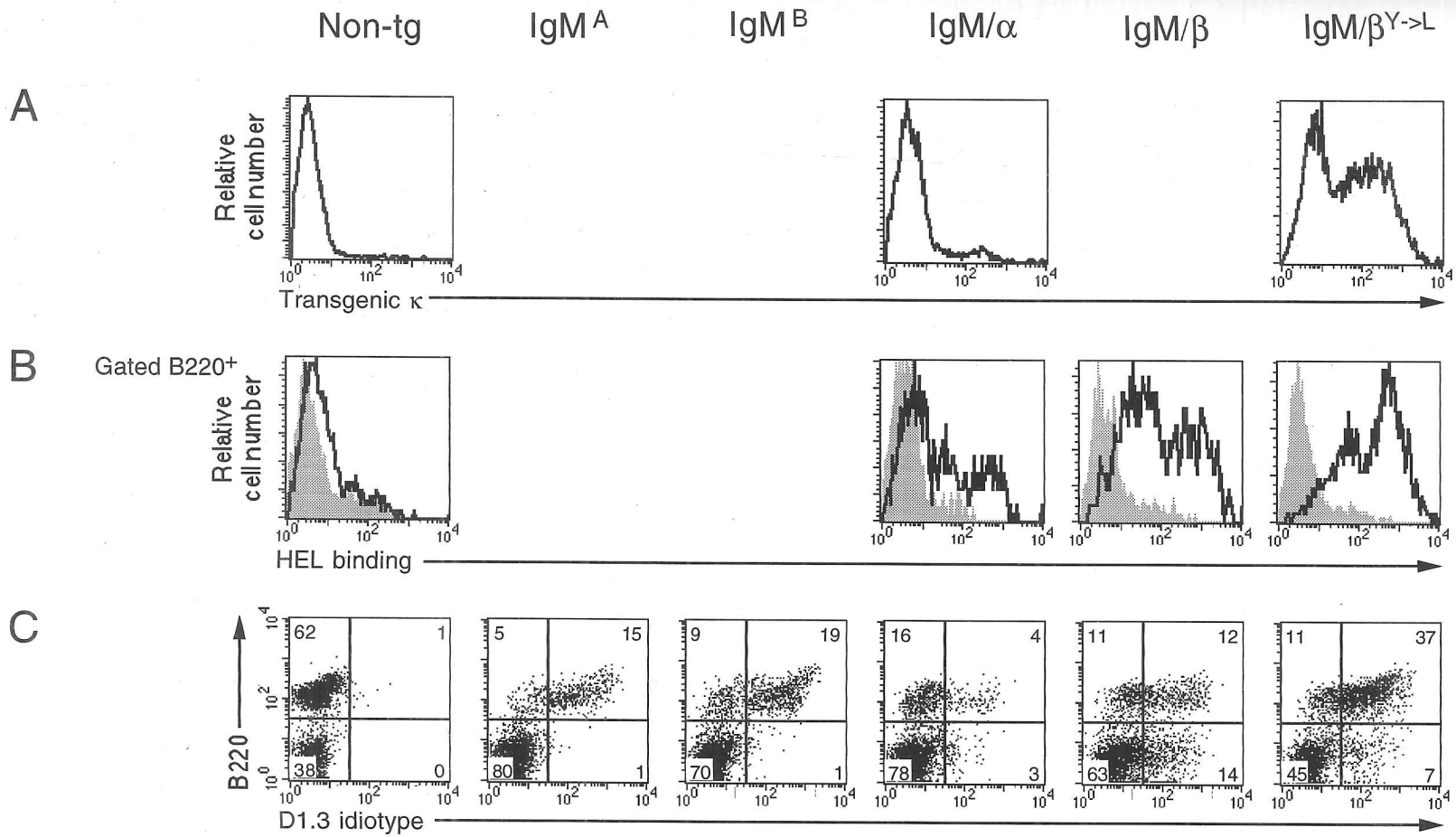

Figure 4.1 Transgene expression in spleen

(A) *Expression of transgenic κ.* Histograms depict splenocytes stained with mouse anti-rat-κ-biotin (revealed by SA-FITC) for flow cytometry and gated by scatter and PI exclusion.

(B) *Transgenic receptors identified by binding of HEL.* Splenocytes were stained with rat anti-B220-PE and HEL (0.1 μg/ml) together with mouse anti-HEL (mAb HyHEL10), visualised by secondary goat anti-IgG1-FITC. Histograms were gated by scatter, PI exclusion and for $B220^+$ cells. Shaded histograms denote secondary antiserum controls (since a few splenic B cells express endogenous IgG1 BCRs).

(C) *Transgenic receptors identified by D1.3 idiotype.* Splenocytes from non-transgenic (Non-tg) and transgenic mice were stained with rat anti-B220-PE and mouse anti-D1.3 idiotype-FITC, and were gated by scatter and PI exclusion. Plots are representative (see Table 4.1) and numbers denote percentage of splenocytes in each quadrant.

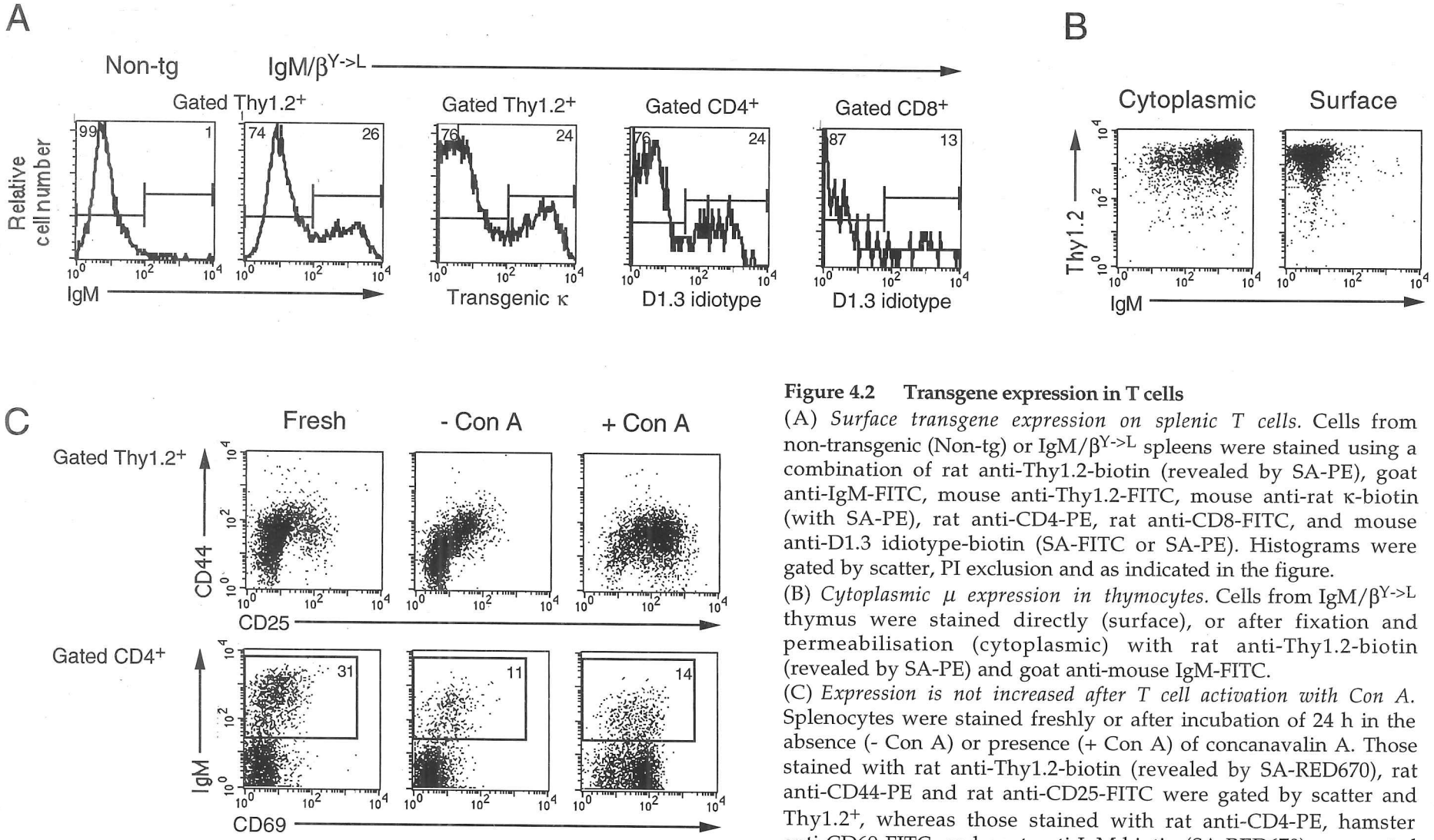

Figure 4.2 Transgene expression in T cells

(A) *Surface transgene expression on splenic T cells.* Cells from non-transgenic (Non-tg) or IgM/$\beta^{Y->L}$ spleens were stained using a combination of rat anti-Thy1.2-biotin (revealed by SA-PE), goat anti-IgM-FITC, mouse anti-Thy1.2-FITC, mouse anti-rat κ-biotin (with SA-PE), rat anti-CD4-PE, rat anti-CD8-FITC, and mouse anti-D1.3 idiotype-biotin (SA-FITC or SA-PE). Histograms were gated by scatter, PI exclusion and as indicated in the figure.

(B) *Cytoplasmic μ expression in thymocytes.* Cells from IgM/$\beta^{Y->L}$ thymus were stained directly (surface), or after fixation and permeabilisation (cytoplasmic) with rat anti-Thy1.2-biotin (revealed by SA-PE) and goat anti-mouse IgM-FITC.

(C) *Expression is not increased after T cell activation with Con A.* Splenocytes were stained freshly or after incubation of 24 h in the absence (- Con A) or presence (+ Con A) of concanavalin A. Those stained with rat anti-Thy1.2-biotin (revealed by SA-RED670), rat anti-CD44-PE and rat anti-CD25-FITC were gated by scatter and Thy1.2+, whereas those stained with rat anti-CD4-PE, hamster anti-CD69-FITC, and goat anti-IgM-biotin (SA-RED670) were gated by scatter and CD4+ (numbers denote percentage within region).

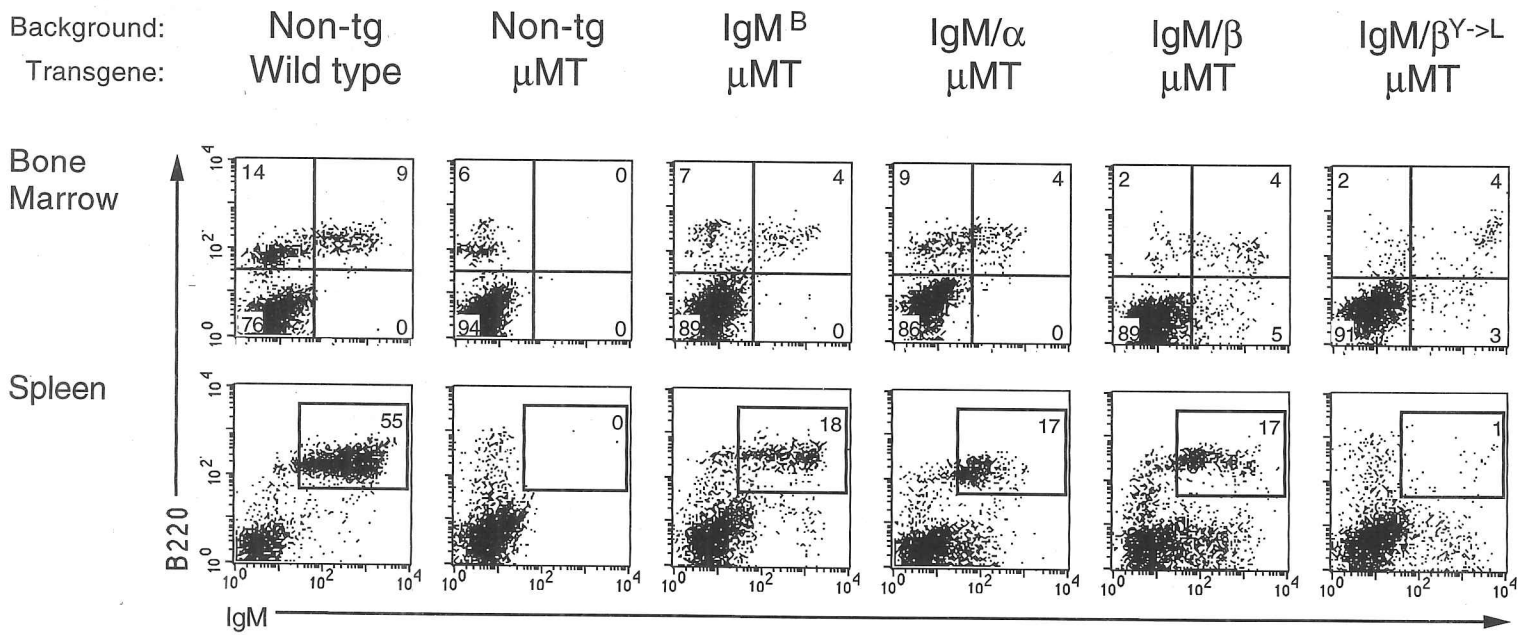

Figure 4.3 **Rescue of B cell development in a *μMT* background**
Cells from bone marrow and spleen of non-transgenic mice (Non-tg) or transgenic mice crossed into a *μMT* background were stained with rat anti-B220-PE and goat anti-IgM-FITC, and the representative flow cytometric analyses (see Table 4.2) were gated by scatter and PI exclusion. Numbers denote percentage in quadrants or region.

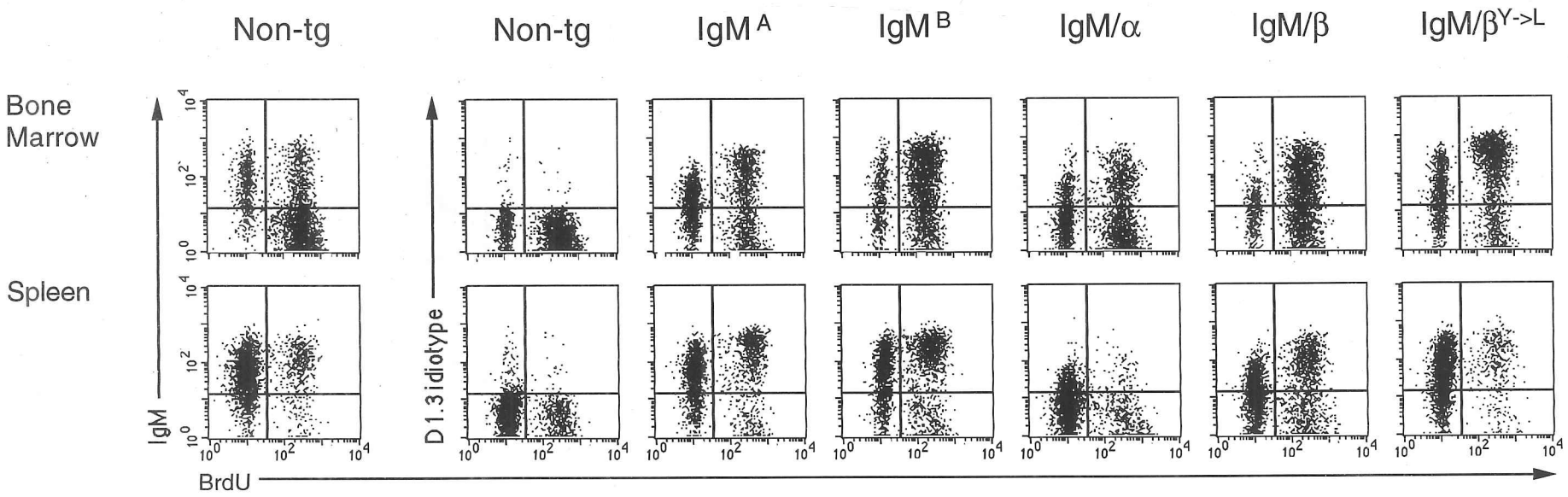

Figure 4.4 More rapid turnover of transgenic B cells *in vivo*

Mice were administered BrdU for 72 h before cells from bone marrow and spleen were surface stained with rat anti-B220-PE and either mouse anti-D1.3 idiotype or goat anti-IgM-biotin (revealed by SA-RED670). Cells were fixed and permeabilised prior to DNaseI digestion and staining with mouse anti-BrdU-FITC. Flow cytometric profiles were gated by scatter and $B220^+$. Whereas 50% of bone marrow IgM^+ and 15% of splenic IgM^+ B cells from the non-transgenic (Non-tg) controls had incorporated BrdU, the proportions increased to 60-90% and 30-50% in bone marrow and spleen respectively of $D1.3^+$ B cells from the transgenic mice (except $IgM/\beta^{Y->L}$ splenic B cells which behaved similarly to non-transgenic).

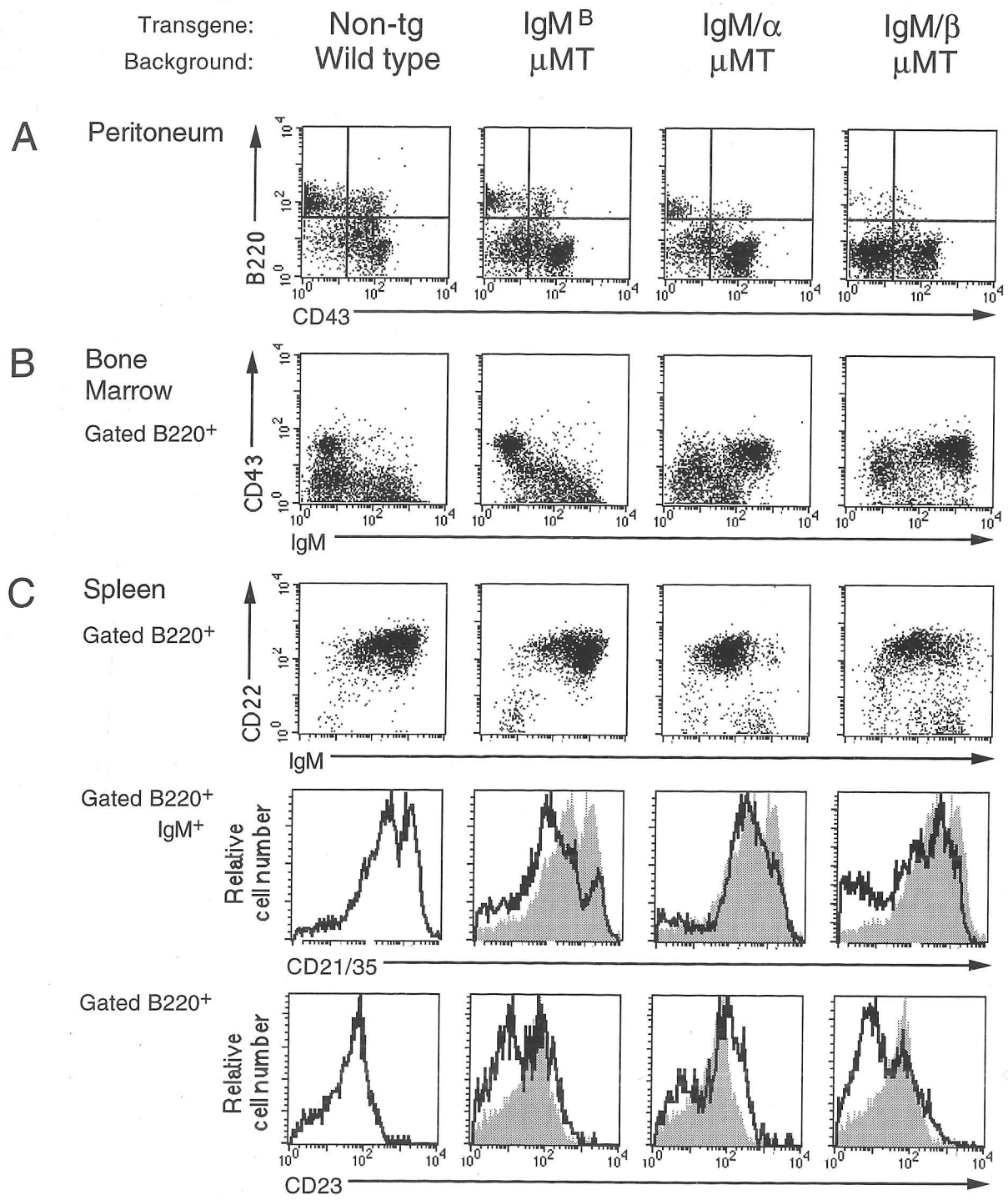

Figure 4.5 Expression of cell differentiation markers on reconstituted B cells

(A) *Peritoneal B-1 population.* Cells washed from the peritoneal cavity of non-transgenic (Non-tg) controls or transgenic mice crossed into a *μMT* background were stained with rat anti-B220-PE and rat anti-CD43-biotin (revealed by SA-RED670) and gated by scatter. The $CD43^+B220^+$ cells represented 20% of the total $B220^+$ cells for all mice.

