Regulated Accessibility of Variable Region Genes May Control Developmentally Ordered T Cell Receptor γ Gene Rearrangement

by

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ABSTRACT

γδ T cells can be divided into subtypes on the basis of Vγ gene expression, receptor diversity and tissue localization. These subtypes arise in "waves" in the thymus during ontogeny. One of the models proposed to explain this phenomenon is the programming model, in which rearrangement of particular Vγ genes is targeted in progenitor cells whose subtype is predetermined.

Earlier studies demonstrated that the T cell receptor Vγ genes rearrange to the Jγ1 segment in a highly ordered fashion during ontogeny. Here, a PCR assay used to quantitate rearrangements in thymocyte DNA from different developmental time points showed that the relative frequencies of the Vγ rearrangements correspond reasonably well to the frequencies of Vγ-expressing subtypes in the thymus at the different time points.

It has been proposed that the accessibility of a gene to the recombinase machinery may be controlled, at least in part, by transcription. In order to determine the relationship, if any, of transcription to ordered Vγ gene rearrangement, an RT-PCR assay was employed to quantitate transcripts of unrearranged Vγ genes (sterile transcripts) in thymocyte RNA from the same developmental time points as used above. This showed that the pattern of Vγ gene sterile transcripts correlates with the timing of their rearrangement.

The accessibility model predicts that not all Vγ genes in the Cγ1 cluster will be accessible in the same progenitor cell. Some transcripts of rearranged Vγ3 genes were found to consist of the Vγ4 leader (L4) spliced onto the Vγ3Jγ1 coding exon, indicating that both the Vγ3 and the Vγ4 genes are accessible on the same chromosome.

The RT-PCR assay was employed to compare L4 usage in Vγ3 rearranged vs. sterile transcripts. Unlike the rearranged transcripts, the sterile transcripts rarely contain L4, suggesting that the Vγ3 and Vγ4 genes are differentially accessible prior to rearrangement.

The first T cell receptor positive thymocytes to appear during ontogeny are $V\gamma3^+$ cells, which can only be detected in the fetal thymus and can only be generated by the combination of fetal stem cells and fetal thymic stroma. The abundance of Vγ3 sterile transcripts in fetal vs. adult stem cell-derived thymocytes in fetal thymic organ cultures was measured to determine if the Vγ3 gene accessibility in these progenitors contributed to the ability to become a $V\gamma3^+$ cell. In every case, Vγ3 sterile transcript levels were higher in RNA from fetal thymic lobes repopulated with fetal liver than in those repopulated with adult bone marrow. Given this apparent contribution of the stem cell origin to the restriction of $V\gamma3^+$ T cell development, four models were proposed describing the possible role of the thymic stroma in the regulation of this process.

Finally, Vγ sterile transcript levels in SCID adult thymocytes were found to be similar to those in normal fetal thymocytes. Examination of fetal liver chimeras of normal cells into SCID mice (AKR-->SCID) showed, however, that the SCID adult thymus lacked the ability to generate $V\gamma3^+$ cells from fetal stem cells. Neither the maturation of the SCID thymus induced by normal cells in these chimeras, nor the introduction of SCID fetal stem cell into a normal thymus in the reciprocal chimeras (SCID-->AKR), caused a decrease in the Vγ3 sterile transcript levels in the SCID cells. However, Vγ3 sterile transcript levels did decrease in *normal* cells from AKR-->SCID and CB17-->SCID chimeras. The result that SCID cells do not produce lower levels of sterile Vγ3 transcripts in response to coexisting with normal developing T cells in an adult thymus, fails to clarify the role of adult thymic stroma in regulation of Vγ3 gene accessibility.

Thesis Supervisor: Dr. David H. Raulet

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INTRODUCTION

T Cell Antigen Receptor

Structure

The T cell receptor (TCR) is composed of a clonally variable, disulfide-linked heterodimer composed of either αβ or γδ chains, which are responsible for the antigen specificity of the T cell (Dembic et al., 1986, Saito et al., 1987). These are non-covalently associated with the CD3 complex, which is composed of five invariant chains: γ, δ ε, and a disulfide linked dimer of either ζ-ζ or ζ–η (reviewed in (Ashwell & Klausner, 1990)). This complex is thought to be involved in intracellular signal transduction upon TCR stimulation (Frank et al., 1990, Irving & Weiss, 1991).

Ligand Recognition and Function

The TCR recognizes a peptide antigen bound to a Major Histocompatibility Complex (MHC) molecule (reviewed in (Rothbard & Gefter, 1991)). Most αβ T cells express either a CD4 or CD8 co-receptor which binds to a non-polymorphic region of MHC Class II or MHC Class I, respectively, and increases the avidity of the TCR-antigen interaction. αβ T cells which are $CD8^+$ are usually cytolytic and kill cells whose antigen/MHC complex they recognize. $CD4^+$ cells secrete a variety of cytokines upon recognition of antigen/MHC

complex which have a "helper" function for cells bearing that complex.

In contrast, most γδ T cells are $CD4^-CD8^-$. The functions and specificities of γδ cells are still being elucidated and will be discussed below.

Generation of Diversity

A diverse repertoire of T cell specificities is required to respond to the wide variety of antigens that may be encountered in the lifetime of an organism (reviewed in (Davis & Bjorkman, 1988)). There are several mechanisms which operate to generate diversity of T cell receptor chains (reviewed in (Schatz et al., 1992)). The exons forming the antigen binding domain of the T cell receptor chains, like immunoglobulin (Ig) genes, are assembled from gene segments consisting of variable (V), diversity (D-in TCR β, δ and IgH genes only) and joining (J) segments. This process of V(D)J recombination is able to generate considerable diversity because of the variety of choices of gene segments and the production of divergent sequences at the junctions of these segments.

One way junctional diversity is achieved is by the loss of nucleotides from the ends of each coding segment, due to the imprecise nature of the joining process. Also, the junctions often contain non-germline-encoded nucleotides (N regions) which are the result of random base additions by the enzyme terminal deoxynucleotidyl transferase (TdT) (Desiderio et al., 1984, Landau et al., 1987). Another type of base addition is found in P (palindromic) regions, which occur next to coding segments in which no base loss

has taken place. These typically consist of one or two nucleotides complimentary to the end of the adjacent gene segment (Lafaille et al., 1989, McCormack et al., 1989).

Somatic hypermutation, used to increase the diversity of immunoglobulin genes, has not been shown to operate in TCR genes.

The mechanism of V(D)J recombination will be discussed in detail below.

TCR loci

Figure 1-1 illustrates the genomic organization of the murine TCR loci. The δ locus is contained in the region between Vα genes and the Jα genes. Thus, the entire δ locus is deleted in the event of a Vα to Jα rearrangement. Approximately ten of the Vα/δ genes are actually used to assemble δ genes. Some of these can be used by either α and δ, while others, particularly Vδ1 and Vδ5, appear to be used only in the assembly of δ chains. The Vα genes rearrange to one of ~80 Jα segments upstream of a single Cα region. Similarly, Vδ and Vβ join to D and then J segment upstream of a single (δ) or two tandem (β) constant region genes.

The γ genes, in contrast, are arranged in four clusters, each of which contains a single Jγ and Cγ segment (reviewed in (Raulet, 1989)). Cγ3 is a pseudogene which is usually unrearranged and is deleted in some mouse strains. The Vγ genes generally rearrange to the adjacent Jγ segment, thus allowing the possibility of three different rearrangements on the same chromosome. In fact, multiple rearrangements of the TCR γ locus in the same cell are commonly observed. However, usually only one of the rearrangements is

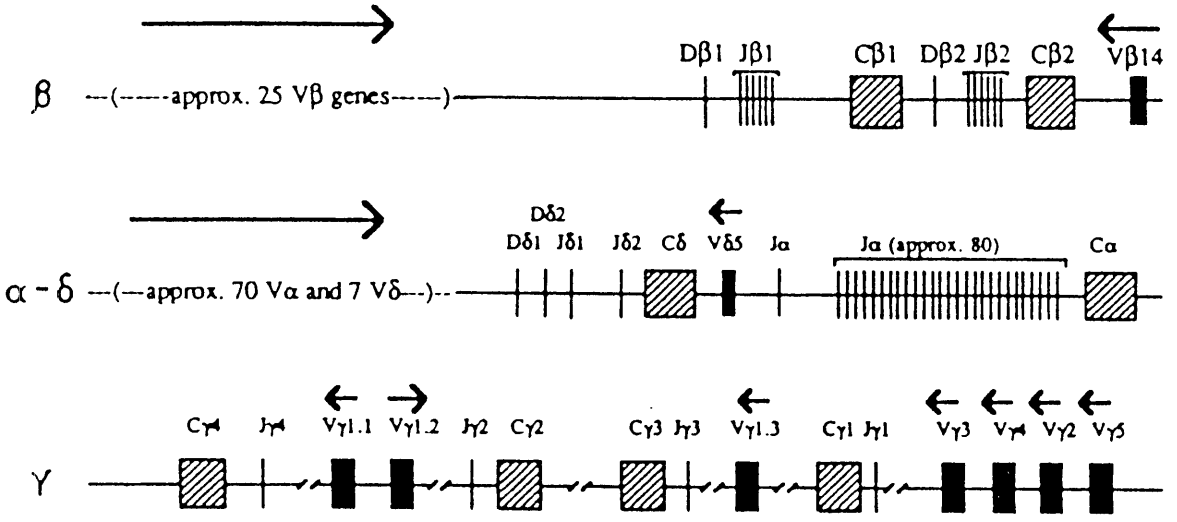


Figure 1-1: Murine T cell Receptor Loci. From Allison & Havran, 1991.

productive (Heilig & Tonegawa, 1987). Degenerate γδ TCR expression may be prevented by preferential pairing with δ chain and allelic exclusion (Korman et al., 1988, McConnell et al., 1989).

Lineage Separation of αβ and γδ T Cells

αβ and γδ T cells can be derived from a common progenitor. Two general models have been proposed regarding the point at which their lineages diverge.

In one model, αβ T cells are derived from cells which have first attempted and failed to produce functional γ and δ rearrangements (Allison & Lanier, 1987). This model is based in part on the timing of the rearrangements, as the δ genes are first to rearrange during ontogeny, followed by γ, β and finally α (Born et al., 1986, Raulet et al., 1985, Snodgrass et al., 1985a, Snodgrass et al., 1985b). Also in support of this model is the observation that γ rearrangements are found in most αβ T cells (Garman et al., 1986, Hayday et al., 1985, Heilig et al., 1985, Heilig & Tonegawa, 1987, Reilly et al., 1986, Traunecker et al., 1986).

The second model proposes that progenitor cells are committed to become either an αβ or a γδ cell before rearrangement takes place. Support for this model comes from the observation that the circular excision products resulting from a Vα-Jα rearrangement contain the δ locus in its germline configuration (Winoto & Baltimore, 1989). This implies that the α gene rearrangements take place in cells which have not undergone δ rearrangement. Another study did find rearranged δ loci in excision circles, but their cloning methods

selected against unrearranged genes, perhaps allowing them to detect rare exceptions (Takeshita et al., 1989). Additional evidence consistent with this model comes from studies in which mice transgenic for rearranged γ and δ genes still had normal numbers of αβ T cells (Dent et al., 1990, Ishida et al., 1990). Thus, the αβ T cells developed regardless of the presence of functionally rearranged γ and δ genes in the progenitor cells.

Currently, the second model is the favored one. However, it does not account for the presence of rearranged γ genes in most αβ T cells. It is possible that lineage commitment occurs after γ rearrangement. Alternatively, γ rearrangement may not be restricted in progenitor cells which are committed to the αβ lineage. Indeed, the rearranged state of the γ genes may not be important as most of these genes are not expressed in αβ T cells (Korman et al., 1988). Similarly, most γδ T cells have partial (Dβ-Jβ) β chain rearrangements (Asarnow et al., 1988, Marusic-Galesic et al., 1988a). Thus the Vβ to DJβ rearrangement step may be the important one in lineage determination.

T Cell Development in the Thymus

T cells originate from hematopoietic stem cells in the bone marrow or fetal liver which migrate to the thymus and, under the influence of the thymic stroma, become mature T cells. There is evidence that some mature T cells are produced in the absence of a thymus, but this discussion will focus on thymus-dependent T cell development.

Thymic architecture

The murine thymus is an organ essentially derived from epithelial tissue formed between embryonic day 11 (E11) and E12 (reviewed in (van Ewijk, 1991)). Shortly thereafter, lymphoid progenitors begin to enter. By E13, the thymus has begun to shape itself into two distinct compartments, the cortex and the medulla. The medulla is in the interior of each thymic lobe and is surrounded by the cortex. The cortex contains a meshwork of epithelial cells with long, branching processes. This region is packed with lymphocytes and has scattered macrophages. The medulla is made up of more conventional type epithelial cells, has fewer lymphocytes and contains bone marrow-derived dendritic cells, particularly near the border with the cortex.

The outer (subcapsular) region of the cortex is distinct from the rest as the location in which the majority of the lymphocytes are actively dividing. The epithelial cells form into baskets which are filled with lymphocytes. This is also where the thymic nurse cells (TNC) are found. TNC are large epithelial cells that bind thymic lymphocytes so tightly that when isolated from the thymus, they are found to have completely engulfed many lymphocytes. These remain viable and can be released in culture, suggesting that *in vivo* the TNC give inductive signals while sequestering the developing lymphocytes. In the deeper cortex, the epithelial cells are organized into sheets perpendicular to the thymic capsule. These may serve to guide the migration of thymocytes from the outer cortex toward the medulla.

Thymus colonization

T cells first begin as pluripotent hematopoietic stem cells (HSC). These are present in the fetal liver and in the bone marrow after E15 (reviewed in (Ikuta et al., 1992)). These cells can give rise to all erythroid, myeloid and lymphoid lineage cells. However, as they differentiate, their developmental potential becomes more limited. HSC-derived T precursor (pro-T) cells migrate into the thymus from the fetal liver during gestation and from the bone marrow starting late in gestation. The seeding of these cells early in life does not suffice to generate T cells indefinitely. Maintaining T cell production requires colonization of the thymus by successive waves of stem cells through adulthood (Jotereau et al., 1987). These cells develop into mature T cells under the influence of the thymic stroma (see below).

Purified pluripotent HSCs injected directly into the thymus can develop into T cells. However, it is not clear if under normal physiological conditions, stem cells are still multipotent when they enter the thymus. They may need to differentiate to the pro-T stage, perhaps to express the appropriate homing receptors. Recently, monoclonal antibodies have identified novel cell surface markers (JORO) that define a population of pro-T cells (Palacios et al., 1990). Staining of embryos revealed that cells expressing these markers are present in the fetal liver at E9 and in adult bone marrow. These cells appear in the thymus at E11, and at E14 they make up almost 100% of thymocytes. This number declines to ~1% by E18 and the cells remain a small percentage of thymocytes in adult (Palacios & Samaridis, 1991). The kinetics of the expression of JORO markers

suggest that HSCs become pro-T cells in the fetal liver and bone marrow prior to migration to the thymus.

Thymocyte differentiation

Pro-T cells enter the thymus and move through it in a specific order which is related to maturational steps performed by different thymic microenvironments (reviewed in (Rothenberg, 1992)). After entering the thymus near the border of the cortex and medulla, pro-T cells slowly migrate to the outer region of the cortex. During this period, cell division begins. In the subcapsular region, proliferation is at its maximum rate. This phase ends with the expression of TCR on the cell surface. These cells then stop proliferating, shrink and move back through the cortex, where they begin to mature. Only T cells with a mature phenotype enter the medulla. Cells may reside here for several days before being exported to the periphery. The role of the medulla is not clear, but it is thought to be involved in selection or functional maturation of T cells.

In the process of becoming mature T cells, thymocytes undergo many stages defined by cell-surface phenotype for markers such as CD4, CD8, TCR/CD3, and HSA, among others (reviewed in (Rothenberg, 1992)). The earliest thymocytes have a cell surface phenotype similar to HSCs, but lack the ability to differentiate into non-T cell lineages and express CD4 at low levels (Wu et al., 1991). These cells are $HSA^{lo}CD4^{lo}CD8^{-}TCR^{-}$, after which they undergo a transition to the $HSA^{+}CD4^{-}CD8^{-}TCR^{-}$ (DN) stage. These cells appear to pause, apparently awaiting an as yet undefined triggering signal from the thymic microenvironment. Once initiated, these cells undergo a

transition to a rapidly dividing state in which they begin to express low levels of CD8 on their surface followed by CD4. At the same time, they rearrange their TCR α genes. When this stage is over, the cells are $HSA^{hi}CD4^{+}CD8^{+}TCR^{+}$ (DP) . It is at this stage that self-reactive cells are deleted by negative selection, and cells with the appropriate MHC-restriction are encouraged to mature by positive selection. After the selection step, these cells attain their mature T cell phenotype of $HSA^{-}CD4^{+}CD8^{-}TCR^{+}$ or $HSA^{-}CD4^{-}CD8^{+}TCR^{+}$ and move to the medulla.

The above discussion refers to the development of αβ T cells. Because γδ T cells are $CD4^{-}CD8^{-}$ even when mature, it is difficult to define maturational steps on the basis of expression of these molecules.

Influence of T cells on the thymic environment

It has been established that the thymic environment has a great influence on developing thymocytes. Recent studies have shown that this influence is bidirectional.

The most compelling examples of the dependence of thymic stromal development on the presence of lymphocytes come from experiments in SCID mice. These mice have a defect in their ability to rearrange Ig and TCR genes that results in a profound lack of B and T cells (Bosma et al., 1983). The thymus of a SCID mouse is almost entirely cortical, with only a few scattered medullary cells, illustrating the need for lymphoid cells to maintain the structure of the medulla.

Various methods have been used to develop a normal thymic architecture in SCID mice. The injection of normal bone marrow cells into SCID mice restored the thymic architecture to a normal morphology (Shores et al., 1991). In addition, SCID mice crossed to TCR αβ transgenic mice had normal thymuses (van Ewijk et al., 1994). Finally, injection of normal lymph node cells into a SCID mouse caused the restoration of the cortex and medulla (Surh et al., 1992). Thus, it appears that the presence of mature T cells is necessary for the establishment and/or maintenance of normal thymic architecture.

Immunobiology of γδ T cells

γδ T cells can be divided into subtypes on the basis of V gene usage, receptor diversity, ontological timing of development and tissue localization. This discussion will focus primarily on T cells with receptors whose γ genes are derived from the Cγ1 cluster (For a summary of Cγ1-derived γδ subtypes see Fig. 1-2).

Ontogeny of γδ T Cell Subtypes

γδ T cell subtypes arise in ordered waves during ontogeny. The first $CD3^+$ thymocytes appear at embryonic day 13 (E13). At this stage, virtually all $CD3^+$ thymocytes express a Vγ3/Vδ1 receptor. The abundance of these cells peaks at ~E15, after which their numbers decline to be undetectable in the thymus by birth (Cron et al., 1988, Havran & Allison, 1988, Itohara et al., 1989). A second, overlapping wave comprising Vγ4/Vδ1-expressing thymocytes is

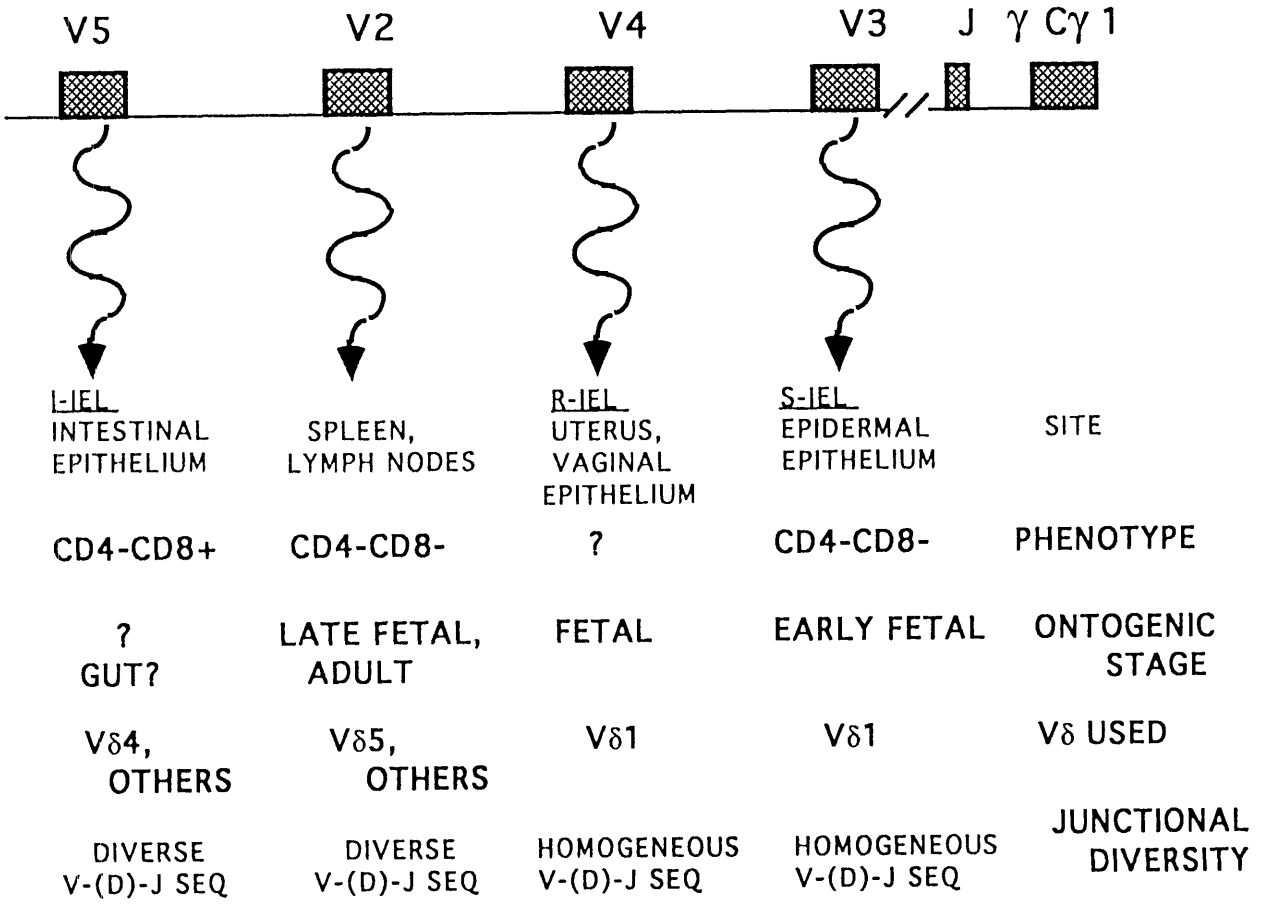


Figure 1-2: γδ T cell subtypes of the Cγ1 cluster. From Raulet, et al, 1991

thought to arise and decline slightly later that the first, although the lack of a Vγ4-specific antibody prevents an exact comparison of the Vγ3 and Vγ4 waves (Ito et al., 1989). Thymocytes bearing Vγ2, paired with various Vδ, particularly Vδ5 (Elliott et al., 1988, Korman et al., 1988), first appear in the fetal thymus at ~ E16 and, along with Vγ1.1 and Vγ1.2-bearing cells, make up most of the γδ T cells in the adult thymus (Houlden et al., 1988, Itohara et al., 1989). Vγ5-expressing cells appear to arise extrathymically (De Geus et al., 1990, Lefrancois et al., 1990).

Receptor Diversity

Vγ3/Vδ1-expressing T cells are unusual in that, as a population, their TCRs are almost completely lacking in junctional diversity (Asarnow et al., 1988, Havran et al., 1989). Similarly, Vγ4-expressing cells are characterized by the invariant junctional sequences of the Vγ4 chain, which is paired with the same Vδ1 chain used by Vγ3-expressing cells (Lafaille et al., 1989). The junctional sequences almost exclusively used by the Vγ3, Vγ4 and Vδ1 chains in early fetal thymocytes are called the "canonical" sequences (Asarnow et al., 1988). These sequences lack N regions, probably due to the low levels of TdT in the early fetal thymus (Landau et al., 1987, Rothenberg & Triglia, 1983).

In contrast, Vγ2-expressing cells have more diverse receptors, with regard to both junctional sequences and pairing with δ chain (Cron et al., 1988, Cron et al., 1989), as do $V\gamma5^+$ cells (Bonneville et al., 1988, Goodman & LeFrancois, 1989, Takagaki et al., 1989a).

Tissue Tropism

Another characteristic distinguishing the various γδ T cell subtypes is their tissue localization in the periphery of the adult mouse. γδ T cells with polymorphic receptors are primarily found in the secondary lymphoid organs ($V\gamma2^+$, $V\gamma1.1^+$ and $V\gamma1.2^+$), the lungs ($V\gamma2^+$) and the lining of the intestine ($V\gamma5^+$, i-IELs) (reviewed in (Haas et al., 1993)).

Cells expressing the canonical Vγ3/Vδ1 receptor are found in the epidermis and are known as skin intraepithelial lymphocytes (s-IELs). Similarly, $V\gamma4^+$ T cells reside in the epithelial layer of the tongue and female reproductive organs and are known as r-IELs (reviewed in (Allison & Havran, 1991, Havran et al., 1991a, Raulet et al., 1991)).

The fact that s-IELs express the same Vγ3/Vδ1 TCR as the early fetal thymocytes suggests that these are the precursors of the s-IELs. Definitive proof was rendered by studies in which nude mice, which are deficient in $V\gamma3^+$ s-IELs, were engrafted with fetal thymic lobes or $V\gamma3^+$ fetal thymocytes (Havran & Allison, 1990). This resulted in the appearance of donor-type $V\gamma3^+$ cells in the skin. Additionally, the depletion of $V\gamma3^+$ cells during fetal development by the administration of anti-Vγ3 antibody *in utero*, resulted in mice that were deficient in s-IELs (Havran & Allison, 1990).

The observation that tissue localization of s-IELs, r-IELs and i-IELs is correlated with the Vγ gene usage suggests that the TCR may act as the homing molecule to direct these cells from the thymus to their peripheral locations. However, the s-IELs and i-IELs in TCR transgenic mice were found to bear the transgene-encoded receptor

(Bonneville et al., 1990), indicating that tissue localization does not depend on the expression of the correct TCR. It is therefore possible that subtype determination involves the coordinate activation of TCR and homing receptor expression during development.

Fetal Origin of Invariant γδ T Cells

As mentioned above, Vγ3 cells are only present in the thymus during fetal ontogeny between ~E14-E17. Two possible explanations are that only fetal stem cells are competent to become Vγ3$^+$ cells, or that only a fetal thymic microenvironment can support the development of Vγ3$^+$ cells. To test this, fetal thymic lobes were repopulated with either fetal liver stem cells or adult bone marrow stem cells. These same stem cell populations were introduced into the adult thymus by intrathymic injection (Ikuta et al., 1990). The only combination which led to the appearance of Vγ3$^+$ cells was the fetal stem cells in the fetal thymus, indicating that the stage of development of both the thymic stroma and progenitor cell are important. This was confirmed in studies which used thymic grafts and stem cells transferred into a nude host in all the combinations described above. Only the mouse grafted with fetal liver and reconstituted with fetal liver stem cells generated Vγ3$^+$ cells in the skin (Havran et al., 1991a).

Antigen Specificity and Function

Although their actual function(s) are unknown, γδ cells appear to possess some of the same functional capabilities as αβ cells

(reviewed in (Haas et al., 1993, Raulet et al., 1991)). γδ T cells capable of lysing tumor targets have been isolated from a variety of tissues including thymus, peripheral blood, spleen and intestine. In addition, γδ cells can secrete a variety of cytokines. Anti-CD3 antibody treatment or lectin stimulation of γδ T cell clones revealed production by each clone of some combination of the following cytokines: IL-2, -3, -4, -5, -10, TNFα, TGFβ, GM-CSF, IFNγ and β.

Antigen specificities of γδ T cell are not well understood. What follows is a discussion of reactivities of various subtypes, although the physiological relevance of these is not clear.

Because of their location in the secondary lymphoid organs and the variability of their receptors, Vγ2-, Vγ1.1-, and Vγ1.2-expressing T cells were thought to have the capacity to respond to conventional antigens. γδ T cell clones and hybridomas have been isolated which recognize a variety of antigens including classical and non-classical MHC, the MHC I-like CD1 molecule, and mycobacterial antigens (reviewed in (Haas et al., 1993, Porcelli et al., 1991)).

Interestingly, many MHC-specific clones are broadly cross-reactive, suggesting that they recognize a relatively non-polymorphic antigenic determinant (Matis et al., 1987). Also, alloreactive cells occur infrequently in populations of γδ T cells, indicating that they may not have the bias for MHC recognition found in populations of αβ cells.

Mycobacteria-reactive γδ cells have been found in immunized mice and also in the lesions of leprosy patients and the synovial fluid of patients with arthritis (Holoshitz et al., 1989, Janis et al., 1989, Modlin et al., 1989, O'Brien et al., 1989). It was found that even γδ T

cells from healthy donors produced a vigorous response against mycobacterial antigens (Pfeffer et al., 1990). In addition, a large fraction of γδ T cells isolated from newborn thymus recognize the highly conserved mycobacterial heat-shock protein HSP65. These were also found to cross-react with the human HSP65 (Born et al., 1990). Although these cells were isolated from newborn thymus, they all express Vγ1.1/Vδ6, a splenic γδ subtype. The observation that these mycobacteria-specific hybridomas spontaneously produce IL-2 in culture suggests that they may be reactive against stressed autologous cells.

