15 T cell receptor V(D)J recombination: mechanisms and developmental regulation

HAYDN M. PROSSER AND SUSUMU TONEGAWA

1 Introduction

The vertebrate immune system has evolved highly variable antigen receptor molecules in order to identify and neutralize the enormous range of invasive organisms which may be encountered (Tonegawa 1983; Blackwell and Alt 1988; Davis 1988). The variability of the two basic forms of receptors, T cell receptor (TCR) and the immunoglobulins (Ig), is dependent upon a common process of site-specific recombination within the encoding DNA. This V(D)J recombination activity enables a specific combination of the multiple variable (V), diversity (D) (in certain cases), and joining (J) region gene segments to fuse into a single genetic unit encoding the variable region of the antigen receptors. Additional variability is accomplished by the imprecise joining of the V, D, and J gene segments due to the loss of and/or addition of nucleotides at the join.

Since its original description V(D)J recombination has remained a subject of intensive investigation. However, the essence of how the mechanism operates remains a mystery. There is no solid evidence as to the identity of the enzymatic component(s) of the V(D)J recombinase; although some promising leads have emerged. Prime among these leads is the cloning of the recombination activating genes, RAG-1 and RAG-2, whose products are capable of making cells competent for V(D)J recombination which are normally lacking in this activity (Schatz *et al.* 1989; Oettinger *et al.* 1990). In the absence of the ability to directly study the biochemistry of the V(D)J recombinase much of our knowledge of the system stems from investigations of the substrates and products of the process.

This chapter discusses what is known about V(D)J recombination within thymocytes which gives rise to the two forms of TCR, αβ, and γδ. However, due to the common nature of V(D)J recombination within both the T and B branches of lymphocyte development much evidence will be drawn from studies on pre-B cells and the rearrangement of Ig genes. In addition to the discussion of the mechanism of recombination there will be a summary of the role of recombination in T cell development with particular reference to evidence from mutant mice with TCRs disrupted by homologous recombination.

2 V(D)J recombination

2.1 Recombination substrates

The T cell receptor (and Ig) V, (D), and J gene segments are flanked by conserved recombination signal sequences (RSS) (Figure 15.1a) (Tonegawa 1983; Hesse *et al.* 1989). The RSSs act as recognition sites for the V(D)J recombinase activity and delineate the points at which somatic recombination occurs. Each RSS is comprised of two consensus sequences, a heptamer and an A/T rich nonamer, which are separated by a non-conserved spacer of either 12(+/− 1 bp) or 23(+/− 1 bp) nucleotides in length. Recombination is governed by the '12–23' rule whereby RSSs with a 12 nucleotide spacer may only recombine with RSSs with a 23 nucleotide spacer and vice versa. The arrangement of RSSs determines which combinations of TCR gene rearrangements are possible (Figure 15.1b). Thus V gene segments, with a 23 bp spacer RSS 3′ of the segment, can recombine with D gene segments or directly with J gene segments, both with a 12 bp spacer RSS 5′ of the segment. The D gene segments of TCR β and δ are flanked by a 5′ 23 bp spacer RSS

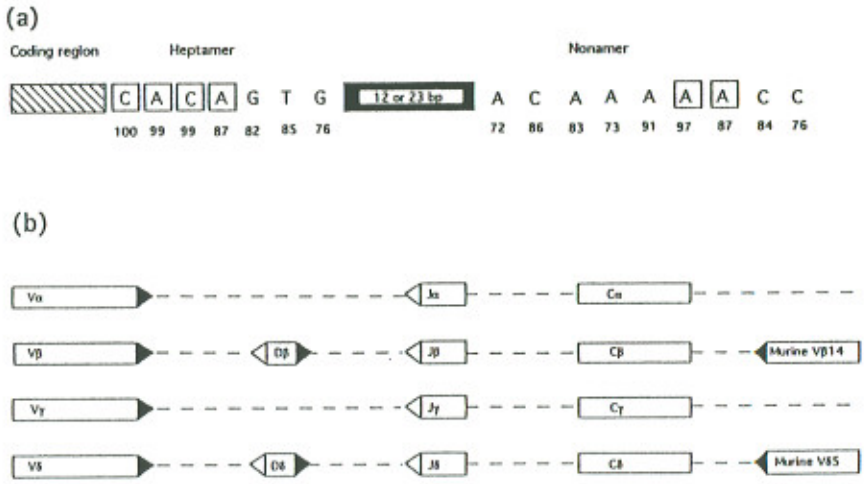


Fig. 15.1 (a) The consensus sequences of the heptamer and nonamer components of the RSS (Hesse *et al.* 1989). The numbers below each nucleotide represent the percentage with which nucleotides are found the RSSs of a large number of antigen receptor loci. The boxed nucleotides are the most important components of the RSS based on mutational analysis. (b) Schematic representation of the arrangement of 12 bp spacer RSSs (clear triangles) and 23 bp spacer RSSs (filled triangles) in relation to the TCR coding sequences (rectangles). The illustration is not to scale and does not represent the TCR loci accurately. The Vβ14 gene found 3′ of the constant region in the murine TCR β locus is indicated.

and a 3′ 12 bp spacer RSS so that they can recombine with both V and J gene segments, and with other D gene segments.

V(D)J recombination can be studied experimentally by the introduction of artificial recombination substrates into appropriate cell types (Akira *et al.* 1987; Hesse *et al.* 1987). Artificial recombination substrates are bacterial plasmid constructs containing RSSs divided by intervening DNA. The development of a quantitative assay for V(D)J recombination activity using artificial recombination substrates has allowed the importance of each nucleotide of a heptamer or nonamer sequence to be assessed by mutagenesis (Hesse *et al.* 1989) (Figure 15.1b). The fact that RSSs taken out of the context of antigen receptor genes are still functional as recombination substrates signifies that they are the only DNA motifs necessary to direct V(D)J recombination. The conservation of the RSSs between TCR and Ig loci and the observation that the same RSS are functional in both B and T cells are strong arguments in favour of a single V(D)J recombinase being shared by all lymphocytes (Yancopoulos *et al.* 1986; Lieber *et al.* 1988*a*).