(B) *Pro- and pre-B cell pools in bone marrow.* Bone marrow cells from mice as above were stained with rat anti-B220-PE,rat anti-CD43-biotin (revealed by SA-RED670) and goat anti-IgM-FITC. Dot plots were gated by scatter and $B220^+$.

(C) *Expression of mature B cell markers CD22, CD21/35 and CD23 on splenic B cells.* Splenocytes from mice as above were analysed: for CD22 expression by staining with rat anti-B220-PE, mouse anti-CD22-biotin (visualised with SA-RED670) and goat anti-IgM-FITC, gating by scatter and $B220^+$; for CD21/35 expression by staining with rat-anti-B220-PE, goat anti-IgM-FITC and CD21/35-biotin (with SA-RED670), gating by scatter and$B220^+IgM^+$; for CD23 expression by staining with rat anti-B220-FITC and rat anti-CD23-PE, gating by scatter and $B220^+$. Shaded histograms represent expression on non-transgenic B cells.

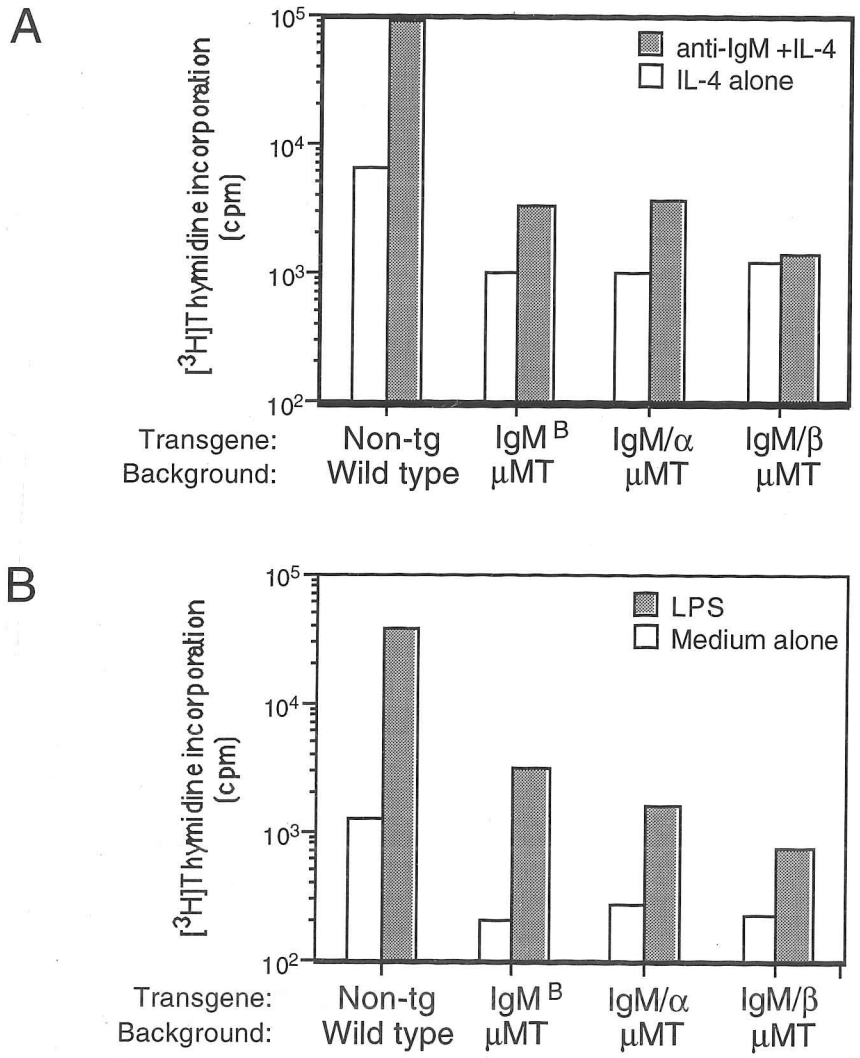

Figure 4.6 Reduced proliferation of transgenic B cells

(A) *Response to anti-IgM and IL-4.* Mean triplicate values of radioactive counts (cpm, counts per minute) were plotted on a logarithmic scale for splenocytes from non-transgenic (Non-tg) controls of transgenic mice in a *μMT* background that were cultured with 10 μg/ml goat anti-IgM $F(ab')_2$ + 100 U/well IL-4 or IL-4 alone. (n=1 in the results shown, however, they are typical of other experiments.)

(B) *Response to LPS.* Mean triplicate values for splenocytes from mice as above cultured in medium alone or supplemented with 1.0 μg/ml LPS.

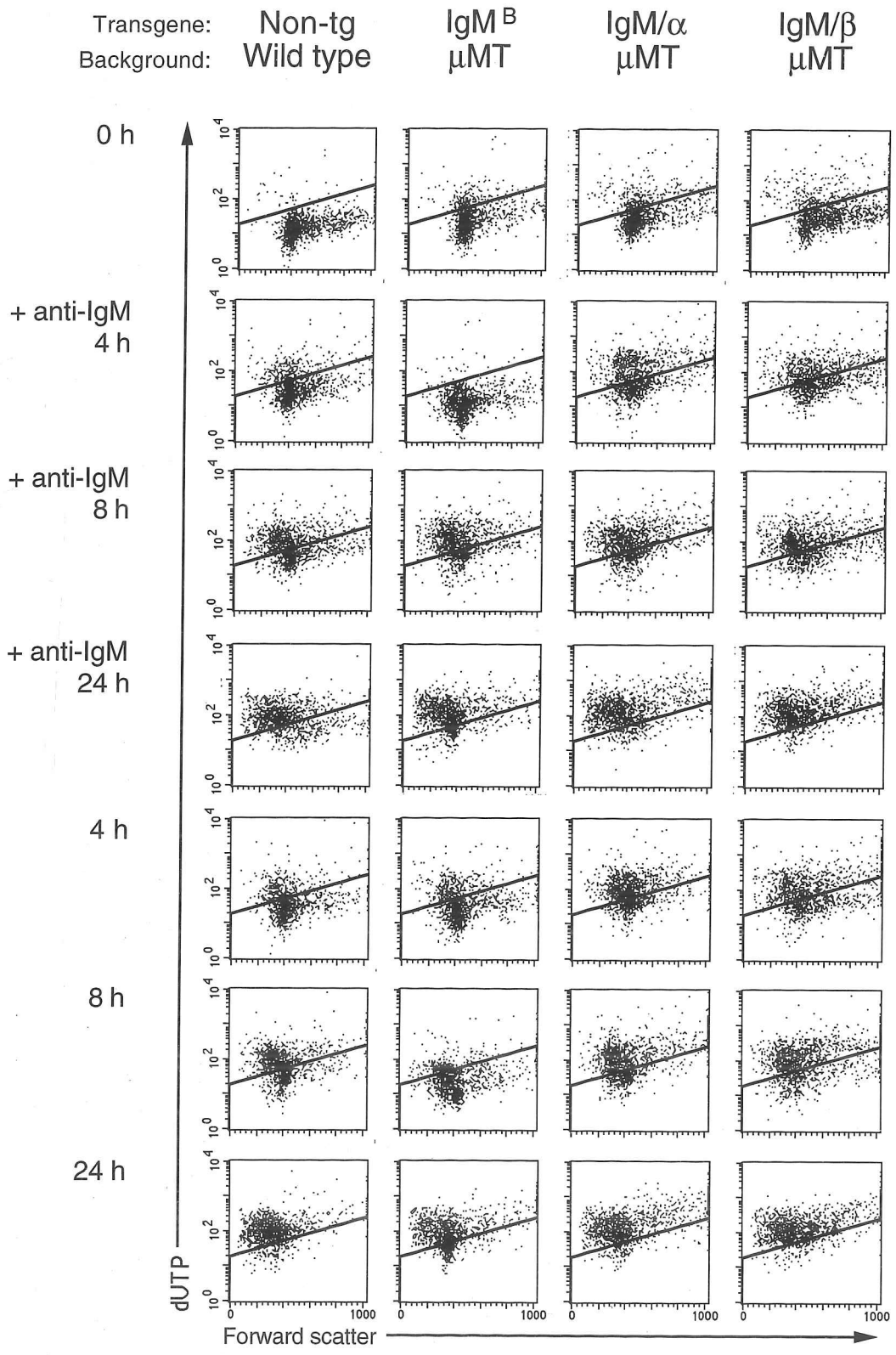

Figure 4.7 **Increased cell death of reconstituted B cells *in vitro***

Splenocytes from non-transgenic (Non-tg) controls or transgenic mice crossed into a *μMT* background were cultured in the absence and presence of 10 μg/ml goat anti-IgM $F(ab')_2$ for indicated times before staining with rat anti-B220-PE and fixation for TUNEL (TdT-mediated dUTP nick end labelling) with dUTP-biotin (revealed by SA-FITC). Dot plots show cells gated as $B220^+$.

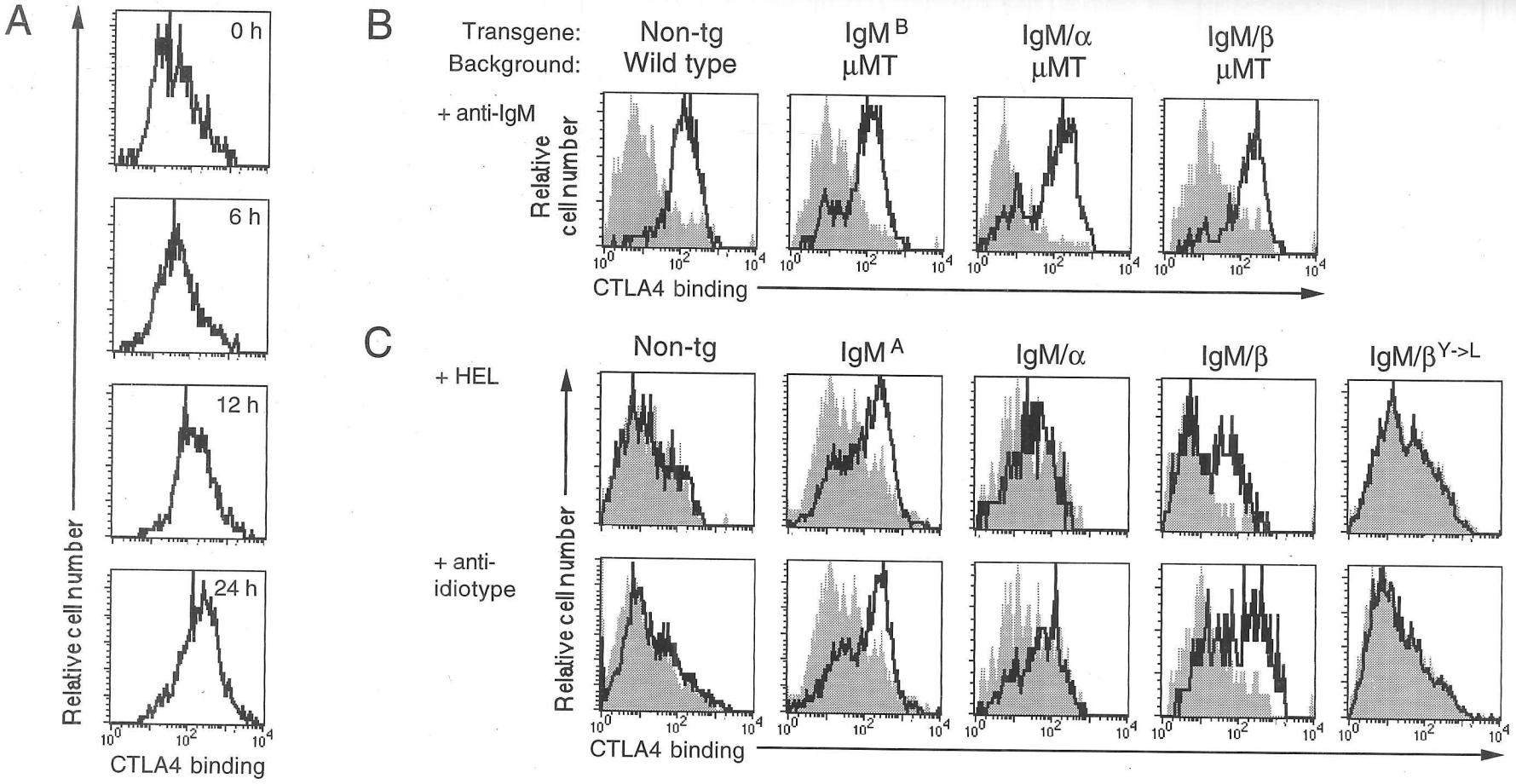

Figure 4.8 Upregulation of B7 mediated by IgM/α and IgM/β, but not IgM/β$^{Y->L}$

(A) *Timecourse in response to LPS.* Splenocytes from non-transgenic mice were cultured for a total of 24 h with the addition of 50 μg/ml LPS for incubation periods of 0, 6, 12 and 24 h, followed by staining for flow cytometric analysis with rat anti-B220-PE and mCTLA4-Hγ1 (revealed by goat anti-human IgG-FITC). Histograms were gated by scatter, PI exclusion and B220$^+$.

(B) *IgM/α and IgM/β upregulate B7 in response to anti-IgM.* Splenocytes from non-transgenic (Non-tg) controls or transgenic mice crossed into a *μMT* background were cultured in the presence of 10 μg/ml goat anti-mouse IgM F(ab')$_2$ for 24 h. Flow cytometric analysis was performed as above and shaded histograms indicate medium alone controls.

(C) *B7 response to HEL and anti-idiotype.* Cells from mice in a wild type (i.e. non-*μMT*) background were analysed as in (B) after incubation with 1.0 μg/ml HEL or 10 μg/ml anti-D1.3 idiotype, mAb E5.2.

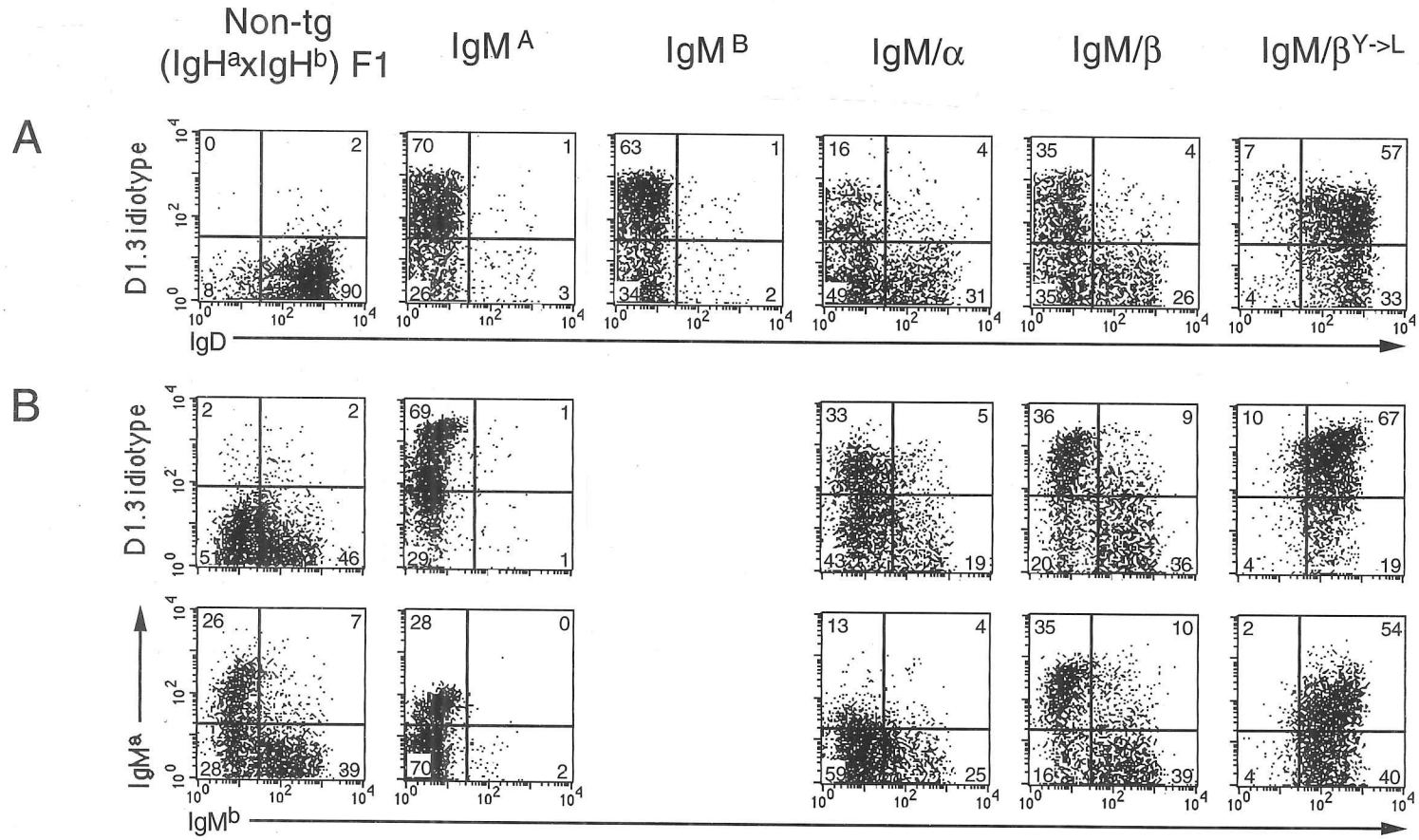

Figure 4.9 Allelic exclusion of endogenous IgH

(A) *Exclusion of IgD.* Splenocytes from transgenic or non-transgenic (Non-tg) mice were stained with rat anti-B220-FITC, mouse anti-D1.3 idiotype-biotin (revealed by SA-RED670) and rat anti-IgD-PE, and flow cytometry plots were gated by scatter and $B220^+$. Numbers denote percentage of splenic B cells within each quadrant.