Even before this discovery, it had been suggested that one function of γδ T cells is immunosurveillance of epithelial tissue to eliminate stressed autologous cells in these locations (Asarnow et al., 1988, Janeway et al., 1988). This theory was initially based on characteristics of Vγ3$^+$ s-IELs. Due to their receptor homogeneity, lack of lateral mobility in the skin and contact with keratinocytes it was proposed that they respond to damage-induced self antigens in keratinocytes. This was supported by the observation that s-IELs in culture with "stressed" keratinocytes responded by producing IL-2 and that exposure of the keratinocytes to heat shock or sodium arsenate increased this stimulation (Havran et al., 1991b).

Antigens for Vγ4-expressing r-IELs have not been determined. However, given their similarity to s-IELs, it is possible that they recognize an analogous antigen in the tissues in which they reside.

Thymic selection

αβ T cells are known to progress through several stages as they mature in the thymus, during which they are subjected to negative selection, to remove self-reactive cells, and positive selection, to produce an MHC-restricted repertoire which can recognize antigen presented by autologous cells. Whether similar selection processes are used to shape the γδ T cell repertoire is currently under investigation.

Studies using MHC-deficient mice indicate that selection on MHC is not required for normal development of most γδ T cells. Mice that are mutant for β2-microglobulin (β2m), and thus are grossly deficient in MHC class I, appear to have normal numbers of γδ T cells in the lymphoid organs, skin, intestines and uterus despite a profound lack of $CD8^+$ αβ T cells (Correa et al., 1992, Zijlstra et al., 1990). Treatment of purified γδ cells from the spleen, thymus lymph node or skin of these mice with anti-γδ antibody resulted in normal proliferative responses and lymphokine secretion. Similar results were obtained from studies examining γδ T cell repertoire and function in MHC Class II deficient mice (Bigby et al., 1993).

In contrast, studies using β2m-deficient mice bred to mice transgenic for a Class I MHC-specific γδ TCR show that T cell development is affected by the lack of MHC (Wells et al., 1991, Wells et al., 1993). In these mice, the transgene-expressing thymocytes were unable to proliferate to TCR stimulation, were HSA positive and did not populate the peripheral lymphoid organs. Similarly, studies using mice transgenic for MHC Class I Tla-specific γδ TCR show that transgene-positive cells are absent from the thymus and periphery

of mice expressing the Tla-encoded determinant (Dent et al., 1990). These studies suggest that some γδ cells may be subject to positive and negative selection on MHC Class I molecules. However, the low frequency of MHC-reactive γδ T cells (Bluestone et al., 1991) indicates that this is probably a minor population in normal mice.

Although most γδ subtypes appear to mature normally in the absence of MHC molecules, it is still possible that a positive selection step involving interaction with a non-MHC ligand is required. This is supported by studies examining the development of Vγ3+ thymocytes either *in vivo* or in fetal thymic organ cultures (Leclercq et al., 1993, Tatsumi et al., 1993). These cells were shown to undergo a change from an immature $V\gamma3^{lo}HSA^{hi}$ to a $V\gamma3^{hi}HSA^{lo}$ phenotype, the same phenotype as s-IELs. This transformation can be blocked by treatment with cyclosporin A, which also inhibits positive selection of αβ thymocytes.

Finally, it does not appear that positive selection is required to insure that $V\gamma3^+$ and $V\gamma4^+$ T cells express receptors with the canonical sequence. The frequency of canonical junctions in s-IELs and r-IELs from MHC Class I-deficient mice was comparable to normal mice, indicating that MHC I molecules do not participate in selecting invariant γδ T cells (Correa et al., 1992). In addition, studies were done in mice which had mutated TCR γ recombination substrates (Asarnow et al., 1993) or mutated Cδ sequences (Itohara et al., 1993) such that no protein products from rearrangements of these genes would reach the surface. Most Vγ3, Vγ4 and Vδ1 rearrangements were found to have the canonical sequence even though it is unlikely that selection of these junctions occurs.

In contrast, an earlier study had found that treatment of fetal thymocytes with an anti-γδ antibody resulted in an increase in the frequency of productive Vγ3, Vγ4, and Vδ1 rearrangements with non-canonical junctions, presumably by somehow bypassing positive selection (Itohara & Tonegawa, 1990). However this result is difficult to reconcile with the above, and may represent an artifactual expansion of rare cells with non-canonical junctions.

Regulation of Ordered γδ T cell Subtype Production

Models proposed to explain the phenomenon of ordered γδ T cell subtype production include the "selection" model and the "targeting" model (reviewed in (Raulet et al., 1991)). The selection model proposes that rearrangement of TCR γ genes is essentially random and that the ordered appearance of γδ T cells bearing distinct Vγ receptors is accomplished by selection of those cells on stage-specific thymic ligands. The molecular targeting model proposes that the lineage of progenitor cells is determined prior to rearrangement, which leads to the targeting of specific V gene segments for rearrangement. It must be pointed out that these models are not mutually exclusive and may actually reinforce each other.

Presently, most evidence weighs in on the side of the targeting model. Many observations suggest that positive selection of most γδ T cells is not absolutely necessary (see above). In addition, it has been found that the rearrangement of Vγ genes is not random, but occurs in a similar order to the appearance of the corresponding Vγ-expressing cells (Garman et al., 1986). Other evidence for gene

targeting is based on observations that the non-productive rearrangement in a panel of well-characterized cell lines is usually the same as the productive one, indicating restriction of rearrangement in the progenitors of these cells (Raulet et al., 1991). Finally, mice which are mutant for Cδ and thus express no detectable γδ TCR, still have the same ordered Vγ and Vδ rearrangement patterns seen in normal mice (Itohara et al., 1993).

The targeting model also proposes that there are various sublineages of T cell precursor cells committed to becoming a particular γδ subtype. The observation that stem cells of adult or fetal origin have different developmental potential fits with this prediction (Havran et al., 1991a, Ikuta et al., 1990, Ikuta & Weissman, 1991).

V(D)J Recombination

The previous section described a model in which regulation of V γ gene rearrangement may be partially responsible for developmentally ordered γδ T cell subtype production. An understanding of the rearrangement mechanism is important to determine its regulation. The assembly of gene segments into functional Ig or TCR genes is a complex process requiring many levels of control. The V(D)J recombination mechanism is discussed below (reviewed in (Gellert, 1992, Lieber, 1991, Schatz et al., 1992).

V(D)J Recombination Signals

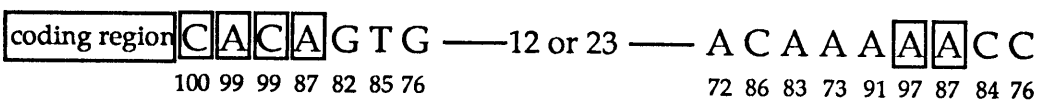
Recombining gene segments of immunoglobulin (Ig) and TCR loci are flanked on one (V, J) or both (D) sides by conserved sequence motifs known as recombination signal sequences (RSS) (Fig. 1-3A). These consist of a palindromic heptamer directly adjacent to the coding sequence and an A/T-rich nonamer which are separated by a non-conserved spacer of 12 or 23 nucleotides. Mutational analyses showed that neither the self-complementarity of the heptamer nor the tract of five A residues in the nonamer are essential for recombination. The most important bases in the heptamer are the four immediately adjacent to the coding segment. The nonamer itself is not absolutely crucial, as a low level of recombination occurs even if it is mutated out of existence. The sequence of the spacer does not appear to be important, but the length does. Reduction of spacer length by more than one base severely reduces joining.

Joining can only occur between gene segments flanked by RSS with different spacer lengths (12-23 rule). In the TCR loci, V and D segments have an RSS with a 23 bp spacer on their 3' end and D and J regions have an RSS with a 12 bp spacer on their 5' ends. This allows joining of V to D, D to D, D to J or V to J. In the IgH locus, the D segments are flanked on both sides by RSS with 12 bp spacers, and the J gene segments by RSS with 23 bp spacers, so direct V-J joining and the use of multiple D segments is not allowed (Fig. 1-3B). Depending on the relative orientation of the gene segments, joining occurs by deletion of the intervening sequences or by inversion (Fig. 1-3C).

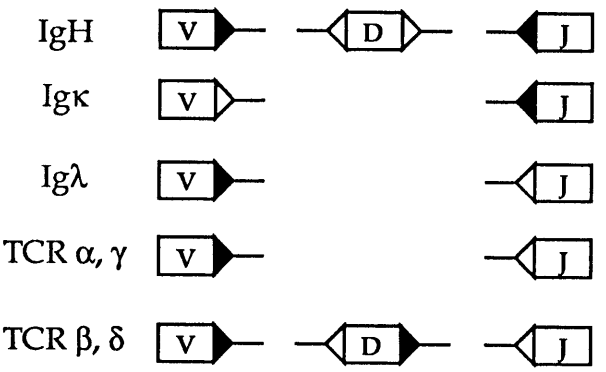
Figure 1-3: Outline of V(D)J recombination. (A) The consensus RSS sequence. The important nucleotides, determined by mutational analyses, are boxed. The number below each nucleotide shows the percentage conservation at each position. (B) The V, D and J segments from the various antigen receptor loci are shown with their type of RSS. Filled triangles indicate an RSS with a 23 bp spacer, open triangles an RSS with a 12 bp spacer. (C) Deletional or inversional recombination.

From Gellert, 1992 and Lieber, 1991

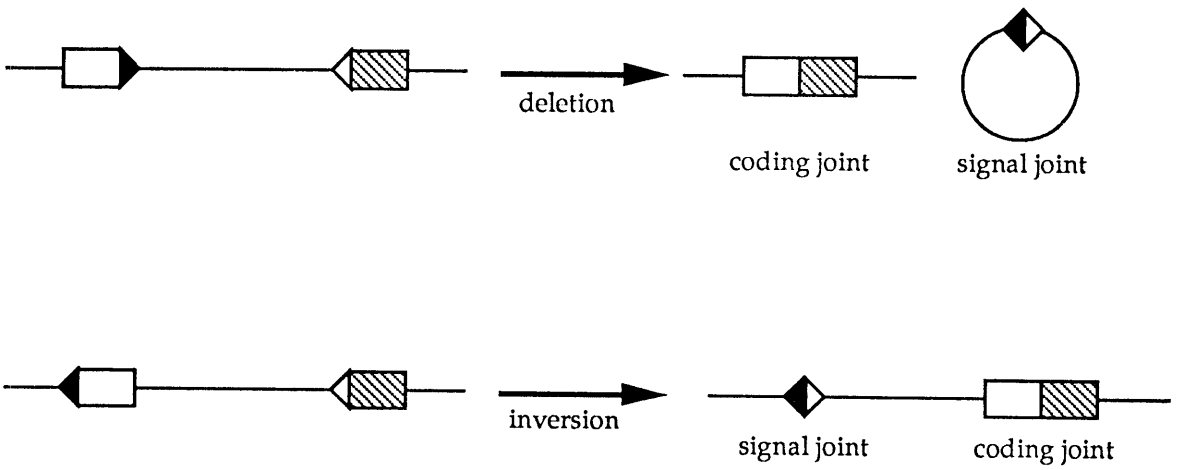
A


coding region C A C A G T G —12 or 23— A C A A A A A C C
100 99 99 87 82 85 76
72 86 83 73 91 97 87 84 76

B


IgH V D J
Igκ V J
Igλ V J
TCR α, γ V J
TCR β, δ V D J

C


deletion
coding joint
signal joint
inversion
signal joint
coding joint

Rearrangement Mechanism

The first step in V(D)J recombination involves site-specific cuts made at the juncture between the coding sequence and the heptamer of the RSS (Roth et al., 1992b). This is followed by resolution of the resulting DNA ends into coding joints and signal joints. Signal joints are formed by precise heptamer to heptamer ligation (Roth et al., 1993), while the joining of the coding ends is much less precise, generally resulting in the loss or addition of 1-10 nucleotides.

The resolution of coding ends has many steps, one of which is thought to be the formation of ends into covalently sealed hairpin loops. This is based, in part, on the accumulation of unresolved coding ends with this conformation in SCID thymocytes (Roth et al., 1992a). Opening of the loop by nicking somewhere other than between the terminal nucleotides would lead to the formation of short inverted repeats. Retention of one or more of these bases after exonucleolytic processing, followed by addition of nucleotides by TdT and subsequent joining would lead to junctional sequences including P and N nucleotide insertions.

However, P nucleotides do not appear in every junction. It is possible that hairpins could be a universal intermediate in V(D)J recombination, but nicking of the hairpin at its terminus would lead to blunt ends. Also, P nucleotides could be removed by exonuclease prior to joining. Potential P nucleotides may also be used as short homologies to aid in the alignment of two coding ends in the joining process, which would mask their presence (Feeney, 1992).

Protein Factors

Only a few of the enzymes involved in V(D)J recombination have been definitively identified (reviewed in (Schatz et al., 1992)). As mentioned above, TdT is responsible for the addition of non-templated nucleotides to the coding junctions. In addition, heptamer- and nonamer-binding proteins and site-specific endonucleases have been detected in extracts from lymphoid cells. The SCID factor has been shown to be important for coding joint formation, although it has yet to be isolated.

To date, the most crucial factors identified in the V(D)J recombination process are the products of two recombinase activating genes RAG-1 and RAG-2. These two linked genes were cloned on the basis of their ability to transfer V(D)J recombination activity to non-lymphoid cells (Oettinger et al., 1990, Schatz et al., 1989). This in itself is surprising because it implies that all other components of the V(D)J recombination machinery are already present in non-lymphoid cells.

That these gene products are necessary for V(D)J recombination is demonstrated by the fact that the deletion of either RAG-1 or RAG-2 genes by homologous recombination results in a complete lack of functional B and T cells (Mombaerts et al., 1992, Shinkai et al., 1992). Co-transfection of RAG-1 and RAG-2 into non-lymphoid cell lines permits rearrangement of recombination substrates with normal signal and coding joints, indicating that these genes are sufficient to activate V(D)J recombination. In addition, RAG-1 and RAG-2 transfected into SCID fibroblasts leads to SCID-like recombination, with normal signal joints and abnormal coding joints,

indicating that neither of these genes codes for the SCID factor (Schatz et al., 1992).

The direct role of RAG-1 and RAG-2 proteins in V(D)J recombination has not been demonstrated. They may actually be a part of the recombination machinery, they may activate other factor involved in V(D)J recombination, or they may recruit ubiquitous factors used for other types of recombination or DNA repair to the V(D)J recombination complex.

Only pre-B and pre-T cells express both RAG-1 and RAG-2. RAG-1 expression is detected in the fetal and postnatal murine central nervous system (Chun et al., 1991), although its importance is questionable, as RAG-1 mutant mice have no obvious nervous system defects (Mombaerts et al., 1992). RAG-2 is expressed in the chicken bursa of Fabricius, where it may be involved in Ig gene conversion (Carlson et al., 1991).

RAG-1 and RAG-2 expression appears to be developmentally regulated. Recent studies have revealed that RAG-1 and RAG-2 expression occurs in two waves during intrathymic development (Wilson et al., 1994). The first occurs at the $CD4^-CD8^-$ stage, coincident with β, γ and δ gene rearrangement, and the second at the early $CD4^+CD8^+$ stage, coincident with α gene rearrangement. In the thymus, levels are high in $TCR^+CD4^+CD8^+$ cells , but not $CD4^+$ or $CD8^+$ single positive cells. Also, crosslinking of the TCR leads to down regulation of RAG-1 and RAG-2 expression (Turka et al., 1991). The timing of RAG-1 and RAG-2 expression implies that the presence of TCR on the surface is not enough to shut off rearrangement, but that

it may be regulated by the processes of positive and negative selection in the thymus.

Regulation of V(D)J recombination

The process of V(D)J recombination must be regulated at many levels to insure that rearrangement occurs in the appropriate lineages (e.g. TCR genes in T cells and Ig genes in B cells), to insure that each B or T cell is monospecific (allelic exclusion), and to account for the developmentally ordered rearrangement of certain Ig and TCR gene segments.

Lineage-related control of IgH and TCRβ genes appears to be limited to the V to DJ step, as various instances of DJ rearrangements of these genes are seen in the inappropriate cells (Asarnow et al., 1988, Ferrier et al., 1990, Marusic-Galesic et al., 1988b, Okada et al., 1994, Serwe & Sablitzky, 1993)

One level of control, allelic exclusion, is the mechanism responsible for each B or T cell having only one antigen specificity. For IgH chains, this is accomplished by the presence of the functional μH protein, which shuts off further IgH rearrangement (reviewed in (Yancopoulos & Alt, 1986)). Once a light chain is produced that can pair with heavy chain, rearrangement of light chain also ceases. In T cells, allelic exclusion may be only partially controlled at the level of rearrangement, and may involve regulation of transcription of rearranged genes and/or selective pairing between chains (reviewed in (Malissen et al., 1992)).

The developmentally ordered rearrangement of certain gene segments represents another restraint on V(D)J recombination. For

example, the most J_H proximal V_H Ig gene segments rearrange earlier in development, and the more 5' segments rearrange later (Malynn et al., 1990, Schroeder et al., 1987, Schroeder et al., 1988, Yancopoulos et al., 1984, Yancopoulos et al., 1988). TCR Vγ genes of the Cγ1 cluster rearrange in a similar developmentally ordered manner (Garman et al., 1986), and Chapter 3).

In all these cases, selective rearrangement occurs even though the V(D)J recombinase is active and all gene segments use the same signal sequences. How then are these various controls exerted on the rearrangement process? The accessibility model (Alt et al., 1986) proposed that rearrangement is regulated by the ability of the locus or of individual gene segments to serve as recombination substrates, which in turn is determined by their accessibility to the recombinase machinery.

Earlier studies have shown a correlation between transcription of unrearranged genes and their subsequent rearrangement. For example, transcripts of unrearranged V_HJ558 genes appear in developing fetal liver pre-B cells immediately before rearrangement of the corresponding genes (Lennon & Perry, 1990, Yancopoulos & Alt, 1985). In the case of a recombination substrate which has been transfected into a pre-B cell line, selection for transcription of a linked gene results in high rates of subsequent rearrangement (Alt et al., 1986). Induction of κ gene rearrangement in pre-B cell lines correlates with expression of unrearranged Cκ and Vκ genes (Martin et al., 1991, Schlissel & Baltimore, 1989). Rearrangement of TCR Vα genes by a cell line in culture correlated with transcription of the unrearranged genes (Fondell & Marcu, 1992). Results of these

studies suggest that accessibility of an unrearranged gene to the recombinase machinery may be controlled, at least in part, by transcription.

This correlation between transcription and rearrangement was strengthened by experiments demonstrating that transcriptional control elements also play a role in regulating rearrangement. For example, rearrangement of a transgenic recombination substrate was found to occur efficiently only in the presence of an active transcriptional enhancer (Ferrier et al., 1990). Rearrangement of a transgenic recombination substrate from the chicken Igλ locus was severely reduced if either the enhancer or the promoter was deleted, but was increased by the removal of an intronic silencer element (Lauster et al., 1993). Finally, mice which have had their IgH intronic enhancer deleted by homologous recombination have severely reduced V-DJ rearrangements (Serwe & Sablitzky, 1993), and κ intronic enhancer knockout mice do not rearrange their κ genes at all (Takeda et al., 1993).

How could transcription be responsible for accessibility? Transcription factors can bind to a promoter site, causing the disruption of the nucleosome structure. Elongation causes displacement of nucleosomes as the polymerase reads through (reviewed in (Adams & Workman, 1993, Felsenfeld, 1992)). However, the fact that transcription is correlated with accessibility in many cases does not mean that transcription *per se* causes accessibility. Accessibility may be controlled at another level which coincidentally allows transcription to occur.

There are examples in which the absence of transcription, due to the lack of promoter elements (Engler et al., 1991) or treatment with transcriptional inhibitors (Hsieh et al., 1992), still allows rearrangement of transfected recombination substrates. These observations suggest that transcription is not absolutely necessary for accessibility. However, the presence of a transcriptional enhancer may be. In all cases thus far, deletion of the enhancer from transgenic recombination substrates or endogenous genes abolishes or severely reduces rearrangement (Chen et al., 1993, Engler et al., 1991, Ferrier et al., 1990, Kallenbach et al., 1993, Okada et al., 1994, Serwe & Sablitzky, 1993, Takeda et al., 1993). In a recent study, the Ig H enhancer was demonstrated to induce chromatin accessibility directly, even when the region responsible for transcription was deleted (Jenuwein et al., 1993).

What role might the enhancer play in accessibility? In mice, the rabbit κ enhancer is not competent to activate transcription in mouse pre-B cells, However, transgenic recombination substrates containing this element rearrange. It was suggested that merely the binding of transcription factors to the enhancer is sufficient to allow accessibility (Kallenbach et al., 1993). Other studies have shown that highly methylated transgenic recombination substrates do not rearrange efficiently, indicating that the degree of methylation may affect a gene's accessibility to recombinase (Engler et al., 1993, Hsieh & Lieber, 1992). Recently, the intronic Ig κ enhancer was demonstrated to cause demethylation of its gene in a cell-type and stage specific manner when transfected into various cell lines

(Lichtenstein et al., 1994), suggesting another way in which enhancers may affect accessibility.

Although enhancers have been clearly implicated in accessibility of TCR and Ig genes to the recombinase machinery, other elements may be necessary to limit the lineage-specificity of this process. For example, transgenic recombination substrates consisting of TCRβ V, D, J segments and an Ig Cμ with either an IgH enhancer or a TCRβ enhancer undergo D to J rearrangement in both B and T cells. However, complete V to DJ rearrangement only occurs in T cells, indicating that the promoters or some other elements may play a role in restricting this to the appropriate lineage (Ferrier et al., 1990, Okada et al., 1994). In support of this is the observation that TCR and Ig enhancers are usually lymphoid-specific, but not lineage-specific (reviewed in (Leiden, 1993, Staudt & Lenardo, 1991)).

In earlier studies, the presence of transcripts of unrearranged genes, although perhaps not essential for rearrangement, were taken to be an indicator of the gene's accessibility. However, more recent studies have shown that high levels of transcript can be detected in the absence of rearrangement, suggesting that in some cases, yet another level of control is being exerted on the rearrangement process (Bottaro et al., 1994, Okada et al., 1994).

In summary, transcription is correlated with rearrangement in many cases, but transcription *per se* may not be responsible for accessibility. Enhancers do appear to be essential in regulating accessibility, but lineage-specificity of rearrangement in some cases may be controlled by other elements, such as promoters. Finally, an

additional step may be required following accessibility of genes to activate rearrangement.

Chapter 2

MATERIALS AND METHODS

Mice. Balb/c-ByJ and AKR/J mice were purchased from Jackson Laboratories, Bar Harbor, ME. Breeding pairs of CB17 wild type and SCID mice were purchased from Taconic, Germantown, NY, and were bred and maintained at University of California, Berkeley. C57BL/6, C57BL/6-Ly5.1 and all fetal mice were bred at the University of California, Berkeley.

Antibodies. Monoclonal anti-CD8 (AD4(15) (Raulet et al., 1980), 3.168.8 (Sarmiento et al., 1980)) and anti-CD4 (GK1.5 (Dialynas et al., 1983), RL172.4 (Ceredig et al., 1985)) antibodies for complement kills were used in the form of culture supernatants. Phycoerythrin-conjugated anti-CD3ε (500-A2), anti-CD4, anti-CD8, FITC-conjugated anti-CD8 and biotinylated anti-Thy1.1 were purchased from Pharmingen, Inc., San Diego, CA. Other antibodies; anti-Vγ3 (536, (Havran et al., 1989)), anti-Thy 1.2 (30H12, (Marshak-Rothstein et al., 1979)), and anti-Ly5.1(A201.7, (Shen et al., 1982)) were provided by the Cancer Research Laboratory, University of California, Berkeley.

Cell lines. The 7.17.A2 cell line ((Kuziel et al., 1987), kindly provided by R. E. Tigelaar) was grown in RPMI-1640 supplemented with 5% FCS, 50μM 2-ME, 0.2 M HEPES, antibiotics and 20 U/ml hu-rIL-2 (Cetus Corp., Emeryville, CA).

Thymocyte Isolation. Gestational age of fetuses was determined as the number of days following the morning of appearance of a vaginal plug (day 0). Total thymocytes were used from embryonic day 14 and 15 (E14, E15) fetuses. $CD4^-CD8^-$ thymocytes were isolated from E18 and adult (3-4 week old) mice, by treatment of the cells with anti-CD4 and anti-CD8 antibodies plus complement (a mixture of rabbit (Cedarlane Laboratories, Ontario, Canada) and guinea pig (Gibco, Grand Island, NY) sera (Holsti & Raulet, 1989)). Fetal thymocytes were pooled from several litters and adult thymocytes from at least 10 animals. For E15, E18 and adult mice, debris from the disrupted thymus capsules was allowed to settle out during thymocyte isolation. This step was omitted when isolating E14 thymocytes to increase cell yield.

$CD3^-$ cells were prepared as follows: total thymocytes from E15 or $CD4^-CD8^-$ thymocytes from adult mice were stained with PE-conjugated antibody against the T cell receptor-associated CD3-ε chain. The $CD3^-$ cells were purified by electronic cell sorting using a FACS IV (Becton Dickinson). Dead cells were gated out on the basis of forward scatter and propidium iodide uptake.

Nucleic Acid Preparation. Genomic DNA was isolated from thymocytes as described (Maniatis et al., 1982). Total RNA was isolated using the guanidinium/CsCl method (Chirgwin et al., 1979). In cases where RNA was isolated from small cell numbers, 20μg E. coli rRNA (Boeringer-Mannheim Biochemicals, Indianapolis, IN) was added as carrier. DNA fragments corresponding to rearranged and

unrearranged Vγ genes were subcloned into Gemini plasmids (Promega Biotech, Madison, WI), to allow the production of DNA and RNA templates of known quantities used to standardize the quantitative PCR experiments. Synthetic RNAs used as standards for absolute quantitation were transcribed in vitro using T7 or SP6 RNA polymerase (Promega Biotech, Madison, WI). All RNA samples were treated by digestion with RQ-1 RNase-free DNase (Promega Biotech, Madison, WI) to remove any contaminating genomic or plasmid DNA. RNA and DNA samples were quantitated spectrophotometrically and their condition observed on ethidium bromide stained agarose gels.

Southern blot analysis. Southern blot analysis was as described (Garman et al., 1986) with the following exceptions: prehybridization wash was omitted and after hybridization, filters were washed twice with 2X SSC, 0.05% SDS at room temp. and twice with 0.1X SSC, 0.05% SDS at 60°C.

PCR primers. Synthetic oligonucleotides used as primers are as follows: CTGGGAATTCAACCTGGCAGATG (L2), GCTAAGAAGGATGTG GGTTG (V2-3'a), CCAGCAGCCACTAAAATGTC (L3), TGGAGGATCCTTGGT GGGTTCA (V3-3'a), ACTGAGGGCACCCAAGGGGATAG (V3-3'c), GGATGG GGATCCTGCTACAAGTC (L4), GGAAGGAATTGTGTGCACAGGT (V4-3'a), GGCGCCCTCTGTGTAGTGGCCTTTGGCCCA (3'β-tubulin), and CAGGCTGGTC AATGTGGCAACCAGATCGGT (5'β-tubulin). V2, V3, V4 and J1 primers have been described previously (Asarnow et al., 1989).

Quantitative PCR of DNA. Serial dilutions of DNA samples were prepared. PCR (Saiki et al., 1988) was carried out in a total volume of 100 ml consisting of 25 pmoles each primer, 200mM each dNTP, 1.5 mM $MgCl_2$, 1x PCR buffer (Cetus Corp., Emeryville, CA) and 2.5 U Amplitaq (Cetus Corp., Emeryville, CA). Samples were heated to 94°C for 3 min. followed by amplification for 35 cycles of 1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C. After the last cycle, a final extension step at 72°C for 10 min was done. 25 ml of each PCR reaction was run on 2% agarose gels in TBE buffer. Products were visualized by ethidium bromide staining. Southern hybridization analysis of the gels with Vγ-specific oligonucleotide probes confirmed the identity of bands corresponding to rearranged Vγ-Jγ genes (data not shown).

Quantitation of the target sequence in the initial nucleic acid sample was accomplished by comparison to the amount of product amplified from titrated quantities of plasmid DNA standards containing the same target sequence. The plasmid DNA standard was prepared by adding 1 pg of plasmid to 1μg of herring sperm DNA, which was then subjected to serial dilution.

Quantitative PCR of RNA. Total RNA was titrated by serial dilution into a solution containing 1mg/ml E. coli rRNA. cDNA was prepared using 200 U MoMuLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), primed with 25 pmoles of the antisense oligonucleotide primer in a total volume of 20 μl containing 1 U/ml RNAsin (Promega Biotech, Madison, WI), 1mM each dNTP, 1.5 mM $MgCl_2$ and 1x PCR buffer. This was incubated for

30-45 min. at 42°C, and the enzyme heat inactivated at 99°C for 5 min. Some reactions were done without reverse transcriptase as a control against the presence of contaminating DNA. PCR was performed using the above cDNA mixture diluted to 100µl by the addition of 1x PCR buffer, 1.5mM $MgCl_2$, 25 pmoles sense primer and 2.5 U Amplitaq. Amplification was carried out as described above.