2.2 Recombination products

V(D)J recombination of the TCR loci most frequently occurs by deletion of the intervening chromosomal DNA separating the oppositely oriented RSSs of the V, D, or J fusion partners (Figure 15.2a). Two products arise from this process, each associated with a particular joint. A 'coding joint' is formed between the coding regions of the rearranged genes within the chromosome. The coding joints exhibit considerable variability due to the loss and/or addition of nucleotides. The second product is a microcircle comprised of deleted intervening DNA including the two RSSs. The two RSSs of the microcircle fuse without junctional variation at their respective heptamer sequences in a head to head fashion to form the 'signal joint'. The existence of microcircle DNA by-products was demonstrated by their isolation and characterization in thymocytes undergoing V(D)J recombination (Fujimoto and Yamaguchi 1987; Okazaki *et al.* 1987; Okazaki and Sakano 1988; Winoto and Baltimore 1989*a*). An alternative form of rearrangement is encountered in situations where the RSS of a V gene segment is oriented in the same direction as the RSS of the J or D gene segment fusion partners (Figure 15.2b). This is true in the case of the murine Vβ4 (Malissen *et al.* 1986) and Vδ5 gene segments (Korman *et al.* 1989; Iwashima *et al.* 1989) which are located 3′ of the D, J, and constant region gene segments. Such V gene segments rearrange by a mechanism of DNA inversion instead of deletion.

Artificial recombination substrates can undergo deletion and inversion depending on the relative orientation of the RSSs. The plasmids can be recovered after exposure to the cells V(D)J recombinase apparatus to facilitate examination of the recombination products by DNA sequencing. Through this approach two aberrant forms of joint were identified at high frequency in the inversion configuration of artificial substrates. 'Hybrid joints' are

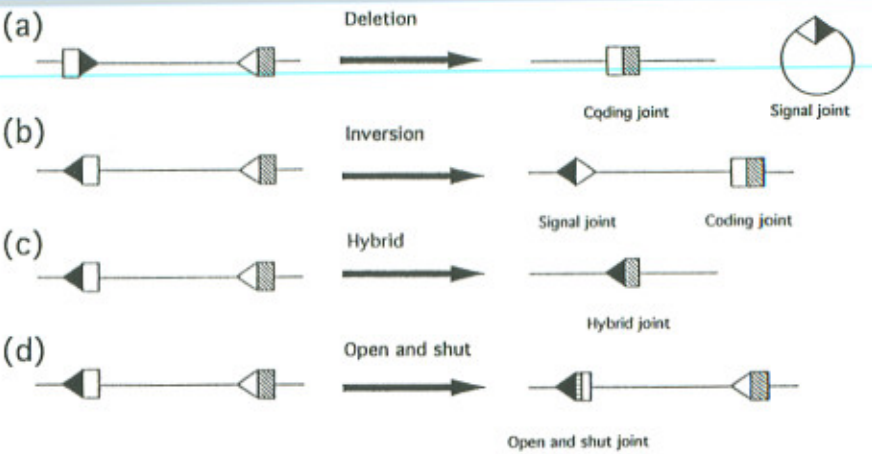
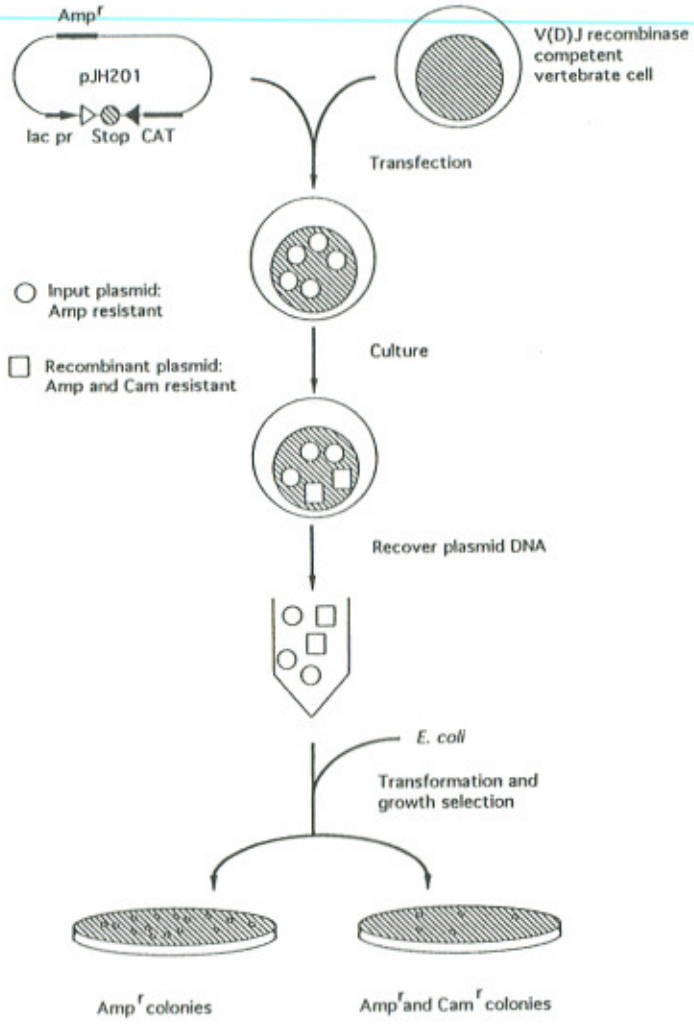


Fig. 15.2 The different forms of V(D)J recombination. The two most common forms of V(D)J recombination at antigen receptor loci are (a) deletion and (b) inversion. Aberrant forms of recombination occur at inversion-type recombination substrates. These are represented by (c) hybrid joints, formed between signal and coding ends, and (d) open and shut joints where horizontal bars indicate the loss and addition of nucleotides at the coding end. Filled triangles represent 23 bp spacer RSSs and clear triangles represent 12 bp spacer RSSs. Coding regions are indicated as rectangles.

formed when a RSS joins to the coding region of its fusion partner (Lewis *et al.* 1988; Moryzycka-Wroblewska *et al.* 1988) (Figure 15.2c). 'Open and shut joints' result when no inversion has taken place so that RSS and coding regions remain in the original configuration (Lewis *et al.* 1988) (Figure 15.2d). However, bases have been lost or added between the coding region and its RSS indicating that this joint must have opened and closed. Artificial recombination substrates have been utilized to develop a quantitative assay for V(D)J recombination (Hesse *et al.* 1987) (Figure 15.3). This assay was useful for purposes such as defining the RSSs by mutagenesis (Hesse *et al.* 1989) and studying the activity of the recombination activating genes RAG-1 and RAG-2 (Oettinger *et al.* 1990).

Aberrant V(D)J recombination events occur in mice possessing the severe combined immunodeficiency (*scid*) mutation (Bosma *et al.* 1983). *scid* is a spontaneous autosomal recessive mutation identified in a laboratory mouse colony. The defect leads to massive reduction in the numbers of mature B and T cells. This is due to the much reduced frequency with which *scid* mice form coding joints (Schuler *et al.* 1986; Lieber *et al.* 1988*b*; Blackwell *et al.* 1989). In contrast the frequency of signal joint formation remains normal; although the joint is less precise than usual. The use of *scid* mice as a tool in dissecting the V(D)J recombination mechanism will be discussed further.