(B) *Allotype exclusion.* Splenocytes from non-transgenic ($IgH^a x IgH^b$) F1 mice or transgenic mice crossed into an IgH^b background were stained with rat anti-B220-PE, either D1.3-idiotype-biotin or mouse anti-IgM^a-biotin (visualised with SA-RED670) and mouse anti-IgM^b-FITC, gating as above.

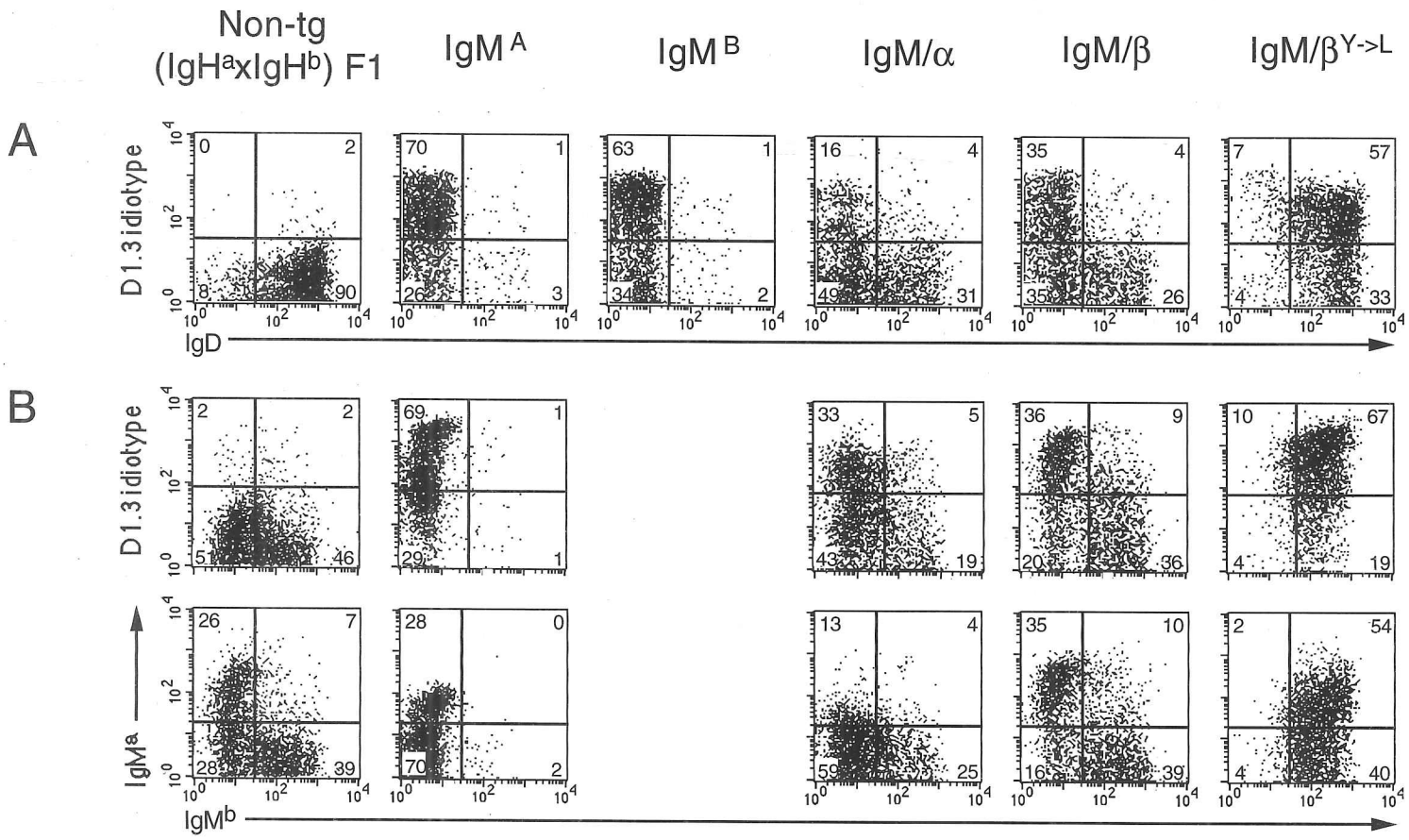

Figure 4.9 Allelic exclusion of endogenous IgH

(A) *Exclusion of IgD.* Splenocytes from transgenic or non-transgenic (Non-tg) mice were stained with rat anti-B220-FITC, mouse anti-D1.3 idiotype-biotin (revealed by SA-RED670) and rat anti-IgD-PE, and flow cytometry plots were gated by scatter and $B220^+$. Numbers denote percentage of splenic B cells within each quadrant.

(B) *Allotype exclusion.* Splenocytes from non-transgenic (IgH^axIgH^b) F1 mice or transgenic mice crossed into an IgH^b background were stained with rat anti-B220-PE, either D1.3-idiotype-biotin or mouse anti-IgM^a-biotin (visualised with SA-RED670) and mouse anti-IgM^b-FITC, gating as above.

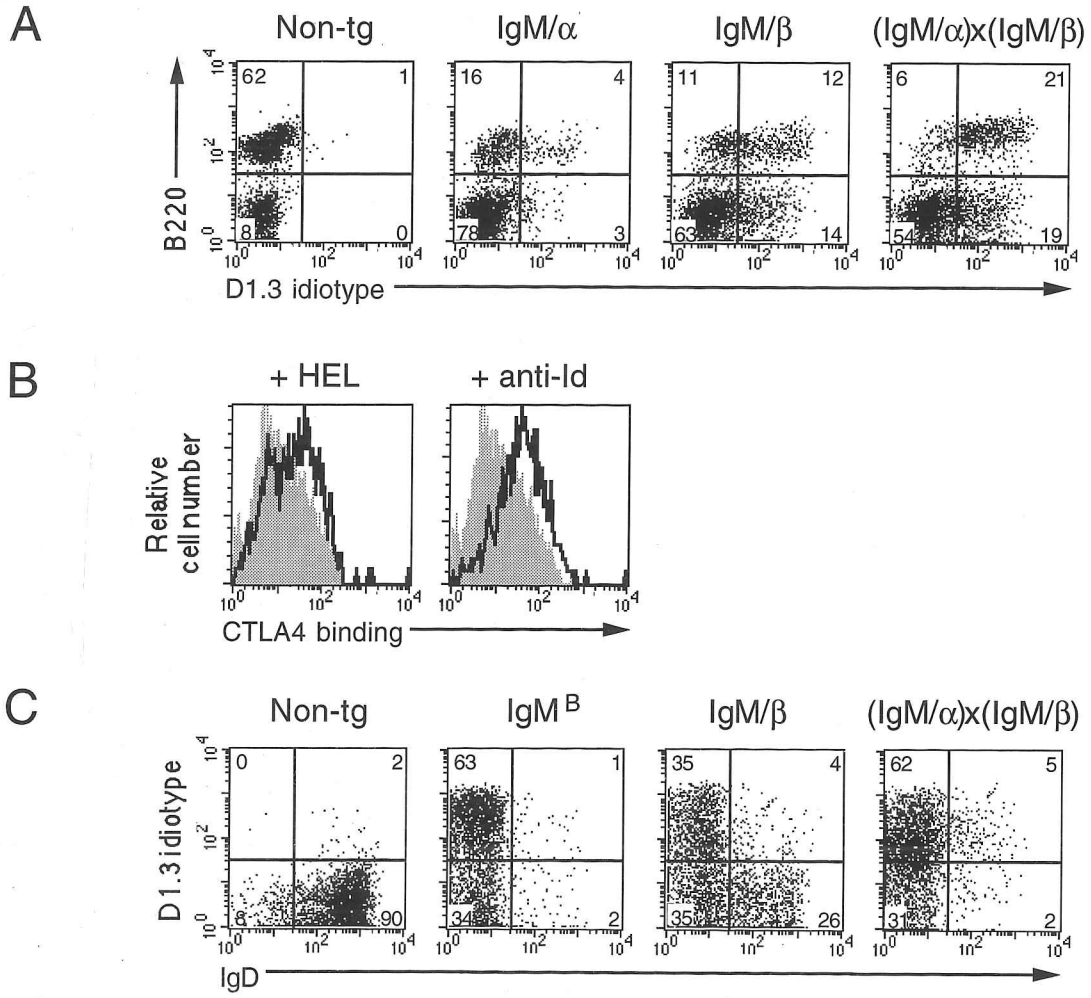

Figure 4.10 The (IgM/α)x(IgM/β) double transgenic mouse

(A) *(IgM/α)x(IgM/β) expresses similar levels of transgenic receptors as the IgM/β parent.* Cells were treated as in Fig. 4.1C, gating by scatter and PI exclusion. Numbers denote percentage of splenocytes in each quadrant.

(B) *Upregulation of B7 in response to HEL and anti-Id by splenic B cells.* Cells were cultured as in Fig. 4.8C with 1.0 μg/ml HEL or 10 μg/ml anti-D1.3 idotype, mAb E5.2, and analysed for binding of mCTLA4-Hγ1 fusion protein. Histograms are gated for $B220^+$ and shaded histograms depict medium alone controls.

(C) *(IgM/α)x(IgM/β) exhibits efficient exclusion of endogenous IgH.* Cells were stained as in Fig. 4.9A and were gated as $B220^+$. Numbers indicate splenic B cells present in quadrants.

Results

Developmental effects of a μ transgene driven by κ enhancer elements

Chapter 5

Chapter 5: Results
Developmental effects of a μ transgene driven by the κ enhancers

In the previous chapter, the chimeric IgM/α and IgM/β transgenes did not inhibit endogenous IgH expression to the same extent as the wild type IgM transgene, suggesting that the compromised BCRs mediated less efficient signalling in allelic exclusion. Thus, the quality of BCR signal appears to be a major factor in fully effective allelic exclusion. However, if allelic exclusion is regarded as a race for productive rearrangement, it is evident that the timing of BCR expression is also an important determinant. Transgenes bearing rearranged V_H regions ought to be expressed as receptors before endogenous V_H rearrangement, resulting in efficient inhibition of endogenous IgH expression. Indeed, transgenes bearing V_H region miniloci seem less effective at mediating allelic exclusion (Brüggemann *et al.*, 1989). Miniloci transgenes, as well as the endogenous IgH, need to undergo V region rearrangement before their expression as receptors in order to inhibit the competing alleles.

In contrast, both rearranged μ transgenes and those bearing V region miniloci can rescue B cell development in a *μMT* B cell-deficient mice blocked at the pro-B cell stage (chapter 4, (Kitamura *et al.*, 1991; Wagner *et al.*, 1994)). The differentation checkpoints of allelic exclusion and pro- to pre-B cell transition both result from pre-BCR signalling, occuring at similar stages in early development. However, the timing of receptor expression may need to be more critical for allelic exclusion than for the pro- to pre-B cell transition. Under the threat of competition from the other allele, transcription of a rearranged μ must occur before the other in order to ensure exclusion. The generation of pre-B cells in *μMT* mice may have more flexible timing restrictions since the pro-B cell may be able to "wait", providing a functionally rearranged μ chain is expressed within the time window before the pro-B cell dies.

In order to test the timing hypothesis, the expression of a rearranged μ transgene was postponed by placing it under the transcriptional regulation of the two enhancers from the κ L chain gene. Thus, the effect of delayed μ expression on early B cell development could be assessed from allelic exclusion and reconstitution of the peripheral B cell pool in *μMT* B cell-deficient mice.

5.1 $V_{NP}C\mu[Ei,E3']^{\kappa}$ does not mediate allelic exclusion.

Transgenic founder lines, that were originally generated for somatic hypermutation experiments (N. Klix *et al.*, in preparation), carry a $V_{NP}C\mu[Ei,E3']^{\kappa}$ transgene (Fig. 5.1A) based on pSV-Vμ1 (a genomic clone of μ with a rearranged V_H region bearing anti-NP specificity) (Neuberger, 1983). The IgH enhancer (Eμ) was replaced by the κ intron enhancer/matrix attachment region (Ei/MAR), and the κ 3′ enhancer (E3′) was placed downstream of the coding regions. κ L chain expression appears not to be required until after μ H chain expression in B cell development, therefore regulation by the κ enhancers might delay the expression a μ transgene. The availability of the $V_{NP}C\mu[Ei,E3']^{\kappa}$ transgenic mice made it possible to address the importance of timing of pre-BCR expression in allelic exclusion and B cell maturation, and also gave an indication of the activation of the κ enhancers during early B cell development.

The ability of the $V_{NP}C\mu[Ei,E3']^{\kappa}$ transgene to mediate allelic exclusion was assayed as in chapter 4. In the absence of specific L chain, the $V_{NP}C\mu[Ei,E3']^{\kappa}$ transgenic μ chain could not be identified by an anti-idiotype (unlike the anti-HEL transgenic mice). However, the transgenic μ (of the IgH^a allotype) could be discriminated from endogenous IgM by breeding $V_{NP}C\mu[Ei,E3']^{\kappa}$ into an IgH^b background. Non-transgenic (IgH^a x IgH^b) F1 mice exhibited efficient allotype exclusion in that the splenic B cells expressed either IgM^a or IgM^b, but not both allotypes (Fig. 5.1B). In contrast, the $V_{NP}C\mu[Ei,E3']^{\kappa}$ transgene (IgM^a) was ineffective at inhibiting the expression of endogenous IgM^b and most of the B cells that expressed the transgene co-expressed the endogenous allotype.

The B cells that expressed endogenous IgH were also be detected by their expression of IgD. Most of the $V_{NP}C\mu[Ei,E3']^{\kappa}$ splenic B cells expressed IgD in a profile similar to the non-transgenic F1 (Fig. 5.1B) and this lack of suppression of IgD expression is consistent with the lack of allotype exclusion.

The effect of the $V_{NP}C\mu[Ei,E3']^{\kappa}$ transgene on allelic exclusion is in contrast to that of the IgM transgene in the previous chapter (see Fig. 4.9A). Thus, $V_{NP}C\mu[Ei,E3']^{\kappa}$ appears not to be expressed early enough in B cell development to prevent the expression of endogenous IgH.

5.2 $V_{NP}C\mu[Ei,E3']^{\kappa}$ does rescue B cell development in a *μMT* background.

Although the lack of allelic exclusion may be attributable to the timing of $V_{NP}C\mu[Ei,E3']^{\kappa}$ expression, it was not clear that the κ enhancers were even

sufficient to activate transcription of the μ transgene at the late pro-/pre-B cell stage for its expression in a pre-BCR. Some Vκ rearrangement has been observed in *μMT* mice (which bear a germline disruption of the membrane exons of μ) (Ehlich *et al.*, 1993; Kitamura et al., 1991), suggesting that the κ regulatory elements can still initiate rearrangement despite the block in pro-/pre-B cell development and in the absence of a pre-BCR. However, the Vκ rearrangment was detected at low frequency, and it has been suggested that pre-BCR signals induce the further activation of the κ locus (Iglesias *et al.*, 1991; Reth *et al.*, 1987; Tsubata and Reth, 1990).

In order to determine whether the κ enhancers were activated early enough to drive the transcription of the rearranged μ transgene for pre-BCR signalling, the mice were crossed into a *μMT* background. Non-transgenic *μMT* mice, which cannot express surface IgM, had no IgM$^+$B220$^+$ cells in the bone marrow (Fig. 5.2). In contrast, the V_{NP}Cμ[Ei,E3′]^κ transgene was able to reconstitute a B220$^+$IgM$^+$ population in the bone marrow. Indeed, it is clear from the B220$^+$IgM$^+$ cells also present in the V_{NP}Cμ[Ei,E3′]^κ spleen (Fig. 5.2) that the transgene rescues the *μMT* phenotype and can effect the maturation of a peripheral B cell pool.

These results indicate that the κ enhancers are active at the late pro-/pre-B cell stage and are able to drive expression of a rearranged μ transgene. In the absence of endogenous competition, V_{NP}Cμ[Ei,E3′]^κ can indeed function as a pre-BCR, suggesting that once a functional μ chain is expressed (even if later than usual), the B cells can continue to mature and migrate into the periphery.

5.3 Discussion

Placing a rearranged μ transgene under the regulatory control of the κ Ei/MAR and E3′ resulted in its expression in B cells, but seemed to modify its timing and therefore its effect on developmental processes. V_{NP}Cμ[Ei,E3′]^κ did not mediate allelic exclusion, but did rescue the *μMT* phenotype, suggesting that the κ enhancers mediated transcription of the rearranged μ transgene at a stage between allelic exclusion and pro- to pre-B cell progression in B cell differentiation.

V_{NP}Cμ[Ei,E3′]^κ was unable to cause exclusion of the endogenous IgH loci. This was not due to a defect in the ability of the transgenic μ to function as a pre-BCR, since in the absence of competing membrane μ, it could rescue B cell development in a *μMT* background. Rather, its inability to mediate allelic

exclusion can be explained by the differential timing of endogenous μ expression (driven by Eμ) compared to the transcriptional activity of the κ enhancers. These results reveal that the κ enhancers do not seem to become active until after V_H rearrrangement, but appeared to be active (presumbly earlier than necessary for κ rearrangement) before the *μMT* block in B cell development (fraction C; Ehlich et al., 1993; Hardy *et al.*, 1991). These indications of κ enhancer activity are in aggreement with the detection of endogenous Vκ rearrangement in *μMT* mice (Ehlich et al., 1993), and with reports of sterile κ transcription in late pro-/pre-B cell subpopulations (Grawunder *et al.*, 1995).

The timing in expression of V_{NP}Cμ[Ei,E3′]$^{\kappa}$ is later than that of μ transgenes driven by Eμ. The delay in transcription appears to be regulated by the κ enhancers, although it was not possible to discriminate the relative contributions of Ei/MAR and E3′. One could only speculate on their roles in transcription of V_{NP}Cμ[Ei,E3′]$^{\kappa}$. From experiments in cell lines, Ei has been thought to play a predominant role in early developmental events such as demethylation and Vκ rearrangement in pre-B cells (Chen *et al.*, 1994; Klug *et al.*, 1994; Lichtenstein *et al.*, 1994). Since the V_{NP}Cμ[Ei,E3′]$^{\kappa}$ transgene already has a rearranged V region, its expression gave an indication of the activities of the κ enhancers in locus accessibility and transcriptional regulation. It would be necessary to monitor the activities of Ei/MAR and E3′ independently in order to determine whether transcription of the κ locus is indeed controlled only by Ei/MAR. Alternatively, if Ei/MAR was essential only for demethylation and Vκ rearrangement, but not transcription, E3′ may be sufficient drive the transcription of a rearranged κ transgene.

The κ Ei/MAR and E3′ together are sufficient to drive the expression of a rearranged μ transgene at a late pro-/pre-B cell stage just after V_H rearrangement, resulting in the rescue of B cell development in *μMT* mice. Their independent contributions to transcriptional regulation in early B cell development should be determined and one approach for discriminating their roles could be to analyse the effects of deleting Ei/MAR or E3′ from a rearranged κ transgene.