25 µl of each PCR reaction was run on 2% agarose gels in TBE buffer. Products were visualized by ethidium bromide staining. In some cases the DNA was transferred to nitrocellulose followed by hybridization (Maniatis et al., 1982) of the blot with ^{32}P-end-labeled unique oligonucleotides internal to the PCR primers. Sequences of the probes are as follows: GAGGCTATTCTGGAAGCTCAG (V2-3'b), TATCCCCTTGGGTGCCCTCAGT (V3-3'b), GCGGGAGTGGGACTTGTCTTGTTT (V3C), TCACCTGCACAGACACCTAG (V4-3'b), ACCTGAGCGAACAGAGTCC ATGGTCCC (β-tubulin).

Quantitation of the target sequence in the initial nucleic acid sample was accomplished by comparison to the amount of product amplified from known quantities of synthetic RNA standards containing the same target sequence. The results were adjusted to account for different content of mRNA, as determined by a parallel analysis of tubulin transcripts in the RNA samples.

The synthetic RNA standards were generated by transcription of corresponding linearized plasmid DNA templates. For the reactions in Chapters 4, 6 and 7, these sequences corresponded to the unrearranged Vγ3, Vγ4 or Vγ2 genes. For the reactions in Fig. 5-3A, the templates corresponded to cloned PCR products of L3-V3-J1 or L4-V3-J1 transcripts. These cloned PCR products were also used as

the templates for the reactions in Fig. 5-3B, except that the Vγ3-3' sequence was substituted for the Jγ1 sequence in each plasmid.

RNase protection assay. The RNase protection assay was a modification of the procedure of Melton (Melton et al., 1984) described previously (Garman et al., 1986).

Repopulation of fetal thymic organ cultures. This procedure was performed as described previously (Ikuta et al., 1990), with minor modifications. Briefly, thymic lobes were dissected from C57BL/6 fetal mice at day 14 of gestation and cultured on polycarbonate filters in Transwell plates (Costar, Cambridge, MA) in DMEM containing 10% FCS, 50μM 2-ME, antibiotics (DMEM-10) and 1.35mM deoxyguanosine (Sigma, St. Louis, MO) for five days. Each lobe was then washed with DMEM-10 without deoxyguanosine, and put into hanging drop culture for 24 hours in 20 μl containing $3X10^5$ fetal liver or adult bone marrow cells from C57BL/6-Ly5.1 mice. Lobes were then placed in culture on Transwell plates for 11 days. Cells isolated from these cultures were subsequently used for staining or isolation of RNA.

Construction of chimeric mice. AKR-->SCID chimeras were constructed as described previously (Shores et al., 1990) with the exception that the unirradiated CB17 SCID were injected i.v. with $3X10^7$ fetal liver, not bone marrow cells. In addition, control chimeras were constructed by injecting SCID mice with SCID fetal liver cells (SCID-->SCID). AKR-->$SCID_{neo}$ chimeras were constructed

by i.p. injection of 0-1 day old SCID neonates with $5X10^6$ AKR fetal liver cells. SCID-->AKR chimeras were constructed by injecting irradiated (1000 rad) 8-12 week old AKR mice with $3x10^7$ SCID E14 fetal liver cells. Control chimeras included lightly irradiated (200 rad) SCID mice injected with SCID fetal liver (SCID-->$SCID_{irr}$) and irradiated AKR injected with CB17wt fetal liver (CB17-->AKR). All chimeras were analyzed 8-12 weeks post-injection.

Analysis of chimeric mice. Peripheral blood lymphocytes from AKR-->SCID were stained with anti-Thy1.1-biotin + streptavidin-PE (Molecular Probes, Eugene, OR) and analyzed on a FACscan (Becton & Dickinson, Mountain View, CA) to determine chimerism. Thymocytes were isolated from the AKR-->SCID chimeras which were positive for Thy-1.1 and pooled. $CD4^-CD8^-$ cells were enriched by treatment with anti-CD4, anti-CD8 and complement, and the resulting cells were stained with anti-Thy1.2-FITC and anti-CD4-PE+anti-CD8-PE. Cells positive for PE (CD4 and/or CD8) were gated out and the host $CD4^-CD8^-Thy\ 1.2^+$ cells were isolated by sorting on an EPICS Elite flow cytometer (Coulter, Hialeah, FL). Cells which had been set aside prior to the complement kill were analyzed on the EPICS after staining with CD4-PE, CD8-FITC and either Thy-1.1-biotin or Thy-1.2-biotin + streptavidin-tricolor.

Thymocytes from SCID-->AKR or CB-->AKR chimeras were pooled and enriched for $CD4^-CD8^-$ cells as above. The remaining cells were stained with anti-CD4-PE+anti-CD8-PE and anti-Thy-1.2-FITC, and $CD4^-CD8^-Thy1.2^+$ cells were isolated by sorting. As above some cells were analyzed prior to the complement kill.

$CD4^-CD8^-$ thymocytes were isolated from the SCID-->SCID and SCID-->$SCID_{irr}$ chimeras by treating with anti-CD4, anti-CD8 and complement.

RNA isolated from these various populations was subjected to the quantitative PCR assay described above.

Epidermal cell preparation. Cells were isolated from the epidermis of AKR-->SCID and AKR-->$SCID_{neo}$ chimeras using a procedure described previously (Sullivan et al., 1985).

Chapter 3

ORDERED REARRANGEMENT OF T CELL RECEPTOR Vγ GENES

Introduction

It is known that γδ T cell subtypes arise in ordered waves during ontogeny. However, the mechanism responsible for this is poorly understood. Two general models can be invoked to explain this phenomenon. In a "selection" model, rearrangement of the T cell receptor genes occurs randomly, and the appearance of temporally restricted waves of particular Vγ-expressing cells are generated by the selection of those receptors on stage-specific thymic ligands. In a "targeting" model, rearrangement is not random, but is programmed on the basis of the fate of the cell. Thus, the sublineage of a progenitor cell is established, and subsequently the appropriate Vγ gene segment is targeted for rearrangement.

A model in which targeted rearrangement of T cell receptor γ genes is responsible for the ordered appearance of γδ T cell subtypes during ontogeny would predict that the rearrangement of these genes at different stages of development would occur in the same order. Previous studies from this lab (Garman et al., 1986) provided evidence that Vγ3 and Vγ4 rearrangements are more abundant in early fetal thymocytes than in adult thymocytes, while Vγ2 rearrangements are more frequent in adult than fetal thymocytes (see Figs. 1-1 and 1-2 for arrangement of Vγ genes). Those studies employed unfractionated preparations of thymocytes at most time points, hence the majority of the cells at the later time points were

$CD4^+CD8^+$ phenotype, most of which are in the $\alpha\beta TCR^+$ lineage. Furthermore, a report from another group challenged the claim that $V\gamma$ genes are differentially rearranged during ontogeny (Carding et al., 1990). This chapter describes quantitative analysis of T cell receptor γ gene rearrangements in $CD4^-CD8^-$ thymocyte populations, in which most $\gamma\delta$ cells and progenitor cells are found.

Results

Initially, $V\gamma$ gene rearrangements during development were quantitated by Southern blot analysis of EcoRI-digested $CD4^-CD8^-$ thymocyte DNA from various stages of development. The observed pattern of $V\gamma$ rearrangements was similar to the pattern that was initially reported: $V\gamma2$ rearrangements are evident by E15 (day 15 of gestation) and increase during development to maximal levels in the adult (17 kb band in Fig. 3-1A). $V\gamma3$ rearrangements are evident by E15 and at E18 (day 18 of gestation), but decrease to undetectable levels by the adult stage (18 kb band in Fig. 3-1B). $V\gamma4$ rearrangements are less abundant than $V\gamma3$ rearrangements, but follow a similar developmental pattern (17 kb band in Fig. 3-1B).

The Southern blot analysis fails to provide sufficient quantitative information to compare the extent of rearrangement of different V genes. Therefore, the $CD4^-CD8^-$ thymocyte DNA samples were also subjected to a quantitative PCR analysis, in which rearranged alleles were amplified from titrated samples of thymocyte DNA using a $J\gamma1$ antisense primer (J1) and a sense strand primer from the leader exon of each $V\gamma$ gene (L3, L4 or L2 primer; for locations and sequences of primers and probes, see Fig. 3-2 and

Figure 3-1: Southern blots of EcoRI-digested genomic Balb/c DNA from E14 and E15 thymocytes, or from E18 and adult $CD4^-$ $CD8^-$ thymocytes. The DNA was gel fractionated, blotted, and probed with: **(A)** a Vγ2-specific probe (a 695 bp Ava1-Cla1 fragment from the Vγ2 gene) or **(B)** a Vγ4- and Vγ3-specific probe (a 2.6kb Pvu2-Hind3 which includes the Vγ4 gene and upstream sequence. Because Vγ4 and Vγ3 are on the same germline EcoRI fragment, the latter probe will detect rearrangements of either Vγ3-Jγ1 (an 18 kb fragment) or Vγ4-Jγ1 (a 17 kb fragment) (Garman, et al, 1986). The origin of the weak bands at 7 kb and 16 kb in adult thymocyte DNA in **(A)** is uncertain, but they have not been seen in other similarly probed blots of adult thymocyte DNA.

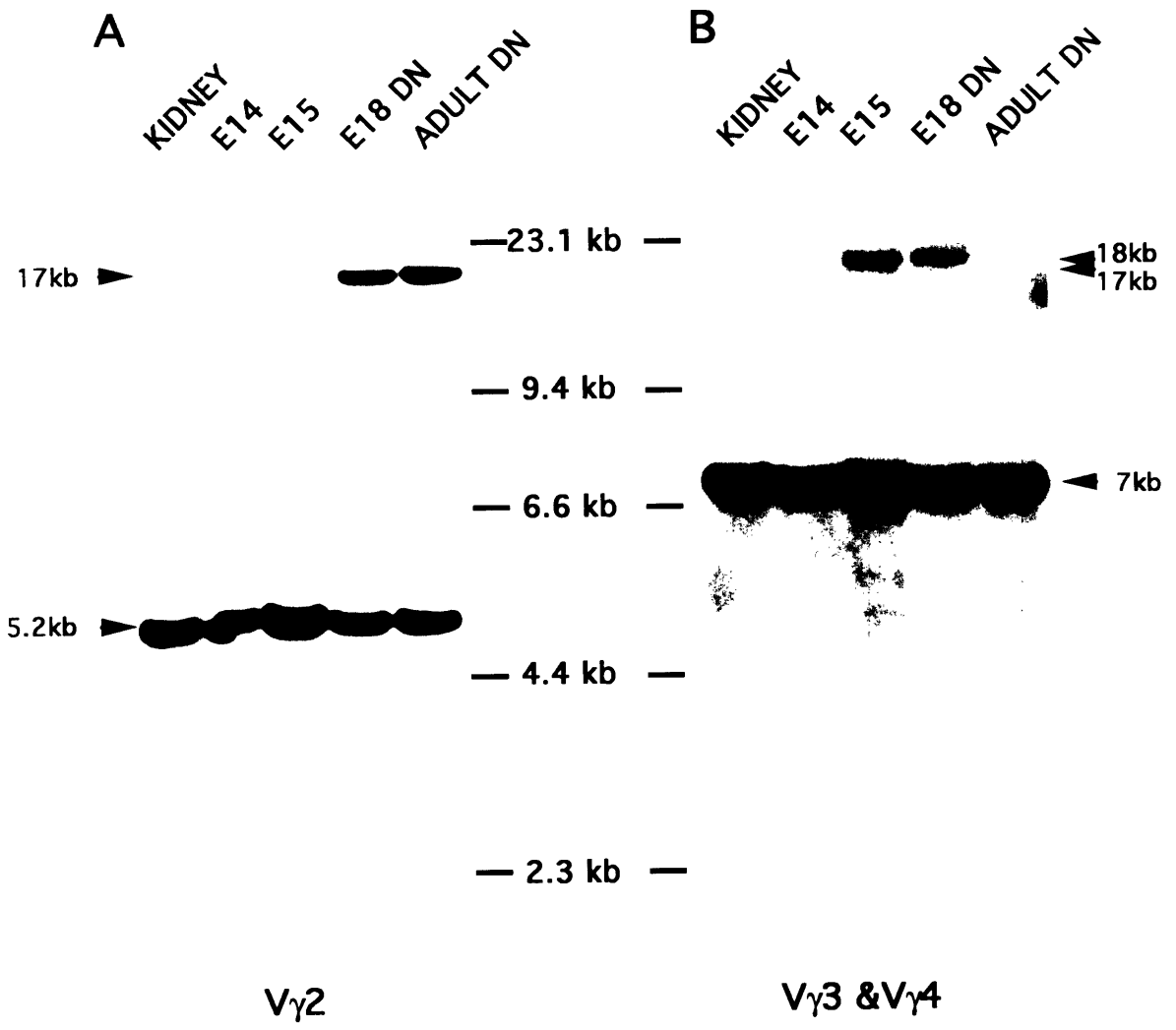
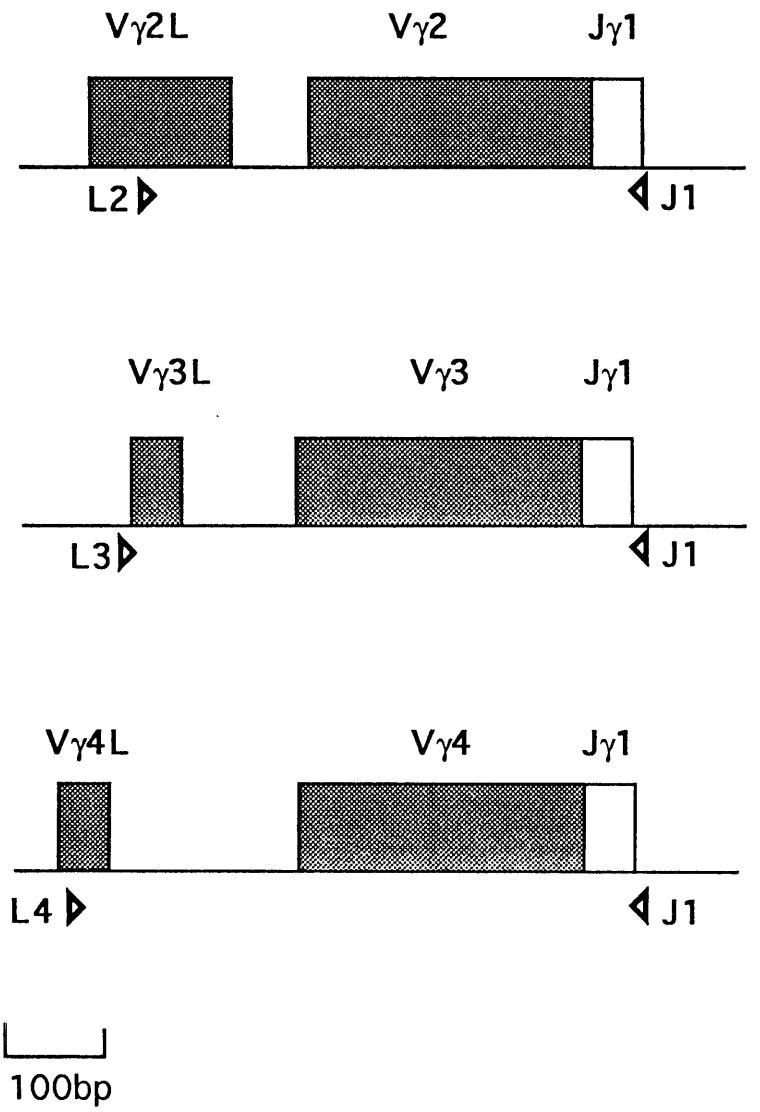

A
KIDNEY
E14
E15
E18 DN
ADULT DN
17kb
5.2kb
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
B
KIDNEY
E14
E15
E18 DN
ADULT DN
18kb
17kb
7kb
Vγ2
Vγ3 &Vγ4

Fig 3-2: Locations of primers used in this study. (▶)PCR sense primer, (◀) PCR anti-sense primer


Vγ2L
Vγ2
Jγ1
L2
J1
Vγ3L
Vγ3
Jγ1
L3
J1
Vγ4L
Vγ4
Jγ1
L4
J1
100bp

Materials and Methods). For the purpose of quantitation, plasmid DNAs containing the corresponding rearranged Vγ-Jγ1 gene were titrated in parallel PCRs. The products of the PCR reactions were separated on an agarose gel, visualized by ethidium bromide staining, and quantitated by comparison to the reference DNA (Fig 3-3). This approach allows the determination of the approximate frequency of each rearranged gene in the genomic DNA population. The identity of the various bands was confirmed by Southern hybridization analysis, with the use of Vγ specific oligonucleotide probes (data not shown).

The PCR analysis revealed that Vγ2 rearrangements are very rare in E14 DNA, and increase ~250-fold during subsequent development to reach a maximum at E18 (Fig. 3-3, plotted in Fig. 3-4A). Vγ3 and Vγ4 rearrangements are also relatively infrequent at E14, and their abundance increases ~20-fold by E15. But unlike Vγ2 rearrangements, Vγ3 and Vγ4 rearrangements decrease later in development, by ~30-fold and ~10-fold, respectively.

The quantitative PCR assay allowed comparison of the frequencies of different Vγ gene rearrangements at given time points (data summarized in Fig. 3-4B as percentages of summed Vγ2, Vγ3 and Vγ4 rearrangements). Vγ2 rearrangements are relatively rare at E14, being ~1/10 as frequent as Vγ3 rearrangements, but subsequently increase to substantially exceed either Vγ3 or Vγ4 rearrangements by the late fetal stage and in the adult. Vγ3 rearrangements, which predominate at E14 and E15, decrease to ~1/50 the level of Vγ2 in the adult. Moreover, the comparisons indicated that Vγ3 rearrangements are more abundant (4-10 fold)

Fig 3-3: PCR analysis of Vγ rearrangements to Jγ1 in thymocyte genomic DNA samples. Dilutions of genomic DNA or plasmid DNA standards were used as templates for PCR. Above each lane is the amount of DNA included in the initial reaction. To amplify rearranged γ genes, the J1 antisense primer and the L3, L4 or L2 sense primers (Fig. 3-2) were used. Control PCR using the 5' and 3' β-tubulin primers were performed to normalize for the amount of genomic DNA in a sample. 25μl of each reaction was run on ethidium bromide-stained 2% agarose gels. The size of each product was as predicted: Vγ3, 520 bp; Vγ4, 630 bp; Vγ2, 550 bp; tubulin, 310 bp. As a control, PCR with each set of primers was also performed using herring sperm DNA instead of template and yielded no detectable product (not shown).

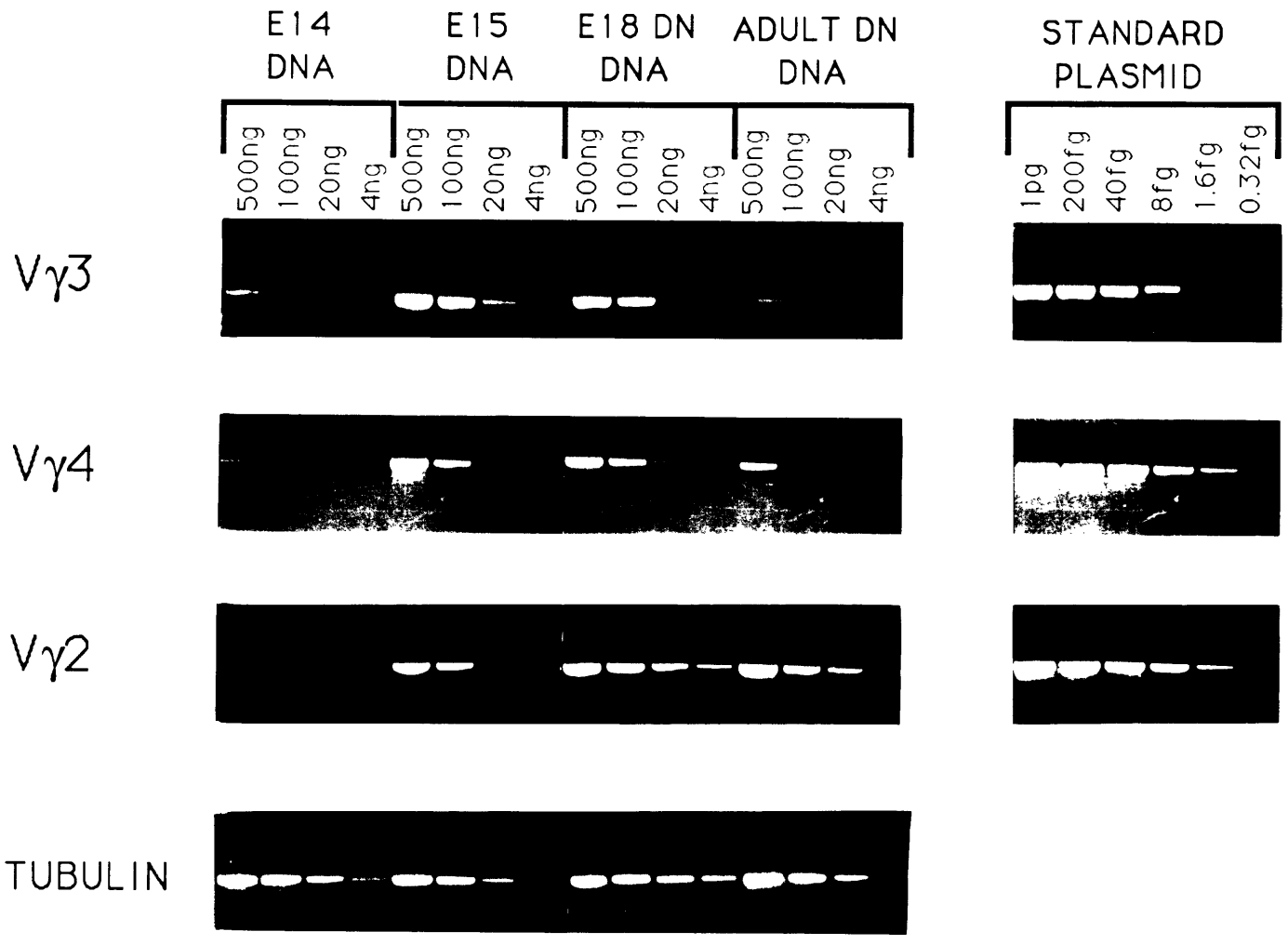
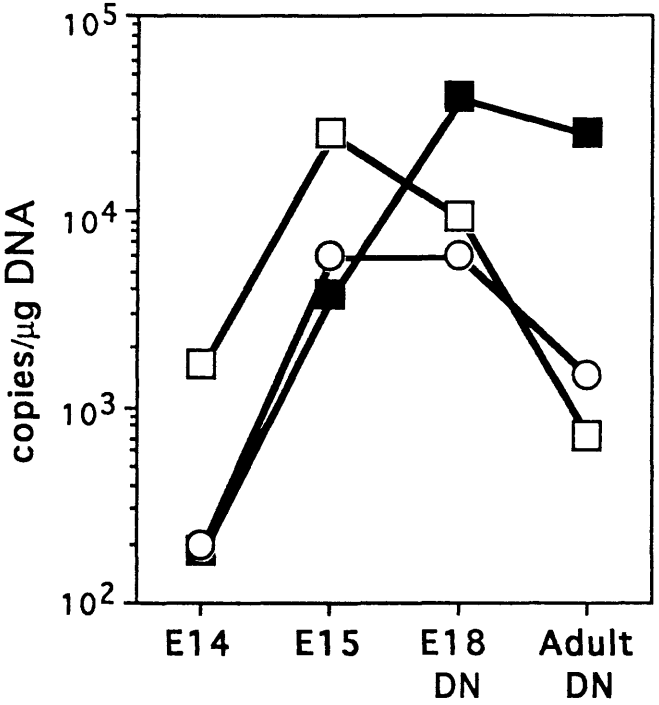
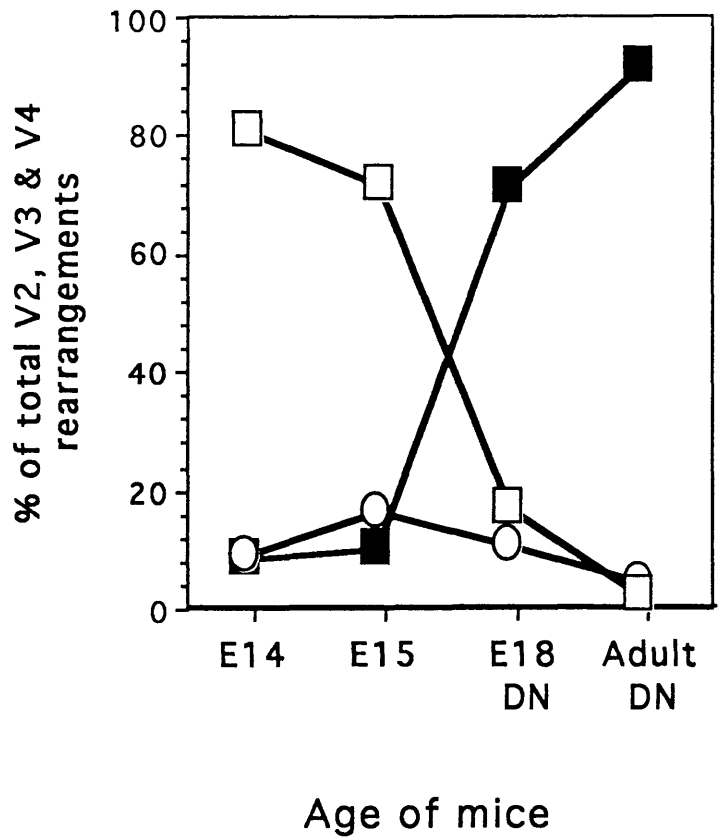

E14 DNA
E15 DNA
E18 DN DNA
ADULT DN DNA
STANDARD PLASMID
500ng
100ng
20ng
4ng
1pg
200fg
40fg
8fg
1.6fg
0.32fg
Vγ3
Vγ4
Vγ2
TUBULIN

Fig 3-4: Summary of rearrangements of Vγ2 (■), Vγ3 (❑), and Vγ4 (○) genes at different stages of ontogeny. The abundance of Vγ rearrangements is expressed either as **(A)** number of copies/μg DNA, or **(B)** as a percent of summed Vγ2, Vγ3 and Vγ4 rearrangements. Quantitative densitometry was used to determine the values from a single experiment, which are depicted in the figure. The results were corroborated in at least one additional complete experiment of each type. The levels of Vγ rearrangements at E14 may be slightly underestimated, due to contamination of this sample with thymic capsule cells (see Materials and Methods).

A


copies/μg DNA
10^5
10^4
10^3
10^2
E14
E15
E18 DN
Adult DN
Age of mice

B


% of total V2, V3 & V4 rearrangements
100
80
60
40
20
0
E14
E15
E18 DN
Adult DN
Age of mice

than Vγ4 rearrangements in the fetal thymocyte DNA samples. Thus, the relative frequencies of the different Vγ rearrangements correspond reasonably well to the frequencies of Vγ expressing cells at the different time points (see Discussion). The analyses presented here corroborate and extend earlier results showing that Vγ genes are rearranged differentially during development.

Discussion

This analysis provides quantitative comparisons of the extent of each Vγ gene rearrangement in the $CD4^-CD8^-$ population at different stages of development. The results fulfill the prediction of the targeting model with regard to ordered rearrangement of Vγ T cell receptor genes, which correlates with the ordered appearance of γδ T cell subtypes during ontogeny.

Discrepancies between the extent of rearrangement of each Vγ gene and the representation of cells expressing a Vγ product on the cell surface might be used to argue for selection events acting on nascent γδ cells. In fact, the extent of Vγ gene rearrangements in the early fetal and adult stages, when adjusted for the rates at which each type of rearrangement is found to be productive, roughly corresponds to the relative frequencies of cells expressing a given Vγ product on the cell surface. The frequencies of Vγ3 and Vγ4 rearrangements in the fetal thymus that are productive (~60%) is much higher than the frequency of Vγ2 rearrangements that are productive (~10%) (Lafaille et al., 1989). Using these approximate values, it can be calculated that the ratios of productive

rearrangements at E14 is ~50Vγ3:6Vγ4:1Vγ2 which fits with the reported predominance of Vγ3+ cells in the early fetal thymus (Havran & Allison, 1988, Itohara et al., 1989). At E18, adjustment of the values for the rates of productive rearrangements yields a ratio of ~2Vγ3:1Vγ4:1Vγ2, consistent with the pattern of Vγ surface expression found in one study (Itohara et al., 1989). In the adult CD4-CD8- population, the ratio, calculated from our data using the reported approximate frequencies of adult thymic Vγ rearrangements that are productive (50% of Vγ3, 37% of Vγ4 and 21% of Vγ2, (Lafaille et al., 1989)), is ~1Vγ3:2Vγ4:15Vγ2. These values fit well with the findings that Vγ2+ cells greatly outnumber Vγ3+ and Vγ4+ cells in the adult (Havran & Allison, 1988, Itohara et al., 1989).