2.3 Junctional diversity and coding joint formation

The loss and/or gain of nucleotides at the coding joint of TCR genes greatly increases the variability of the TCR. The imprecision of the coding joint means that only one-third of all V(D)J recombination events will produce a TCR gene in the correct reading frame. The imprecise joining encountered at the coding joints provides some interesting insights into the V(D)J recombination process, particularly with regard to intermediate DNA structures which are formed. The loss of nucleotides can be explained by exonucleolytic

Fig. 15.3 The essential features of the quantitative assay for V(D)J recombination developed by Hesse *et al.* (1987) are outlined. A bacterial plasmid (pJH201) contains two selectable markers for bacterial growth; the β-lactamase (Amp^r) gene, providing ampicillin resistance, and the chloramphenicol acetyltransferase (CAT) gene, providing chloramphenicol (Cam) resistance. Transcription of the CAT gene from the lac promoter is blocked by the presence of a prokaryotic transcription terminator (circle) which is flanked by a 12 bp RSS (clear triangle) and a 23 bp RSS (filled triangle). On transfection of JH201 into V(D)J recombinase competent cells a fraction of the plasmids will recombine (proportional to the V(D)J recombination rate), deleting the transcription terminator and enabling the plasmid to confer Cam resistence in *E. coli*. After culturing, the plasmid is recovered and used to transform *E. coli* which are grown on medium containing ampicillin alone and on medium containing ampicillin and chloramphenicol. The ratio of the number of *E. coli* colonies on ampicillin and chloramphenicol medium against the number of colonies on ampicillin medium alone is a measurement of the rate of V(D)J recombination.

activity. The gain of nucleotides at the coding joint is due to both a non-templated and a templated mechanism. The non-templated addition is accomplished by terminal deoxynucloetide transferase (TdT) activity within early lymphoid cells (Landau *et al.* 1987; Kallenbach *et al.* 1992; Gilfillan *et al.* 1993; Komori *et al.* 1993). The nucleotides, termed N nucleotides, are added in a random pattern to the free ends of the coding strands prior to joining.

The templated form of nucleotide addition was originally identified in the TCR loci by analysis of the junctional sequences in fetal thymic TCR γδ T cells (Lafaille *et al.* 1989). The identification was aided by the fact that fetal thymic TCR γδ T cell populations are very homogeneous in TCR junctional sequence, in contrast to the case in adult TCR γδ T cells. One or two additional nucleotides, termed P nucleotides, were found at the coding joints which were specific to the junctional borders of particular TCR gene segments. The P nucleotide(s) were complementary to the last base(s) of the coding end thus forming one-half of a palindromic sequence. A model of templated nucleotide addition was proposed in which transfer of nucleotides from one strand of a double-stranded DNA break to the other leads to the creation of the palindromic sequence. Evidence of P nucleotide addition has been found in other antigen receptor loci at coding ends where no nucleotide loss has occurred. P nucleotide addition appears to be an early event in processing of the coding joint as, in the majority of cases, subsequent nucleotide loss masks the existence of this process.

The existence of recombination substrates at an intermediate stage of V(D)J recombination has been documented *in vivo* by analysis of the TCR δ locus of thymocytes (Figure 15.4) (Roth *et al.* 1992*a*). Southern analysis performed on thymus DNA using probes specific for the Dδ2 and Jδ1 regions of the TCR δ locus revealed that 10–20% of the restriction fragments hybridizing to these probes were broken at points corresponding to the RSSs. The double-stranded breaks resulted in truncation of the expected full-length

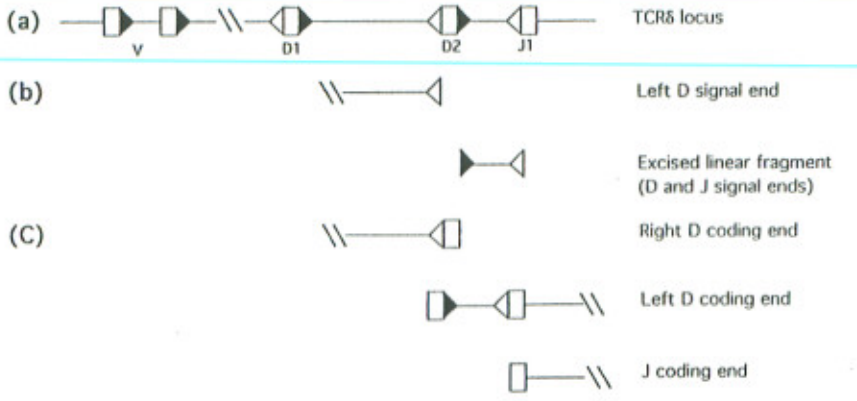


Fig. 15.4 (a) Representation of the TCR δ locus used in the study of recombination intermediate structures (Roth *et al.* 1992*a*). The distances are not to scale and only two V regions are shown. (b) The broken DNA fragments found at the thymic Dδ2 and Jδ1 loci of wild-type mice. All fragments end in RSSs. (c) In *scid* mice additional broken DNA fragments are found in thymic DNA with coding sequences at their ends. Filled triangles represent 23 bp spacer RSSs and clear triangles represent 12 bp spacer RSSs. Coding regions are indicated as rectangles.

hybridizing band. The abundance of these linear DNA molecules suggests that the double-stranded ends are very stable, perhaps as a result of protection by binding of components of the V(D)J recombinase. In wild-type mice the only intermediaries identified were the RSS end 5′ of Dδ2 and the excised linear DNA between Dδ2 and Jδ1 with a RSS at each extremity. Thus, only signal ends were identified and coding end intermediates were not observed. This anomaly was ascribed to the rate of coding end ligation being higher than that of RSS ends so that the abundance of DNA fragments with coding ends was below the level of detection. As previously discussed, mice with the *scid* mutation are defective in coding joint formation. Repetition of the Southern analysis on thymus DNA from *scid* mice enabled the coding joints to be detected. It therefore appears that the *scid* mutation imposes a block on the processing of the intermediate structures which form the coding joint resulting in their accumulation within the thymocyte.

Further analysis of the TCR δ coding joints produced in the *scid* thymus yielded more information on the structure of the intermediate products and on how the coding joint may be processed (Roth *et al.* 1992*b*). Thymic genomic DNA was subjected to two-dimensional electrophoreses and Southern blotted. The second electrophoresis proceeded under denaturing conditions which separated the two strands of a DNA fragment. Different sizes of DNA fragments with double-stranded breaks would fall on a diagonal under these conditions. However, DNA fragments with *scid* TCR δ coding joints exhibited reduced electrophoretic mobility in the second dimension indicating

a doubling of size. This result was explained by the two DNA strands being joined at the coding end in a hairpin loop structure.