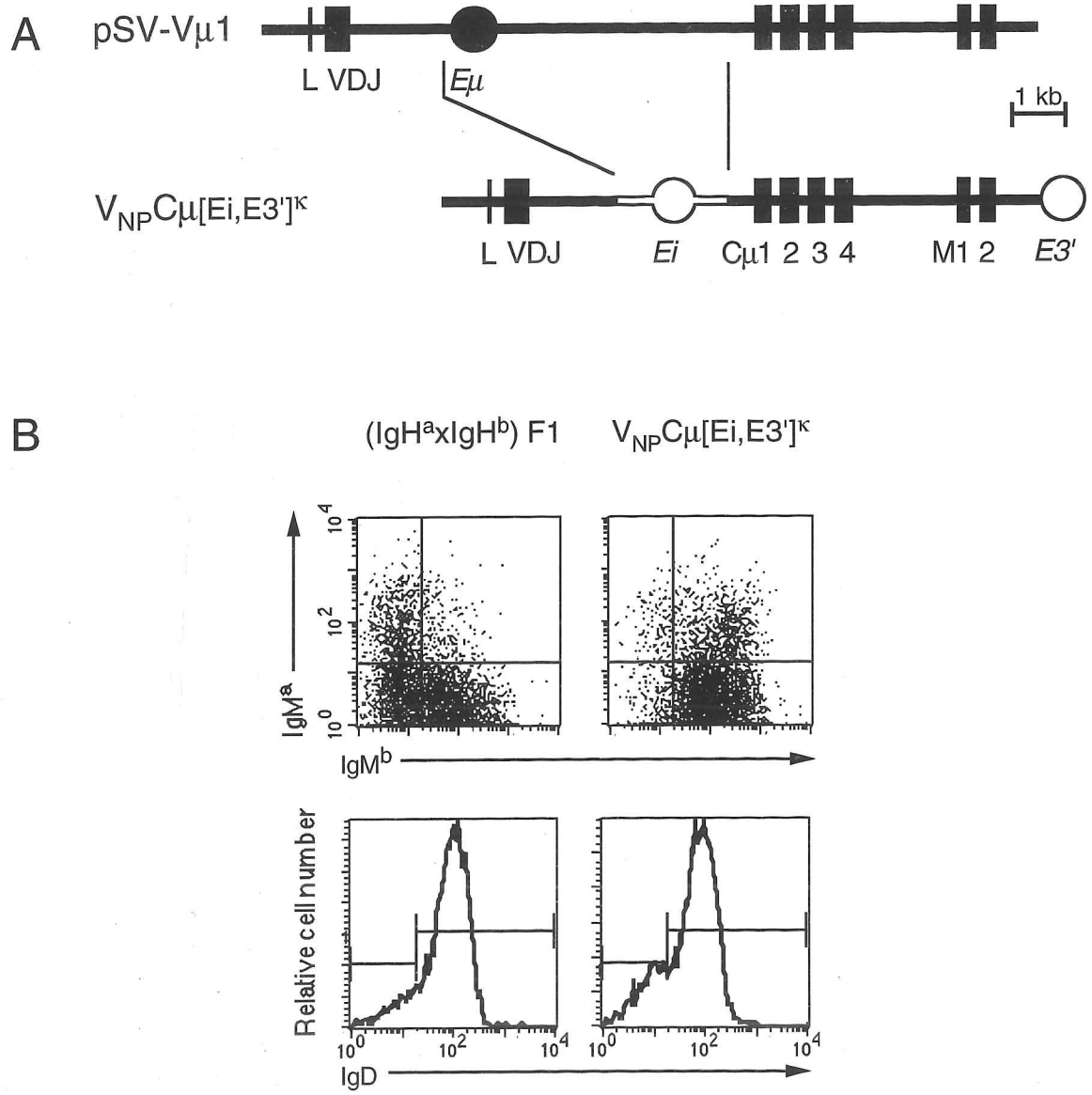

Figure 5.1 Lack of allelic exclusion by $V_{NP}C\mu[Ei,E3']^{\kappa}$

(A) *$V_{NP}C\mu[Ei,E3']^{\kappa}$ transgene construct based on pSV-Vμ1.* The IgH enhancer (Eμ) in the J-C intron of pSV-Vμ1 was exchanged for the κ intron enhancer (Ei), and the κ 3' enhancer (E3') was inserted 3' of the μ coding regions.

(B) *$V_{NP}C\mu[Ei,E3']^{\kappa}$ does not inhibit endogenous IgH expression.* Splenocytes from ($IgH^a x IgH^b$) F1 mice or $V_{NP}C\mu[Ei,E3']^{\kappa}$ mice were stained with rat anti-B220-PE, and either mouse anti-IgM^a-biotin (revealed by SA-RED670) with mouse anti-IgM^b-FITC, or sheep anti-mouse IgD-FITC. Dot plots and histograms were gated by scatter and $B220^+$.

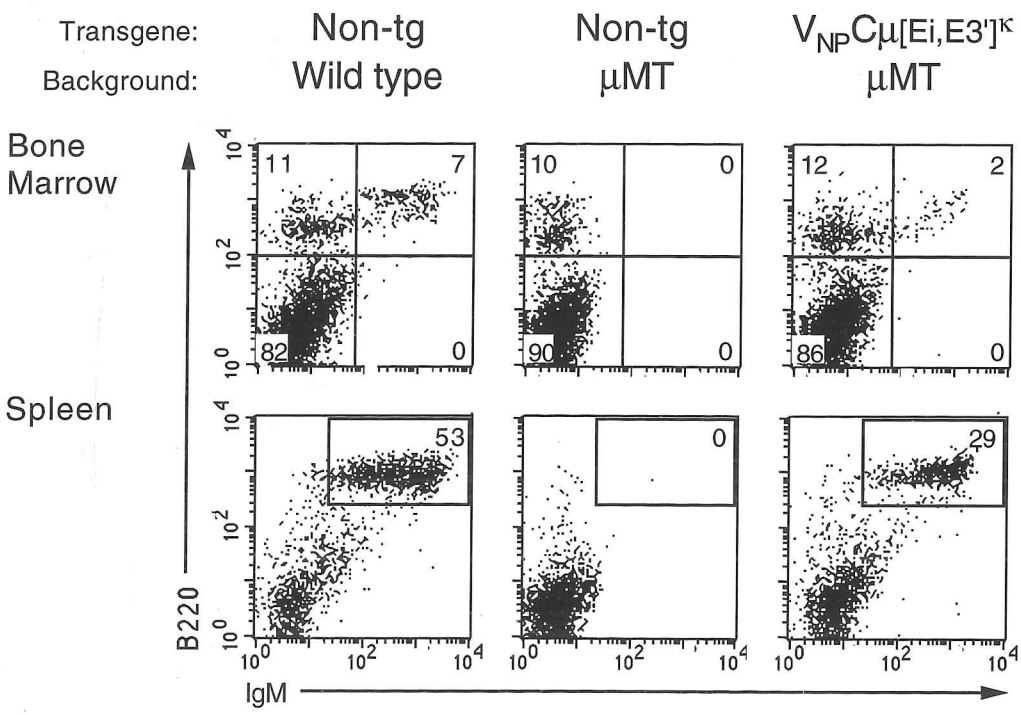

Figure 5.2 Rescue of B cell development by V_{NP}Cμ[Ei,E3']^{κ}

Cells from bone marrow and spleen of non-transgenic (Non-tg) wild type or *μMT* controls or V_{NP}Cμ[Ei,E3']^{κ} crossed into a *μMT* background were stained with rat anti-B220-PE and goat anti-IgM-FITC for flow cytometric analysis, gating by scatter and PI exclusion. Numbers denote percentage of cells in quadrant or region.

Results

Transcriptional regulation by the κ enhancers during B cell development

Chapter 6

Chapter 6: Results
Transcriptional regulation by the κ enhancers during B cell development

The previous chapter showed that the two enhancers from the immunoglobulin κ light chain gene were able to drive the expression of a rearranged μ transgene ($V_{NP}C\mu[Ei,E3']^{\kappa}$), leading to reconstitution of peripheral B cells in a *μMT* background, but not allelic exclusion of endogenous IgH alleles. These findings suggested that the κ enhancers become activated at a precise time in pro-/pre-B cell development after V_H rearrangment. However, it was not clear what the relative contributions of the two κ enhancers were to the transcriptional activity at that differentiation stage. The κ intron enhancer (Ei), located in the Jκ-Cκ intron (Picard and Schaffner, 1984), and the κ 3′ enhancer (E3′), situated 9 kb downstream of Cκ (Meyer and Neuberger, 1989), bind different sets of transcription factors (Pongubala and Atchison, 1991; Staudt and Lenardo, 1991). Thus, the two enhancers presumbly play distinct roles in controlling different events at the κ gene locus thoughout B cell development.

Prior to commencement of this study, the literature might have suggested that the transcriptional regulation at the pre-B cell stage was mostly attributable to Ei, since Ei seemed to be involved primarily in processes early in B cell development. Cell line transfection experiments suggested that Ei is essential not only for transcription, but for demethylation, germline κ expression (sterile transcription), and Vκ rearrangement (Chen *et al.*, 1994; Klug *et al.*, 1994; Lichtenstein *et al.*, 1994). Indeed gene-targetted disruption of Ei by insertion of a *neo* gene in mice abolished Vκ rearrangement (Takeda *et al.*, 1993). In contrast, E3′ has been assumed not to become important until later stages in B cell development. E3′ activated reporter gene expression only weakly in a mature B cell line, yet greatly enhanced expression in plasmacytomas (Fulton and Van Ness, 1993; Meyer *et al.*, 1990). Furthermore, E3′ has been shown to be neccessary for high levels of expression in transgenic mice (Betz *et al.*, 1994; Meyer *et al.*, 1990).

In this chapter, the relative roles of Ei and E3′ in the transcriptional regulation of rearranged κ could be addressed in mice expressing of κ transgenes containing either Ei/MAR or E3′. The results described here indicate that E3′ seems to be the more important of the two enhancers for transcription, becoming active at the pre-B cell stage when the locus is accessible for sterile transcription prior to Vκ

rearrangement.

6.1 The κ enhancers have been shown to be important for somatic hypermutation in transgenic mice and expression in hybridomas.

Transgenic mice, established previously for somatic hypermutation experiments, were used in this study to investigate the relative importance of the two κ enhancers in regulating transcription and expression during B cell development. The different transgene constructs were derived from Lκ, composed of a rearranged mouse VκOx exon linked to a rat Cκ exon (to facilitate discrimination from endogenous mouse κ)and regulated by Ei and the adjacent matrix attachment region (Ei/MAR) and E3′ (Fig. 6.1) (Meyer et al., 1990). Ei/MAR and E3′ were deleted independently to form LκΔ[Ei/MAR] and LκΔ[E3′] respectively (Betz et al., 1994).

Founder lines differing in transgene copy number (denoted in superscript) have been analysed in a previous report (Betz et al., 1994) (notation in different reports is clarified in Materials and Methods). Lines $L\kappa^3$ and $L\kappa^6$, $L\kappa\Delta[Ei/MAR]^4$ and $L\kappa\Delta[Ei/MAR]^7$, and $L\kappa\Delta[E3']^2$ are also presented in this study. The second $L\kappa\Delta[E3']^5$ founder was lost, but a third $L\kappa\Delta[E3']^6$ founder was analysed here instead. The founder lines bearing the Lκ and LκΔ[Ei/MAR] transgenes gave consistent results. Interestingly, although $L\kappa\Delta[E3']^5$ has previously been shown to behave similarly to $L\kappa\Delta[E3']^2$, $L\kappa\Delta[E3']^6$ differed in this chapter, and this line-to-line variation will be discussed.

It has been shown that these transgenes differ in their abilities to undergo somatic hypermutation and the conclusions are summarised in Fig. 6.1 (Betz *et al.*, 1994). Although Lκ contained sufficient information to hypermutate, deletion of either Ei/MAR or E3′ abrogated mutation, suggesting that both enhancers are necessary for targetting the hypermutation process.

If high levels of expression are required to drive the hypermutation machinery, loss of mutation could merely be due to reduced transcriptional activity. Therefore hybridomas generated from the transgenic mice were analysed for transcription of the transgenes. The levels of transgenic mRNA from the LκΔ[E3′] hybridomas were greatly reduced indicating that E3′ is necessary for high level κ expression. In contrast, transgene expression of LκΔ[Ei/MAR] was similar to Lκ, suggesting that deletion of Ei/MAR had little effect on transcription, unlike hypermutation, at the terminal stage in differentiation.

The obvious importance of Ei/MAR to the somatic hypermutation process is under investigation by others, however, these findings also suggest that E3′ is both essential and sufficient (in the absence of Ei/MAR) for transcription in hybridomas. Since hybridomas are only a model for the terminal antibody-secreting plasma cell stage in B cell differentiation, it was obviously interesting to determine whether E3′ also plays a critical role in κ expression earlier in B cell development.

6.2 κ transgene expression in germinal centre cells from Peyer's patches

In the previous studies of the κ transgenic mice, hybridomas were established in order to correlate transcription of the transgenes with their ability to hypermutate. However, hybridomas are believed to represent terminally-differentiated plasma cells and may not accurately reflect processes that occur in the mature B cells undergoing somatic hypermutation within the germinal centre (GC).

Peyer's patches, lymphoid tissue in the gut containing a large proportion of somatically hypermutating B cells, were dissected, and the GC B cells were identified by binding high levels of the lectin peanut agglutinin (PNA). All the transgenic mice revealed good expression of their transgenes in the PNA^{hi} cells (Fig. 6.2), although the levels might differ slightly. The few cells that expressed endogenous κ also expressed it to high levels. It was most noticeable, however, that different lines varied in the proportions of cells expressing transgenic versus endogenous κ. In Lκ mice, almost all the PNA^{hi} cells expressed transgenic κ, but very few expressed endogenous κ. Most of the PNA^{hi} cells in mice from both LκΔ[Ei/MAR] founder lines also expressed the transgene, indicating that Ei/MAR is not necessary for good expression in GC B cells.

The LκΔ[E3′] mice gave conflicting results (Fig. 6.2). $L\kappa\Delta[E3']^6$ behaved like $L\kappa^6$, whereas $L\kappa\Delta[E3']^2$ had almost reversed ratios of transgene$^+$: endogenous κ^+ cells. These findings suggest that E3′ may be required for efficient expression of κ, but that integration effects may compensate for its absence.

6.3 Lκ and LκΔ[Ei/MAR] exclude endogenous κ expression on splenic B cells.

Although GC B cells are at a mature B cell stage in development, they are specialised cells that have been activated by antigen and have migrated into the GC to hypermutate. They may have undergone proliferation, class-switching or differentiation into memory B cells, and thus are a population selected by

antigen. Since most B cells in the periphery are waiting to interact with antigen, spleens from the transgenic mice were analysed to determine the activity of E3′ on κ expression in resting mature B cells.

Splenic B cells (identified by IgM) from both Lκ founder lines expressed high levels of transgenic κ, but very few expressed endogenous κ (Fig. 6.3). Similarly, the absence of Ei/MAR had little effect on the expression levels of transgenic κ, although a few more cells expressed endogenous κ with only slight variation between the two founders. Furthermore, double-staining for both transgenic and endogenous κ revealed that the Lκ and LκΔ[Ei/MAR] cells exhibited allelic exclusion, suggesting that expression of the endogenous κ genes was inhibited by Lκ and also by LκΔ[Ei/MAR], to a slightly lesser extent. The good expression (similar to Lκ) of the LκΔ[Ei/MAR] transgene containing E3′ in the absence of Ei/MAR, indicates that Ei/MAR is not required, but that the presence of just E3′ alone seems sufficient for high levels of transcription of a rearranged κ transgene. Additionally, E3′ appears to be active early in B cell development, driving expression to cause allelic exclusion.

In contrast to these findings, the deletion of E3′ dramatically affected the levels of expression of the transgene in LκΔ[E3′]^2 and also its ability to inhibit expression of the endogenous alleles (Fig. 6.3). Thus, transgenic κ expression suggests that in the LκΔ[E3′]^2 founder line, E3′ is essential for both transcription and allelic exclusion. However, the LκΔ[E3′]^6 founder behaved more similarly to Lκ^6 than even Lκ^3.

(Although three out of six profiles in Fig. 6.3 were obtained with flow cytometry settings differing from the other three, additional experiments have demonstrated that the flourescence intensities of Lκ^3 and Lκ^6 [positive controls for the other transgenes] are equivalent.)

6.4 κ expression in LPS-activated B cell blasts

Having observed the effect of the κ transgenes in mature splenic B cells, the transgenes were analysed in splenic B cell populations that had differentiated into antibody-secreting blasts in order to monitor any changes in expression pattern mediated by the κ enhancers following cellular activation.

After culturing splenocytes with LPS to induce terminal differentation, cytoplasmic expression of the transgenes did not reveal any significantly altered

distribution (Fig. 6.4), reinforcing the observation that the ratio of transgene- to endogenous κ-expressing cells seems to reflect the ability of the transgenes to inhibit expression of the endogenous κ alleles.

Transgene expression was high in cells from all transgenic lines, except Lκ[E3′]^2 (Fig. 6.4). It was not possible, however, to discriminate whether deletion of E3′ in Lκ[E3′]^2 affected the proportion of cells that expressed transgenic κ in a heterogeneous population, or affected the levels of expression within each cell. Indeed, analysis of cloned hybridomas from these mice suggested that E3′ is necessary for high levels expression at this terminal stage in differentiation (Betz *et al.*, 1994).

In each transgenic line, the expression pattern of that particular transgene was consistent in GC, spleen and LPS-activated spleen from that line. The major differences observed between the different transgenes seemed to be their abilities to inhibit endogenous κ expression, that is, to mediate allelic exclusion. Since the LκΔ[Ei/MAR] transgene behaved almost as efficiently as Lκ, Ei/MAR does not appear to be necessary for expression of a rearranged κ transgene and in fact, despite suggestions that E3′ is important only later in B cell development, E3′ seems to be active early enough for driving transcription at sufficient levels to effect the exclusion of endogenous κ.

Further characterisation of the transcriptional activity of E3′ proceeded as a collaboration with Kerstin Meyer: I prepared the lymphoid tissues for FACS, whilst she performed the RT-PCR analysis.

6.5 E3′ drives the lymphoid-specific transcription of a heterologous reporter gene.
It is clear that Ei/MAR is not necessary for either the efficient expression of a rearranged κ transgene or the inhibition of endogenous κ expression, suggesting that the activity of E3′ (in the absence of Ei/MAR) is sufficient. In order to further analyse the role of E3′ in transcription, a transgene bearing a heterologous human β-globin reporter gene with E3′ placed downstream of the coding sequences (βG[E3′]) (Fig. 6.5A) was introduced into mice. This approach facilitated the characterisation of E3′ activity in isolation of any other κ sequence that may contribute to transcriptional regulation or mRNA stability. Thus, E3′ could be tested for tissue-specificity, and the stage at which it is activated could be

determined.

The initial analysis of tissues from βG[E3′] mice by RT-PCR (reverse transcriptase-polymerase chain reaction) analysis, using HPRT (hypoxanthine phosphoribosyl transferase) transcripts as an internal controls, revealed that, although the transgene was expressed in spleen and bone marrow, βG[E3′] transcripts were not detected in liver (Fig. 6.5B). Thus, E3′ was able to drive the lymphoid-specific expression of the reporter gene *in vivo*.