Hence, there is a good concordance between the extent of V gene rearrangement and the abundance of Vγ+ cells. In contrast, previous studies demonstrated that the levels of rearranged Vγ/Jγ1 transcripts correlate poorly with the representation of cells expressing a given Vγ product on the cell surface (Carding et al., 1990, Garman et al., 1986). However, these studies examined the levels of transcripts of the rearranged genes which can be influenced by numerous variables and are not necessarily correlated with the extent of DNA rearrangements. For example, Vγ2 genes are frequently rearranged in αβ T cells, but are usually transcriptionally silent (Garman et al., 1986, Heilig & Tonegawa, 1986).

This is not the only study to offer evidence in favor of the molecular targeting model. A prediction of this model is that rearrangement in individual T progenitor cells is somehow restricted to a particular Vγ gene segment. This is supported by the

observation that in a small panel of well-characterized T cell lines, the rearrangement on the second, unused chromosome is either unrearranged or usually of the same type as the functional rearrangement (Raulet et al., 1991). Additionally, an analysis of rearrangements in freshly isolated $V\gamma3^+$ s-IELs using the quantitative PCR assay described here demonstrated that the vast majority of second alleles also have $V\gamma3$ rearrangements, with only a small fraction carrying $V\gamma2$ and $V\gamma4$ rearrangements (J. Baker and D Raulet, unpublished results). Finally, $V\gamma$ rearrangements were determined in thymocytes derived from mice in which the T cell receptor $C\delta$ gene was disrupted by homologous recombination (Itohara et al., 1993). Because no $\gamma\delta$ receptor appears on the surface of these cells, the levels of $V\gamma$ rearrangement would not be affected by selection. In these thymocytes, patterns of $V\gamma$ rearrangements are the same as in normal mice.

There appears to be a preponderance of evidence in support of programmed gene rearrangement. While our results represent an argument for regulation of gene rearrangment, it should be pointed out that this is not incompatible with the possibility that $\gamma\delta$ T cells are also subject to developmental selection.

Chapter 4

ORDERED PRODUCTION OF T CELL RECEPTOR Vγ STERILE TRANSCRIPTS

Introduction

The previous chapter described experiments which demonstrate that T cell receptor Vγ genes are rearranged differentially during development and that the pattern of rearrangements closely correlates with the ordered appearance of the concomitant Vγ-expressing γδ T cells in the thymus. The ordered pattern of Vγ gene rearrangement in developing T cells is similar in some respects to the rearrangement of immunoglobulin V_H genes in developing B cells. V_H gene rearrangement also occurs in an ordered manner, with the most 3' segments rearranging earlier and 5' segments later (Malynn et al., 1990, Schroeder et al., 1987, Schroeder et al., 1988, Yancopoulos et al., 1984, Yancopoulos et al., 1988). The generality of this phenomenon suggests that similar mechanisms may regulate rearrangement in the different gene families.

The accessibility model (Alt et al., 1986) attributes the control of V-(D)-J rearrangement to the regulation of access to the gene segments by the recombination machinery. Transcriptional activity of a gene can be a reflection of its accessibility, and correlations between transcription of particular gene segments and their rearrangement have been demonstrated in many instances.

No previous study has correlated differential V gene rearrangement with transcription of the genes. In order to

investigate the possibility that transcription of Vγ gene segments is related to the ordered manner in which they rearrange, I have examined transcript levels of unrearranged TCR Vγ gene segments at different developmental time points.

Results

Quantitative RT-PCR of Unrearranged Vγ Genes.

To determine if transcription is correlated with ordered Vγ gene rearrangement, I used a PCR-based assay to detect transcripts of unrearranged Vγ genes (subsequently called sterile Vγ transcripts) in fetal and adult thymocytes. Total RNA was isolated from populations of thymocytes prepared identically to those used for the DNA analysis described in Chapter 3. Using titrated samples of RNA, reverse transcription of cDNA corresponding to sterile Vγ transcripts was primed with antisense oligonucleotides corresponding to sequences 3' of the unrearranged Vγ2, Vγ3 or Vγ4 genes (V2-3'a, V3-3'a, and V4-3'a, Fig. 4-1), which are absent from the corresponding rearranged transcripts. The sterile transcripts were amplified with the same downstream primer and a corresponding upstream sense primer (either in the Vγ coding exon or in the case of Vγ3, the leader exon of the Vγ3 gene). In order to determine the amounts of the transcripts, synthetic RNAs corresponding to each unrearranged Vγ gene were titrated in parallel reverse-transcription/PCRs (see Materials and Methods). PCR products were visualized on Southern blots probed with end-labeled oligonucleotides corresponding to sequences 3' of the Vγ gene but internal to the primers used for the PCRs (V2-3'b, V3-3'b, and V4-3'b, Fig. 4-1). In addition, the level of

Figure 4-1: Locations of primers and probes used in this study. (▷)PCR sense primer, (◁) PCR anti-sense primer, (▬) oligonucleotide probe for Southerns, (▶) 7-mer/9mer recombination signal.

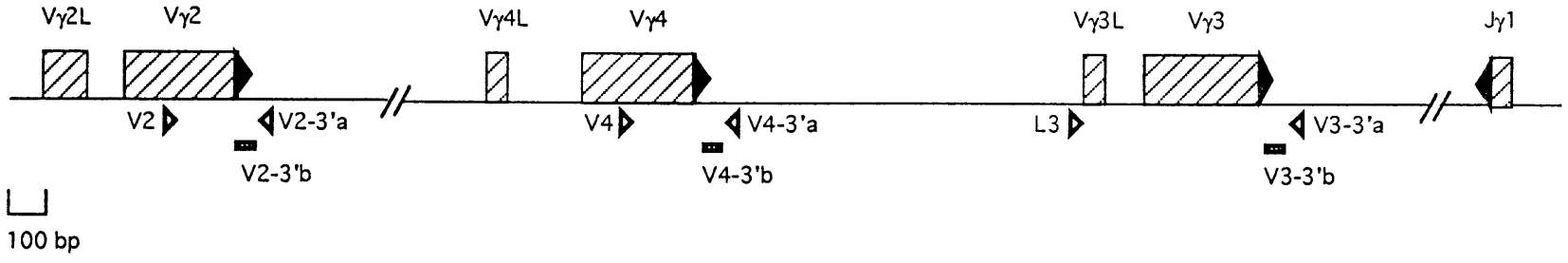

Vγ2L
Vγ2
Vγ4L
Vγ4
Vγ3L
Vγ3
Jγ1
V2
V2-3'a
V2-3'b
V4
V4-3'a
V4-3'b
L3
V3-3'a
V3-3'b
100 bp

Figure 4-2A: RT-PCR analysis of sterile Vγ transcripts in total RNA from thymocytes (see text and Materials and Methods).

Dilutions of total RNA from thymocytes, or of *in vitro* synthesized standard RNAs, were used as templates, with anti-sense primers 3' of each unrearranged Vγ gene (-3'a primers, Fig. 4-1), to direct cDNA synthesis. The sterile transcripts were amplified with the same downstream primer and an upstream V-specific primer (L3 for Vγ3, V4 for Vγ4, and V2 for Vγ2, see Fig. 1A). The products were gel-fractionated, blotted, and hybridized with oligonucleotide probes corresponding to sequences 3' of the V gene but 5' of the primer used for PCR (-3'b probes in Fig. 4-1). The sizes of the products as predicted from the sequences are: Vγ3, 450 bp; Vγ4, 334 bp; Vγ2, 288 bp; TUB, 310 bp. The size of the larger V3 product (450 bp) indicates it is derived from a correctly spliced transcript. DNA contamination of the RNA sample would have yielded a larger (550 bp) fragment, due to the presence of the intron. The standard plasmid for Vγ3 in this experiment is constructed from genomic DNA, and therefore includes the intron.

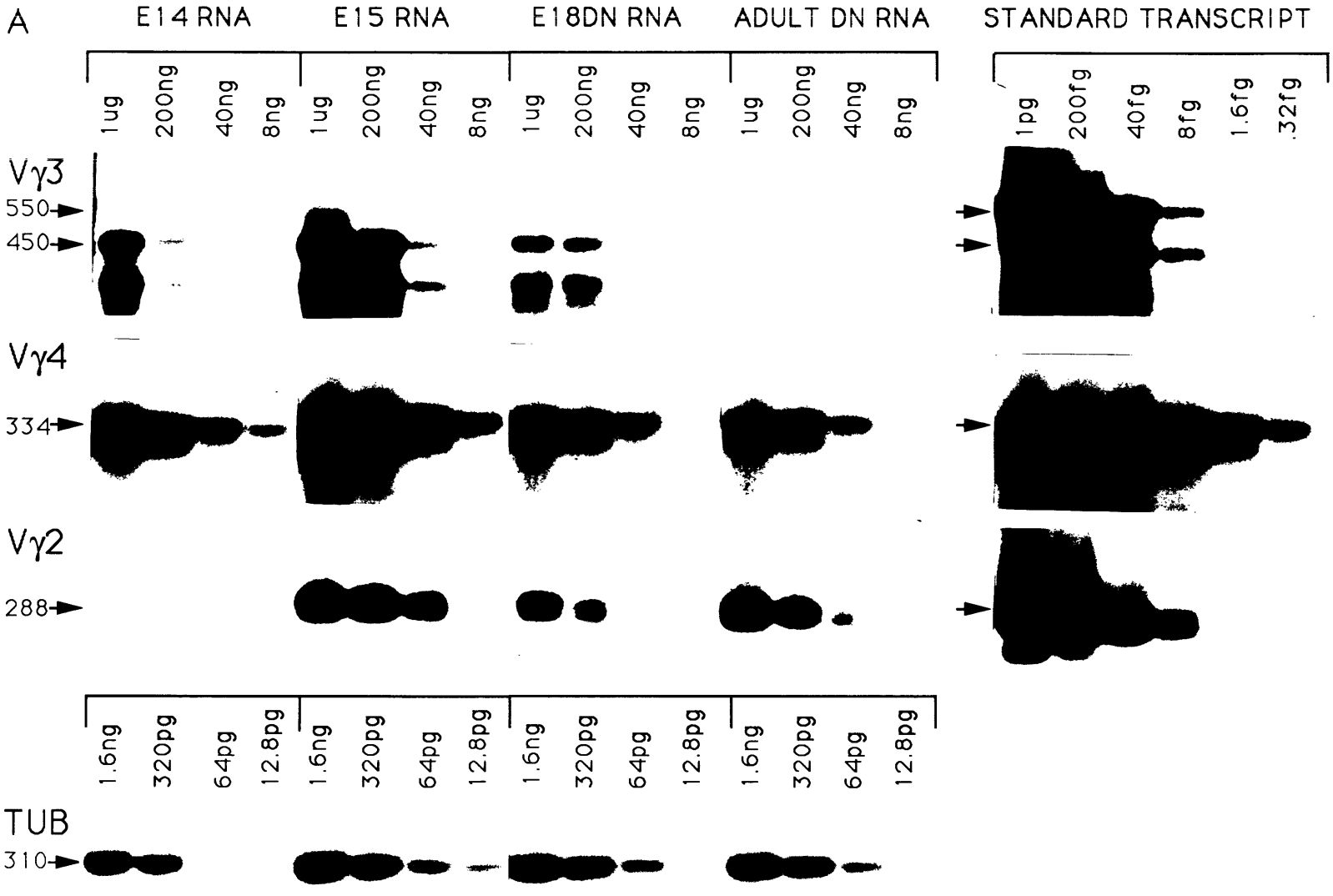
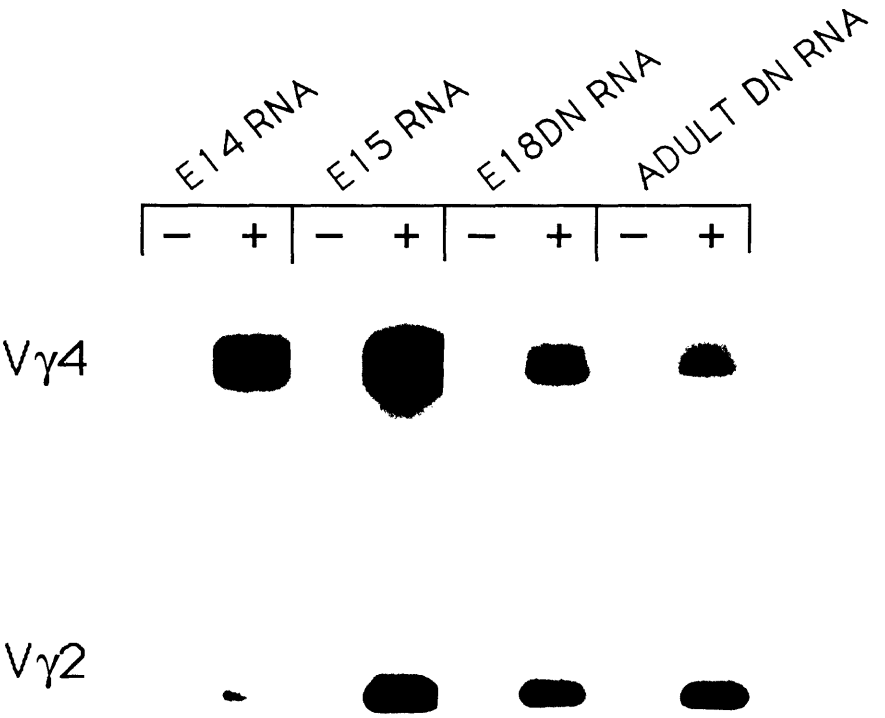

A
E14 RNA
E15 RNA
E18DN RNA
ADULT DN RNA
STANDARD TRANSCRIPT
1ug
200ng
40ng
8ng
1pg
200fg
40fg
8fg
1.6fg
.32fg
Vγ3
550
450
Vγ4
334
Vγ2
288
1.6ng
320pg
64pg
12.8pg
TUB
310

Figure 4-2B: Dependence of the PCRs on reverse transcriptase. For Vγ2 and Vγ4 PCRs, reactions were performed with 1 μg total RNA (same samples as used in Fig. 4-2A) with (+) or without (-) the addition of reverse transcriptase in the initial reaction.

B



the ubiquitously expressed β-tubulin transcript in each RNA sample was determined in parallel quantitative PCRs, in order to normalize for the amount of mRNA initially added to the PCR reaction.

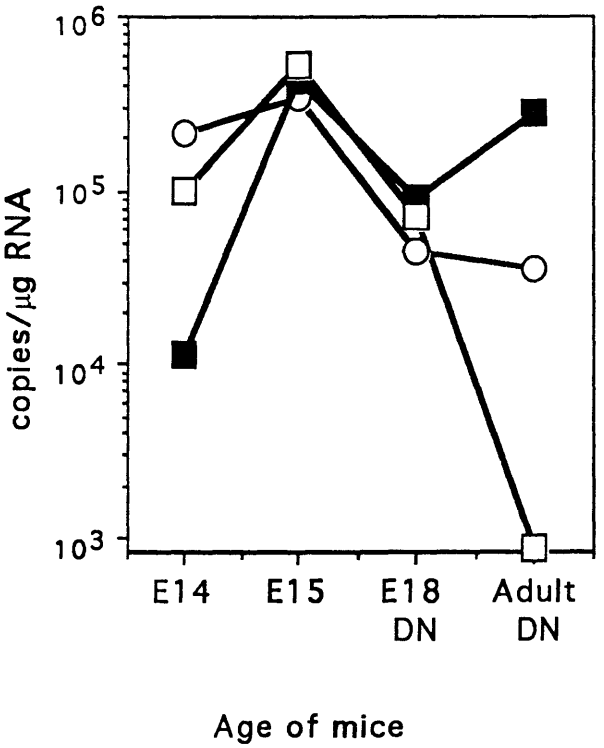
Two control experiments indicate that contamination of the RNA samples with genomic DNA was not responsible for the observed bands. In the case of Vγ2 and Vγ4, PCRs run in parallel in which reverse transcriptase was omitted in the initial step yielded no bands (Fig. 4-2B). In the case of the Vγ3 PCR, in which the primers spanned an intron, no product of the size of the unspliced 550 bp genomic DNA fragment was observed (Fig. 4-2A). Note that in both Vγ3 and Vγ4 PCR, fragments smaller than the expected fragments were observed in addition to the expected product. This has been reported in other reverse transcription/PCR experiments (Kyes, 1989, Takagaki et al., 1989b) and may result from aberrant initiation of reverse transcription within the transcript.

Sterile Vγ Transcript Levels Correlate With Rearrangement Patterns.

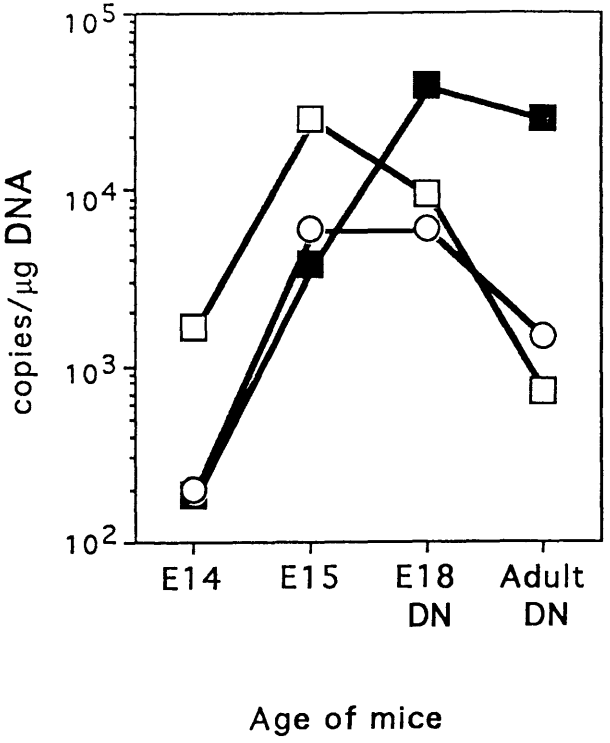
As shown in Figure 4-2A and summarized in Figure 4-3, sterile Vγ3 transcripts are relatively abundant in E14 thymocytes, increase several-fold at E15, and diminish dramatically (>100-fold) to undetectable levels by the adult stage. The sterile Vγ4 transcript shows a similar pattern, although the decline in levels between the early fetal and adult stage is less precipitous (~10-fold) than that of the sterile Vγ3 transcript. In contrast, sterile Vγ2 transcripts show the opposite pattern, in that they are very low at E14 and increased

Figure 4-3: Summary of sterile transcripts (**A**) and rearrangements (**B**) of Vγ2 (■), Vγ3 (❑), and Vγ4 (○) genes at different stages of ontogeny. Sterile transcripts are presented as the number of copies/μg total RNA. Quantitative densitometry was used to determine the values from a single experiment, which are depicted in the figure. The results were corroborated in at least one additional complete experiment of each type. The levels of sterile Vγ rearrangements and transcripts at E14 may be slightly underestimated, due to contamination of this sample with thymic capsule cells (see Materials and Methods). Note that (**B**) is identical to Figure 3-3(A) and is included here for comparison.

A transcripts of unrearranged Vγ genes



B Vγ gene rearrangements



~20-30-fold by E15 to the level they maintain in the adult stage. Considering that increased accessibility of an unrearranged gene is expected to precede rearrangement of the gene, the changes in abundance of sterile transcripts correlate very well with the observed patterns of subsequent Vγ2, Vγ3 and Vγ4 rearrangements during ontogeny (compare Figs. 4A and 4B).

It might be argued that the virtual absence of sterile Vγ3 transcripts in adult DN thymocytes is due to the deletion of most of the unrearranged Vγ3 gene segments from the thymocyte population, which would result from rearrangement of Vγ2 (or Vγ4) to Jγ1 in most of the cells. This does not appear to be the case, since a strong band corresponding to the unrearranged Vγ3 and (Vγ4) gene is present in Southern blots of DNA from adult $CD4^-CD8^-$ thymocytes (Fig. 3-1). Using the quantitative PCR assay, only a two-fold or smaller reduction was found in germline-configuration Vγ3 genes in adult $CD4^-CD8^-$ thymocyte DNA compared to fetal thymocyte DNA (data not shown).

It might also be argued that the sterile Vγ transcripts originate in mature $\gamma\delta^+$ cells, from alleles that remain unrearranged, rather than in the progenitors of $\gamma\delta^+$ cells. To address this possibility, the PCR analysis was repeated with samples of RNA from E15 and adult DN thymocyte populations from which $CD3^+$ cells had been removed by cell sorting. The pattern and amounts of sterile transcripts in these cells was similar to that in the corresponding unsorted thymocyte populations (Table 4-1). These results indicate that TCR-negative cells produce most of the sterile transcripts detected.

age of mice	copies/μg	
	Vγ2	Vγ3
E15	$5.7X10^5$	$3.4X10^5$
Adult DN	$1.2X10^5$	$<3.6X10^3$

Table 4-1. Sterile Transcript Levels in $CD3^-$ Thymocyte RNA.

$CD3^-$ cells were isolated from E15 and Adult DN thymocytes, and sterile transcript levels in the RNA from both of these populations were assessed by RT-PCR as in Fig. 4-2.

Discussion

The developmental pattern of Vγ gene rearrangements corresponds well to the pattern of sterile Vγ transcript levels. The decrease of rearrangement is correlated with the decline of the corresponding sterile transcript, as in the case of Vγ3 and Vγ4 rearrangements. Previous examples have documented correlations between the onset of rearrangements and the appearance of sterile transcripts (see Introduction), as is the case for Vγ2 rearrangements. The fact that different Vγ gene rearrangements follow opposite trends during development, which are positively correlated with the abundance of the corresponding sterile Vγ transcripts, argues strongly against a chance relationship. Differences in the steady state level of a given transcript at different stages of development are likely to reflect differences in transcription rates, although the possibility that degradation rates change specifically during development has not been ruled out.

The correlation between transcription and rearrangement argues in favor of a model in which differential accessibility regulates Vγ gene rearrangement. However, this study provides no clue as to whether transcription is a cause or merely a consequence of increased accessibility. Examples given in the introduction to this thesis appear to support the idea that transcription of a gene is actually a requirement for its rearrangement. Specifically, in all these cases, the removal of the transcriptional enhancer from either a transgenic recombination substrate (Ferrier et al., 1990) or from endogenous genes either abolishes (Takeda et al., 1993) or severely reduces (Serwe & Sablitzky, 1993) rearrangement. However, it must

be pointed out that enhancers, in addition to upregulating transcription, may also play a role in either establishing or maintaining an open chromatin configuration.(Ariizumi et al., 1993, Engler et al., 1991, Hsieh et al., 1992, Jenuwein et al., 1993) Other studies have suggested that replication or demethylation may play a role in regulating gene accessibility. Thus, a gene may be rendered accessible by a mechanism other than transcription, and transcription may or may not accompany it.

Another possibility is that the sterile transcripts themselves (or their products, if any) may be responsible for increased accessibility. For example, sterile transcripts of immunoglobulin C_H regions involved in class-switching include one or more I exons spliced to the first C_H exon. When the Iε was replaced with an Eμ enhancer/V_H promoter combination, very little lymphokine-induced switching occurred even though it was demonstrated that transcription through this region did take place (Bottaro et al., 1994). One interpretation of these results is that the actual sequence of the sterile transcript is important in this case. Additional evidence for such a mechanism was reported in the case of *in vitro* studies of a test plasmid containing an Ig switch recombination signal sequence (Reaban & Griffin, 1990). In this experiment, transcription of the sequence, as well as the presence of the transcripts themselves, was required to stabilize an unusual conformation which caused a relaxation of supercoils in the plasmid (i.e. increased accessibility). However, this mechanism appears to be dependent on the repetitive nature of the switch recombination signal and may not be applicable to the sequences of the V-(D)-J heptamer-nonamer recombination signals.

Chapter 4A

COMPARISON OF PCR ASSAYS

Introduction

The RT-PCR assay described in Chapter 4 demonstrated a striking difference in Vγ3 sterile transcript levels between adult DN and E15 fetal thymocytes. This experiment was repeated many times and always showed a difference of 50 to 100-fold. In the experiments described in later chapters, it was necessary to modify the PCR conditions to increase the sensitivity because of the use of limiting amounts of input RNA. In control experiments comparing Vγ3 sterile transcript levels in E15 and adult DN RNA, the magnitude of the difference was less striking than was previously observed. This chapter describes comparisons of the different PCR approaches in an attempt to determine the actual difference in levels of Vγ3 sterile transcripts between these populations.

Results

The small cell numbers recovered from fetal thymic organ cultures (Chapter 6) or sorted populations from SCID chimeras (Chapter 7) necessitated using considerably less input RNA in each PCR. As a result it was necessary to increase the sensitivity of the RT-PCR. The first attempt to improve the assay was to use a "hot start" technique, which involved heating all the elements of the PCR to 72°C before mixing. This is reported to prevent the production of extraneous products due to low stringency hybridization of primers

in the first round of amplification (Chou et al., 1992, D'Aquila et al., 1991). Another version of the PCR assay involved the use of a new primer pair, V3/V3-3'c (Fig. 4A-1). In both cases, these altered conditions resulted in strongly increased sensitivity of the PCR. In fact, PCR products of sterile Vγ3 transcripts were readily detectable in RNA from the adult DN thymocyte population, whereas these products were almost undetectable in earlier PCRs. Quantitation in various experiments revealed a 6-18-fold difference in Vγ3 sterile transcript levels between E15 and adult DN RNA samples. Examples are shown in Figure 4A-2. The same samples were analyzed using either hot start or standard RT-PCR. The standard PCR revealed an ~80-fold difference in Vγ3 sterile transcripts between E15 and adult RNA samples (Fig. 4A-2A), while the difference using hot start only appeared to be ~10-fold (Fig. 4A-2B). An RT-PCR assay using the new primer pair resulted in only a 6-fold difference between E15 and adult DN RNA with regard to Vγ3 sterile transcript levels (Fig. 4A-2C).

After this was discovered, the condition of the RNA samples was checked on a formaldehyde agarose gel. Both the E15 and adult DN RNA samples were somewhat degraded but appeared to contain comparable levels of 18s and 28s rRNA (data not shown). Additionally, both yielded the same amount of product when amplified with β-tubulin primers (Fig. 4A-2D). The apparent increase in Vγ3 sterile transcripts is not due to contamination of the adult DN RNA as this effect was seen with a number of independent adult DN samples (data not shown). In addition, no Vγ3 PCR product was ever detected when using a control RNA from the fibroblast

Figure 4A-1: Location of new Vγ3 primers and probe. Sense (▷) and anti-sense (◁) PCR primers; oligonucleotide probe (▬); ▶ 7-mer/9-mer recombination signal. PCR primers (▷, ◁) and probe (▭), used in Chapter 4 are included for comparison.