The existence of a hairpin loop at the coding end DNA intermediates provides a highly plausible explanation for the creation of P nucleotides (Figure 15.5). A symmetrical nicking of the hairpin loop by some component of V(D)J recombinase complex followed by filling-in of the single-stranded product would produce P nucleotide addition to the coding end. The *scid* defect might therefore represent an inability to correctly nick the hairpin loop in order to create a product suitable for ligation. In wild-type mice one or two P nucleotide additions are seen after formation of the coding joint. This may indicate that nicking of the hairpin loop in normal mice is only marginally offset from the apex. However, it is also possible that P nucleotide addition greater than two nucleotides length normally occur but are deleted by exonucleolytic activity. The very rare coding joints found in *scid* mice can possess long P nucleotide additions of 12 to 15 nucleotides in length (Ferrier *et al.* 1990*a*; Kienker *et al.* 1991; Schuler *et al.* 1991). This occurrence may represent an aberrant nicking of the hairpin loop due to the *scid* mutation or alternatively a fortuitous nicking in the vicinity of the hairpin loop due to DNA damage unrelated to V(D)J recombination, leading to an intermediate which is then processed as normal.

2.4 Factors responsible for V(D)J recombination

The evidence reviewed thus far from analysis of recombination substrates, intermediate structures, and recombination products provides an outline of the probable activities involved in V(D)J recombination. The RSSs are recognized by components of the V(D)J recombinase producing double-stranded breaks in the DNA at the border of the RSS. The free coding ends form an intermediate hairpin loop structure which is later nicked, frequently off-centre, and the single-stranded product is filled-in with P nucleotides. Nucleotides are lost from or added to the coding ends before ligation to form the variable coding joint. In contrast the signal joints normally lack variability being formed by the precise fusion of two heptamer sequences.

Little is known about the protein factors responsible for most of these activities. The best characterized factor which plays a role in V(D)J recombination is the enzyme terminal deoxynucleotide transferase (TdT). TdT was proposed to be responsible for N nucleotide addition to the coding joints (Landau *et al.* 1987; Kallenbach *et al.* 1992). The function of TdT was unequivocally demonstrated by the creating of mutant mice lacking TdT which were deficient in N region diversity (Gilfillan *et al.* 1993; Komori *et al.* 1993). Though TdT is important for the variability, and so functioning, of the TCR it should be noted that it is not essential for V(D)J recombination.

As previously mentioned, the factor rendered defective by the *scid* mutation is vital to the processing of the coding junction intermediates (Schuler *et al.* 1986; Lieber *et al.* 1988*b*; Blackwell *et al.* 1989). In addition to the

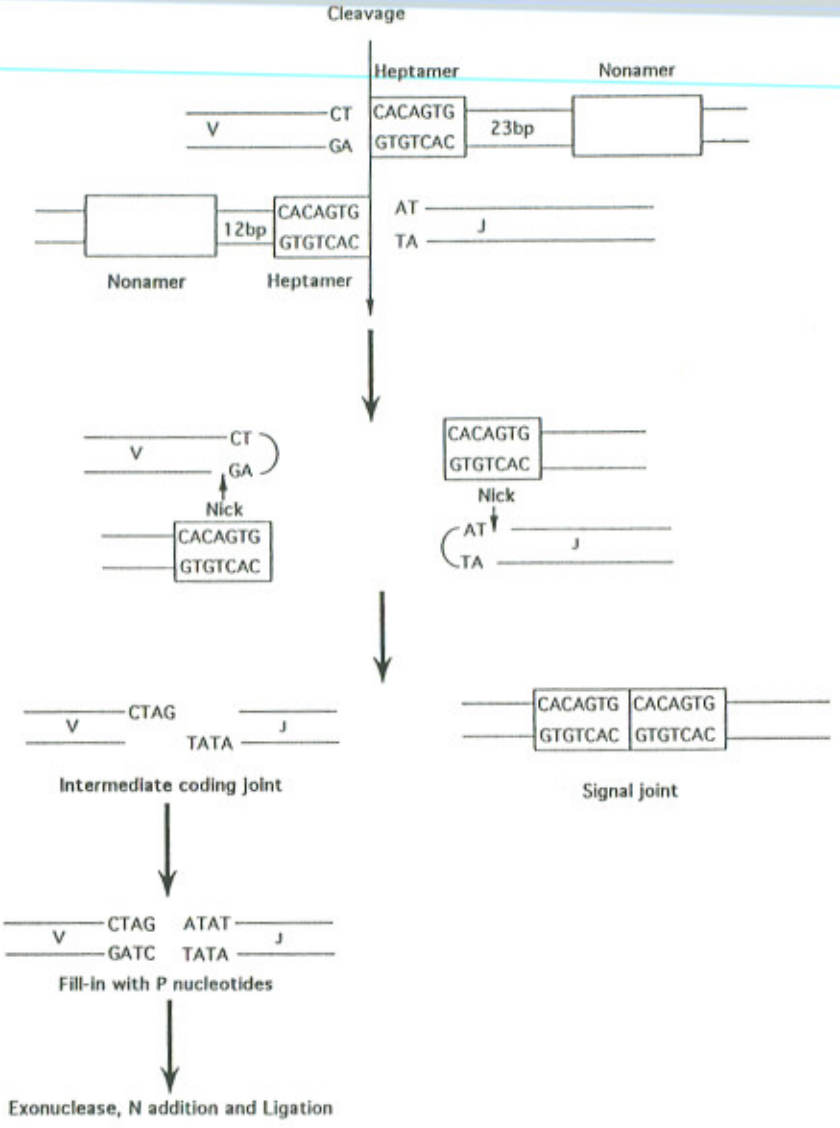


Fig. 15.5 A model for the events during antigen receptor recombination. Hairpin loop structural intermediates are formed at the coding ends which are later nicked and filled-in to create templated, or P, nucleotides. A two nucleotide addition is indicated, although longer additions are possible. Subsequently the coding ends are subjected to exonuclease digestion and N nucleotide addition prior to ligation to form the coding joint. Recombination between V and J coding regions is represented.