6.6 The transcriptional activity of E3′ is B cell-specific.

Although E3′ was able to drive expression in lymphocytes, it was necessary to establish that its activity was restricted to the B cell lineage. Although IgH is normally expressed only in a B cell-specific manner, Eμ can drive the expression of transgenes in the T cell lineage (see Fig. 4.2; Cook *et al.*, 1995). Indeed, there have been suggestions that E3′ exhibits activity in thymocytes (Hiramatsu *et al.*, 1995) and that κ transgenes are expressed in the thymus (Storb *et al.*, 1996; Lamers *et al.*, 1989; Xu *et al.*, 1981).

When βG[E3′] splenocytes were fractionated by FACS (fluorescence-activated cell sorting) into $B220^+$ and $Thy1.2^+$ populations (Fig.6.6A), transgenic transcripts were expressed in the splenic B cells but not in the T cells (Fig. 6.6B). Additionally, transgene expression was not detected in newborn thymus, although newborn spleen contained transcripts (Fig. 6.6C). (Newborns were analysed to minimise the risk of contamination of the thymus by lymph node B cells at that age.) Therefore E3′ does not seem to drive transcription in the T cell lineage, but is transcriptionally active only in B cells.

6.7 E3′ is transcriptionally active in pre-B cells.

The inhibition of endogenous κ expression evident in LκΔ[Ei/MAR] mice, as well as Lκ mice, indicated that expression of a rearranged κ transgene driven by E3′ was effective enough to mediate allelic exclusion. E3′ seemed to be not only sufficient for good expression, but more importantly, to become activated before most endogenous Vκ rearrangement, rather than only in the later stages of B cell development. In order to determine the differentiation stage at which E3′ becomes active, bone marrow B cell precursors were sorted by FACS.

BP-1 is a cell differentiation molecule whose surface expression at a discrete stage in B cell development, fractions C and D (Hardy *et al.*, 1991), defines the late pro-

ut which is lost on progression to the
e marrow from βG[E3′] mice were
based on the levels of BP-1 expression
xpected to contain βG[E3′] transcripts
ent, as well as pro-B cells (Fig. 6.7B).
ed in the B220+BP-1+ pre-B cell
e transcription at the pre-B cell stage.

...ctive transcription in pro-B cells.

E3′ appears to be active in pre-B cells at a time when the κ locus is preparing for Vκ rearrangement. At the earlier pro-B cell stages, though, only V_H rearrangement is necessary. Nevertheless, it is possible that E3′ becomes active earlier, since the LκΔ[Ei/MAR] seemed to be expressed before rearrangement of the endogenous Vκ alleles.

CD43 is another cell surface marker that has been used in the fractionation of B cell subpopulations in the bone marrow, allowing discrimination of the CD43+ pro-B cells (fractions A to C; Hardy et al., 1991) from the later stages of B cell differentiation that have lost CD43 expression (fraction D onwards), the only exception being the expression of CD43 on the B-1 cells in the peritoneum. The analysis of βG[E3′] bone marrow that was enriched for the presence or absence of CD43 on B subpopulations (Fig. 6.8A) revealed that although transcription of the transgene was detected on B220+CD43- pre-B and B cells, βG[E3′] was not transcribed in the pro-B cells that were B220+CD43+ (Fig. 6.8B). Thus, E3′ appears to become transcriptionally active around the pro- to pre-B transition before Vκ rearrangement occurs, but at a time when the cells no longer express CD43 (see Fig. 1.3 in Introduction).

6.9 Discussion

The expression of rearranged κ transgenes and the inhibition of endogenous κ in the mature B cells of Lκ, LκΔ[Ei/MAR] and LκΔ[E3′] mice suggested that E3′ was sufficient to direct high levels of transgene expression causing allelic exclusion early in B cell development. Further characterisation of the transcriptional activity of E3′ linked to a β-globin reporter gene indicated that E3′ does indeed become active in pre-B cells at the stage when sterile κ transcripts are detected and when *RAG* expression is upregulated (Grawunder *et al.*, 1995), suggesting a role in regulating locus accessibility prior to Vκ rearrangement.

and pre-B cells in the bone marrow, but which is lost on progression to the immature B cell stage. Therefore, bone marrow from βG[E3′] mice were fractionated into B lineage subpopulations based on the levels of BP-1 expression (Fig. 6.7A). The B220$^+$BP-1$^-$ fraction was expected to contain βG[E3′] transcripts since immature/mature B cells were present, as well as pro-B cells (Fig. 6.7B). Transgene expression was also detected in the B220$^+$BP-1$^+$ pre-B cell compartment, indicating that E3′ can induce transcription at the pre-B cell stage.

6.8 E3′ does not drive transcription in pro-B cells.

E3′ appears to be active in pre-B cells at a time when the κ locus is preparing for Vκ rearrangement. At the earlier pro-B cell stages, though, only V_H rearrangement is necessary. Nevertheless, it is possible that E3′ becomes active earlier, since the LκΔ[Ei/MAR] seemed to be expressed before rearrangement of the endogenous Vκ alleles.

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6.9 Discussion

The expression of rearranged κ transgenes and the inhibition of endogenous κ in the mature B cells of Lκ, LκΔ[Ei/MAR] and LκΔ[E3′] mice suggested that E3′ was sufficient to direct high levels of transgene expression causing allelic exclusion early in B cell development. Further characterisation of the transcriptional activity of E3′ linked to a β-globin reporter gene indicated that E3′ does indeed become active in pre-B cells at the stage when sterile κ transcripts are detected and when *RAG* expression is upregulated (Grawunder *et al.*, 1995), suggesting a role in regulating locus accessibility prior to Vκ rearrangement.

The expression of the LκΔ[Ei/MAR] and LκΔ[E3′] transgenes parallels experiments using gene-targetted mice where the κ enhancers were deleted by replacement with a recombined *LoxP* site. LκΔ[Ei/MAR] was expressed almost as well as Lκ and similarly, there was no apparent change in the level of basal κ expression in *EκND* mice which lacked the genomic region containing Ei and its associated MAR (Xu *et al.*, 1996). However, germline deletion of E3′ in *3′EκD* mice resulted in a two- to three-fold decrease in expression levels in resting B cells (Gorman *et al.*, 1996), consistent with the requirement for E3′ activity as shown by low level expression in LκΔ[E3′]^{2} mice (and LκΔ[E3′]^{5} in Betz *et al.*, 1994) and may possibly suggest that E3′ behaves analogously to a locus control region (LCR) as described originally for the human β-globin gene (Grosveld *et al.*, 1987). LCRs direct high-level and tissue-specific expression of transgenes, independent of their site of integration, perhaps by establishing an open chromatin structure (Dillon and Grosveld, 1993). Thus, a κ transgene lacking the putative E3′ LCR, would essentially be poorly expressed. However, position effects at the integration site may compensate for absence of an LCR, providing a possible explanation for the behaviour of the other LκΔ[E3′]^{6} founder whose transgene expression was very similar to that of the intact Lκ construct.

Consistent with the detection of different proportions of B cells that express transgene versus endogenous κ, the *EκND* and *3′EκD* mice exhibited altered ratios of κ- to λ-expressing B cells (of 1:1 and 2:1 respectively instead of 20:1 in non-transgenic mice), indicating impaired Vκ rearrangement and suggesting that Ei/MAR is more important than E3′ for this event. Although the results described in this chapter compare the roles of Ei/MAR and E3′ in the distinct processes of transcription of rearranged transgenes and rearrangment of endogenous Vκ, taken together, they shown that both κ enhancers are involved in the activation of the κ locus at this pre-B cell stage in development.

Activation for sterile transcription and Vκ rearrangement correlate with recently characterised molecular events at the κ locus, and at E3′ in particular. Although the interaction of the NFκB transcription factor with Ei seemed to be an attractive mechanism for regulation in early B cell development, *in vivo* footprinting of primary cells revealed that the NFκB binding site of Ei was occupied at both pro- and pre-B stages (Shaffer *et al.*, 1997). In contrast, at E3′, an early type of chromatin structure was detected in pro- and pre-B cell lines, that persisted until the mature B cell stage (Roque *et al.*, 1996). These results agreed with the genomic footprints of transcription factors already shown to bind E3′,

such as CREM/ATF-1 (Pogubala and Atchison, 1995) and PU.1/NF-EM5 (*Pip-1*) (Eisenbeis *et al.*, 1995; Pongubala *et al.*, 1992), and additionally showed the association of BSAP (*Pax-5*) (Busslinger and Urbánek, 1995). Changes in binding at E3′ across the pro- to pre-B cell transition were further dissected in primary cells and suggested that although NFκB binding may be neccesary, activation of the κ locus is regulated by factors that associate with E3′ (Shaffer *et al.*, 1997). Moreover, nuclear extracts from primary cells analysed by gel mobility shift revealed that BSAP and PU.1 compete for binding. This led to the speculation that bound BSAP might inhibit PU.1 binding in pro-B cells until the differentiative signal (via the pre-BCR) for progression to the pre-B cell stage when BSAP binding decreases and allows PU.1 binding to increase.

The chromatin structure and genomic footprinting of E3′ in cells lines has also revealed a change on the transition from mature B cells to plasma cells (Roque *et al.*, 1996). These findings are consistent with indications that E3′ might be responsible for the increased expression in plasma cells that are necessary for antibody secretion (Blasquez *et al.*, 1989; Meyer and Neuberger, 1989), and with our findings (not included in this chapter) that the activity of E3′ in regulating the βG[E3′] transgene is enhanced on LPS activation (Meyer *et al.*, 1996). In contrast, a surprising result to arise from the germline deletion of E3′ in *3′EκND* mice, was that although expression was reduced in resting B cells, it was normal in LPS-activated B cells from these mice (Gorman *et al.*, 1996). Whilst Ei/MAR may be partially compensating for the loss of E3' activity in *3′EκND* , these results could point to the existence of an additional endogenous element, absent from transgenic constructs, that drives high level endogenous κ expression (and cooperates with Ei/MAR) in the absence of E3′.

There have also been indications that E3′, although originally identified as a transcriptional enhancer, acts as a negative regulator in determining both T/B specificity and pro-B/pre-B specificity of Vκ rearrangment in transgenic mice (Hiramatsu et al., 1995). When E3′ was deleted, the transgene containing germline Vκ and Jκ segments was observed to undergo rearrangement in splenic T cells. Premature Vκ rearrangement of the transgene was also observed in early pro-B cells, not only by the detection of rearranged Vκ substrates, but also by the presence of non-germline (N) nucleotides at recombination junctions which are only normally incorporated in V_H rearrangement since TdT activity is absent in pro-B cells. However, no indications of E3′ activity in pro-B cells or T cells were observed in this chapter. Whilst any negative regulation of the transcriptional

readout would have gone undetected, it is possible that differences in the precise nature of the transcription factors involved in these processes, or their recruitment by the transgene substrates, result in alternative outcomes.

In conclusion, it is clear that the activities of E3′ are sufficient to direct the high level transcription of a rearranged κ at a time in B cell development when it can effect allelic exclusion. Although E3′ seems to be dominant in driving transcriptional activity in this study, the gene-targetted deletions in *EκND* and *3′EκD* mice suggest that Ei/MAR and E3′ may have overlapping functions, with their relative contributions varying in different processes (Gorman *et al.*, 1996; Xu *et al.*, 1996). Whilst Ei may primarily influence rearrangement, E3′ seems to be important both in rearrangement and expression. However, neither element appears to be necessary for the expression of κ in the gene-targetted mice and additional elements regulating the κ locus remain to be identified.

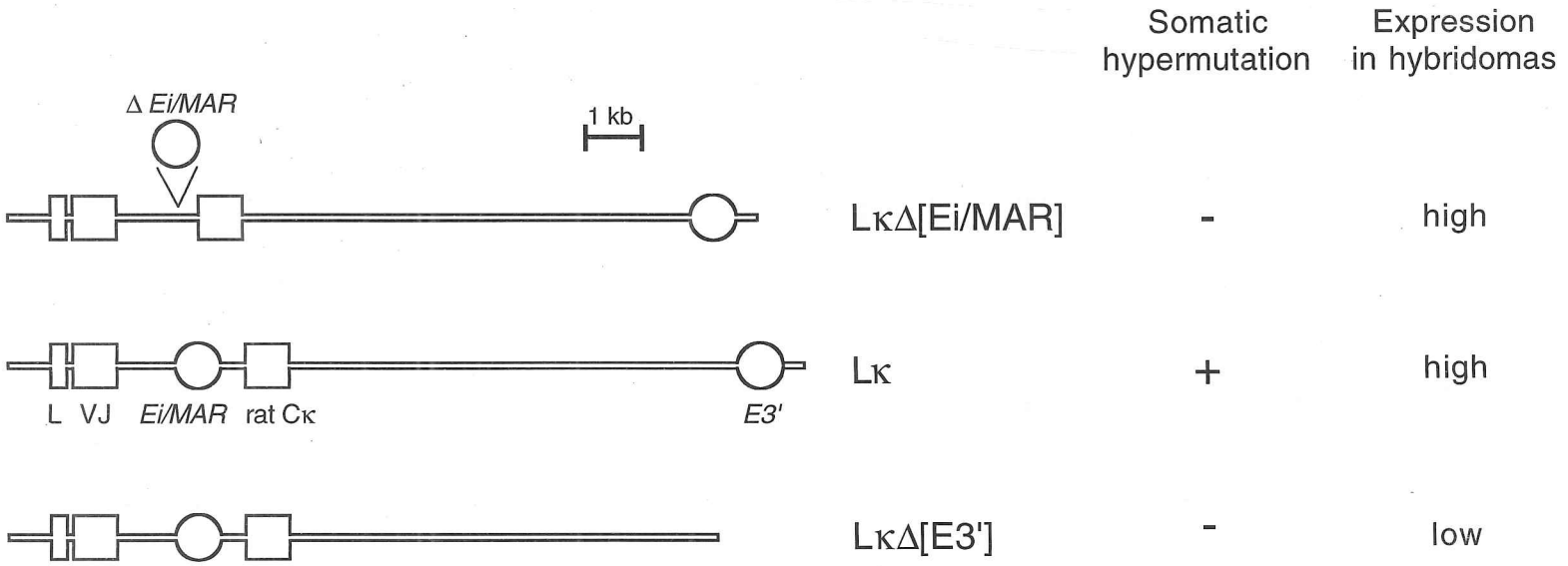

Figure 6.1 History of the κ transgenic mice

The transgenes were based on Lκ composed of rearranged VκOx and rat Cκ (to discriminate from endogenous mouse κ) regions regulated by the κ intron enhancer/matrix attachment region (Ei/MAR) and Igκ 3'enhancer (E3'). LκΔ[Ei/MAR] and LκΔ[E3'] were derived from the deletion of Ei/MAR and E3' respectively.

The abilities of the transgenes to undergo somatic hypermutation (assessed by sequences derived from hybridomas and Peyer's patches) and their levels of transcription in hybridomas (detected by Northern analysis) as described by Betz *et al.* (1994) is summarised.

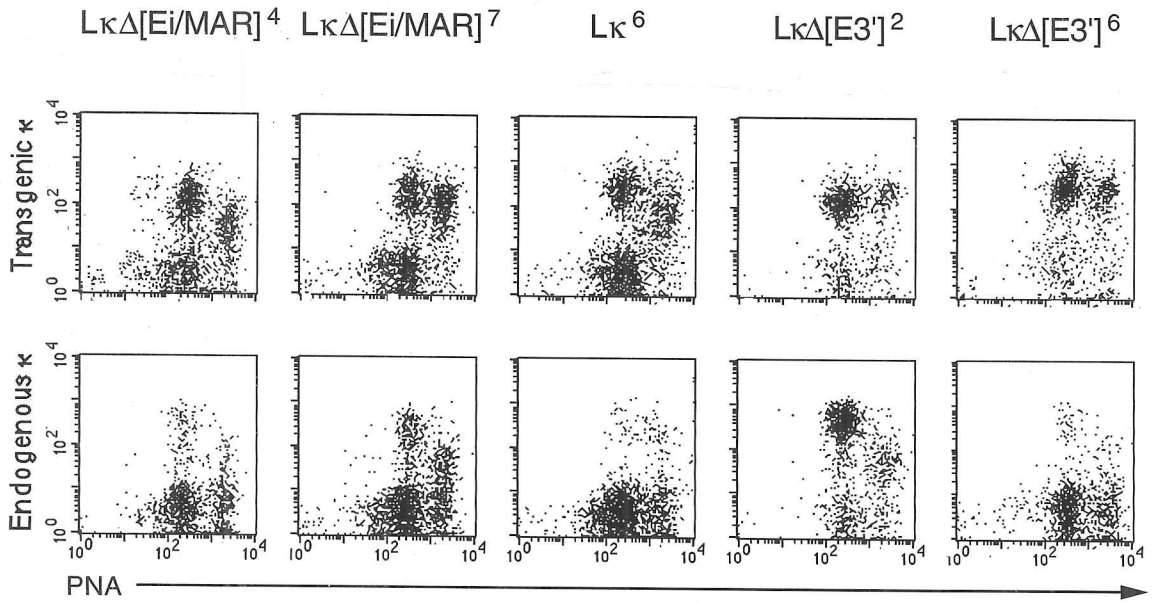

Figure 6.2 κ expression in germinal centre B cells
Cells from Peyer's patches of transgenic mice were stained with either mouse anti-rat κ-biotin (transgenic κ) or rat anti-mouse κ-biotin (endogenous κ) (both revealed by SA-PE) and peanut agglutinin (PNA)-FITC. Dot plots were gated by scatter and PI exclusion.