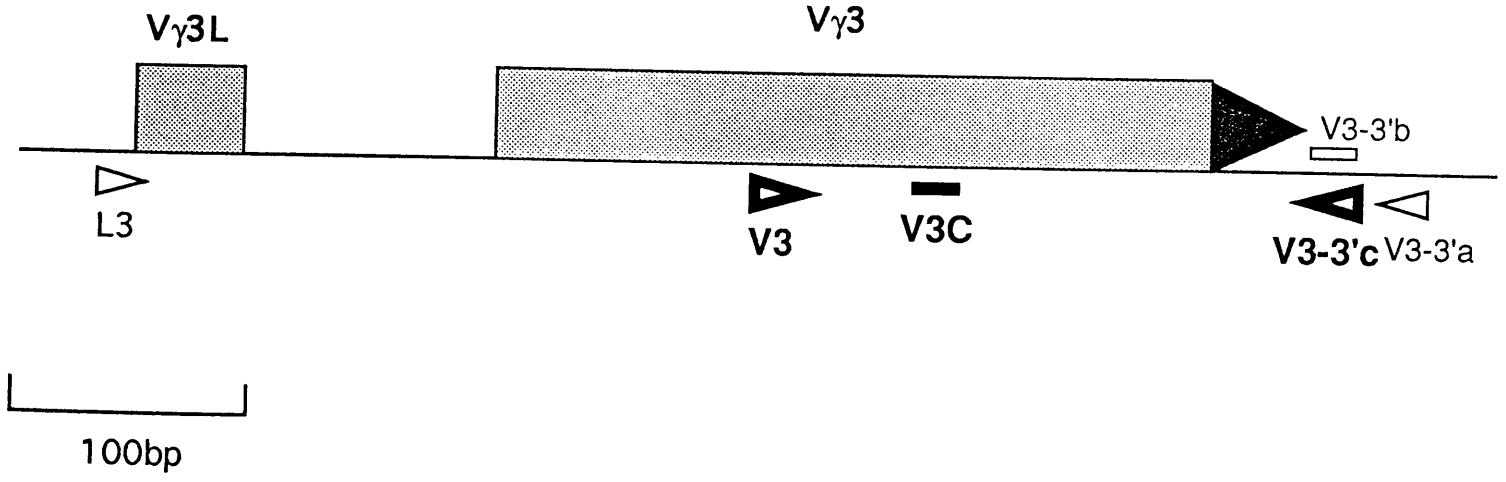
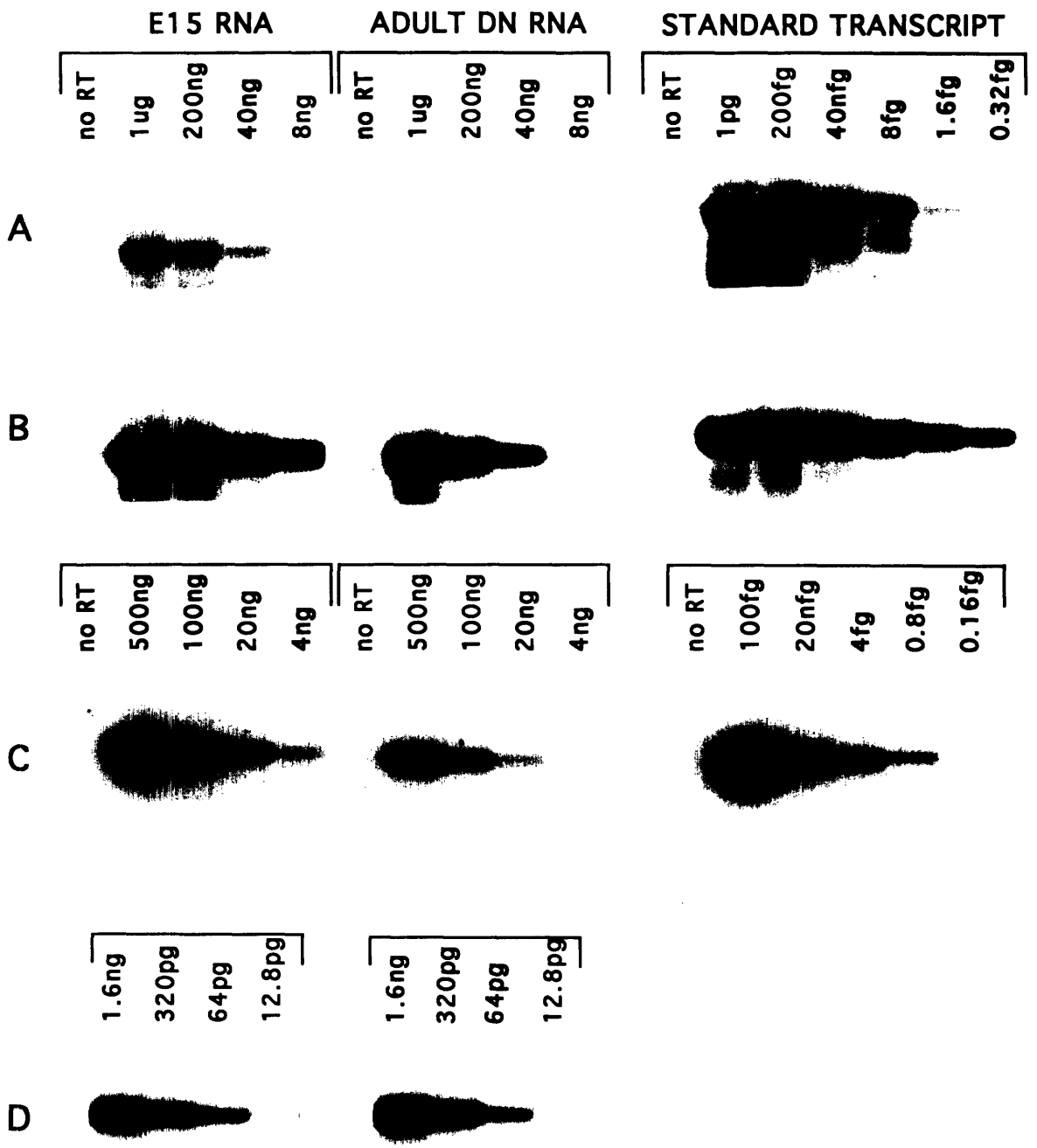

Vγ3L
Vγ3
V3-3'b
L3
V3
V3C
V3-3'c
V3-3'a
100bp

Figure 4A-2: Comparison of variations of the RT-PCR assay. RNA from E15 or adult DN thymocytes was titrated and subjected to RT-PCR to detect Vγ3 sterile transcripts (**A, B, C**) or β-tubulin transcripts (**D**). The amount of input RNA is indicated above each lane. Lanes designated "no RT" refer to reactions with the highest titration of input RNA from which the reverse transcriptase was omitted. (**A**) RT-PCR was performed as described in Figure 4-2 using the V3L/V3-3'a primer and the V3-3'b probe. (**B**) RT-PCR performed as in (A), except that all components of the PCR reaction were heated to 72°C prior to mixing ("hot start"). (**C**) RT-PCR performed using the V3/V3-3'c primer pair and the V3C probe (see Fig. 4A-1 for locations). (**D**) RT-PCR using the β-tubulin primers and probe.

The sizes of the products in all reactions were correct as predicted from the sequences. (A, B) 450 bp (E15 and adult DN) and 550 bp (standard transcript) (C) 253 bp (D) 310 bp.

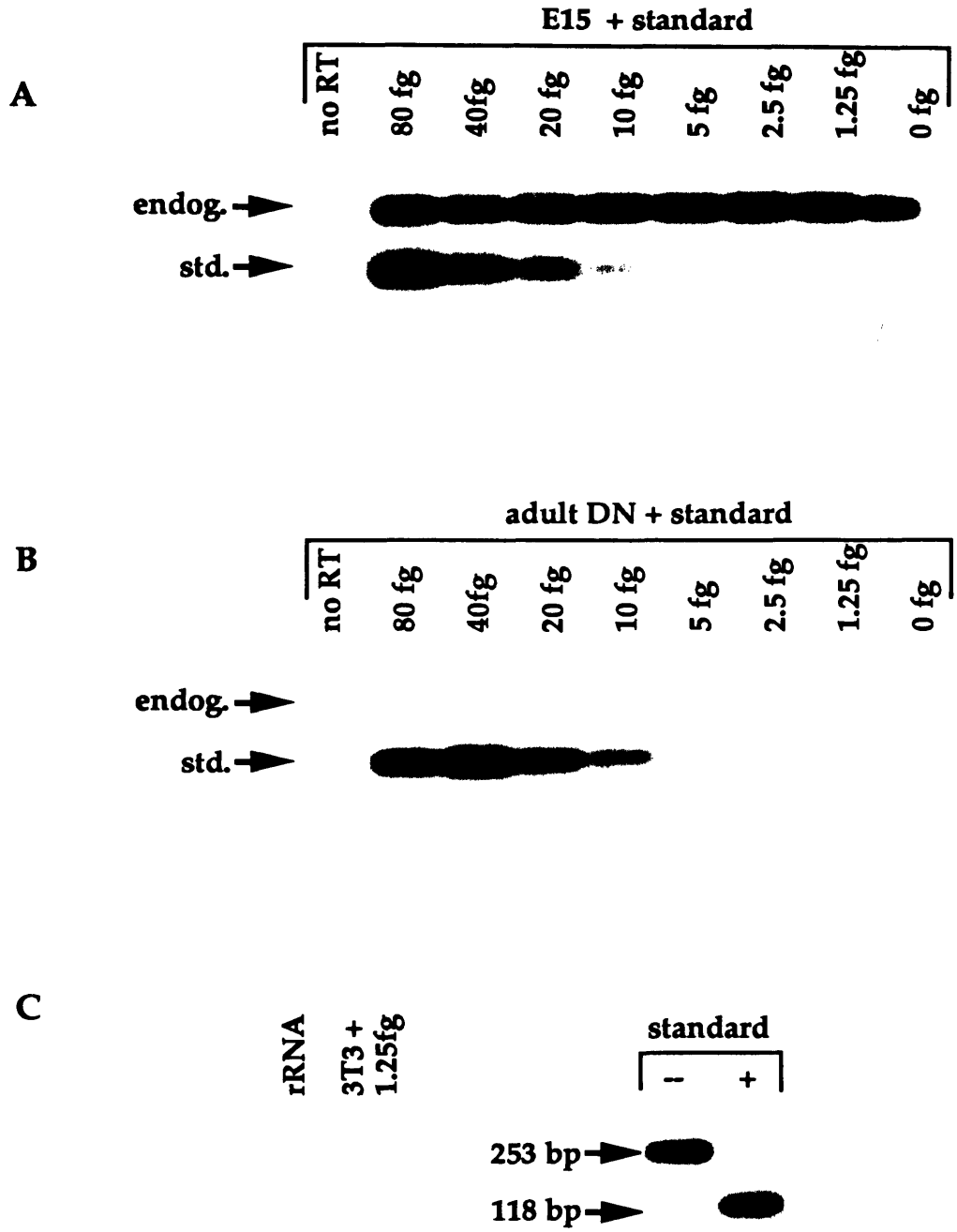

E15 RNA
ADULT DN RNA
STANDARD TRANSCRIPT
no RT
1ug
200ng
40ng
8ng
no RT
1ug
200ng
40ng
8ng
no RT
1pg
200fg
40nfg
8fg
1.6fg
0.32fg
A
B
no RT
500ng
100ng
20ng
4ng
no RT
500ng
100ng
20ng
4ng
no RT
100fg
20nfg
4fg
0.8fg
0.16fg
C
1.6ng
320pg
64pg
12.8pg
1.6ng
320pg
64pg
12.8pg
D

NIH3T3 cell line or E. coli ribosomal RNA as template (data not shown).

In order to determine which results were correct, I employed a competitive PCR assay, which, because it is internally controlled, is reported to be the most reliable quantitative PCR assay (Becker-Andre & Hahlbrock, 1989, Siebert & Larrick, 1992). In this assay, a constant amount of sample is used in every PCR and each is mixed with graded doses of a synthetic competitor transcript which yields a distinguishable PCR product. Both templates compete for the primers during each cycle. The reaction that contains equimolar amounts of standard transcript and endogenous sequence yields the same amount of product from each. The competitor in this case is the *in vitro* synthesized Vγ3 transcript used as a quantitation standard in previous assays. The plasmid from which it was transcribed contains an insertion of an EcoR1 linker. Thus, the PCR products of the standard and endogenous transcripts can be distinguished by subjecting them to EcoR1 digestion prior to gel electrophoresis.

Samples of either E15 or adult DN RNA were spiked with different amounts of standard transcript in two-fold dilutions. RT-PCR was performed using the V3/V3-3'c primer pair for 25 cycles. After amplification, a portion of each reaction was digested with EcoR1, run on gels, blotted, and the blot hybridized with the V3C probe (Fig. 4A-1). The results, shown in Fig. 4A-3, reveal an ~10-fold difference in Vγ3 sterile transcript levels between E15 and adult DN RNA samples.

Figure 4A-3: Vγ3 sterile transcript levels determined by competitive RT-PCR. Competitive RT-PCR was performed on **(A)** E15 and **(B)** adult DN RNA samples using the V3/V3-3'c primer pair. A series of reactions was performed on 200 ng of thymocyte RNA which included graded (two-fold dilutions) amounts of Vγ3 standard transcript as indicated above each lane. After amplification, 20 µl from each reaction was digested with EcoR1, gel-fractionated, blotted, and the blots hybridized with the V3C probe. The bands corresponding to products from the endogenous transcripts (endog., 253 bp) or standard transcripts (std., 118 bp) are indicated by arrows. **(C)** Control reactions using as template E. coli rRNA only, RNA from the NIH3T3 fibroblast cell line +1.25 fg standard transcript or standard transcript only either intact (-) or EcoR1 digested (+), as indicated.


A
E15 + standard
no RT
80 fg
40fg
20 fg
10 fg
5 fg
2.5 fg
1.25 fg
0 fg
endog.
std.
B
adult DN + standard
no RT
80 fg
40fg
20 fg
10 fg
5 fg
2.5 fg
1.25 fg
0 fg
endog.
std.
C
rRNA
3T3 + 1.25fg
standard
-
+
253 bp
118 bp

Discussion

Results presented here show that the magnitude of the difference in Vγ3 sterile transcript levels between E15 and adult DN RNA varies depending on the assay conditions used. The standard RT-PCR used in Chapter 4 yields a 50-100-fold greater amount of product from E15 RNA than from adult DN RNA, while this difference is 6-18-fold using either a "hot-start" technique or a more efficient primer pair. The problem of how to determine which result is the correct one was resolved using a competitive RT-PCR assay. This is generally acknowledged to be reliable because each reaction is internally controlled. Using this assay, the level of Vγ3 sterile transcript in E15 thymocyte RNA was shown to be ~10-fold greater than in adult DN RNA. Because of this, the difference determined using the hot start or the V3/V3-3'c primer pair was designated as the correct one.

The original data could be interpreted to mean that there is an ON/OFF difference between E15 and adult cells with regard to Vγ3 germline transcription. The results using the more sensitive PCR assays suggest a more subtle difference. This difference, although smaller than originally determined, is reproducible and still represents a significant difference in sterile Vγ3 transcript levels between fetal and adult thymocyte precursors, consistent with a significant difference in the accessibility of the Vγ3 gene in these populations.

Although we believe that of all the PCR techniques, the competitive RT-PCR assay is the most reliable way to measure small differences in transcript levels, it has a major drawback. Because the

target RNA sample is not titrated, a large amount of each sample is required to cover a useful range of standard transcript concentrations. The small amounts of RNA recovered from fetal thymic organ cultures or sorted cells from chimeras precluded the use of a competitive PCR assay in subsequent studies. However, subsequent RT-PCR experiments were performed using the V3/V3-3'c primer pair which gives approximately the same results as the competitive PCR assay.

In Chapter 3, the level of rearrangement of Vγ3 genes in E15 thymocyte DNA was shown to be ~30-fold higher than in adult DN thymocyte DNA (Fig. 3-3 and 3-4). It is possible that the difference in levels of rearranged Vγ3 genes had been overestimated, because, like the sterile transcript levels, rearrangements were quantitated using a PCR assay. However, the V3L/J1 primer pair appears to work quite efficiently as indicated by the absence of incorrectly sized products on the ethidium bromide-stained gels. Also, this primer pair can quite efficiently amplify transcripts from as little as 800 cell equivalents of E15 RNA in an RT-PCR assay (data not shown). Therefore, we believe the 30-fold difference observed in Chapter 3 to be reasonably accurate. With the results presented in this chapter, this difference can not be completely accounted for by the difference in levels of Vγ3 sterile transcripts. How can this discrepancy be explained?

One possibility is that E15 and adult DN thymocyte populations both contain cells which transcribe the unrearranged Vγ3 gene, but the fetal cells transcribe it at a higher rate which is above some threshold required for rearrangement of the gene.

Alternatively, the *rate* of rearrangement of the Vγ3 gene in E15 thymocytes may in fact only be 10-fold greater than in adult DN thymocytes. However, the cells which rearrange and express the Vγ3 gene may persist longer in the fetal thymus due to a signal which is only provided by the fetal thymic stroma. This is supported by recent studies showing development of fetal thymocytes from a $V\gamma3^{low}HSA^{high}$ to a $V\gamma3^{high}HSA^{low}$ stage (Leclercq et al., 1993, Tatsumi et al., 1993). This transition was inhibited by cyclosporin A, suggesting that a signal from the fetal thymic stroma may be necessary for the survival and/or maturation of these cells. Perhaps in the adult thymus, the $V\gamma3^{+}$ cells do not accumulate due to the absence of this signal.

Possible explanation for results using different assay conditions.

It would seem that the use of several dilutions of each sample prior to RT-PCR would control for efficiency of cDNA production and amplification in samples which contain different amounts of target transcript. An improvement in the efficiency of the amplification would be expected to affect each sample equally. Why then did the original assay conditions overestimate the difference between the populations?

In the original PCR assay, when the primer/Taq polymerase mixture is added at relatively low temperature, the primers may bind to imperfectly matched sequence in addition to the target sequence. Addition of a few nucleotides to the 3' end of the primer by Taq polymerase would then allow the primer to remain bound at

the imperfectly matched sites at the higher annealing temperature (Innis et al., 1988). This would occur in the first round of the PCR, so that aberrently initiated templates would be amplified in subsequent rounds, and thereby interfere with the amplification of the desired product. In fact, in ethidium bromide stained gels of PCR products amplified with the V3L/V3-3'a primers, many bands were present which did not hybridize to the oligonucleotide probe located between the two primers (data not shown). These bands do not appear on gels of PCR products generated using the newer methods.

In this scenario, the "hot start" conditions, or the use of the new primer pair, increase the efficiency of amplification of the desired product by not allowing initiation of polymerization at aberrant sites. Because the adult DN sample has 10-fold fewer sterile transcripts, it has a higher ratio of non-target/target sequences. This would result in increased amounts of incorrect PCR products which would compete for limiting components in the reaction mixtures. Therefore, the content of Vγ3 sterile transcripts in the adult DN sample would be underestimated.

Chapter 5

REGULATED ACCESSIBILITY OF Vγ3 AND Vγ4 T CELL RECEPTOR GENES

Introduction

The previous chapter examined transcription of Cγ1 cluster genes and the possible role of differential accessibility in controlling their ordered rearrangement. Clearly, the timing of Vγ3 and Vγ2 accessibility is different, matching their rearrangement patterns. However, Vγ3 and Vγ4 genes are both rearranged and expressed in the fetus and they are only separated by ~1 kb. This raises the question as to whether differential accessibility leads to separate lineages of progenitor cells, or if $V\gamma3^+$ and $V\gamma4^+$ cells can be derived from the same immediate progenitor. The accessibility model, when applied to Vγ gene rearrangement, predicts that only one of the Vγ genes in the Cγ1 cluster will be accessible in a given progenitor cell. The model makes no predictions concerning whether all the Vγ genes in a cell become accessible later, after the cell has differentiated into a mature $\gamma\delta^+$ cell. In this chapter, evidence is presented that the Vγ4 gene is accessible in mature $V\gamma3^+$ cells, but may be inaccessible in progenitors of $V\gamma3^+$ cells.

Results

Some Vγ3 transcripts use the Vγ4 leader in the 7.17A2 cell line.

Experiments to determine the start sites of Vγ transcripts in a Vγ3$^+$ cell line, 7.17A2, found evidence of transcripts containing the Vγ4 leader exon (L4) sequences, but not the Vγ4 coding exon sequences (Spencer, 1991). Since Vγ4 is not rearranged in 7.17A2, we suspected that these transcripts might consist of L4 sequences spliced to the Vγ3 coding exon sequences. To check this, cDNA from 7.17A2 RNA was subjected to PCR amplification using either the L3 (V3 leader exon) or L4 sense primer and the J1 antisense primer (Fig. 5-1). Products of both reactions (ie. using the L3/J1 or L4/J1 primer pairs) were easily detected and examples were cloned and sequenced (data not shown). The Vγ3-Jγ1 junctional sequences of each product corresponded to the functional, canonical s-IEL sequence (Asarnow et al., 1988). As expected, in the L3/J1 product, L3 was correctly spliced to Vγ3. The L4/J1 product consisted of L4 correctly spliced to the Vγ3 sequences (Spencer, 1991). The origin of the L4-Vγ3-Jγ1 transcript is presumably a large primary transcript that originates upstream of L4 and extends through Vγ3 and the Jγ1-Cγ1 regions (Fig. 5-1).

The relative abundance of transcripts of the L4-Vγ3-Jγ1 and L3-Vγ3-Jγ1 types in 7.17A2 were quantitated with the use of the RNase protection assay, employing *in vitro* synthesized RNA probes corresponding to each cloned PCR product described above (results of D. Spencer). As shown in Figure 5-2, fragments consistent with protection of the probes by both L3-Vγ3 and L4-Vγ3 transcripts were

Figure 5-1: Schematic representation of rearranged Vγ3+ transcripts employing the L4 or L3 sequences. Shown are the spliced products which would result from transcripts originating upstream of either the Vγ3 or the Vγ4 leader. Beneath each spliced product are the primers to amplify products for PCR analysis and cloning of probes used in the RNase protection assay in Figure 5-2. Also shown is the oligonucleotide used to probe blots in Fig.5-3.

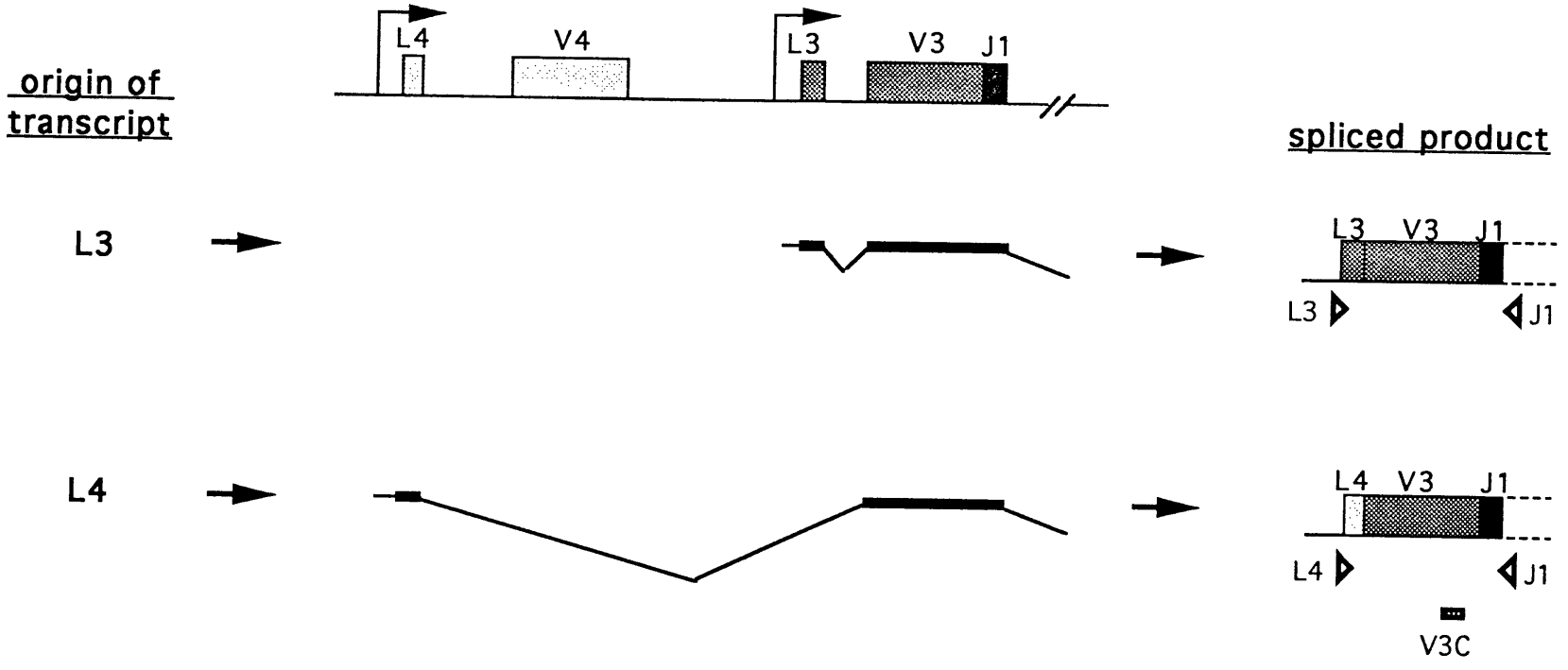
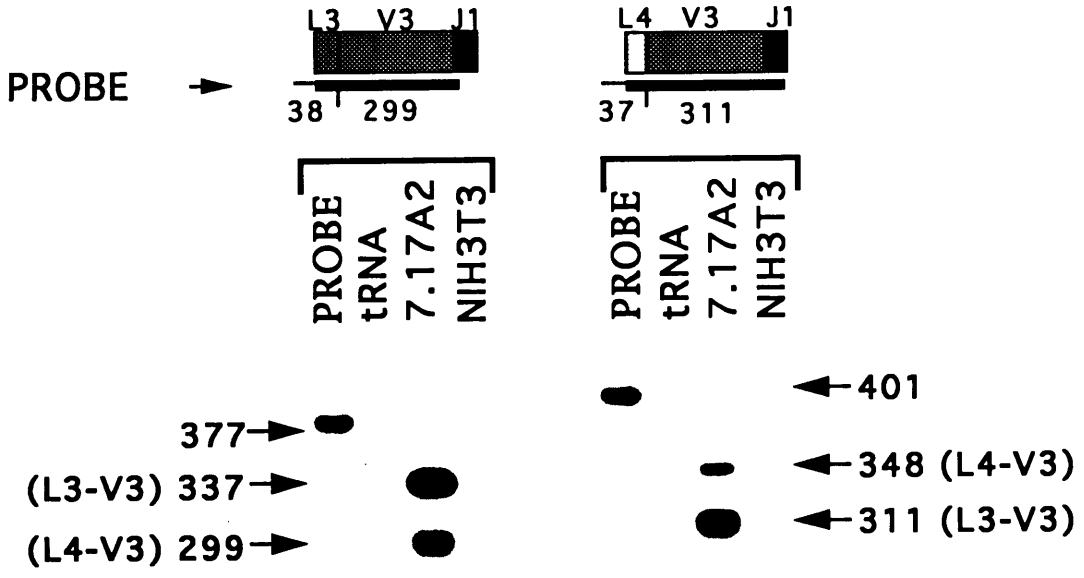

origin of transcript
L4
V4
L3
V3
J1
spliced product
L3
L3
V3
J1
L3
J1
L4
L4
V3
J1
L4
J1
V3C

Figure 5-2: RNase protection assay to detect L3-V3 and L4-V4 transcripts in the 7.17A2 s-IEL line. The protected bands, corresponding to the indicated products, are indicated by arrows, as are the undigested probes. The probes used for RNase protection are shown above the corresponding lanes, with the size of the exons indicated in base pairs. Labeled probes were hybridized with 2 μg of total RNA from 7.17A2 cells line, from negative control NIH3T3 fibroblastic cells or with 2 μg yeast tRNA, and digested with RNase.

Note that the smaller band in each case corresponds to protection of the V3 exon alone. During cloning of the probes, exonuclease digestion removed different amounts from the 3' end, leaving the L3-V3 probe with 4 and the L4V3 probe with 16 bases of Jγ1 sequence. This accounts for the difference in size between the lower bands protected with each probe.

(Experiment performed by D. Spencer)


PROBE
L3
V3
J1
38
299
L4
V3
J1
37
311
PROBE
tRNA
7.17A2
NIH3T3
PROBE
tRNA
7.17A2
NIH3T3
401
377
(L3-V3) 337
(L4-V3) 299
348 (L4-V3)
311 (L3-V3)

observed with both probes. As determined by densitometric analysis, approximately 19% of all the Vγ3-Jγ1 transcripts use L4, while most of the remaining transcripts use L3. The results indicate that in this cell line an appreciable fraction of Vγ3 transcripts originate from a larger primary transcript that includes L4. Hence, the Vγ3 and Vγ4 genes on the same chromosome must both be accessible to RNA polymerase.

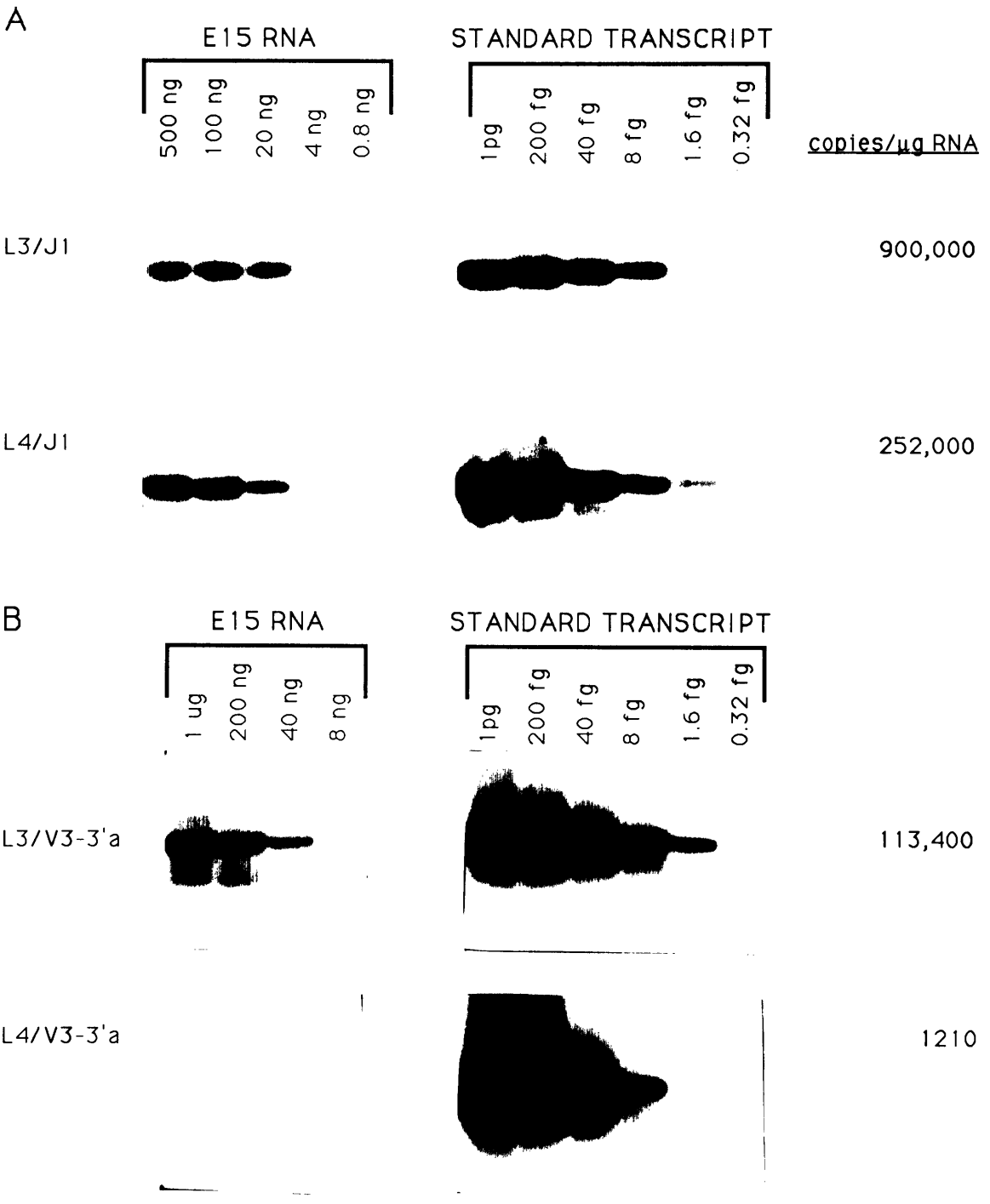
L3 vs. L4 usage in Vγ3Jγ1 transcripts in freshly isolated thymocytes

To determine if transcripts of rearranged Vγ3 genes frequently use L4 in freshly isolated thymocytes, cDNA from E15 thymocyte RNA was subjected to quantitative PCR using J1 as a downstream primer and L3 or L4 as upstream primers. Vγ3-containing PCR products were detected by hybridization with a Vγ3-oligonucleotide probe (V3C, Fig. 5-1). The analysis revealed that ~22% of rearranged Vγ3 transcripts in E15 cells are of the L4-Vγ3-Jγ1 type (Fig. 5-3A). Therefore, as in the 7.17A2 cell line, a substantial fraction of rearranged Vγ3 transcripts from E15 thymocytes appear to result from processing of a transcript that initiates upstream of the Vγ4 gene, indicating that both the Vγ3 and Vγ4 genes must be accessible to RNA polymerase on a significant fraction of the rearranged chromosomes.