exhibit a mutant phenotype, being more sensitive to X- and γ-irradiation than normal cells (Fulop and Phillips 1990; Biederman *et al.* 1991; Hendrickson *et al.* 1991). The *scid* factor was recently identified as the catalytic subunit of the DNA dependent protein kinase (DNA-PK) (Blunt *et al.* 1995). The nature of the *scid* factor was indicated by the observation that mutant Chinese hamster ovary cell lines with defects in DNA double-strand break repair (X-ray sensitivity) also lacked the ability to direct V(D)J recombination when co-transfected with the recombinase activating genes (RAG-1 and RAG-2) and artificial recombination substrates (Alt *et al.* 1992; Taccioli *et al.* 1993, 1994*a*). One of the DNA double-strand break repair mutants, V3, showed a preferential impairment in the joining of coding joints, as in the *scid* mutation, whereas a further two of these lines (xrs-6 and XR-1) were deficient in the formation of coding and signal joints (Alt *et al.* 1992; Pergola *et al.* 1993; Taccioli *et al.* 1993, 1994*a*). All three mutant cell lines fell into different complementation groups (Taccioli *et al.* 1994*a*). Additionally the DNA repair defect of V3 and *scid* cells, and the V(D)J recombination defect of *scid* cells are complemented by human chromosome 8 (Itoh *et al.* 1993; Kirchgessner *et al.* 1993; Komatsu *et al.* 1993; Banga *et al.* 1994). Several lines of evidence indicate that the V3 and *scid* mutations are mutations of the same gene and that this gene is the catalytic subunit of DNA-PK (Blunt *et al.* 1995). Both V3 and *scid* cells lack DNA-PK activity and, in extracts of these cells, the activity can be restored by the addition of purified DNA-PK. Yeast artificial chromosome containing the DNA-PK catalytic subunit gene restored DNA-PK activity to V3 cells and complemented the radiosensitivity and V(D)J recombination competence of V3 cells.

The DNA-PK catalytic subunit associates with two nuclear DNA end binding subunits of approximately 70 kDa and 80 kDa size (Ku70 and Ku80 respectively). The xrs-6 cell line is defective in functional Ku (Getts and Stamato 1994; Rathmell and Chu 1994; Taccioli *et al.* 1994*b*), lacks detectable DNA-PK activity (Finnie *et al.* 1995) and is complemented by expression of Ku80 cDNA (Smider *et al.* 1994; Taccioli *et al.* 1994*b*). Thus both *scid*/V3 and XR-6 cells possess mutations in components of DNA-PK. The precise role that DNA-PK plays in DNA double-strand break repair and V(D)J recombination remains to be determined.

As the heptamer and nonamer sequences of the RSS represent probable DNA binding sites for components of the V(D)J recombinase efforts have been made to identify and clone factors which bind these sequences. Biochemical evidence for heptamer binding (Aguilera *et al.* 1987; Hamaguchi *et al.* 1989) and nonamer binding (Halligan and Desiderio 1987; Li *et al.* 1989) activities has been found. Furthermore, a heptamer binding protein (RBP-Jκ) has been purified and cloned (Hamaguchi *et al.* 1989; Matsunami *et al.* 1989). The predicted amino acid sequence of RBP-Jκ contains a 40 amino acid region of homology with the conserved motif of integrase proteins responsible for site-specific recombination in yeast, bacteria, and bacterio-

phage. RBP-Jκ is therefore a strong candidate for a factor recognizing the heptamer in V(D)J recombination.

It is apparent from the evidence presented thus far on the make-up of the V(D)J recombinase that there is a gap between our knowledge of activities and our knowledge of the factors responsible. Thus, in some cases V(D)J recombinase activities have been identified in the absence of any candidate factors. In other cases factors have been identified but conclusive proof of their involvement in V(D)J recombination is lacking due to the absence of a suitable experimental system to study their proposed role. However, there are two factors, the RAG-1 and RAG-2 proteins with an indisputable role in V(D)J recombination, though their exact function remains unknown.

3 The recombination activating genes, RAG-1 and RAG-2

The proposition that a single genetic locus might be capable of activating V(D)J recombinase activity stemmed from the surprising observation that transfection of NIH3T3 cells with sheared human or murine genomic DNA could induce occasional V(D)J recombination (Schatz and Baltimore 1988). Although the recombination events were rare (about 0.1% of transfectants) it was possible to identify them because V(D)J recombination led to activation of a drug resistance gene contained within an artificial substrate integrated into chromosomal DNA. The region encoding the V(D)J recombinase activity was narrowed down by serial genomic transfection of oligonucleotide-tagged genomic DNA. The RAG-1 gene was finally identified as a probe which detected a single 6.6 kb mRNA in transfectants, as well as pre-B and pre-T cells (Schatz *et al.* 1989). However, transfection of the RAG-1 gene alone, as a 18 kb genomic clone (clone 12C.2), or as independently isolated cDNAs, activated recombination in NIH3T3 cells at a rate 1000-fold lower than that expected if RAG-1 were the only gene responsible. This observation led to speculation that a second recombination activating gene (RAG-2), located close to RAG-1 but not fully contained within clone 12C.2 was necessary for full V(D)J recombination activity. Indeed, a probe isolated from 12C.2 did detect a predominant 2.2 kb mRNA species with the appropriate tissue specificity (Oettinger *et al.* 1990).

Thus, two genes in close proximity, RAG-1 and RAG-2, were identified which, when co-expressed, act synergistically to activate V(D)J recombination in NIH3T3 cells. RAG-1 and RAG-2 have since been shown to activate V(D)J recombination in other cells where this activity is not normally present, including CHO cells (Kallenbach *et al.* 1992) and neuronally differentiated p19 embryonal carcinoma cells (Schatz *et al.* 1992). The products of recombination within RAG-1 and RAG-2 expressing NIH3T3 cells are normal; an imprecise coding joint with frequent base loss and an invariable signal joint. The absolute necessity of RAG-1 and RAG-2 for V(D)J recombination is underscored by gene disruption in mice by homologous recombination

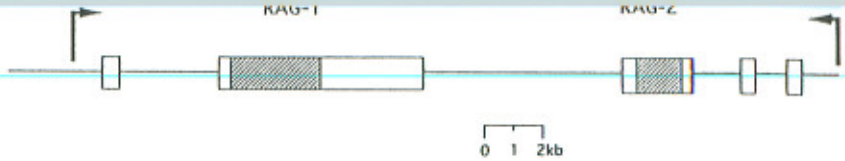


Fig. 15.6 The genomic locus containing RAG-1 and RAG-2. Coding regions of the RAG-1 and RAG-2 genes are hatched and untranslated regions are clear. RAG-2 has multiple 5′ untranslated exons of which only two are represented. *Arrows* represent the direction of transcription.

(Mombaerts *et al.* 1992*b*; Shinkai *et al.* 1992). Inactivation of either RAG-1 or RAG-2 by this method prevents V(D)J recombination at any TCR or Ig locus. The low frequency of V(D)J recombination seen in NIH3T3 cells on transfection of RAG-1 alone was therefore ascribed to low level expression of the endogenous RAG-2 gene.