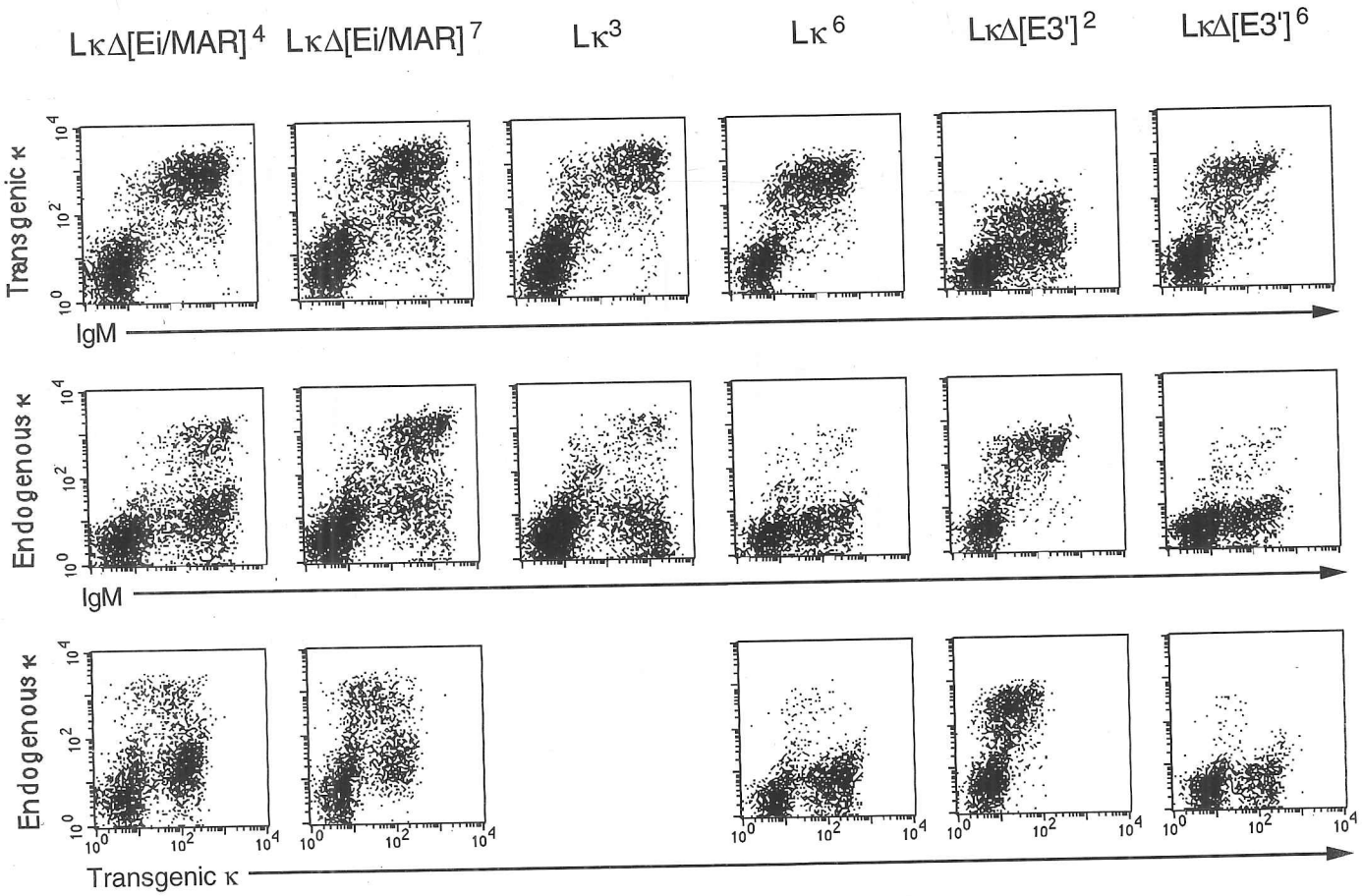

Figure 6.3 κ expression and allelic exclusion in splenic B cells
Splenocytes from transgenic mice were stained with either mouse anti-rat κ-biotin (transgenic κ) or rat anti-mouse κ biotin (endogenous κ) (both revealed by SA-PE) and goat anti-IgM-FITC. For double-staining of κ, goat anti-rat Ig-FITC was used to detect transgenic κ. The plots (gated by scatter and PI exclusion) shown were analysed on two two different settings for flow cytometric analysis (LκΔ[Ei/MAR]^4, LκΔ[Ei/MAR]^7 and Lκ^3; Lκ^6, LκΔ[E3']^2,LκΔ[Ei/MAR]^6) but are representative of multiple individuals and other experiments have shown Lκ to be expressed to the same levels in both founders.

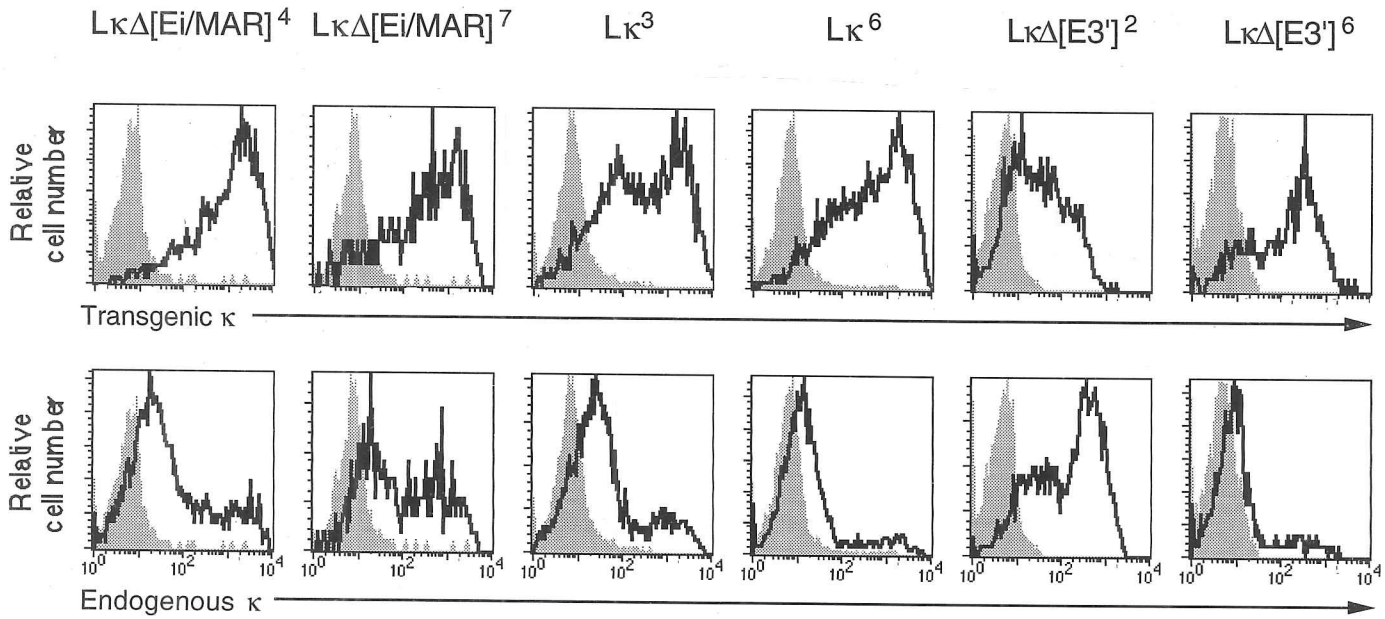

Figure 6.4 **κ expression in LPS-activated spleen**
After incubation of splenocytes from transgenic mice for 48 h in the presence of 50 μg/ml LPS, the cells were fixed and permeabilised prior to staining with either mouse anti-rat κ-biotin (transgenic κ) or rat anti-mouse κ-biotin (endogenous κ) detected by SA-PE. Gates for histograms were set for activated lymphocyte scatter and shaded histograms indicate SA-PE alone controls.

Figure 6.5 Lymphoid-specific transcription of the βG[E3'] transgene
(A) *Schematic representation of βG[E3'] transgene.* The human β-globin reporter gene (coding regions shown as rectangles) is regulated by the Igκ 3' enhancer (E3') placed downstream.
(B) *Transcription of βG[E3'] in lymphoid tissues.* cDNA prepared from liver (Li), spleen (Sp) and bone marrow (BM) of βG[E3'] mice were analysed by RT-PCR detecting β-globin (βG) transcripts or HPRT as an internal control. (M, molecular weight markers)

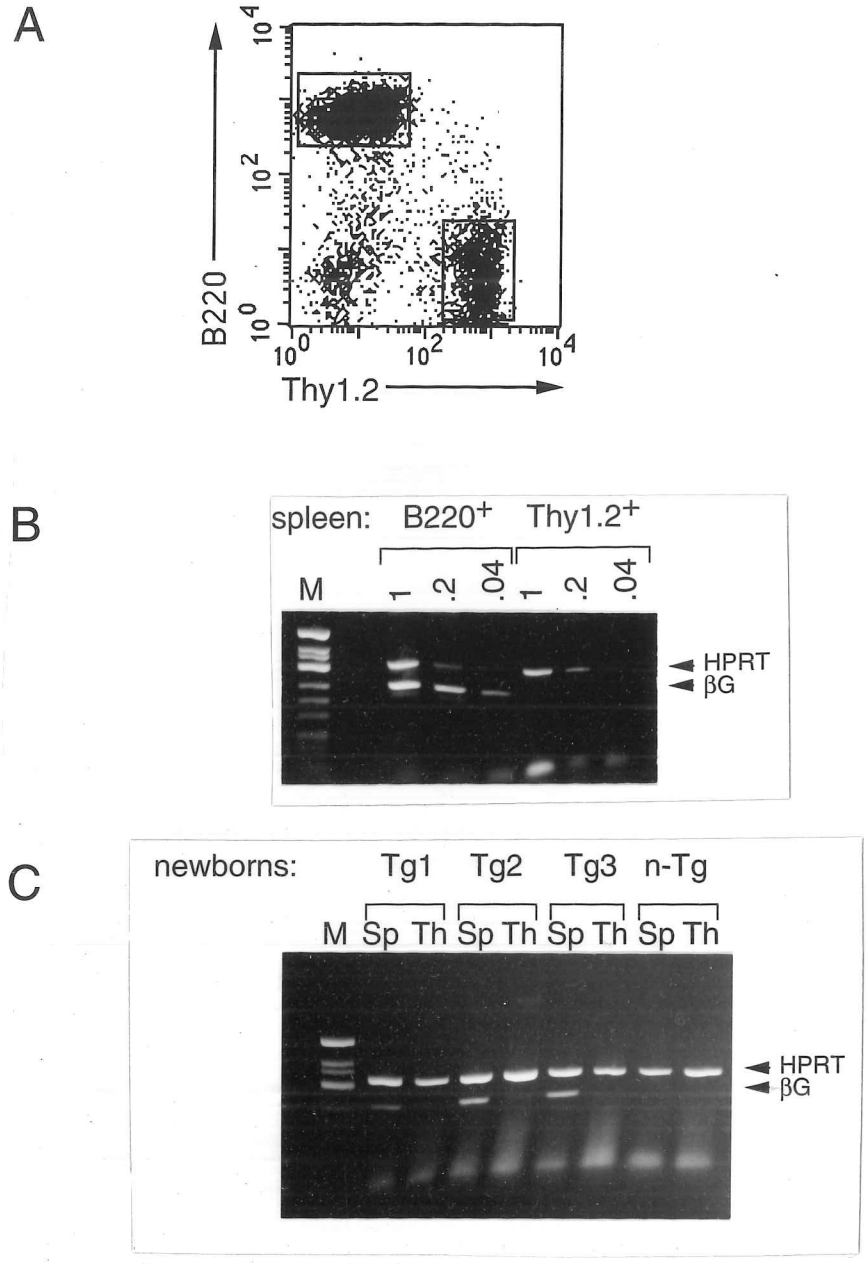

Figure 6.6 Transcription of βG[E3'] is B cell-specific.

(A) *Splenic B220+ B cell and Thy1.2+ T cell populations fractionated by FACS.* Splenocytes from βG[E3'] transgenic mice were stained with rat anti-B220-PE and mouse anti-Thy1.2-FITC and sorted by viable lymphocyte scatter and gated regions as indicated.

(B) *Expression of βG[E3'] in splenic B cells but not T cells.* β-globin (βG) transcripts were detected by RT-PCR analysis of serial dilutions of cDNA derived from the above FACS populations using HPRT as an internal control. (M, molecular weight markers)

(C) *βG[E3'] is not expressed in thymus.* Spleen (Sp) and thymus (Th) from newborn (day 0) βG[E3'] transgenic (Tg) mice or non-trangenic (Non-tg) littermates were analysed for β-globin transcripts as in (B).

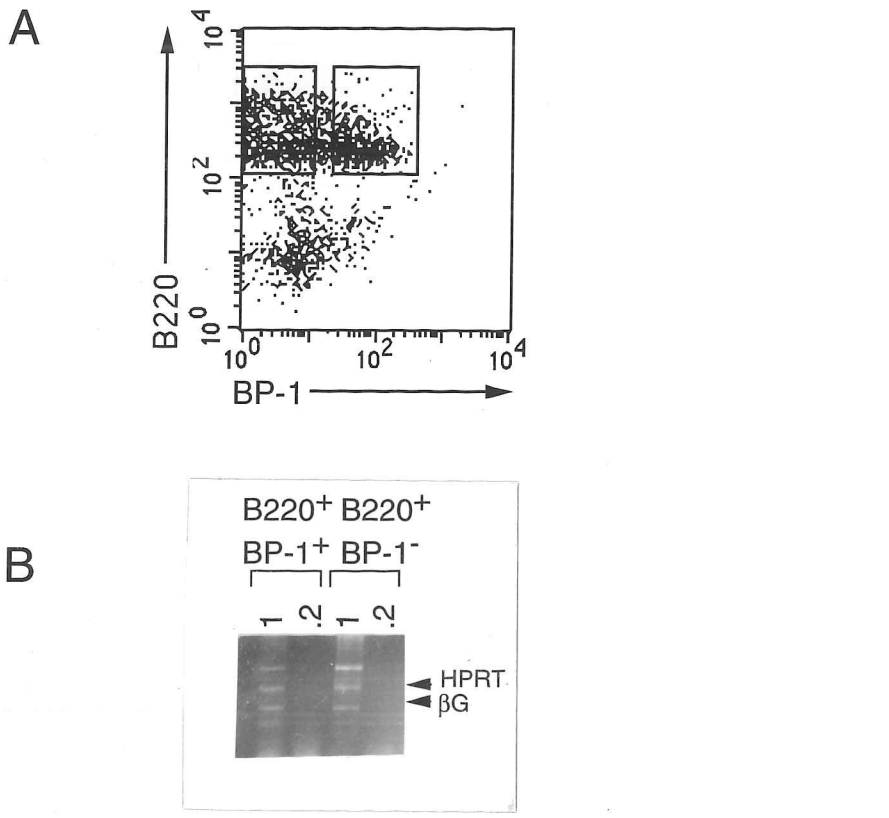

Figure 6.7 **βG[E3'] is transcribed in pre-B cells.**

(A) *Enrichment of BP-1$^+$ pre-B cells by FACS.* Bone marrow cells from βG[E3'] mice were stained with rat anti-B220-FITC and mouse anti-BP-1-biotin (revealed by SA-PE) and sorted by viable lymphocyte scatter and by gates as indicated.

(B) *βG[E3'] is detected in both B220$^+$BP-1$^+$ and B220$^+$BP-1$^-$ subpopulations.* Serial dilutions of cDNA prepared from the above bone marrow sorts were analysed by RT-PCR for the presence of β-globlin (βG) transcripts or HPRT as an internal control.

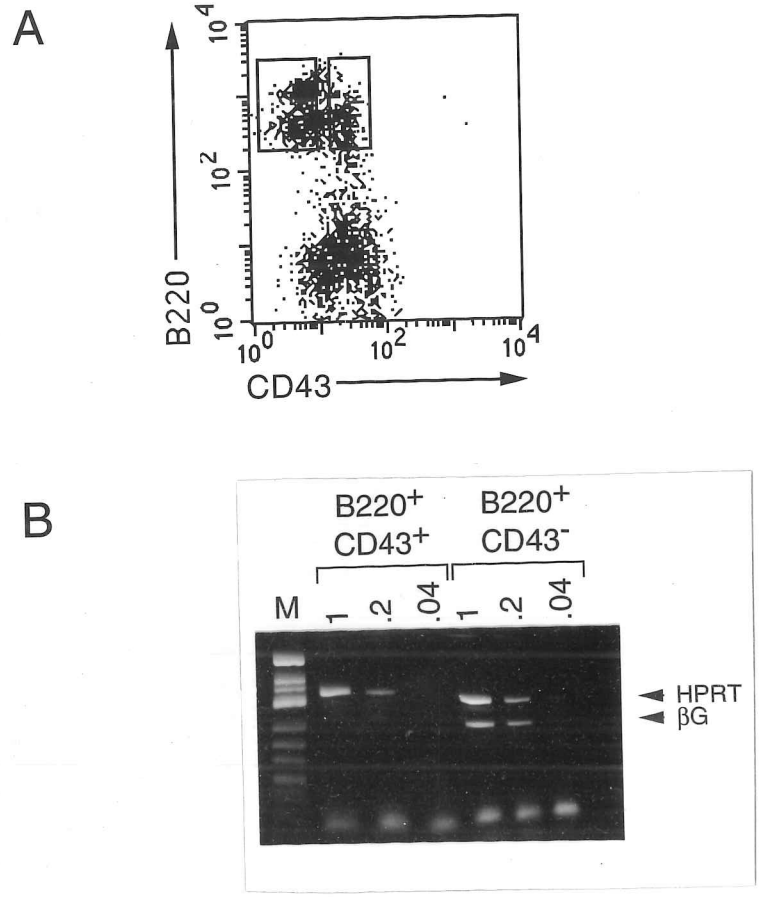

Figure 6.8 **βG[E3'] is not transcribed in pro-B cells.**
(A) *Enrichment of CD43$^+$ pro-B cells by FACS.* Bone marrow cells from βG[E3'] mice were stained with rat anti-B220-PE and rat anti-CD43-biotin (revealed by SA-FITC) and gates were set for viable lymphocytes by scatter and regions as indicated.
(B) *βG[E3'] is only detected in the B220$^+$CD43$^-$ subpopulation.* RT-PCR analysis for the presence of β-glolin (βG) transcripts in serial dilutions of cDNA prepared from the above bone marrow sorts using HPRT as an internal control. (M, molecular weight markers)

Conclusion and Prospect

Chapter 7

Chapter 7: Conclusion and Prospect

The immune system can generate and regulate the production of functional antibodies bearing diverse specificities for antigen, but not self-antigen. This remarkable process centres on the BCR which is fundamental to the B cell's existence. As a pre-BCR complex of surface μ and surrogate light chain associated with the Ig-α/Ig-β heterodimer, it mediates antigen-independent events in the bone marrow (such as allelic exclusion) necessary for B cell development. Following maturation and migration of the B cell into the periphery, the BCR can mediate antigen-dependent activation by transmembrane signalling and intracellular targetting for antigen presentation.

Using a transgenic approach, some of these checkpoints in B cell differentiation were addressed, from the expression of Ig to subsequent signalling events mediated by BCR. The cytoplasmic domains of Ig-α and Ig-β, but not an Ig-β with a mutant ITAM, could independently mediate B cell activation *in vitro*, and effect the maturation of peripheral B cells *in vivo*. However, the allelic exclusion assay revealed that the individual tails were not adequate on their own to fully inhibit endogenous IgH expression, and allelic exclusion was optimal only when both were expressed together, suggesting that the Ig-α/Ig-β heterodimeric structure exhibits cooperativity and bears additional features not present in the homodimers. The findings that the signal requirements for efficient allelic exclusion differed from those driving B cell maturation in the anti-HEL transgenic mice were also observed in mice expressing $V_{NP}C\mu[Ei,E3']^{\kappa}$, a rearranged μ transgene regulated by the κ enhancers. Whilst the μ transgene rescued B cell development in *μMT* B cell-deficient mice, the delay in its expression led to defective IgH exclusion, indicating that the timing of expression can be critical, as well as the quality of signal (via Ig-α and Ig-β), for pre-BCR function. Although both Ei/MAR and E3′ were present in the $V_{NP}C\mu[Ei,E3']^{\kappa}$ transgene construct, the transcriptional activity at the pre-B cell stage seemed largely attributable to regulation by E3′ since further characterisation suggested that E3′ may regulate locus accessibility and sterile transcription leading to Vκ rearrangement in the absence of Ei/MAR.

These studies have highlighted the complexity of B cell differentiation, suggesting several discrete processes in development. For example, one interesting checkpoint is that of selection into the periphery since the bone marrow produces many more B cells than those that mature and exit. *mb-1*$^{\Delta c/\Delta c}$

mice, bearing germline truncation of the Ig-α cytoplasmic domain, have greatly reduced peripheral B cell numbers, and *3'EκD* mice lacking E3' due to targetted deletion have decreased numbers of κ^+ splenic B cells. These observations cannot be accounted for solely by the reduction in B cell generation exhibited in the bone marrow of these mice. Selection may depend on the strength of signal generated via the BCR, however, additional mechanisms are likely to influence B cell maturation, and certainly the selection mechanism for the discrimination between self and non-self is a major question in immunology that endures.