L3 vs. L4 usage in Vγ3 sterile transcripts in freshly isolated thymocytes

The preceding results raise the question whether the Vγ4 and Vγ3 genes are both accessible on the same chromosome *before*

Figure 5-3: Usage of L4 and L3 in transcripts of unrearranged genes in E15 fetal thymocytes. Transcripts of rearranged (**A**), or unrearranged (**B**) Vγ3 genes were detected by PCR amplification of Jγ1-primed (**A**) or V3-3'a-primed (**B**) cDNA with L4 or L3 upstream primers. Gel-fractionated products were blotted and hybridized with the Vγ3 oligonucleotide probe (V3C). Comparison of titrated E15 RNA with titrated *in vitro* synthesized standard transcripts (see Materials and Methods) allowed the determination of the number of copies/μg total RNA, indicated to the right of the figure.


A
E15 RNA
500 ng
100 ng
20 ng
4 ng
0.8 ng
STANDARD TRANSCRIPT
1pg
200 fg
40 fg
8 fg
1.6 fg
0.32 fg
copies/µg RNA
L3/J1
900,000
L4/J1
252,000
B
E15 RNA
1 ug
200 ng
40 ng
8 ng
STANDARD TRANSCRIPT
1pg
200 fg
40 fg
8 fg
1.6 fg
0.32 fg
L3/V3-3'a
113,400
L4/V3-3'a
1210

rearrangement, which would argue that rearrangement of Vγ3 vs. Vγ4 is not controlled by differential accessibility. If both genes are accessible on the same chromosome, it might be expected that the sterile Vγ3 transcripts in fetal thymocytes, like the rearranged transcripts, would commonly initiate upstream of the Vγ4 gene and include the Vγ4 leader exon. To examine this possibility, a similar PCR assay was used as in the previous experiment, except that in order to amplify transcripts of the unrearranged genes, the V3-3a' downstream primer (Fig. 4-1) was used instead of the J1 primer. As before, the upstream primers were either the L3 or the L4 primers. Using this method allowed determination of the abundance of *sterile* transcripts containing L4 spliced to Vγ3 relative to those containing L3 spliced to Vγ3. In contrast to what was observed in the case of rearranged transcripts, L4-Vγ3 sterile transcripts were much rarer (~1/100th the level) than L3-Vγ3 sterile transcripts in E15 thymocytes (Fig. 5-3B). These data indicate that very few of the sterile Vγ3 transcripts arise by splicing of L4 to Vγ3. A possible explanation is that the long primary transcripts that initiate upstream of Vγ4 and include Vγ3 are not synthesized in progenitors of Vγ3 cells, perhaps because the Vγ4 gene is inaccessible in these cells. Thus the Vγ4 gene may become accessible in Vγ3 cells only following rearrangement and/or maturation of the cells.

Discussion

The results presented in this chapter of studies of rearranged versus sterile Vγ3 transcription are consistent with the accessibility model. Initially, we found that rearranged Vγ3 transcripts in an s-

IEL cell line and in fetal thymocytes often employ the upstream Vγ4 leader exon, indicating that both the Vγ3 and Vγ4 genes must be accessible in many of the cells with rearranged Vγ3 genes. In contrast, sterile Vγ3 transcripts in fetal thymocytes employ the Vγ4 leader exon only very rarely. As an explanation for these results we propose that essentially all Vγ3 sterile transcripts derive from primary transcripts initiating at the Vγ3 promoter, rather than from larger transcripts that include L4. The larger sterile transcripts would not be produced if the L4/Vγ4 gene is inaccessible in the progenitors of Vγ3 cells, and/or if the Vγ4 promoter is inactive in these cells. An alternative possibility, that has not been ruled out, is that the large sterile transcript is produced, but L4 fails to be spliced to Vγ3. We think this is unlikely, however, since other splicing events occur normally in sterile transcripts, for example L3 to Vγ3 (Figs 4-2 and 5-3B) and L4 to Vγ4 (data not shown).

It may seem unlikely that regulation by differential accessibility of genes segments separated by only 1 kb could occur. However, a recent study of the human T cell receptor δ locus (Lauzurica & Krangel, 1994) demonstrated that the step of V to D rearrangement of a transgenic recombination substrate is regulated separately from VD to J rearrangement even though there is only 0.9 kb between the D and J segments.

An attractive version of the accessibility model as applied to Vγ gene rearrangement is that Vγ gene accessibility is controlled by selective activation of the Vγ gene promoters. Gene accessibility, in turn, controls differential gene rearrangement. The fact that the promoter regions of Vγ2, Vγ3 and Vγ4 show little sequence

relatedness (Doherty and Raulet, unpublished), is consistent with the possibility that they are independently regulated. Following gene rearrangement and/or maturation of the γδ cell, transcription of other Vγ genes in the cell apparently occurs, as shown by the splicing of the Vγ4 leader exon to Vγ3 in rearranged transcripts. Among other possibilities, this may occur because rearrangement brings the downstream Cγ1 enhancer (Kappes et al., 1991, Spencer et al., 1991) nearer to the previously silent promoters, thus activating them. Another possibility is that each V gene has two types of promoter elements, one of which is selectively activated to produce the sterile transcripts, and a second promoter element, active in all mature γδ cells, used to produce the mature transcripts.

Unfortunately, it is difficult to directly assess the whether Vγ gene promoters are differentially active during development. For example, we have found in transient transfection allays that the Vγ4 promoter is active in several γδ T cell lines and hybridomas, even ones which do not express a Vγ4 receptor on their surface (J. Goldman, C. Thut and K. Guenther, unpublished results). However, the activity of promoters in mature cells is no indication of their regulation during development. At present, no appropriate precursor cell lines exist which can differentiate into particular Vγ-expressing T cells *in vitro*. This rules out the possibility of using the transient transfection/reporter gene assays normally employed to assess transcriptional element functions.

Recent examination of the Vγ3 promoter has revealed a transcriptional repressor region between Vγ3 and Vγ4 which acts on both the Vγ3 and heterologous promoters in a γδ, but not an αβ T cell

line (Clausell & Tucker, 1993). This may be involved somehow in the regulation of Vγ3 vs. Vγ4 rearrangement in developing T cells. However, it is difficult to assign this repressor element a role in regulating differential Vγ3/Vγ4 rearrangement as it has only been tested in mature T cell lines.

Chapter 6

Vγ STERILE TRANSCRIPTS IN THYMOCYTES FROM DIFFERENT STEM CELL SOURCES

Introduction

The previous chapter(s) demonstrated a correlation between the transcription of unrearranged T cell receptor Vγ gene segments and their rearrangement. This correlation is particularly strong with regard to the Vγ3 gene segment as both its transcription and rearrangement decline contemporaneously. Cells which express a $V\gamma3^+$ T cell receptor on their surface are interesting in that they only appear in the thymus for a restricted period of time during fetal ontogeny (days 14-18). In the adult mouse they are found only in the skin as intraepithelial lymphocytes (s-IEL). It has been demonstrated that the generation of $V\gamma3^+$ T cells requires that precursor cells of fetal origin develop in a fetal thymus (Havran et al., 1991a, Ikuta et al., 1990). Any other combination of stem cells and thymus (e.g. fetal liver/adult thymus, adult bone marrow/ fetal or adult thymus) fails to generate $V\gamma3^+$ cells either in the thymus or the skin. This suggests that the targeted rearrangement of Vγ3 genes is programmed by the thymic microenvironment and/or the origin of the progenitor cells. This chapter describes experiments to determine whether the production of Vγ3 sterile transcripts, a correlate of targeted rearrangement, is a property inherent in fetal progenitor cells, by comparing sterile transcript levels in fetal liver-derived vs. bone marrow-derived thymocytes differentiating in a fetal thymus.

Results

Repopulation of fetal thymic organ cultures (rFTOC)

The analysis illustrated in Figure 6-1 has been previously demonstrated to support the growth and development of T cells from their precursors in vitro (Ikuta et al., 1990). Thymic lobes from C57BL/6 mice (Ly-5.2) were dissected from fetuses on day 14 of gestation (E14), and treated with deoxyguanosine, which destroys resident lymphoid cells but leaves the thymic stroma intact. These "empty" lobes were then repopulated with either E14 fetal liver (FL) or adult bone marrow (BM) cells from C57BL/6-Ly5.1 congenic donors in hanging drop cultures. The repopulated lobes were grown for eleven days to allow for thymocyte development and the resulting cells were harvested for RNA preparation.

A fraction of cells from both types of rFTOC was set aside for staining to ascertain if the repopulation was successful. As shown in Figure 6-2A, the repopulations worked as expected (Ikuta, et al, 1990). The resultant cells are virtually all donor-derived (Ly-5.1$^+$) indicating that the deoxyguanosine depletion of host thymocytes was successful. Also, as demonstrated previously, Vγ3$^+$ cells are only produced in fetal thymic lobes which were repopulated with fetal liver stem cells (6.4% of total), whereas lobes repopulated with adult bone marrow had only background staining for Vγ3 (Fig. 6-2A).

Analysis of Vγ3 sterile transcript levels

An RT-PCR assay similar to the one used in Chapters 4 and 5 was employed to analyze the level of Vγ3 sterile transcripts in total

Figure 6-1: Repopulation of fetal thymic organ cultures. See text and Materials and Methods for details.

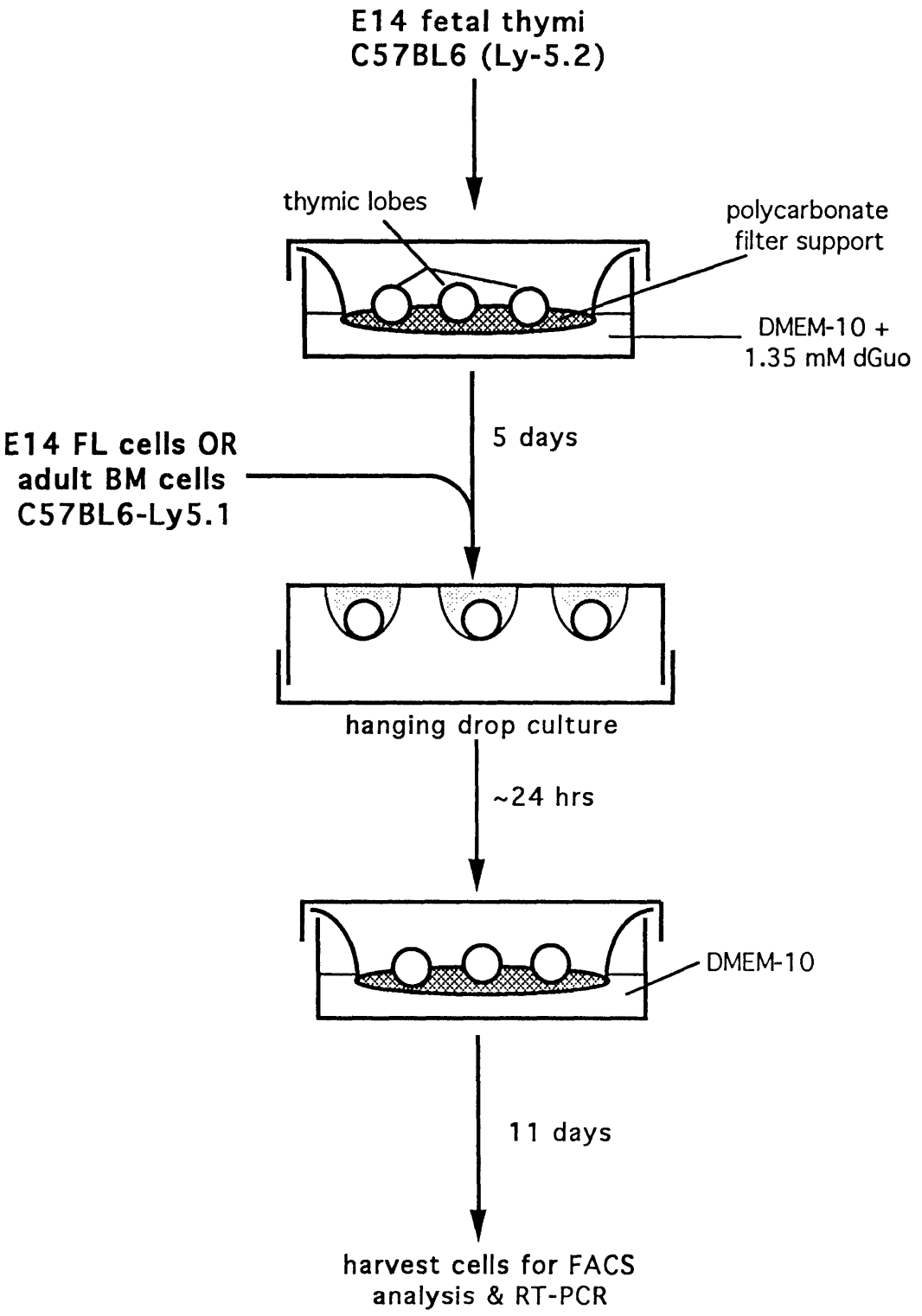
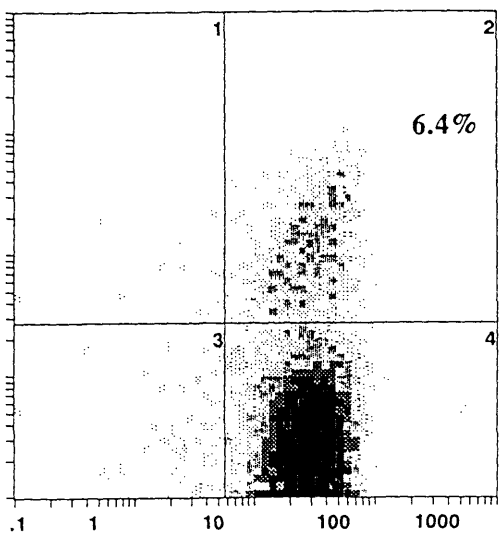

E14 fetal thymi
C57BL6 (Ly-5.2)
thymic lobes
polycarbonate
filter support
DMEM-10 +
1.35 mM dGuo
5 days
E14 FL cells OR
adult BM cells
C57BL6-Ly5.1
hanging drop culture
~24 hrs
DMEM-10
11 days
harvest cells for FACS
analysis & RT-PCR

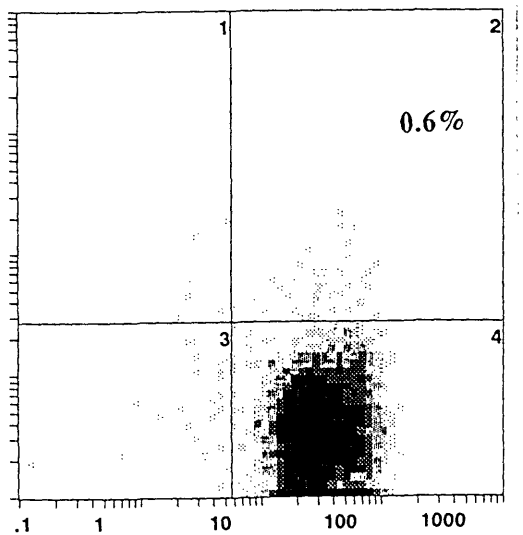
Figure 6-2: FACS analysis of cells from repopulated fetal thymic organ cultures. Cells isolated from FL- or BM-repopulated fetal thymic lobes after 11 days in culture were stained with (**A**) anti-Ly5.1-FITC and anti-Vγ3-biotin + streptavidin-APC and (**B**) anti-CD4-PE + anti-CD8-PE.

A

fetal liver repopulated



bone marrow repopulated

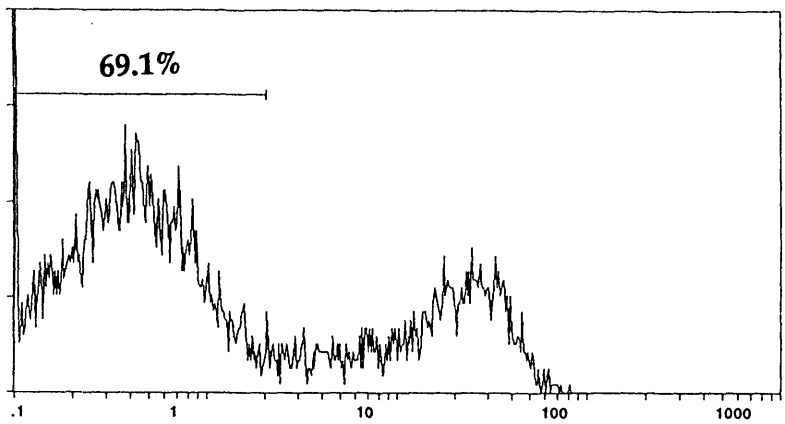


Vγ3

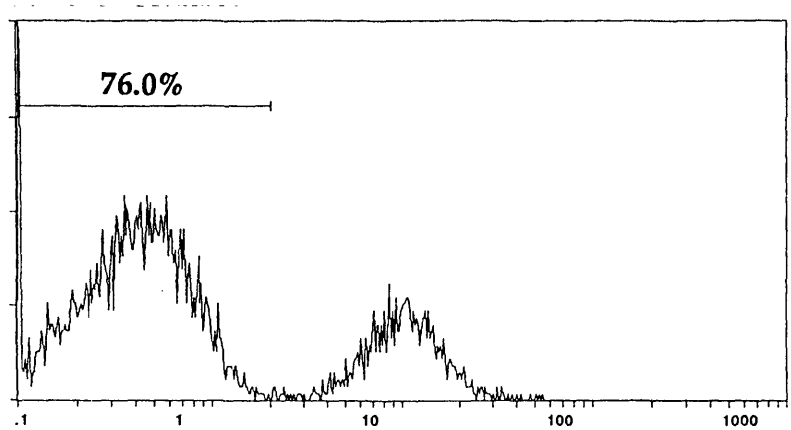
Ly-5.1

B

fetal liver repopulated



bone marrow repopulated



CD4 +CD8

RNA from rFTOC cells. However, several changes were made. RNA used in earlier analyses was prepared from $CD4^-CD8^-$ thymocytes, while RNA from the rFTOCs was prepared from unfractionated cells. These repopulations took place over the course of 2-3 weeks. As a result, the cells were harvested in small pools, not all at once. Because only a small number of cells could be recovered from thymic lobes on any given day, it was not feasible to isolate $CD4^-CD8^-$ cells either by complement kills or sorting. However, staining for CD4 and CD8 revealed similar proportions of double negative cells in pools of cells derived from fetal liver or bone marrow repopulated thymic lobes (Figure 6-2B).

Because the number of cells in each pool was small (generally $<10^6$), RNA was isolated using E. coli rRNA as carrier. This precluded quantitation of sample RNA by spectrophotometer, so PCR assays were performed using cell-equivalents of RNA (rather than μg or ng quantities). The starting samples were titrated, reverse-transcribed to produce cDNA, the cDNA amplified, and the products visualized on Southern blots. Quantitation was accomplished as before by parallel amplification of known quantities of an *in vitro* synthesized transcript. However, the primer pair (L3/V3-3'a) used to amplify sterile transcripts from thymocyte RNA in earlier assays did not work efficiently enough to reliably amplify transcripts from the small quantities of RNA used here. To remedy this, a new primer pair was used (V3/V3-3'c) (for location and sequences of primers and probes, see Fig. 4A-1 and Chapter 2) which resulted in a smaller product (253 bp vs. 450 bp) and allowed linear amplification of Vγ3 sterile transcripts across the range of RNA concentrations used.

RNA from independent pools of either fetal liver- or bone marrow-repopulated thymic lobes were subject to RT-PCR as described above. RNA from these pools was shown to produce similar amounts of product when amplified with primers specific for β-tubulin (Fig. 6-3C). As shown in figure 6-3A and summarized in Table 6-1, Vγ3 sterile transcript levels were higher in fetal liver than in bone marrow rFTOCs in every case. The difference varied from 3-fold (e.g. FL-1 vs. BM-3) to 14-fold (e.g. FL-3 vs. BM-1) with the average difference of 5-7-fold. This concurs reasonably well with the relative difference in Vγ3 sterile transcript levels between fetal and adult $CD4^-CD8^-$ thymocytes using this primer pair (see Fig. 4A-2C).

The same pools had different absolute values for Vγ3 sterile transcript levels in the different experiments (Table 6-1). Thus, it is not possible to compare absolute levels between experiments. However, in every case the *relative* levels of Vγ3 sterile transcripts in each pool are comparable from one experiment to the next.

The difference in sterile Vγ3 transcript levels between FL-rFTOCs and BM-rFTOCs, is consistent, though small. To insure that the observed 3-14 fold difference is reliable, 30,000 cell equivalents of RNA from each individual pool in Expt 2 (Table 6-1) were combined into larger pools of either FL- or BM-derived cells. These were titrated in *two-fold* dilutions and subjected to the same PCR assay as above. In concordance with earlier observations, the difference in Vγ3 sterile transcript levels was five-fold greater in RNA from FL than BM (Fig. 6-4A and Table 6-1).

Figure 6-3: Sterile transcripts levels in RNA from rFTOCs. RNA from independent pools of rFTOC cells (or *in vitro* synthesized standard transcript) was titrated (by 5-fold dilutions) and reverse-transcribed using an anti-sense primer. The cDNA was then amplified using the same anti-sense primer and a sense primer. 25µl from each reaction was gel-fractionated, blotted, and the blots probed with ^{32}P-labeled oligonucleotide. Above each lane is the amount of input RNA in cell equivalents (or fg for the standard transcript). Lanes labeled "No RT" represent PCRs with the highest titration of input RNA in which the reverse transcriptase was omitted. RNA samples are designated either FL (from fetal liver-repopulated thymic lobes) or BM (from bone marrow-repopulated thymic lobes). **(A)** Vγ3 sterile transcripts detected using the V3/V3-3'c primer pair and V3C probe (Fig 4A-1). **(B)** Vγ2 sterile transcripts detected using the V2/V2-3'a primer pair and V2-3'b probe (Fig. 4-1). **(C)** Tubulin transcripts. Note that these are the results of two different experiments. Product for the sample BM-2 is shown for both experiments to allow comparison.

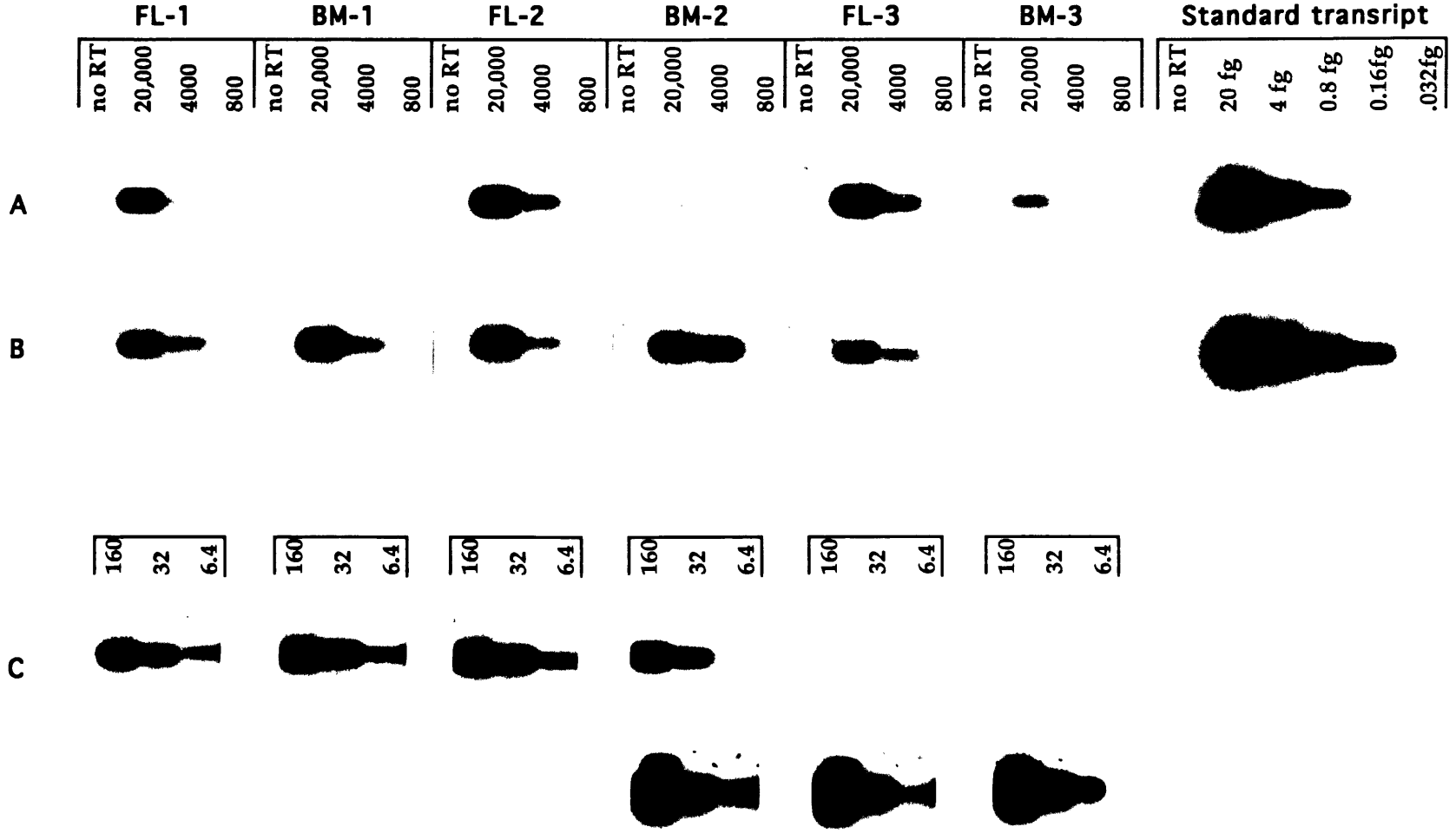

FL-1
BM-1
FL-2
BM-2
FL-3
BM-3
Standard transript
no RT
20,000
4000
800
20 fg
4 fg
0.8 fg
0.16fg
.032fg
A
B
160
32
6.4
C

Figure 6-4: Sterile Vγ transcript levels in RNA from pooled rFTOCs. RT-PCR was performed as in Figure 6-4 using as template combined pools of RNA from either fetal liver rFTOCs (FL-pool) or bone marrow rFTOCs (BM-pool), with two-fold dilutions. **(A)** Vγ3 sterile transcripts detected using the V3/V3-3'c primer pair and V3C probe (Fig 4A-1). **(B)** Vγ2 sterile transcripts detected using the V2/V2-3'a primer pair and V2-3'a probe (Fig. 4-1).
(C) Tubulin transcripts.

A
B
C
FL-pool
BM-pool
standard transcript
no RT
10,000
5000
2500
1250
625
no RT
10,000
5000
2500
1250
625
no RT
10 fg
5 fg
2.5 fg
1.25fg
.625fg
312.5
156.3
78.1
39.1
312.5
156.3
78.1
39.1

Table 6-1: Summary of sterile transcript levels in rFTOC RNA. PCR gels were scanned on a densitometer, and values expressed as copies/10,000 cell equivalents were calculated by comparing amount of PCR product from each sample to the amount produced from known amounts of standard transcript.

a) PCR shown in Fig. 6-3A

b) PCR shown in Fig. 6-4A

c) PCR shown in Fig. 6-3B

d) PCR shown in Fig. 6-4B

e) calculated as average of BM samples vs. average of FL samples

	STERILE Vγ3 TRANSCRIPT LEVELS copies/10,000 cell equiv.			STERILE Vγ2 TRANSCRIPT LEVELS copies/10,000 cell equiv.	
SAMPLE #	Expt. 1	Expt. 2[a]	Expt. 3[b]	Expt. 4[c]	Expt. 5[d]
BM-1	1026	182		456	
BM-2	1140	239		798	
BM-3		342			
BM-pool			570		1303
FL-1	3534	1140		342	
FL-2	6270	2280		570	
FL-3	7410	2508		251	
FL-pool			2850		928
AVERAGE DIFFERENCE[e]	FL>BM 5.3X	FL>BM 7.7X	FL>BM 5X	BM>FL 1.6X	BM>FL 1.4X

Analysis of Vγ2 sterile transcript levels.