The RAG-1/RAG-2 locus has been mapped to chromosome 2 in the mouse and the syntenic chromosome 11p in humans (Oettinger *et al.* 1992). The locus has an unusual structure (Figure 15.6). RAG-1 and RAG-2 are positioned within 8 kb of each other and are transcribed in opposite orientations (Oettinger *et al.* 1990). The entire coding and 3′ untranslated regions of RAG-1 and RAG-2 are each contained within one exon. RAG-1 possesses one exon containing 5′ untranslated sequence whereas RAG-2 contains multiple undetermined 5′ untranslated exons (Schatz *et al.* 1992). The structure of the RAG-1/RAG-2 locus is conserved in species as diverse as chicken (Carlson *et al.* 1991) and *Xenopus* (Greenhalgh *et al.* 1993) suggesting that selective pressure might act to maintain this arrangement. Such selective pressure might act if, for example, RAG-1 and RAG-2 shared some *cis*-acting transcriptional regulatory components. It is unlikely that the RAG-1 and RAG-2 genomic structure originated as a gene duplication event as the two genes are completely unrelated in DNA sequence. The unusually compact nature of the RAG-1/RAG-2 locus has led to speculation that it may have originated as a part of a viral or fungal recombination mechanism system which integrated into the ancestral genome and evolved to perform its present function in V(D)J recombination (Oettinger *et al.* 1990).

In addition to the structure of the genetic locus, the predicted amino acid sequences of RAG-1 and RAG-2 are also highly conserved between species. Upon low stringency hybridization the coding sequences of the RAG-1 and RAG-2 genes cross-hybridize with all vertebrate species tested (Schatz *et al.* 1989; Oettinger *et al.* 1990). The amino acid sequences of mouse and human RAG-1 and RAG-2 are 90% identical. The identity between mouse and chicken is 75% for RAG-1 and 70% for RAG-2 (Carlson *et al.* 1991). RAG-1 possesses no known DNA binding motifs. RAG-1 does, however, contain a 50 amino acid cysteine-rich sequence, termed the RING finger, with homology to motifs in a number of otherwise unrelated proteins of widely

varying function including *S. cerevisiae* RAD-18 gene, human rel transforming gene, the human RING-1 gene, and the human rpt-1 interleukin-2 receptor regulator gene (Freemont *et al.* 1991; Lovering *et al.* 1993). This sequence is related to known zinc finger motifs and may represent a DNA binding domain (Lovering *et al.* 1993). Mutational analysis of RAG-1 has demonstrated that the RING finger motif is dispensible for V(D)J recombination of extracellular substrates in fibroblast cells (Sadofsky *et al.* 1993; Silver *et al.*, 1993). A particularly interesting homology is found between the C-terminal half of RAG-1 and the *S. cerevisiae* gene HPR1 (Wang *et al.* 1990). The HPR1 gene functions in yeast to suppress excision recombination. HPR1 itself has homology with yeast topoisomerase, although there is no evidence that HPR1 or RAG-1 function is related to topoisomerase activity (Aguilera and Klein 1990). Deletion of the HPR1 homologous region leads to inactivation of RAG-1 V(D)J recombinase activity (Sadofsky *et al.* 1993; Silver *et al.* 1993). However, point mutation of a potential topoisomerase active site (tyrosine 998) did not drastically effect recombinase activity (Kallenbach *et al.* 1993; Sadofsky *et al.* 1993; Silver *et al.* 1993). The RAG-2 gene and protein have no homologies with any known sequence.

Both RAG-1 and RAG-2 proteins localize to the nuclei of thymocytes and of transfected fibroblasts (Lin and Desiderio, 1993; Sadofsky *et al.* 1993; Silver *et al.* 1993*)*. Within the nucleus expression of the RAG-2 protein, and thus recombinase activity, is regulated in the cell cycle dependent manner. RAG-2 protein accumulates in the G0/G1 phase, is rapidly degraded before entry into S phase and remains at a low level through the S, G2, and M phases (Lin and Desiderio 1994). The pattern of expression of the RAG-2 protein during the cell cycle corresponds to the appearance of broken ended signal sequences (Schlissel *et al.* 1993). A possible mechanism for this regulation is the destabilization of the RAG-2 protein by a cyclin dependent kinase. Several lines of evidence support this view. RAG-2 is phosphorylated *in vivo* at position threonine 490 leading to destabilization of the protein (Lin and Desiderio, 1993). Mutation of residue threonine 490 extends the half life of RAG-2 *in vivo*. Furthermore threonine 490 of RAG-2 is phosphorylated *in vitro* by $p34^{cdc2}$. Restriction of V(D)J recombinase activity to the G0/G1 phase of the cell cycle may be important to avoid the deleterious effects of DNA double-strand breaks occurring during mitosis or S phase. Indeed, universal mechanisms exist to prevent a delay passage through the cell cycle in response to DNA double-strand breaks (Kastan *et al.* 1992; Murray 1992).

Despite their obvious central roles in V(D)J recombination the function of RAG-1 and RAG-2 remains unknown. There is no evidence to suggest that RAG-1 and RAG-2 interact with each other to achieve their activity. Similarly there is no evidence that RAG-1 or RAG-2 bind DNA. Using a genetic screen a protein named Rch1 has recently been shown to interact with the RAG-1 protein and thus may potentially be a component of a recombinase complex (Cuomo *et al.* 1994). Rch1 bears similarity to the yeast nuclear envelope protein SRP1. There are two possible ways of envisaging RAG-1

and RAG-2 action. The first possibility is that RAG-1 and RAG-2 encode the lineage-specific components of the V(D)J recombinase enzyme. The alternative view is that RAG-1 and RAG-2 may be regulatory proteins which function to activate the V(D)J recombinase either by post-translational modification or by controlling gene expression. What limited circumstantial evidence exists has been proposed to support the former assertion and is detailed below (Oettinger 1992; Schatz *et al.* 1992).

1. An essential component of the V(D)J recombinase mechanism present throughout vertebrate species would be expected to display high evolutionary conservation. RAG-1 and RAG-2 are highly conserved throughout their length as might be expected if they have to interact with other components of the V(D)J recombinase and possibly DNA. A regulatory protein might have more leeway for variation in primary structure while retaining its function.
2. Following transfection of NIH3T3 cells with RAG-1 and RAG-2 the only discernible phenotype gained is V(D)J recombination activity (Oettinger *et al.* 1990). If RAG-1 and RAG-2 were regulatory proteins it might be expected that other lymphoid markers such as TdT, Oct-2, or B220 might be expressed.
3. The primary phenotype detected in RAG-1 or RAG-2 knock-out mice is the loss of V(D)J recombination (Mombaerts *et al.* 1992*b*; Shinkai *et al.* 1992). If RAG-1 and RAG-2 were regulators, other developmental abnormalities might be apparent in these mutant mice.
4. RAG-1 has homology to a *S. cerevisiae* gene, HPR1, which is known to function in a recombination process, albeit in the suppression of this recombination (Wang *et al.* 1990).
5. The expression of RAG-2 has been correlated with a second vertebrate recombination mechanism, that of Ig gene conversion in the bursa of Fabricus in chickens (Carlson *et al.* 1991). However, it does not appear to be essential for this process as gene disruption of RAG-2 in cell lines does not abolish this process (Takeda *et al.* 1992).