The presence of an anti-HEL transgene bearing the D1.3 V region conferred a phenotype, possibly arising from selection against the specificity. Although transgenic mice were used to generate primary B cells expressing the same BCRs and to gain insight into B cell events *in vivo*, there are dangers in generating a monoclonal population of B cells. An essential feature of the antibody response is its diversity, and mechansims may operate that act against monoclonality. One solution may be to use transgenes bearing miniloci and thus, events such as rearrangement, selection on the basis of specificity or affinity, or class switching could be analysed.

However, the transgenic system has its limitations in general. Much remains to be learnt about the regulation of the genome and major concerns in the analysis of transgenic mice are not only artefacts such as specificity or abberrant tissue expression (as observed for the anti-HEL chimeric receptors), but founder effects due to integration site (LκΔ[E3'] transgenic mice). Transgene constructs may lack some important, but as yet unidentified, regulatory elements such as those suggested by the gene-targetted *EκND* and *3'EκD* mice in which neither Ei nor E3' is an absolute requirement for κ expression.

An alternative approach for addressing development and selection of a diverse, yet non-self repertoire of B cells into the periphery, would be to target the gene directly, eliminating transgene integration effects. Although any experimental system is subject to artefacts, gene-targetting technology is gradually becoming more versatile, facilitating the dissection, deletion, reconstitution and tissue-specific regulation of genomic factors.

Differentiation processes occurring *in vivo* need to be correlated with studies of the biochemical mechanisms operating from sites proximal to the BCR, such as the recruitment of effectors to Ig-α and Ig-β, to the occupancy of genomic

regulatory sites by transcription factors. Whilst these experiments are an immense undertaking, and as yet most easily pursued in homogeneous cell lines, the means for enriching primary B cell subpopulations are continually improving, combining physical sorting techniques with the exploitation of gene-targetted and transgenic mice.

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Publications

International Immunology, Vol. 8, No. 10, pp. 1561–1568

The Igκ 3′-enhancer triggers gene expression in early B lymphocytes but its activity is enhanced on B cell activation

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Keywords: allelic exclusion, B cell development, transcription, transgenes

Abstract

Igκ gene expression is controlled by two enhancers, one located within the major intron (Ei) and the other located downstream of C_κ (E3′). Whereas loss of E3′ has previously been shown to diminish κ expression, we show here that a rearranged κ transgene lacking Ei is well expressed, even at the pre-B cell stage. This suggests that E3′ alone might be sufficient to give properly regulated transcription throughout B cell development. Indeed, we show that a transgene composed of a β-globin reporter linked to E3′ is expressed in a B cell-specific manner, becoming activated at the late pro-B to pre-B cell stage but with dramatically enhanced activity on B cell activation. Thus, E3′ becomes active as a transcription enhancer at the stage when V_κ–J_κ rearrangement is being initiated and is sufficient to yield an expression pattern in a linked reporter gene similar to that of fully rearranged κ genes.

Introduction

Ig genes have been widely studied in order to further our understanding of the developmental regulation of gene expression. The productive rearrangement and transcription of both Ig heavy and light chain genes occurs early in B cell development leading to the production of a repertoire of B lymphocytes displaying membrane Ig on their surface. Encounter with antigen can lead to terminal differentiation of a B lymphocyte into an antibody-secreting plasma cell and is accompanied by a large increase in the steady-state levels of Ig mRNA.

Three major regulatory regions have been identified that control the expression of rearranged Igκ genes: the V_κ promoter at the 5′-end of the gene, the intron-enhancer/matrix attachment region (Ei/MAR) located in the major J_κ–C_κ intron and the 3′-enhancer (E3′) located downstream of the constant region. All three regions exhibit B cell-specific activity as judged by transfection assays (reviewed in 1). However, their different roles in regulating light chain gene expression during B cell development remain unclear.

Here, we find that a rearranged κ transgene containing E3′ but not Ei/MAR is nevertheless well expressed during B cell development. The transgene effects allelic exclusion of endogenous κ expression suggesting that Ei/MAR is not required even for transgene expression at the pre-B cell stage; E3′ appears sufficient. We therefore sought to confirm this by charting the expression of E3′ during lymphoid development, analysing the expression pattern of an E3′-linked β-globin reporter gene. The results indicate that E3′ is activated early in B cell development but its activity is substantially enhanced on B cell activation.

Methods

Transgenes and transgenic mice

Mice carrying the L_κ, $L_\kappa\Delta$[Ei/MAR] and $L_\kappa\Delta$[E3′] transgenes (Fig. 1A) have been described previously (2).

The βG[E3′] transgene (Fig. 4A) was assembled by inserting a 1 kb *Xba*I–*Sac*I fragment including the mouse κ E3′ (3) into a Bluescript derivative in which a human β-globin gene had been inserted between the *Kpn*I and *Xba*I sites. The *Kpn*I–

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Transmitting editor: J.-C. Weill

Received 18 May 1996, *accepted* 9 July 1996

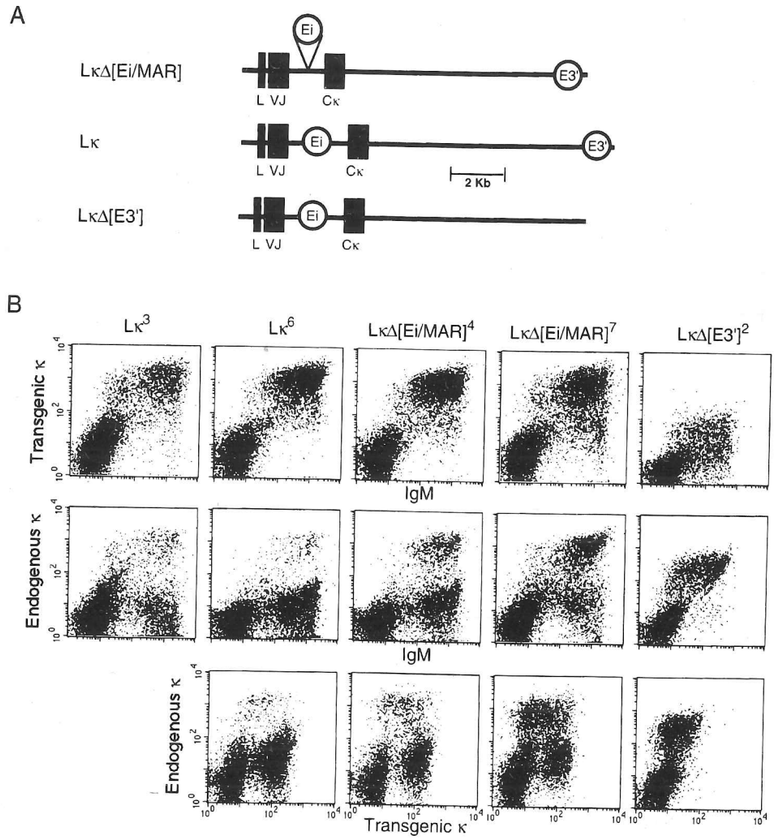

Fig. 1. Flow cytometry analysis of transgenic and endogenous mouse κ expression on the surface of spleen cells of various κ-transgenic lines. (A) The transgenes. (B) Two-dimensional FACS plots. Spleen cells from 8- to 12-week-old mice were stained with biotinylated monoclonals (revealed by phycoerythrin–streptavidin) specific for the transgenic (rat) or endogenous (mouse) κ chains together with FITC-conjugated goat anti-mouse IgM antiserum. Double staining for transgenic and endogenous κ chains was performed using FITC-conjugated goat anti-rat Ig antiserum and biotinylated anti-mouse κ. The data are representative of multiple experiments although the slightly reduced brightness of IgM staining on $L_\kappa\Delta$[E3′] mice seen here was not always observed.

*Xba*I β-globin fragment was generated by PCR from human genomic DNA using oligonucleotides priming 105 nucleotides 5′ of the transcription start site (introducing a 5′ *Kpn*I site) and 340 nucleotides 3′ of the polyadenylation signal (introducing a 3′ *Xba*I site). The amplified β-globin gene (which extends from 5′ of the promoter to 3′ of the polyadenylation site) was assembled from two subfragments; the strategy involved creating a *Spe*I site in the second β-globin intron, 460 nucleotides downstream of the splice donor site.

The $V_{NP}C_\mu$[Ei, E3′]$^\kappa$ transgene was obtained from a derivative of plasmid pSV-Vμ1 (4) in which the region of DNA located between the most 5′ and 3′ *Xba*I sites in the J_H-Cμ intron was replaced by a 2.1 kb section of the mouse J_κ–C_κ intron spanning κEi/MAR obtained by PCR using oligonucleotides GGACTAGTGATAGGAACAGAGCCACT and GCTCTAGACAGAGATTCAGACCAGTTTA. The *Xba*I–*Sac*I 802 bp fragment containing the κ E3′ was then introduced into the unique *Xho*I site located just 3′ of the C_μM exons of pSV-V_μ1.

Transgenic mice were created by injecting vector-free DNA fragments into the nuclei of (C57BL/6×CBA/Ca)F_1 zygotes and founder animals were bred further against F_1s. The number of transgene copies was estimated from Southern blots (not shown) and, for κ transgenic lines, is denoted by a superscript following the transgene designation. Offspring transgenic for L_κ-derived transgenes were screened by ELISA detecting rat κ determinants. Mice transgenic for βG[E3′] were screened by PCR of tail DNA.

Immunofluorescence analysis

Following removal of erythrocytes, samples comprising single cell suspensions were stained with various combinations of FITC-conjugated goat anti-mouse IgM or goat anti-rat Ig antisera (Southern Biotechnology, Birmingham, AL), FITC-conjugated peanut agglutinin (Sigma, Dorset, UK), FITC-conjugated anti-Thy-1.2 (CD90) (TS; Sigma), FITC-conjugated anti-IgM^b [MB86 (5)] mAb, biotinylated anti-rat κ [RG-7/9.1 (6)], anti-mouse κ [187.1 (7)], anti-BP-1 (8), anti-Thy-1.2 (CD90) (53-2.1; PharMingen), anti-CD43 (S7; PharMingen) and anti-IgM^a (DS-1; PharMingen) mAb, and phycoerythrin-conjugated anti-CD45R(B220) antibody (RA3-6B2; Gibco). The biotin conjugates were revealed using FITC–streptavidin (Amersham, Buckinghamshire, UK), phycoerythrin–streptavidin (Jackson, Baltimore, PA) or Red670–streptavidin (Gibco). Flow cytometric analysis and cell sorting were performed on a Becton Dickinson (San Jose, CA) FACScan and FACStar Plus respectively, using Lysys II and CELLQuest software; viable lymphocytes were gated by forward/side scatter and by exclusion of propidium iodide.

Preparation of RNA and analysis of gene expression

RNA was extracted by the guanidine isothiocyanate method (9) and transgene expression analysed by ribonuclease protection using rat κ or human β-globin probes with a heat shock cognate gene probe acting as internal reference as previously described (10). Densitometry tracing was performed on Molecular Devices Computing Densitometer Model 300A using ImageQuant software. For RT-PCR analysis, 0.1–1 μg RNA was reverse transcribed with MMLV reverse transcriptase (Gibco/BRL, Gaithersburg, MD) using 100 ng $(dT)_{15}$ primer for 1 h at 37°C. Aliquots of the reaction were then used in PCR. The β-globin forward and reverse primers (CCTGAGGAGAAGTCTGCC and AGCACACAGACCAGCACG) yield a 328 bp product; the HPRT forward and reverse primers used as internal control (TGCCGACCCGCAGTCCCAGCGTCG and GCTGTAC-TGCTTAACCAGGGAAAG) amplify a 461 bp product. PCR were annealed at 60°C for 1 min, extended at 72°C for 1 min and denatured at 92°C for 1 min for a total of 30 cycles.

Results

Igκ gene expression in splenic B cells in the absence of Ei/MAR

We have used transgenic mice in order to address the relative importance of the two Igκ enhancers (Ei and E3′) in regulating κ gene expression at the different stages of B cell development. The Igκ transgenes are derived from L_κ (Fig. 1A). L_κ is composed of a mouse V_κOx-1 segment rearranged to J_κ5 and linked to C_κ. The entire construct is of mouse origin except for a 470 nucleotide stretch extending from the *Hpa*I site in the C_κ exon to a *Bgl*II site 3′ of C_κ, this region having been replaced by the corresponding portion of rat C_κ. This means that the transgenic light chains can be serologically distinguished from endogenous mouse κ. The $L_\kappa\Delta$[Ei/MAR] and $L_\kappa\Delta$[E3′] transgenes differ from L_κ by deletion of Ei/MAR or E3′ respectively.

Spleen cells from the transgenic mice were stained for transgenic κ or endogenous κ together with IgM; they were also double-stained for both transgenic and endogenous κ chains (Fig. 1B). In two different L_κ lines (L_κ^3 and L_κ^6, with the superscript denoting copy number), the transgene stains brightly on the surface of most IgM^+ B cells, apparently inhibiting expression of the endogenous alleles since few cells express endogenous κ chains. The pattern of κ chain expression in mice carrying the $L_\kappa\Delta$[Ei/MAR] transgene is essentially similar although the degree of inhibition of endogenous light chain expression is somewhat less than that observed with the L_κ mice. This is particularly true in the case of the $L_\kappa\Delta[Ei/MAR]^7$ line. Nevertheless, it is evident that, even in the absence of Ei/MAR, the light chain transgene is expressed at a good level on splenic B cells and can mediate allelic exclusion.

In contrast to these findings, the transgene in $L_\kappa\Delta[E3']^2$ mice is relatively poorly expressed on splenic B cells. It is also co-expressed with endogenous light chain, there being little evidence of an inhibition of endogenous κ rearrangement/expression. Interestingly, $L_\kappa\Delta$[E3′] mice exhibit significant line-to-line variation in expression levels that is not simply attributable to transgene copy number (2). We have not so far observed this with L_κ or $L_\kappa\Delta$[Ei/MAR] transgenic lines. Thus, of three $L_\kappa\Delta$[E3′] lines examined, two (containing two and six transgene copies) expressed badly whereas a third line (harbouring six copies) expressed well (data not shown). This suggests that E3′ behaves analogously to a locus control region as originally described for the human β-globin gene (11). Thus, in the absence of E3′, the κ transgene is essentially poorly expressed although, in some mouse lines, position effects at the integration site can compensate for its absence.

Igκ gene expression in germinal centre cells in the absence of Ei/MAR

We have previously noted (2) that removal of Ei/MAR resulted in a substantial drop in the accumulation of somatic mutations in Igκ transgenes. The mechanism of hypermutation is unknown but one possible explanation of this finding is that the mutation rate drops as a consequence of decreased transgene expression. To test the feasibility of this explanation, we compared the expression of the L_κ and $L_\kappa\Delta$[Ei/MAR] in the germinal centre cells of Peyer's patches (identified as staining brightly with peanut agglutinin). The results (Fig. 2) show that, as in the splenic cells, the transgene is expressed well in the $L_\kappa\Delta$[Ei/MAR] mice although neither of the lines studied manifests as high a degree of exclusion of endogenous κ expression as is observed in L_κ mice. Furthermore, transgene expression in the $L_\kappa\Delta[Ei/MAR]^7$ line is slightly higher than in the $L_\kappa\Delta[Ei/MAR]^4$ line. It is nevertheless clear that despite removal of Ei/MAR, transgene expression in these cells is good.

Igκ gene expression in pre-B cells in the absence of Ei/MAR

The fact that the presence of the $L_\kappa\Delta$[Ei/MAR] transgene leads to diminished expression of endogenous mouse κ chains suggests that, even in the absence of Ei/MAR, the transgene is activated early enough in B cell ontogeny for it to inhibit rearrangement of the endogenous mouse κ locus. Furthermore, removal of Ei/MAR does not affect the expression of the κ transgene in bone marrow as judged by ribonuclease protection assays (Fig. 3A). As expected the κ transgene is also expressed in spleen. A much weaker signal is seen in both kidney and thymus. However, both cytoplasmic immunofluorescence (not shown) and FACS analysis (Fig. 3C) have

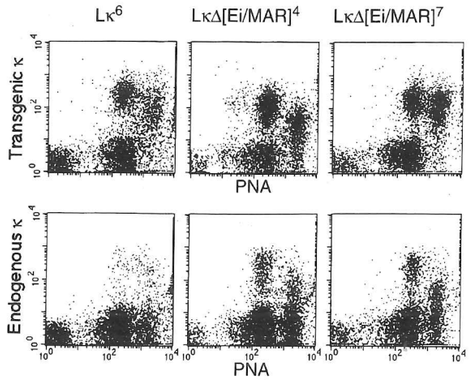

Fig. 2. Flow cytometry analysis of transgenic and endogenous κ staining of Peyer's patch cells from L_κ and $L_\kappa\Delta$[Ei/MAR] mice. Cells from 11- to 14-week-old mice were stained with biotinylated anti-κ antibodies (as in Fig. 1) together with FITC-conjugated peanut agglutinin (PNA).

confirmed that the Thy-1^+ population in thymus is in fact negative for rat κ polypeptide. The weak C_κ RNA signal detected in thymus may be due to contaminating plasma cells (Fig. 3C).

To confirm that $L_\kappa\Delta$[Ei/MAR] is well expressed in pre-B cells we crossed the $L_\kappa\Delta$[Ei/MAR] transgene into a μMT/μMT background. The μMT/μMT mice (12) carry a targeted *neo* insertion into the C_μM1 exon and are blocked in B cell development since they cannot make membrane IgM. The bone marrow B lineage cells in these mice therefore all belong to the pro-B fractions A–C (in the classification of Hardy *et al.*) or pro-B/pre-B-I fractions (classification of Rolink *et al.*) (13–15). The almost complete absence of transgene expression in spleen confirms that B cell development in $L_\kappa\Delta$[Ei/MAR] μMT/μMT mice is blocked before the mature B cell stage. The abundance of transgene RNA in the bone marrows of L_κ μMT/μMT (not shown) and $L_\kappa\Delta$[Ei/MAR] μMT/μMT mice (Fig. 3B) strongly supports the proposal that, even in the absence of Ei/MAR, the κ transgene is well expressed in the early stages of B cell development.

E3′ activates expression of a heterologous reporter transgene in pre-B cells

The studies on the L_κ, $L_\kappa\Delta$[Ei/MAR] and $L_\kappa\Delta$[E3′] mice not only demonstrate that Ei/MAR is not essential for the activation of rearranged κ gene expression early in B cell differentiation but also point to an important role for E3′ throughout B cell development, since it is sufficient to drive expression in the absence of Ei/MAR. We therefore linked E3′ to the human β-globin gene (to create βG[E3′], Fig. 4A) to test whether E3′ would be sufficient to confer lymphoid-specific expression *in vivo* and, if so, to determine the stage at which E3′ is activated. From ribonuclease protection assays, it is clear that E3′ conferred lymphoid specificity of expression in that β-globin mRNA was detected in spleen and bone marrow but not in kidney or liver (Fig. 4B).