To investigate whether the differences observed were a general phenomenon or specific to the Vγ3 transcript, Vγ2 sterile transcript levels were also determined. As shown in Figure 6-3B and summarized in Table 6-1 there is no consistent difference in Vγ2 sterile transcript levels between FL and BM rFTOCs Some FL rFTOCs had higher levels than BM rFTOCs (e.g. FL-2 vs. BM-1), while others were lower (e.g. FL-1,3 vs. BM-2), with the average difference BM>FL by 1.6-fold. As above, an additional RT-PCR experiment was performed using the pooled RNA samples from FL or BM rFTOCs with two-fold dilutions. As shown in Figure 6-4B and Table 6-1, the pooled samples resulted in a difference in Vγ2 sterile transcript levels between BM- and FL-derived thymocytes of 1.4-fold.

Discussion

Previous studies have demonstrated a requirement for the combination of fetal stem cells and a fetal thymic microenvironment to generate Vγ3$^+$ T cells. RT-PCR assays used here show that the level of Vγ3 sterile transcripts is higher in RNA samples from FL-derived thymocytes than from BM-derived thymocytes, reflecting the difference in developmental potential between fetal and adult thymocyte precursors in the ability to develop into Vγ3$^+$ cells. Furthermore, the difference in Vγ3 sterile transcript levels between FL and BM progenitors is enough to account for the difference observed between E15 and adult DN thymocytes.

Because the thymic lobes were repopulated with unfractionated FL or BM cells, there could be a difference in total

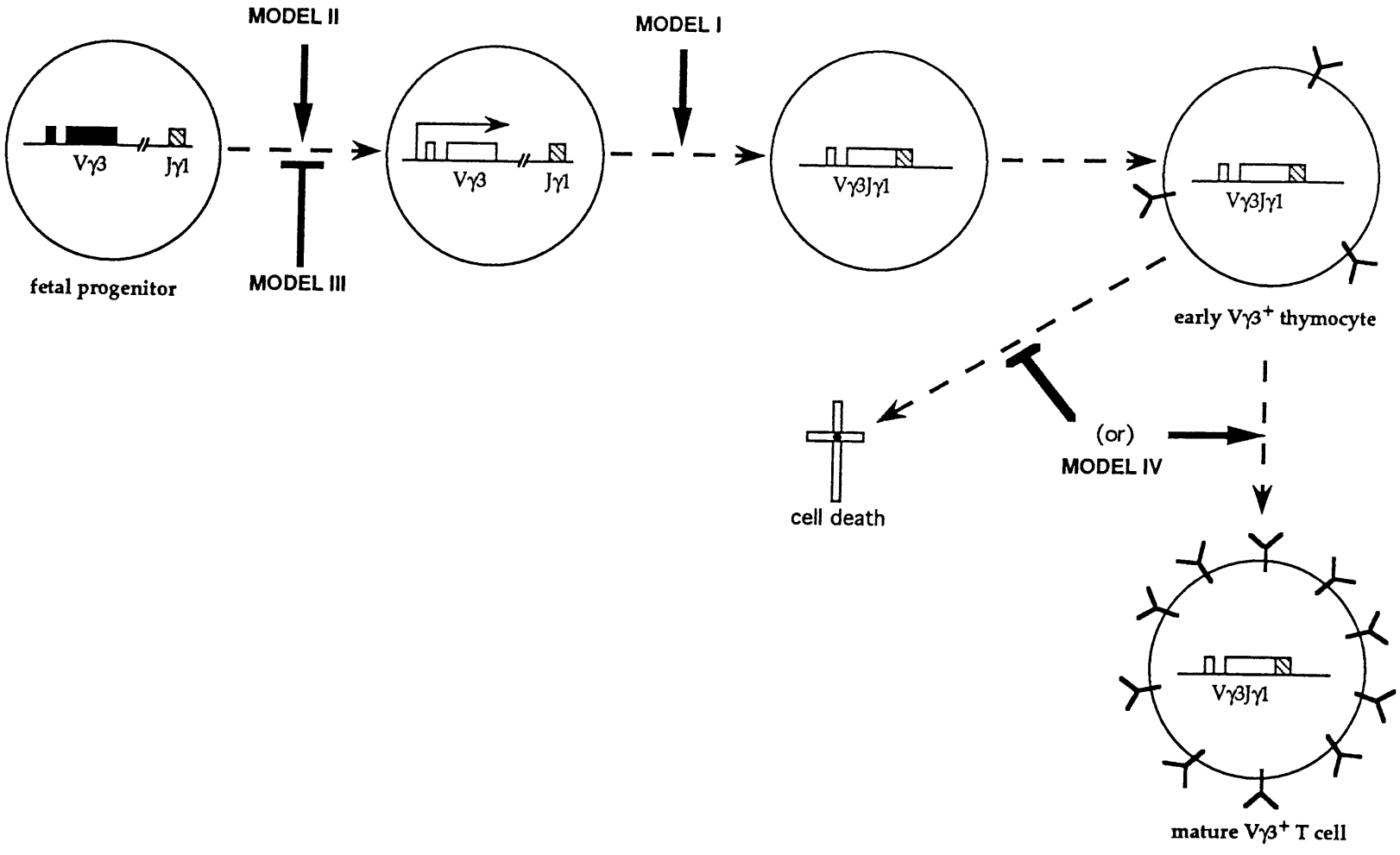
numbers of T cell progenitors, resulting in the observed difference in Vγ3 sterile transcript levels. That this is not the case is supported by the observation that there is no consistent difference between FL and BM repopulated thymic lobes with regard to Vγ2 sterile transcript levels.

If the accessibility of Vγ3 genes is controlled at the level of stem cell origin, what role is played by the thymic microenvironment in controlling the generation of $V\gamma3^+$ T cells? At least four models can be envisioned which incorporate our observations with the earlier results on this issue (Fig. 6-5).

In the first model, the fetal progenitor cell destined to become a Vγ3 cell transcribes the germline Vγ3 gene independently of interaction with the thymus, reflecting an increased accessibility of the gene inherent in these cells. However, a signal which can be provided by the fetal thymus, but not the adult thymus, is required to activate actual rearrangement of the gene. This possibility is supported by recent studies showing that transcription and/or demethylation of transgenic recombination substrates can occur in cells in the absence of high levels of rearrangement, indicating that an additional step may be required (Bottaro et al., 1994, Chen et al., 1993, Okada et al., 1994).

In the second model, a signal from the fetal thymic stroma initiates transcription and/or accessibility of germline Vγ3 genes allowing their rearrangement. According to this model, the signal can only be delivered by the fetal thymic stroma, and only fetal stem cells are receptive to this signal.

Figure 6-5: Possible role of thymic signals in Vγ3$^+$ T cell production. Steps in the rearrangement of a Vγ3 gene in a fetal progenitor cell are shown as well as the points at which positive (↓) or negative (⊤) signals are delivered by the fetal (Models I, II, IV) or adult (Model III) thymic stroma according to each of the four models described in the text.


MODEL II
MODEL I
Vγ3
Jγ1
fetal progenitor
MODEL III
Vγ3
Jγ1
Vγ3Jγ1
Vγ3Jγ1
early Vγ3+ thymocyte
cell death
(or)
MODEL IV
Vγ3Jγ1
mature Vγ3+ T cell

A third possibility is that fetal progenitors have the potential to spontaneously transcribe and rearrange their Vγ3 genes. The adult thymus, but not the fetal thymus, delivers a negative signal which actively shuts off the production of Vγ3 sterile transcripts and/or Vγ3 gene rearrangement

Finally, in the fourth model, the occurrence of both germline transcription and rearrangement of the Vγ3 gene segment in the fetal progenitor cell is independent of the thymic microenvironment. However, the fetal thymus, but not the adult thymus, may provide other signals which are required for the survival and/or maturation of the $V\gamma3^+$ cells. This is supported by studies which show that phenotype maturation of Vγ3 expressing cells is promoted by interactions within the fetal thymus (Leclercq et al., 1993, Tatsumi et al., 1993). Whether these interaction involve the T cell receptor has not been established. However, in one study, crosslinking of the γδ T cell receptor on HSA^+ thymocytes resulted in downregulation of RAG-1 expression (Tatsumi et al., 1993), which is associated with T cell maturation (Brandle et al., 1992).

An important aspect of each of the above models is that the rearrangement of the Vγ3 gene is a programmed event in a particular subset of fetal progenitor cells. Thus, the signal(s) delivered by the fetal thymus to support, or by the adult thymus to repress, the development of Vγ3 cells only act on progenitors in which this lineage is predetermined.

The rearrangement of the Vγ3 gene has been demonstrated to proceed correctly both with regard to developmental timing and production of canonical junctional sequence in the absence of

selection, probably as the result of molecular constraints (Asarnow et al., 1993, Itohara et al., 1993). While this and other evidence suggest that programmed rearrangement of Vγ genes plays an important role in the ordered appearance of γδ T cell subtypes during development, this does not imply that selection events in the thymus are not also a part of the normal physiological process of γδ T cell development.

With regard to activating or repressing Vγ3 gene transcription/rearrangement, the nature of any putative signals between the thymus and stem cells colonizing it is unknown. However, cytokines are possible candidates. For example, cytokines can differentially induce transcription of specific immunoglobulin heavy chain constant regions prior to switch recombination to that C_H segment in B cells (Lutzker et al., 1988, Stavnezer et al., 1988). IL-10 and IL-7 have been demonstrated to be involved in γδ T cell development in the fetal thymus (Fine et al., 1994, Tomana et al., 1993). However, no cytokine has been determined to preferentially support the outgrowth of a particular γδ T cell subtype. An alternative possibility is that receptors on stem cells interact with counter-receptors on the surface of thymic stromal cells, resulting in either the activation or repression of Vγ3 gene rearrangement.

Chapter 7

Vγ STERILE TRANSCRIPTS IN THYMOCYTES FROM SCID MICE AND CHIMERAS

Introduction

The previous chapters examined the role of Vγ gene targeting in the ordered appearance of γδ T cell subtypes. The results suggested that differential accessibility of Vγ genes may be responsible for the changing rearrangement patterns during ontogeny. To further study the importance of sterile Vγ transcripts in development, I wished to look at patterns in cells which do not rearrange their T cell receptor genes, namely in thymocytes of SCID mice.

SCID mice have a defect in their recombinatorial machinery such that they are unable to correctly complete V-(D)-J rearrangement (Schuler et al., 1986). As a result, they are severely deficient in B and T cells (Bosma et al., 1983). The thymus of the SCID mouse reflects this deficiency in that it is not organized into distinct cortical and medullary regions and it has only a fraction of the cellularity of a normal thymus. The thymocytes that are present are large in size, IL-2R$^+$, CD4$^-$, CD8$^-$ and TCR$^-$ (Shores et al., 1990). These cells most resemble early fetal thymocytes of a normal mouse. Cells of this phenotype are also present in small numbers as T cell precursors in the adult murine thymus. (Scollay et al., 1988).

Introduction of normal cells into a SCID thymus, either by exogenously added stem cells (bone marrow chimeras) or through

the spontaneous reversion of the SCID mutation in a small subset of endogenous cells ("leakiness"), changes the thymus to a more normal adult morphology. The thymus develops distinct cortical and medullary regions (Shores et al., 1991), and a portion of the SCID thymocytes advance to the $CD4^+CD8^+$ stage (double positive-DP) (Shores et al., 1990). It is important to note that cells which become DP do not necessarily express T cell receptor themselves, but merely require the presence of these normal cells.

Because the adult SCID thymus in some respects resembles a normal fetal thymus, it was of interest to examine whether the similarity extends to the presence of high levels of Vγ3 sterile transcript. If so, I wished to know if the maturation of the SCID thymus, induced by the presence of normal cells, would cause the level of Vγ3 sterile transcripts to decrease in SCID thymocytes. It was also of interest to determine whether the SCID thymus, given its resemblance to a normal fetal thymus, could support the development of $V\gamma3^+$ T cells. This chapter describes studies to address these questions.

Results

Vγ Sterile Transcript Levels In SCID Thymocytes

First it was important to determine Vγ sterile transcript levels in thymocytes of unmanipulated SCID mice. Because they are arrested prior to γ gene rearrangement (Carroll & Bosma, 1991), adult SCID thymocytes may have similar progenitor pools as normal adult $CD4^-CD8^-$ thymocytes prior to rearrangement of T cell receptor genes. Thus, they may also have similar patterns of Vγ gene sterile

source of thymocyte RNA	sterile transcripts copies/μg Vγ2	Vγ3
E15 $CD3^-$	$5.7X10^5$	$3.4X10^5$
Adult DN $CD3^-$	$1.2X10^5$	$<3.6X10^3$
SCID E15	$4.6X10^5$	$2.3X10^5$
SCID Adult	$4.6X10^5$	$2.3X10^5$

Table 7-1: RNA from E15 or adult SCID thymocytes was subjected to quantitative RT-PCR as described in Chapter 4. Values for E15 $CD3^-$ and adult DN $CD3^-$ sterile transcript levels from Table 4-1 are included for comparison

transcription. Alternatively, it is possible that they have the same pattern of sterile transcripts as fetal thymocytes, because of their resemblance with regard to cell surface markers and thymic architecture. To test this, RNA was isolated from thymocytes of 3-4 week old adult and E15 fetal CB17 SCID mice and subjected to the quantitative RT-PCR assay described in Chapter 4.

The results, summarized in Table 7-1, compare the levels of Vγ2 and Vγ3 sterile transcripts in SCID E15 and adult thymocytes to levels in normal $CD3^-$ thymocytes. First, it is important to point out that with regard to either Vγ2 or Vγ3 sterile transcript levels, there is no discernible difference between SCID adult and SCID E15 fetal thymocytes. It also appears that the pattern of Vγ3 sterile transcripts in either population closely resembles that of normal $CD3^-$ E15 fetal thymocytes. Sterile Vγ2 transcript levels also are similar between SCID adult, SCID E15 and normal E15 $CD3^-$ thymocytes. Thus, with regard to sterile transcript levels, the adult SCID thymus is comparable to a normal fetal thymus.

Construction of Chimeras

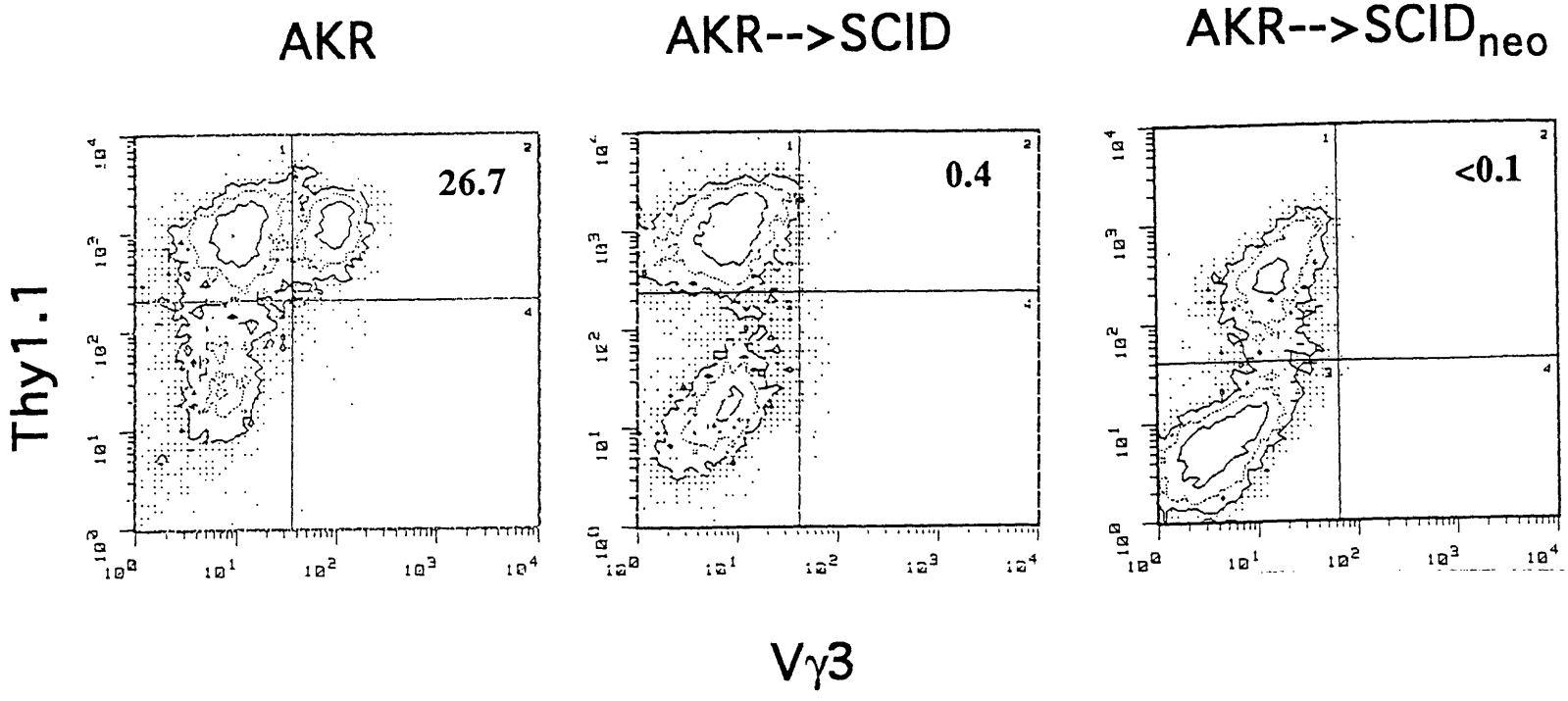
Experiments by Shores, et al (Shores et al., 1990, Shores et al., 1991) demonstrated that the introduction of bone marrow cells from a normal mouse into a SCID mouse would induce the thymus to change to a more normal state. I made similar chimeras to test whether the Vγ3 sterile transcript levels in SCID thymocytes would decrease in response to residing in a thymus which changed from a fetal to an adult state.

First, potential CB17 SCID (Thy1.2) hosts were tested for leakiness by performing ELISAs to determine the IgG content of their serum. Only IgG-negative hosts were used. They were injected i.v. with E14 fetal liver cells from AKR (Thy1.1) donors (AKR-->SCID). Fetal liver was chosen as the source of stem cells because this would allow the determination of whether the environment of the SCID thymus is sufficiently "fetal" to support the development of Vγ3-expressing T cells. Unlike most stem cell chimeras, the hosts were unirradiated to allow observation of the effect on residual SCID host thymocytes. Control chimeras were also constructed by injecting SCID hosts with E14 fetal liver from CB17 SCID donors (SCID-->SCID).

s-IELs in chimeras

As discussed in the previous chapter, Vγ3$^+$ T cells can only be generated from the combination of fetal stem cells and a fetal thymic microenvironment (Havran et al., 1991a, Ikuta et al., 1990). Because of the resemblance of the adult SCID thymus to a normal fetal thymus, its potential to support the development of Vγ3$^+$ T cells from fetal progenitors was examined. If these cells are in fact produced, they may only be present in the thymus for a limited time, which could easily be missed. It was assumed that these cells would be capable of homing to the skin, as Vγ3$^+$ s-IELs were recovered from adult (nude) hosts reconstituted with fetal liver and fetal thymic stroma (Havran et al., 1991a). Therefore, the presence of donor-type s-IELs was determined by staining epidermal cells for Thy-1.1 and Vγ3. However, no Vγ3$^+$ cells were found in the skin of these mice (Fig 7-1).

Figure 7-1: s-IELs in SCID chimeras. Epidermal cells isolated from the skin of AKR, AKR-->SCID chimeras or AKR-->$SCID_{neo}$ chimeras were stained with antibodies against Vγ3 and Thy1.1. Percent of donor-type s-IEL is indicated.


AKR
AKR-->SCID
AKR-->SCID$_{neo}$
26.7
0.4
<0.1
Thy1.1
Vγ3

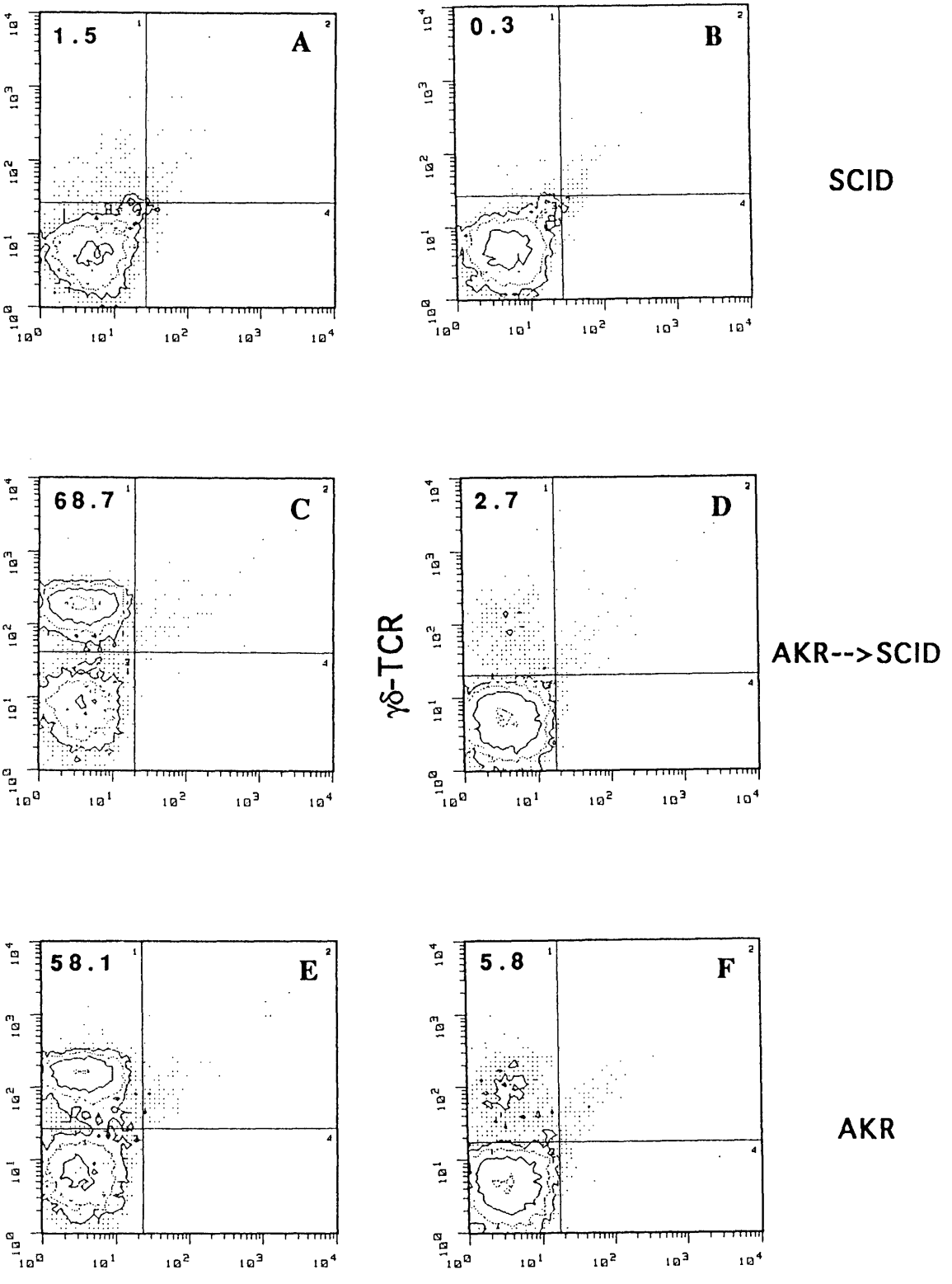
Additional chimeras were constructed using fetal liver cells from AKR donors and neonatal (0-1 day) SCID recipients (AKR--> $SCID_{neo}$) in the hope that the fetal stem cells would have a better chance of developing into $V\gamma3^+$ cells in a SCID thymus which has not been subject to the effects of aging. Also, neonates have been shown to be the best hosts for complete reconstitution of unirradiated SCID mice with allogeneic stem cells (Cowing & Gilmore, 1992). However, staining of epidermal cells from these chimeras did not detect $V\gamma3^+$ cells (Fig. 7-1).

The reconstitution looked to be complete as demonstrated by the normal distribution of CD4/CD8 subsets in the thymus (data not shown). Additionally, the spleens of these chimeras were removed, enriched for $\gamma\delta$ T cells by treatment with antibodies against heat-stable antigen (to remove B cells), CD4 and CD8 plus complement. Staining of surviving cells with pan-$\alpha\beta$ or pan-$\gamma\delta$ antibodies revealed the presence of both $\alpha\beta^+$ and $\gamma\delta^+$ T cells in the spleens of these chimeras (Fig. 7-2) which have the donor Thy1.1 type (data not shown). This indicated that the lack of $V\gamma3^+$ cells in the skin was not due to some general defect in the ability of these mice to generate T cells from donor stem cells.

Isolation of thymocytes from chimeras

These chimeras were designed to examine the effect of the presence of normal cells on SCID thymocytes. First, host cells had to be isolated from the chimeras. This was done by exploiting the Thy1 allelic difference between donor (AKR-Thy1.1) and host (CB17 SCID-Thy1.2) cells. Isolation of SCID cells from these thymuses was first

Figure 7-2: T cells in spleen of AKR-->$SCID_{neo}$ chimeras. Spleen cells from SCID (**A, B**), AKR-->SCIDneo (**C, D**) or AKR (**E, F**) mice were depleted of B cells and stained with antibodies against αβ T cell receptor (**A, C, E**) or γδ T cell receptor (**B, D, F**).


1.5
A
0.3
B
SCID
68.7
C
2.7
D
αβ-TCR
γδ-TCR
AKR-->SCID
58.1
E
5.8
F
AKR

attempted by two rounds of depletion with anti-Thy1.1 antibody plus complement. Because the SCID cells made up such a small proportion of the thymocytes in the chimeras, it was difficult to achieve a high level of purity using this technique. Staining of the resultant cells revealed that only 20% were Thy1.2$^+$ (data not shown). Thus, in order to isolate a pure population of host cells it was necessary to sort them away from the thymocytes which had expanded from the donor stem cells.

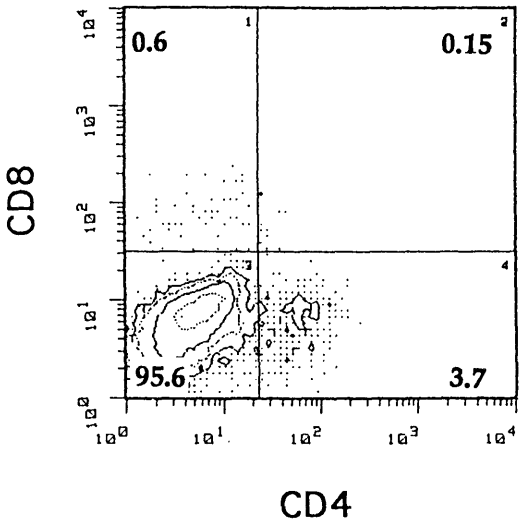
Eight to ten weeks post-injection, mice were tested for chimerism by staining of peripheral blood lymphocytes (PBLs) with anti-Thy1.1 antibody. All thymuses from mice which were chimeric by PBL phenotype (i.e. Thy1.1$^+$) had normal cellularity (average $1.5x10^8$ cells/thymus) compared to thymuses from mice which were Thy1.1$^-$ (average $3.4x10^6$ cells/thymus). Thymocytes from chimeric mice were pooled, and a portion of them were stained for CD4, CD8 and Thy1.1 or Thy1.2. The SCID (Thy1.2$^+$) cells made up 0.5% of total thymocytes. As expected, the donor cells had differentiated into the normal CD4/CD8 subsets (Fig. 7-3B). In addition, some of the resident SCID thymocytes (29.8%) had adopted the CD4$^+$CD8$^+$ phenotype (Fig. 7-3B), in accordance with earlier findings (Shores et al., 1990).

The remaining cells were subjected to one round of treatment with anti-CD4 and anti-CD8 antibodies plus complement to enrich for CD4$^-$CD8$^-$ cells. The resultant cells were stained with a mixture of anti-CD4/CD8-PE, anti-Thy1.2-FITC and anti-Thy 1.1-biotin + streptavidin-tricolor. CD4$^-$CD8$^-$ (PE$^-$) cells were sorted into host (Thy1.2$^+$) and donor (Thy1.1$^+$) populations. Post-sort analysis

Figure 7-3: CD4/8 phenotypes of thymocytes from SCID chimeras. Percent of each CD4/CD8 subset is indicated in the appropriate quadrant. **(A)** Total thymocytes from SCID-->SCID chimeras were stained with CD4- and CD8-specific antibodies. **(B)** Total thymocytes from AKR-->SCID chimeras were stained with anti-CD4, anti-CD8 and either Thy1.1- or Thy1.2-specific antibody. CD4/CD8 staining from either host (SCID) or donor (AKR) cells is displayed by gating on $Thy1^+$ cells, as indicated. **(C, D)** Total thymocytes from SCID-->AKR **(C)** or CB17-->AKR **(D)** chimeras were stained with anti-CD4, anti-CD8 and anti-Thy1.2 antibodies. CD4/CD8 subsets from donor cells are displayed by gating on $Thy1.2^+$ cells.