Whatever the function of RAG-1 and RAG-2 proves to be, their coordinated expression in pre-T and pre-B cells is essential for V(D)J activity. There is no data available on the *cis*-elements responsible for regulating expression. However, recent studies have yielded useful data on the control of RAG-1 and RAG-2 expression in T and B lineages; data from both lineages will be discussed because of probable general applicability of control mechanisms.

4 Regulation of TCR V(D)J recombination in thymocyte development

The onset of V(D)J recombination is strictly controlled in different T cell

rearrangement precedes Vβ to Dβ rearrangement which in turn precedes TCR α rearrangement. Both TCR γ and TCR δ rearrangements occur at about the same time as TCR β rearrangement leading to expression of TCR γδ on a distinct set of T cells. No full V(D)J rearrangement of TCR genes has been detected in B cells. However, there are examples of incomplete Dβ to Jβ rearrangements in B cells and conversely Ig D_H to J_H rearrangements in T cells (Forster *et al.* 1980; Kurosawa *et al.* 1981; Cook and Balaton 1987). As all examples of V(D)J recombination share the same mechanism but not all TCR and Ig loci rearrange concurrently the control of onset of rearrangement cannot be at the level of the recombinase. Control of V(D)J recombination must therefore depend on the particular loci. An accessibility model has been proposed to explain how different loci are selectively presented as substrates for the V(D)J recombinase (Alt *et al.* 1987; Blackwell and Alt 1988). In this model a locus is made receptive to the V(D)J recombinase by transcription from the unrearranged gene segments. The mode of operation of transcription is unknown but may involve a relaxation of chromatin structure. The first evidence in favour of the accessibility model came from the study of pre-B cells where germline transcripts were found in loci undergoing V(D)J recombination (van Ness *et al.* 1981; Reth and Alt 1984; Yancopoulos and Alt 1985; Lennon and Perry 1985, 1990; Schlissel *et al.* 1991*a*). Germline transcripts from unrearranged TCR genes are also found in thymocytes (Calman and Peterlin 1986; Pardoll *et al.* 1987). More recently, the introduction of heat shock inducible RAG-1 and RAG-2 genes into a recombinationally inert B cell line has demonstrated the importance of transcriptional enhancers in promoting the accessibility of V(D)J segments for recombination (Oltz *et al.* 1993). Heat shock of the cell line lead to expression of the recombinase activating genes and rapid rearrangement of chromasomally integrated V(D)J recombination substrates, but only when these substrate constructs contained transcriptional enhancers.

The accessibility model therefore implicates *cis*- and *trans*-acting components of the transcriptional apparatus of the antigen receptor genes as the controlling mechanism for initiation of V(D)J recombination. Indeed, induction of transcription at the Igκ locus in pre-B cells by treatment with LPS and at the Igμ locus of a pre-T cell line by transfection of a transcription factor binding the Ig heavy chain gene enhancer do increase the rate of recombination at these loci (Schlissel and Baltimore 1989; Schlissel *et al.* 1991*b*). Various *cis*-acting elements of the TCR genes have been identified. These include transcriptional enhancers 3′ of the TCR α (Winoto and Baltimore 1989*b*; Ho *et al.* 1989), TCR β (Krimpenfort *et al.* 1988; McDougall *et al.* 1988; Gottschalk and Leiden 1990; Takeda *et al.* 1990; Prosser *et al.* 1991), and TCR γ (Kappes *et al.* 1991; Spencer *et al.* 1991) constant regions, and in the J to constant region intron of the TCR δ gene (Bories *et al.* 1990; Redondo *et al.* 1990), and promoter elements of the murine Vβ genes (Anderson *et al.* 1988, 1989). Interestingly, a deletion of the TCR β locus by

although the TCR β enhancer remained intact (Mombaerts *et al.* 1992*b*). This finding suggests that the TCR β enhancer is not in isolation responsible for directing TCR β recombination. The TCR β deletion does, however, remove the Jβ2 to Cβ2 intron where a potential regulatory element of unknown function has been identified as a DNaseI hypersensitive site (Hashimoto *et al.* 1990). Transcriptional silencer activities have been described for the TCR α (Winoto and Baltimore 1989*c*), TCR β (Krimpenfort *et al.* 1988; Takeda *et al.* 1990; Prosser *et al.* 1991), and TCR γ (Bonneville *et al.* 1990; Ishida *et al.* 1990) loci. However, the role of these activities in controlling TCR rearrangement and/or transcription remains unproven.

The importance of the specificity of transcriptional activation in the regulation of tissue-specific recombination has been revealed by the generation of transgenic mice using chimeric minigene constructs (Ferrier *et al.* 1990*b*). These constructs contained unrearranged TCR β V, D, and J gene segments in association with the Cμ gene in the presence and absence of the Igμ enhancer. The presence of the Igμ enhancer was necessary for recombination to occur and allowed incomplete D to J recombination in both B cells and T cells. However, complete V to DJ recombination was restricted to T cells implying that tissue-specific transcription elements associated with the Vβ gene were responsible for directing the tissue-specific rearrangement. Incorporation of the TCRβ and TCRα enhancers into this minigene construct in place of the Igμ enhancer underlined the importance of the enhancer in directing appropriate V(D)J recombination (Capone *et al.* 1993). In this case all recombination, including D to J recombination was predominantly T cell specific. Also recombination occurred stage specifically. Thus, the TCRα and TCRβ enhancer containing minigene constructs rearranged respectively when the endogenous TCRα and TCRβ gene segments rearranged. The intronic immunoglobulin heavy chain enhancer plays a similar role in directing endogenous heavy chain rearrangement (Chen *et al.* 1993). Thus, replacement of the enhancer region with the Neo^r gene by gene targeting caused a *cis*-acting block of endogenous J_H rearrangement.

A possible scenario for control of the ordered rearrangement of TCR genes is that recombination of one TCR gene is necessary for the induction of the recombination of its partner TCR gene. By analogy evidence has been presented suggesting that Ig heavy chain recombination induces Igκ chain rearrangement (Reth and Alt 1984), although there is also evidence to the contrary (Blackwell *et al.* 1989). However, in TCR β knock-out mice rearrangement of the TCR α locus proceeded as normal (Mombaerts *et al.* 1992*a*). Therefore TCR α rearrangement is not dependent on prior TCR β rearrangement.