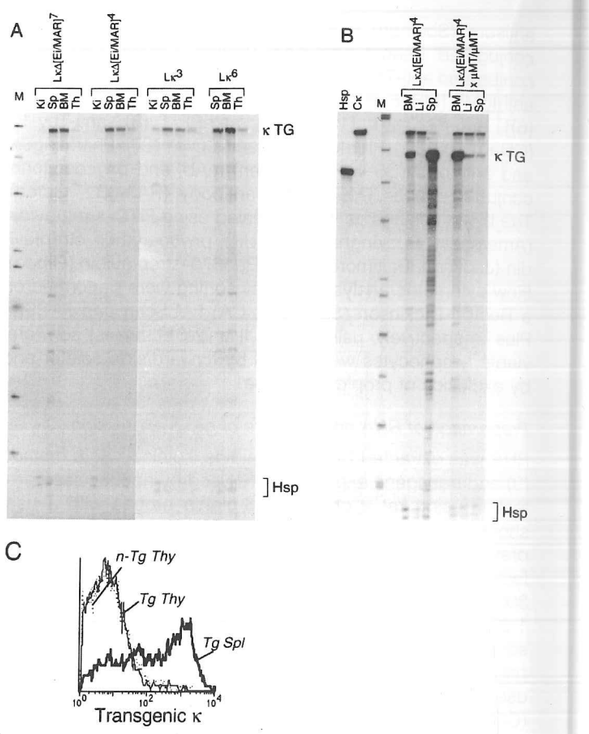

Fig. 3. Ribonuclease protection assays of transgene expression in $L_\kappa\Delta$[Ei/MAR] and L_κ mice. (A) RNA (10 μg) from kidney (Ki), spleen (Sp), bone marrow (BM) and thymus (Th) from two lines of $L_\kappa\Delta$[Ei/MAR] and two lines of L_κ mice were mapped with a κ transgene-specific probe as well as a probe for an hsp70 heat shock cognate (Hsp) as an internal reference. (B) RNA (10 μg) from bone marrow, liver (Li) and spleen from an $L_\kappa\Delta$[Ei/MAR]^4 mouse and from an $L_\kappa\Delta$[Ei/MAR]^4 mouse that had been backcrossed into a homozygous μMT/μMT background were mapped as in (A). The mobilities of the undigested C_κ and Hsp cognate probes are illustrated. M, DNA size markers. (C) Flow cytometry analysis of transgenic (Tg) κ expression in permeabilized spleen (Spl) and thymus (Thy) samples from an L_κ mouse as well as from a non-transgenic (n-Tg) control. The spleen cells have been gated for Thy-1.2^- whereas the thymus samples are gated for Thy-1.2^+.

We then used RT-PCR assays to assess expression of the βG[E3′] transgene within the lymphoid sub-compartments. For these assays, we used HPRT transcripts as internal control, initially confirming that the assay gave the same results as the ribonuclease protection experiments with respect to transgene expression in liver, spleen and bone marrow [Fig. 5A(i)]. Fractionation of splenocytes into CD45R(B220)$^+$ and Thy-1^+ subpopulations reveals that transgene expression is restricted to B as opposed to T cells [Fig. 5A(ii)] and indeed transcripts are detected in newborn spleen but not newborn thymus [Fig. 5A(iii)]. With regard to expression in bone marrow, expression is found in the BP-1^+ CD45R(B220)$^+$ fraction (indicating that the transgene is already activated at the late pro-B/pre-B cell

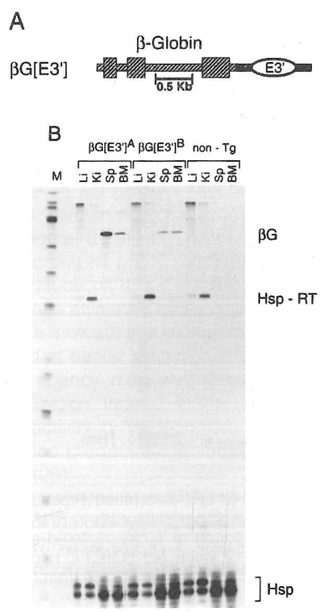

Fig. 4. Lymphoid-specific expression of the κE3′-linked human β globin transgene. (A) Structure of the βG[E3′] transgene. (B) Ribonuclease protection mapping of RNA (10 μg) from liver (Li), kidney (Ki), spleen (Sp) and bone marrow (BM) from two lines of βG[E3′] mice and a non-transgenic littermate. The βG[E3′]^A line contains 10–20 transgene copies whereas βG[E3′]^B harbours five to 10 copies. 'Hsp' denotes bands derived from the hsp70 cognate internal reference probe; RNA from kidney, as opposed to that from many other tissues, usually gives a full length, readthrough protected fragment (Hsp-RT) with this probe.

stage) as well as in the BP-1^- CD45R(B220)$^+$ fraction (which includes early pro-B as well as mature and recirculating B cells) (Fig. 5B). [BP-1 (8) is an aminopeptidase whose expression within the B lineage is restricted to the pre-B cell stage.] However, enriching the bone marrow B-lineage cells for CD43$^+$ and CD43$^-$ subfractions suggests that the transgene is not fully active at the early pro-B cell stage.

Timing of activation of an IgH transgene linked to κEi/E3′

The results regarding expression of the βG[E3′] transgene in B cell precursors as well as that of L_κ-derived transgenes in μMT/μMT bone marrow suggest that reporter genes linked to the Igκ enhancers become transcriptionally activated at a stage late in pro-B cell development when CD43 expression levels are decreasing, but prior to the major expansion at the pre-B cell stage [fraction C′ of Hardy *et al.* (13)] which is absent in μMT/μMT mice (15). This predicts that a Igμ transgene driven by the Igκ enhancers will be expressed prior to the block in μMT/μMT mice (and will therefore be able to rescue B cell development in these animals) but subsequent to V–DJ rearrangement at the IgH locus (and will therefore not mediate IgH chain allelic

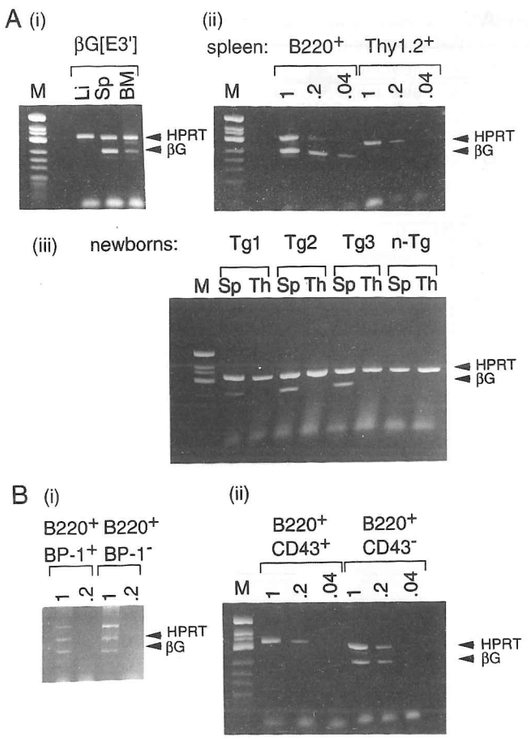

Fig. 5. Expression of the κE3′-linked human β globin transgene in lymphoid subsets of βG[E3′]^A mice. The presence of transgene-derived human β-globin transcripts was analysed by RT-PCR using HPRT as an internal control. (A) Expression is largely restricted to the B lineage. Comparison of β-globin expression levels in: (i) Liver, spleen and bone marrow; (ii) Serial dilutions of cDNA derived from sorted splenic CD45(B220)$^+$ and Thy-1.2^+ cells; (iii) Spleen and thymus of newborn (day 0) mice (Tg, transgenic, n-Tg, non-transgenic litter mate). (B) Transgene expression within the B lineage. Bone marrow from βG[E3′]^A mice (8–11 weeks old) was sorted into: (i) CD45R(B220)$^+$/BP-1^+ and CD45R(B220)$^+$/BP-1^- subpopulations; (ii) CD45R(B220)$^+$/CD43$^+$ and CD45R(B220)$^+$/CD43$^-$ subpopulations. M, molecular weight markers.

exclusion). We tested this prediction by examining the expression of a transgene, $V_{NP}C\mu$[Ei, E3′]$^\kappa$ (Fig. 6A), containing a mouse Igμ chain gene but in which (i) the IgH intron-enhancer has been replaced by κEi/MAR enhancer and (ii) κE3′ has been placed 3′ of the transcription unit. The results are entirely consistent with our predictions. The $V_{NP}C_\mu$[Ei, E3′]$^\kappa$ transgene effectively rescues B cell development in μMT/μMT mice and is well expressed on the surface of the splenic B cells (Fig. 6B). However, despite this good expression, the same transgene (which is of IgM^a allotype) does not inhibit endogenous μ expression in a wild-type IgH^b background; the splenic B cells that express the transgene (IgM^a) also express endogenous (IgM^b) alleles, in comparison to allelic exclusion demonstrated by (CBA/Ca×C57BL/6)F_1 mice (Fig. 6C) or other μ transgenes driven by IgH enhancers.

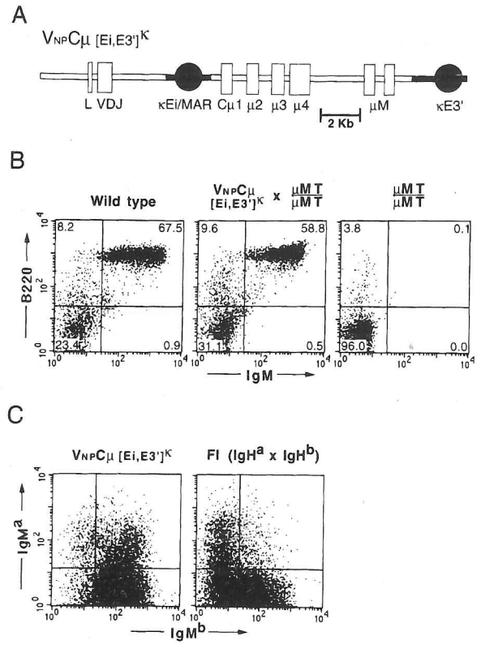

Fig. 6. An Igμ transgene linked to κ[Ei/MAR, E3′] rescues B cell development in μMT/μMT mice but does not mediate IgH allelic exclusion. (A) The $V_{NP}C\mu$[Ei, E3′]$^{\kappa}$ construct. (B) The abundance of splenic B cells in 7–9 week old μMT/μMT, $V_{NP}C\mu$[Ei, E3′]$^{\kappa}$ μMT/μMT and wild-type litter mate mice was assessed by double staining with phycoerythrin-conjugated anti-CD45R(B220) and FITC-conjugated goat anti-mouse IgM antiserum. Viable lymphocytes were gated as judged by forward and side scatter and exclusion of propidium iodide, the figures denoting the percentage in each quadrant. (C) Allelic exclusion of IgH expression was assessed by staining spleen cells from 8- to 9-week-old mice with phycoerythrin-conjugated anti-CD45R(B220), FITC-conjugated anti-IgM^b and biotinylated anti-IgM^a, revealed by Red670–streptavidin, and gating on CD45R(B220)$^+$ lymphocytes (as judged by scatter). The mice analysed were $V_{NP}C\mu$[Ei, E3′]$^{\kappa}$ transgenics crossed into an IgH^b background with (CBA/Ca×C57BL/6)F1 mice (IgH^a/IgH^b) staining as a control.

κE3′ activity increases dramatically on lipopolysaccharide (LPS) activation

Ig mRNA levels increase considerably on activation of mature B cells. In the case of the IgH locus, this has been shown to be attributable in part to increased stability of IgH mRNA (16,17) and in part—in both IgH and κ light chain loci—to increased transcription (18,19). In order to test whether κE3′ might contribute to effecting the increased κ mRNA abundance in plasma cells, splenic B cells from βG[E3′] mice were cultured in the presence of 50 μg/ml bacterial LPS and RNA extracted at various times. A dramatic up-regulation of β-globin mRNA levels is obtained as judged by ribonuclease protection assays (Fig. 7). Densitometry tracing controlled by the heat shock cognate internal reference reveals that the LPS treatment lead to a 10- to 20-fold increase in β-globin mRNA levels after 48 h.

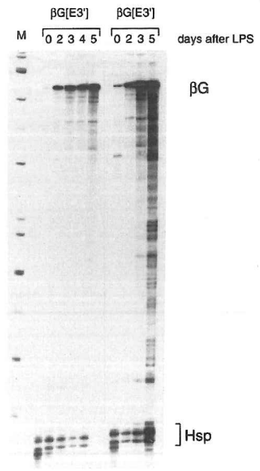

Fig. 7. κE3′ is activated on LPS stimulation. Ribonuclease protection mapping of transgenic β-globin transcripts in RNA (5 μg) from total spleen cells (left hand panel) or T cell-depleted spleen cells (right hand panel) from two 12 week old βG[E3′]^A mice that had been cultured in the presence of bacterial LPS (50 μg/ml) for up to 5 days.

Discussion

The results presented here indicate that, assayed as a transcription enhancer, E3′ is a B cell-specific element that is activated at the late pro-B/pre-B cell stage of differentiation but with its activity being significantly enhanced on B cell activation. This pattern of enhancer activity nicely parallels the differentiation-specific changes in chromatin structure around the κ 3′-enhancer that have very recently been noted by Roque *et al.* (20). The early activation of the enhancer correlates with the stage at which sterile κ transcripts are detected and the cells up-regulate *RAG* expression for V_{κ}–J_{κ} rearrangement (21). It is tempting therefore to speculate that E3′ may activate V_{κ}–J_{κ} rearrangement at the pre-B cell stage.

Recent experiments by Hiramatsu *et al.* (22) using transgenic κ rearranging substrates have revealed that the presence of E3′ suppresses V_{κ}–J_{κ} rearrangement in developing thymocytes. There is an apparent contrast between these observations and our own findings that the E3′-linked β-globin transgene is not detectably expressed in T cells. The resolution of the paradox probably lies in the fact that suppression of V_{κ}–J_{κ} rearrangement and potentiation of β-globin transcription depend on distinct constellations of E3′-binding factors. Indeed, like a locus activating region, E3′ might serve as a focus to alter local chromatin structure early in lymphoid ontogeny (possibly before B/T lineage separation). The precise constellation of DNA-binding factors subsequently recruited would then depend on the differentiation stage of the host cell, leading to an inhibition of V_{κ}–J_{κ} rearrangement in T cells but an activation of transcription and rearrangement in late pro-/pre-B cells. Later, the activity of E3′ increases on B cell activation to contribute to the increase in Igκ mRNA levels.

That E3′ plays a role in Igκ gene expression at the later stages of B cell development is entirely consistent with previous work which has revealed a dependence of transgenic κ expression on sequences 3′ of C_{κ} (2,10) as well as from transfection assays revealing E3′ to be a potent enhancer in myeloma cells (3,23). Its role at earlier stages of B cell development is less clearly anticipated. Thus, E3′ was found to be very weak in activating a reporter gene in the A20 B cell lymphoma unless acting synergistically with Ei (24). Furthermore, some E3′-linked reporter genes transfected into AMLV-immortalized pre-B cell lines were only activated following LPS induction (10,25) leading to the suggestion that E3′ only becomes activated at the pre-B to B cell transition. Our results here indicate that this is not the case, rather that E3′ is activated at earlier stages in B cell development. The apparent discrepancy may well result from the effects of AMLV immortalization. Indeed, in the case of Ei, it has been shown that its activity in AMLV-immortalized cells is inhibited by the v-*abl* gene product, an inhibition which is relieved by LPS treatment (26).

How can the cell-type specificity of E3′ activity be understood in terms of the cell-type specificity of its known cognate binding factors? While the κE3′ was initially defined as an 808 bp fragment (3), subsequent analysis has mapped most of its activity to a 132 bp core region (10,25) which is already active in pre-B cells (25). Although, rabbit, human and mouse E3′ are highly conserved even outside the core (27,28) with functionally important sites being apparently located within the flanking regions (29), the cell-type specificity of βG[E3′] expression is fully consistent with what we know of the transcription factors recognizing the core. Thus, three functional nuclear factor binding sites have been identified in the core. One is a cAMP response element that is bound by the widely expressed CREM/ATF-1 transcription factors (30). The other two are binding sites for E2A and PU.1/NF-EM5(Pip-1) (31,32), sites which are recognized by B cell-specific complexes. Equivalent sites have also been identified in the human E3′ (33). Whilst PU.1 is expressed in both B and myeloid cells (34), Pip-1 (which is thought to encode NF-EM5) is lymphoid specific, being well expressed in pre-B and B cells and present at increased levels in plasma cells but only weakly detectable in T cells (32). Thus, the complex which forms between phosphorylated PU.1 and Pip is restricted to the B-lineage. In fact, the transcriptional activation of the E3′-linked β-globin reporter at the pro-pre-B cell stage with no detectable activity in T cells correlates well not only with cell-type specificity of the PU.1/Pip-1 complex but also with that of the E12/E47 dimer whose levels rise at the pro- to a pre-B cell transition (35,36). However, the suppressive effect of E3′ on V_{κ}–J_{κ} rearrangement in T cells is less easily rationalized in terms of known E3′-binding factors. The effect depends on the PU.1 binding site present in E3′ but cannot reflect an involvement of PU.1 in the repression (since PU.1 is unlikely to be present at this stage) but has rather been interpreted in terms of an as yet unidentified rearrangement repressor that binds the same site as PU.1 and is displaced when PU.1 becomes available (22).

With regard to the activity of E3′ at later stages in B cell development, it is notable that mutating any of the three identified sites in the mouse core enhancer diminishes transcriptional activity in pre-B as well as in plasma cell lines (30). This suggests that these factors play a role throughout the pre-B, B and plasma cell stage. It is unclear which of the nuclear factors mediate the up-regulation seen on B cell activation. Thus, PU.1 can be phosphorylated by casein kinase II, JNK and as yet unidentified kinases (37) which may affect its transcriptional activity. It also interacts with retinoblastoma protein *in vitro* (38) suggesting that its transcriptional activity might be repressed in resting cells. Similarly, CREM has been shown to be inducible by cross-linking of surface Ig in a B cell line (39).

Finally we come to the issue whether Ei and E3′ fulfil distinct roles. Considered as transcription enhancers, it is notable that although Ei/MAR affects κ gene transcription (since the $L_{\kappa}\Delta$[Ei/MAR] transgene is not as consistently well expressed as L_{κ}; see also Xu *et al.* (40)), E3′ nevertheless appears the more important of the two enhancers since its removal clearly has a more severe effect on transgene expression at all stages of B cell differentiation than does deletion of Ei/MAR. Very recent confirmation that Ei/MAR is also not needed for good expression of rearranged κ genes in the context of the endogenous locus has come from gene targeting experiments (41). Thus, both Ei and E3′ are likely to contribute to κ gene expression with the E3′ being the more potent and necessary transcriptional activator. However, both enhancers are involved in the regulation of somatic hypermutation (2) and V_{κ}–J_{κ} rearrangement (22,41,42) and it will be interesting to ascertain the relative importance of the two enhancers in these two processes.

Acknowledgements

We thank Gareth Williams for constructing the βG[E3′] and $V_{NP}C_{\mu}$[Ei, E3′]$^{\kappa}$ transgenes, Sarah Davies for microinjection of zygotes, Theresa Langford, Gareth King and Mike Norris for animal handling, David Gilmore and Andy Riddell for help with flow cytometry, and Cristina Rada, Beatriz Goyenechea and César Milstein for discussion. The work was in part supported by a grant from the Wellcome Trust to K. B. M. and by an International Research Scholars award from the Howard Hughes Medical Institute to M. S. N.

Abbreviations

LPS	lipopolysaccharide
MAR	matrix attachment region

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