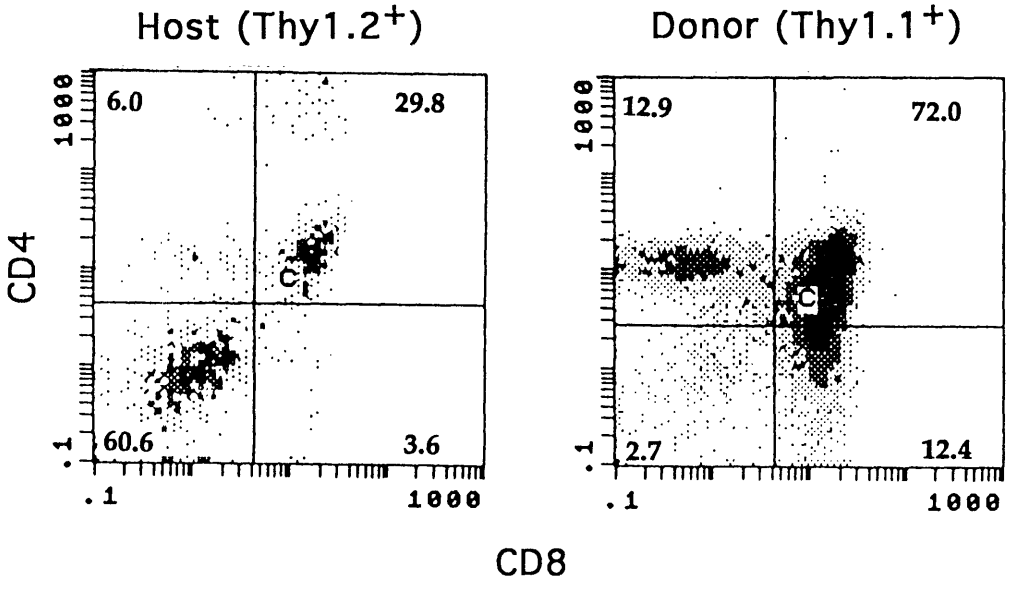
A

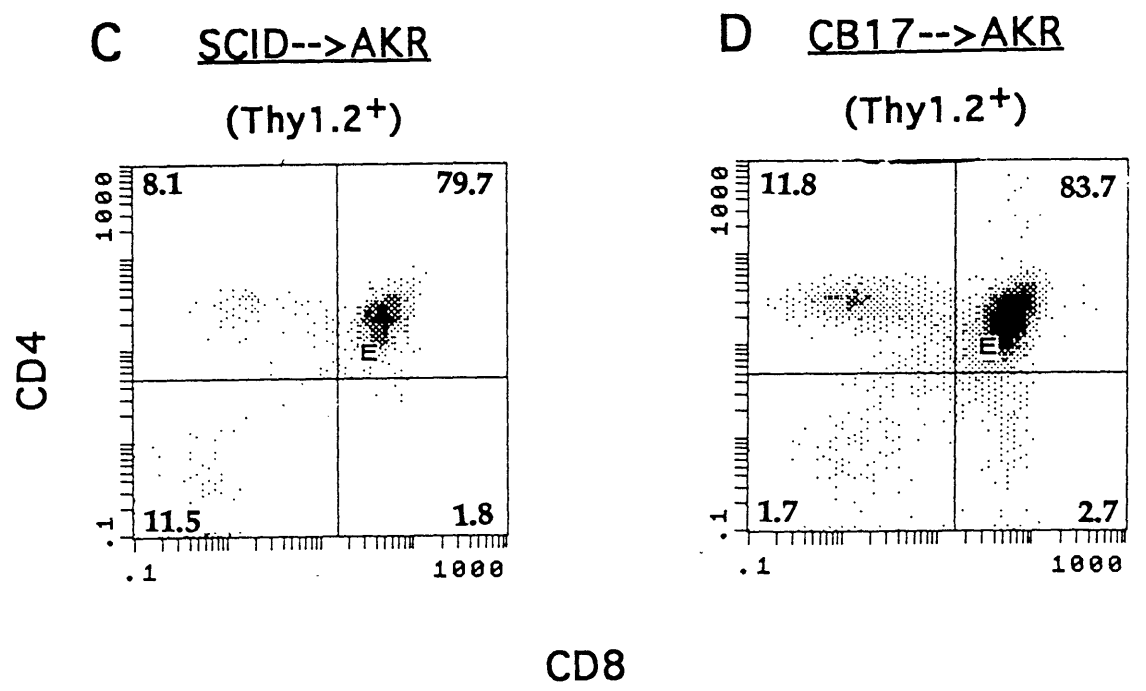
SCID-->SCID



B

AKR-->SCID




C
SCID-->AKR
(Thy1.2+)
8.1
79.7
11.5
1.8
D
CB17-->AKR
(Thy1.2+)
11.8
83.7
1.7
2.7
1000
.1
1000
.1
CD4
CD8

revealed the purity of the sorted cells was 98.7% (SCID) and 98.8% (AKR).

Vγ3 sterile transcripts in AKR-->SCID chimeras.

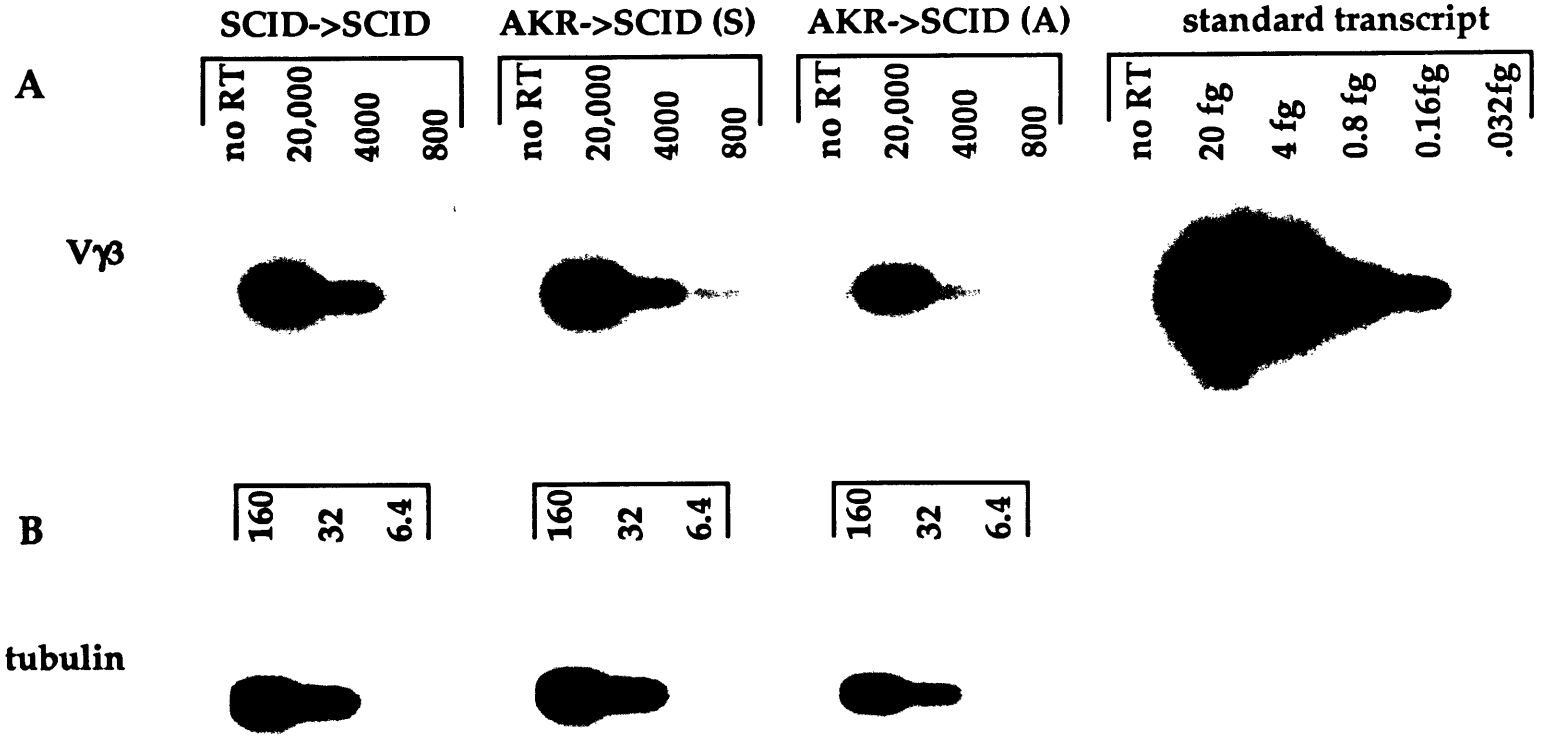
As demonstrated above, the Vγ3 sterile transcript levels in SCID thymocytes are comparable to those present in normal fetal thymocytes. In order to determine if these levels were influenced by the maturation of the thymus induced by the presence of normal cells, RNA was isolated from SCID-->SCID thymocytes and sorted SCID host and AKR donor thymocytes from AKR-->SCID chimeras. As in Chapter 6, the small cell numbers required the use of rRNA as carrier. Thus, the amount of RNA used for the RT-PCR was measured in cell equivalents. This RNA was amplified using the V3/V3-3'c primer pair (Fig. 4A-1). Earlier experiments had shown that SCID-->SCID thymocyte RNA generated the same amount of Vγ3 product as SCID thymocyte RNA (data not shown). As illustrated in Figure 7-4, it is apparent that there is little change in the levels of Vγ3 sterile transcript in SCID thymocytes from the AKR-->SCID chimeras. There is, however, an ~5-fold decrease in Vγ3 sterile transcript levels in AKR thymocytes from these chimeras, as expected. These data indicate that the "adult" thymic environment is unable to cause a decrease in Vγ3 sterile transcript levels in SCID progenitor cells.

SCID-->AKR chimeras.

Another way to test the effect of a mature thymic microenvironment on SCID thymocytes was to introduce them into a normal thymus. This was accomplished by injecting CB17 SCID fetal

Figure 7-4: Vγ3 sterile transcripts in AKR-->SCID chimeras.

(A) RNA from SCID-->SCID chimeras, sorted populations of cells from AKR-->SCID chimeras (AKR-->SCID(S): SCID host cells or AKR-->SCID(A): AKR donor cells) or *in vitro* synthesized standard transcript was titrated (by 5-fold dilutions) and reverse-transcribed using the V3-3'c primer. The cDNA was then amplified using the V3/V3-3'c primer pair. 25μl from each reaction was gel-fractionated, blotted, and the blots probed with ^{32}P-labeled V3C oligonucleotide. (For locations of primers and probe, see Fig. 4A-1). Above each lane is the input RNA in cell equivalents (or fg for the standard transcript). Lanes labeled "no RT" represent PCRs with the highest titration of input RNA in which the reverse transcriptase was omitted. **(B)** RT-PCR product from the same RNA samples using tubulin primers and probe.


SCID->SCID
AKR->SCID (S)
AKR->SCID (A)
standard transcript
A
no RT
20,000
4000
800
no RT
20 fg
4 fg
0.8 fg
0.16fg
.032fg
Vγ3
B
160
32
6.4
tubulin

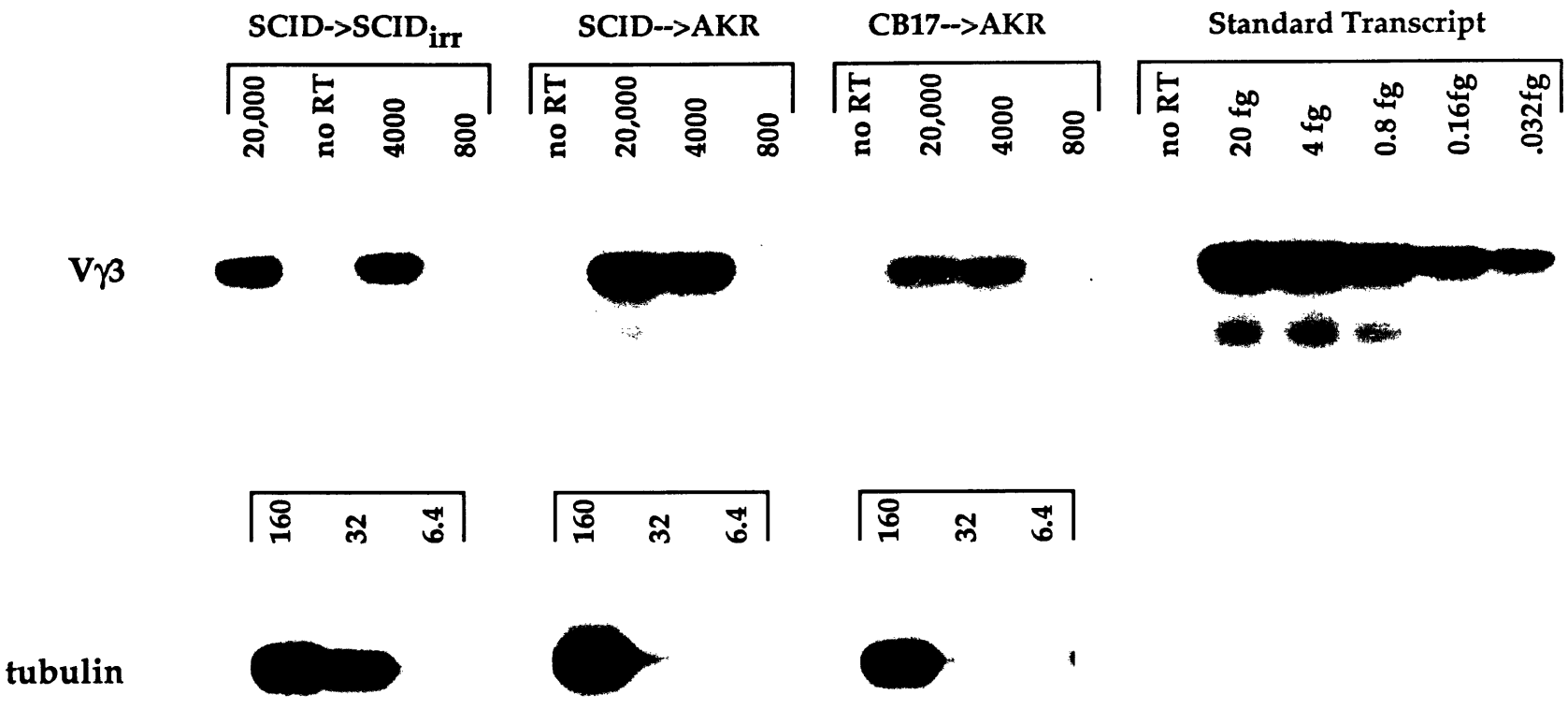
liver cells into irradiated AKR hosts and observing the effect on CD4/CD8 subsets and Vγ3 sterile transcript levels. Control chimeras were also constructed by injecting CB17 wild type fetal liver into AKR hosts (CB17-->AKR), and CB17 SCID fetal liver into lightly irradiated SCID hosts (SCID-->$SCID_{irr}$).

Analysis of the CD4/CD8 subsets demonstrated similar results as the reciprocal chimeras. Donor cells from CB17-->AKR chimeras differentiated into all four subsets in relatively normal proportions (Fig. 7-3D). A portion of SCID thymocytes (79%) from SCID-->AKR chimeras became $CD4^+CD8^+$ as predicted (Fig. 7-3C).

Donor thymocytes from SCID-->AKR or CB17-->AKR chimeras were pooled and treated with one round of anti-CD4 and anti-CD8 antibodies plus complement. The resulting cells were stained with CD-4/CD8-PE and Thy1.2-FITC. Donor DN cells were sorted as PE^- $FITC^+$ and were found to be 95% (SCID) and 98.5% (CB17) $CD4^-CD8^-$ $Thy1.2^+$. Vγ3 sterile transcript content in the RNA from these cells was assessed as above using RT-PCR (Fig. 7-5). Again there was little apparent difference between SCID-->SCID and SCID-->AKR with respect to sterile Vγ3 transcript levels. In contrast, levels were ~5 -25-fold lower in CB17-->AKR thymocytes as would be expected. Thus, thymocytes derived from CB17 stem cells produce low levels of Vγ3 sterile transcript in the adult thymic microenvironment. However, the adult thymus does not appear to exert this effect on stem cells from SCID mice.

Fig. 7-5: Vγ3 sterile transcript levels in -->AKR chimeras. RNA from SCID-->$SCID_{irr}$, sorted DN donor thymocytes from SCID- ->AKR or CB17-->AKR chimeras, or *in vitro* synthesized transcript was subjected to RT-PCR to detect Vγ3 sterile transcripts or tubulin transcripts as in Figure 7-4.

Note that the first two lanes of SCID-->$SCID_{irr}$ products were loaded in reverse order.


SCID->SCID$_{irr}$
SCID-->AKR
CB17-->AKR
Standard Transcript
20,000
no RT
4000
800
no RT
20,000
4000
800
no RT
20,000
4000
800
no RT
20 fg
4 fg
0.8 fg
0.16fg
.032fg
Vγ3
160
32
6.4
160
32
6.4
160
32
6.4
tubulin

Discussion

An RT-PCR assay determined that E15 and adult SCID thymocytes express transcripts of unrearranged Vγ2 and Vγ3 genes in a pattern similar to normal E15 fetal thymocytes, suggesting that the adult SCID thymus resembles a fetal thymus with respect to Vγ3 gene accessibility.

Previous studies demonstrated that the SCID thymus is essentially not defective and evidence presented here supports this. For example, upon the introduction of normal stem cells, it is able to direct the differentiation of thymocytes into normal CD4/CD8 subsets. In addition, it is able to produce γδ and αβ T cells from these stem cells as evidenced by their presence in the spleen. Despite the effects of normal AKR cells on the thymic architecture and CD4/CD8 expression by SCID thymocytes in AKR-->SCID chimeras, there did not appear to be any effect on SCID thymocytes in these chimeras with regard to reduction of Vγ3 sterile transcript levels.

Another attempt to investigate the effect of a normal adult thymic environment of SCID thymocytes involved the introduction of SCID stem cells directly into a normal thymus using SCID-->AKR chimeras. Since these cells would never have been exposed to a fetal thymic environment, the influence of the adult thymus can be evaluated. SCID cells isolated from SCID-->AKR chimeras still had high levels of Vγ3 sterile transcripts indicating that, like SCID cells from AKR-->SCID chimeras, the environment in which these cells mature does not affect the transcription of the Vγ3 genes.

In normal mice fetal liver stem cells colonize the fetal thymus and a some of these cells express high levels of Vγ3 sterile

transcripts in preparation to become Vγ3$^+$ cells. These thymocytes would then presumably rearrange their genes and migrate to the skin. As development progresses, these cells would be displaced by thymocytes derived from bone marrow cells which would not produce high levels of Vγ3 sterile transcripts. Vγ3 progenitor cells among SCID thymocytes, being unable to rearrange their genes may survive and become trapped in the thymus, remaining through adulthood. This may not seem likely because some studies indicate that SCID thymocytes have a high rate of turnover (Rothenberg et al., 1993, Schuler et al., 1988). However it is possible that the small subset of thymocytes which are transcribing unrearranged Vγ3 genes are long-lived in the thymus. Thus, in the chimeras, these cells would remain regardless of the state of maturation of the thymus.

Another possibility is that the normal adult thymus is capable of providing signals which actively shut off the production of Vγ3 sterile transcripts. This could explain the observation that thymocytes derived from CB17 fetal stem cells introduced into a normal thymus have reduced levels of Vγ3 sterile transcripts. The reduced levels of Vγ3 sterile transcript in *donor* derived cells from the AKR-->SCID chimeras demonstrates that if this signaling does occur, it is working properly in the adult-like thymus of these chimeras. The observation that the Vγ3 sterile transcript levels in SCID thymocytes from both AKR-->SCID and SCID-->AKR chimeras remain high indicates that these cells may be arrested at a developmental point at which they are unresponsive to the putative negative signals from the adult thymic stroma.

Results presented in this chapter show that, although the unmanipulated adult SCID thymus resembles a normal fetal thymus with regard to Vγ3 sterile transcript levels, it does not appear to have the ability to support the development of $V\gamma3^+$ T cells from normal fetal progenitors. Why is this so?

One possibility is that the signal provided by the normal fetal thymus may be required to induce rearrangement of the Vγ3 gene whose accessibility, as evidenced by high levels of Vγ3 sterile transcript, may be an inherent property of the fetal progenitor cell. This is supported by the observation that SCID thymocytes in the SCID-->AKR chimeras express high levels of Vγ3 sterile transcripts in spite of the fact that they are derived from fetal stem cells which have never been exposed to fetal thymic stroma. Thus, the SCID thymus may essentially play a passive role with regard to the production of Vγ3 sterile transcripts but would not provide signals, given by the normal fetal thymus, to induce rearrangement of Vγ3 genes.

Alternatively, it is possible that the adult SCID thymus is different from a fetal thymus in that it can not deliver a signal which induces maturation of $V\gamma3^+$ cells (perhaps from the HSA^+ to the HSA^- stage) and which may be required for the export of these cells to the periphery. Thus, $V\gamma3^+$ cells, even if generated in the thymus, may not appear in the skin of these mice.

CHAPTER 8

CONCLUDING REMARKS

The Accessibility Model and TCR γ Gene Rearrangement

The accessibility model has been invoked to explain regulation of rearrangement in situations such as sequential rearrangement of IgH chain genes or cytokine-mediated class switching. In this model, transcription of unrearranged gene segments, which precedes their rearrangement, is thought to be an indicator of the accessibility of the gene segments to the recombinase machinery. Consistent with this model are my observations that the pattern of sterile Vγ gene transcript levels during ontogeny correlates with the timing of their rearrangement.

A prediction of the accessibility model is that only one Vγ gene of the Cγ1 cluster will be accessible in a given progenitor cell. We observed that, in contrast to rearranged Vγ3 transcripts, *sterile* Vγ3 transcripts rarely have the Vγ4 leader exon spliced to the Vγ3 coding exon. This suggests that Vγ4 and Vγ3 genes may be differentially accessible prior to rearrangement.

Taken together, the results in Chapters 3-5 suggest that ordered Vγ gene rearrangement is accomplished, at least in part, by the selective induction of V gene accessibility, which is correlated with transcription of the unrearranged genes. Although this and other work provide strong correlations between transcription and rearrangement, this does not imply that transcription *per se* is responsible for accessibility of the genes. The presence of sterile

transcripts may merely be a reflection of the chromatin accessibility mediated by a different mechanism. Experiments showing that high levels of sterile transcript can be produced without appreciable levels of rearrangement have been used to argue that transcription is not sufficient to cause accessibility of these genes. However, in those studies, the measure of accessibility was rearrangement. Thus, it is possible that transcription *is* sufficient for accessibility, but that accessibility is not sufficient for rearrangement. For example, local control of gene accessibility may be regulated by certain factors, while activation of rearrangement may require other locus or gene-specific factors necessary to assemble the recombinase complex. Identification of cis-acting elements that regulate accessibility should help to sort out the various levels at which recombination is controlled.

It has not been determined which cis-acting elements are responsible for the various steps in rearrangement as opposed to those responsible for transcription. The immunoglobulin heavy chain enhancer is an excellent system for analysis of this problem as it is well-characterized and known to be important for both transcription and rearrangement. Selective mutation of discrete sites within the enhancer might allow the dissociation of transcription, accessibility and rearrangement.

Previous research involving accessibility and rearrangement has usually focused on immunoglobulin, rather than T cell receptor genes. These studies have the advantage that rearrangement occurs *in vitro* in pre-B cell lines, the counterparts of which are not available for the γδ T cell lineage. Studies of developmental

regulation of immunoglobulin and TCR gene rearrangement have recently moved to in vivo models such as mice transgenic for rearrangement substrates or with specific control elements deleted or mutated. These models allow the most stringent analysis of this issue. Ongoing studies with Vγ gene transgenic recombination substrates suggest that it is not simply the promotor elements nor the relative position of the genes which determine the order of rearrangement in this system (J. Baker and D. H. Raulet, unpublished results).

Relative Contribution of Stem Cell and Thymus Origin to Vγ3$^+$ Cell Development

All the aspects of the control of differential transcription and/or accessibility of Vγ genes are not fully known. It could be the property of the origin of the stem cell (i.e. fetal liver vs. adult bone marrow) and/or of differential signaling by fetal vs. adult thymic stromal cells. My results showed that fetal liver stem cells, but not adult bone marrow stem cells generated high levels of Vγ3 sterile transcripts in a fetal thymus. This fits with earlier results showing that only fetal stem cells can give rise to Vγ3$^+$ cells, which were corroborated in my experiments.

Taking these results into account, I proposed four models to explain the restricted development of Vγ3$^+$ thymocytes. In the first model, fetal Vγ3 progenitor cells spontaneously transcribe the unrearranged Vγ3 gene, indicating its accessibility. A signal from the fetal thymus is then required to activate rearrangement. The second model states that the Vγ3 gene rearrangement is a spontaneous

process after the accessibility of the Vγ3 gene in the fetal progenitor is triggered by signals from the fetal thymus. The third model describes a mechanism in which the spontaneous transcription and/or rearrangement of Vγ3 genes in progenitor cells is actively switched off by a signal delivered by the *adult* thymus. Finally, in the fourth model, transcription and/or accessibility, as well as rearrangement, are spontaneous processes. After expression of the T cell receptor on the surface, the fetal thymic stroma delivers a signal to allow survival and/or to induce maturation of the Vγ3$^+$ cell.

It is possible that some aspects of each model play a role in the production of Vγ3$^+$ cells. This is a multi-step process which may be regulated at every stage. Thus, distinct thymic signals may be necessary to induce accessibility, rearrangement and maturation of these cells.

Certain aspects of these models can be readily tested. For example, if Vγ3 sterile transcript production in fetal progenitors is independent of thymic signals, it may be possible to detect these transcripts in purified populations of stem cells or pro-T cells from fetal and adult bone marrow, allowing a comparison between the two. It may also be possible to incubate stem cells with separated populations of fetal thymic stromal cells in reaggregation cultures. This would be useful in determining which stromal cell types might be important for the signaling involved in development of Vγ3$^+$ cells.

Recently, it has been shown that thymic stromal cells from young (day 7) mice can be used in reaggregation cultures (J. P. Allison, unpublished results). Reaggregation experiments using day 7 thymic stroma with fetal or adult stem cells could be used to

determine if Vγ3 sterile transcript levels are reduced, as predicted by Model III. In addition, mixing experiments with adult and fetal thymic stroma could be performed to determine if the effect of one or the other is dominant.

Another way to test whether the adult thymus has a negative effect on Vγ3 gene transcription, would be to introduce fetal or adult stem cells into an adult thymus by intrathymic injection, and determine Vγ3 sterile transcript levels at various time points after transfer.

Vγ3 Gene Accessibility in SCID Chimeras

For the final series of experiments I started by determining the pattern of sterile Vγ transcripts in SCID thymocytes to observe the pattern of expression in the absence of rearrangement. I found that the sterile transcript patterns resembled those of E15 fetal thymocytes. This concurs with the fetal appearance of the SCID adult thymus with regard to thymocyte cell surface markers and thymic architecture.

I made AKR-->SCID fetal liver chimeras to examine the effect of the maturation of the SCID thymus, induced by the presence of normal cells, on Vγ3 sterile transcript levels in the resident SCID thymocytes. I also made SCID-->AKR chimeras to examine the effect of introducing the SCID fetal stem cells into an adult thymus. In both types of chimeras, little change was observed in Vγ3 sterile transcript levels in SCID thymocytes. The lack of response of SCID stem cells to changes in the thymic microenvironment suggests that

they have limited usefulness for the understanding the mechanism of regulated rearrangement of the Vγ3 gene.

AKR thymocytes from the AKR-->SCID, and wild type CB17 thymocytes from CB17-->AKR chimeras did express lower levels of Vγ3 sterile transcripts. However, the role of the thymus in the downregulation of Vγ3 sterile transcripts is difficult to ascertain. Events may have occurred between the injection of the fetal liver cells and the determination of the sterile transcript levels in the thymus weeks later. The relationship between fetal and adult stem cells is not clear. It is possible that the fetal stem cells injected into the chimeras home to the bone marrow where they are somehow converted to "adult" stem cells prior to migration into the thymus. More direct experiments to determine if negative signals are given by the adult thymus are described above.

Although the adult SCID thymus resembles a normal fetal thymus, my results suggest that it lacks the ability to deliver the appropriate signals necessary to support development of $V\gamma3^+$ T cells from fetal progenitors. Perhaps the differences between normal fetal and SCID adult thymus can be examined to provide clues as to what is necessary for the production of $V\gamma3^+$ cells.

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