Inactivation of rearrangement is important both to prevent multiple, and unselected, specificities in lymphocytes, and to avoid the possibility of aberrant chromosomal rearrangements leading to immortalization of the cell. It has been shown that RAG-1 and RAG-2 mRNA expression is actively regulated

during thymic development thus restricting recombinase activity to specific developmental stages. Expression of RAG-1 and RAG-2 peaks in two waves *in vivo*. The first occurs at the double negative ($CD4^-CD8^-$) thymocyte stage corresponding to the appearance of TCRβ, γ, and δ gene transcripts, and the second occurs at the double positive ($CD4^+CD8^+$) thymocyte stage when full-length TCRa transcripts appear (Wilson *et al.* 1994). Between these two stages RAG-1 and RAG-2 expression is down-regulated. Down-regulation at the double-negative stage can be mimicked by phorbol ester and calcium ionophore treatment of thymocytes *in vitro*. At the $CD4^-CD8^{lo}$ thymocyte stage (the immediate progenitor to the double-negative stage) RAG-1 and RAG-2 messenger RNA was reduced by a post-transcriptional mechanism upon either cross-linking the TCR or treatment with phorbol esters *in vitro* (Takahama and Singer 1992). Down-regulation of RAG-1 and RAG-2 messenger RNA at the double positive stage of thymocyte development may be induced either by (1) binding of antigen and MHC to the TCR during the process of positive selection (Borgulya *et al.* 1992; Brandle *et al.* 1992) or (2) by non-specific stimuli such as cross-linking TCR with antibodies and treatment with phorbol esters and calcium ionophore (Turka *et al.* 1991).

However, other mechanisms must operate to restrict the availability of loci for V(D)J recombination. For example, productive rearrangement of one TCRβ gene prevents rearrangement of the allelic gene. Additionally, mice made transgenic for a rearranged TCR β gene allelically excluded endogenous TCR β rearrangement although the recombination apparatus remained functional, as testified by the normal rearrangement of the TCR α loci (Uematsu *et al.* 1988; von Boehmer 1990). Thus, allelic exclusion operates at the level of the TCR β gene itself. The mechanism by which allelic exclusion operates to shut down recombination is unknown. Transgenesis using frame shifted and/or truncated TCR β genes indicate that translation of the TCR β constant region gene segment is required for allelic exclusion of the endogenous TCR β genes (Krimpenfort *et al.* 1989). It is not yet established whether TCR β must be expressed at the cell surface to achieve allelic exclusion. The T cell-specific protein-tyrosine kinase $p56^{lck}$, which is physically associated with the cytoplasmic tails of the CD4 or CD8 molecules and is activated upon antigenic stimulation of the TCR, is likely to be involved in allelic exclusion of TCR β (Anderson *et al.* 1992). Over-expression of *lck* in transgenic mice reduced Vβ to Dβ segment joining while other TCR genes rearranged as normal. This suggests that a signal transduced via $p56^{lck}$ may cause TCR β allelic exclusion.

4.1 Thymocyte development in mice with disrupted TCR genes

Gene knock-out experiments have revealed the important role of TCR rearrangements in directing the development of thymocytes. It is well established that transition from the TCR $\alpha\beta^+$ double-positive ($CD4^+CD8^+$) to

TCR $\alpha\beta^+$ single-positive ($CD4^+$ or $CD8^+$) stage of thymocyte development is dependent on binding of TCR αβ to thymic MHC molecules and is associated with the process of positive selection. The earlier transition from double-negative ($CD4^-CD8^-$) to double-positive ($CD4^+CD8^+$) thymocytes has been shown to be dependent on productive rearrangement of the TCR β gene. Disruption of the TCR β gene by homologous recombination prevents this transition, which can be reconstituted by introduction of a TCR β transgene (Mombaerts *et al.* 1992*a*). Analogous disruption of the TCR α locus has no effect on the double-negative to double-positive transition (Mombaerts *et al.* 1992*a*). Disruption of the *lck* gene in mice also prevents the transition of thymocytes from the double-negative to double-positive stage (Molina *et al.* 1992). This result suggests $p56^{lck}$ is important in transducing the signal responsible for the maturation of thymocytes to the double-positive stage.

Developmental mechanisms in murine fetal TCR γ and TCR δ gene rearrangement have been studied using homologous recombination (Itohara *et al.* 1993) and transgenic techniques (Asarnow *et al.* 1993). Murine fetal TCR γ and TCR δ genes possess several distinguishing characteristics. They are divided into subsets each with a specific combination of V, (D), and J genes segments. Each subset develops at a distinctive time and is expressed in specific tissues. Fetal TCR γδ T cells are also very limited in their TCR V(D)J junctional sequences in contrast to adult TCR γ and TCR δ genes. There are two interpretations of how these features are developed. Either they are intrinsic features of the fetal TCR γδ T cells which are programmed intracellularly to develop this way, or only these types of TCR γδ T cell populations are selected to proliferate by some extracellular system akin to thymic selection. Despite disruption of the TCR δ locus by homologous recombination fetal TCR γ transcripts showed the normal features of VJ combinations, tissue specificity, and limited junctional diversity (Itohara *et al.* 1993). As no extracellular mechanism of selection can operate on T cells with a disrupted TCR δ gene, because there is no cell surface expression of the TCR, the features associated with the recombination of fetal TCR γδ T cells must be programmed intracellularly. The limited junctional diversity of fetal TCR γ genes was also studied using a transgene comprised of a minilocus of unrearranged Vγ and Jγ genes in which the Vγ genes were mutated by the generation of a frame shift in the DNA sequence (Asarnow *et al.* 1993). Although no TCR γ protein was available for selection from outside the cell, the coding junctions formed upon rearrangement of the transgene in newborn mice were characteristically lacking in diversity. Hence, this study also supports the notion of an intracellular mechanism controlling murine fetal TCR γδ sequences.

5 Summary

Much has been learnt about the activities involved in V(D)J recombination by studying recombination substrates, intermediates, and products. In con-

trast progress in identifying and elucidating the function of the essential components of the V(D)J recombinase itself has been limited. Intuitively, it seems likely that the RAG-1 and RAG-2 proteins represent the essential tissue-specific components of the V(D)J recombinase, although the evidence in favour of this proposition is very weak at present. In the absence of any other leads, the most productive route to understanding the V(D)J recombinase mechanism most probably lies in studying the roles of RAG-1 and RAG-2. Progress in this endeavour has been slow, due in a large part to technical difficulties involved in working with RAG-1 and RAG-2. No doubt studies addressing questions such as whether RAG-1 and RAG-2 proteins interact with each other, with other components of the V(D)J recombinase, or with DNA substrates, are likely to warrant research for some time to come. The ultimate goal of such research would be to establish a cell-free system for V(D)J recombination in order to study the biochemical functioning of the V(D)J recombinase component(s).

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