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Review

V Gene Replacement in T and B Lymphocytes: Illicit or Regimented Rearrangement?

RACHEL GOLUB*

Unité du Développement des Lymphocytes, Institut Pasteur, 75724 Paris Cedex 15, France

Abstract. Lymphocytes can undergo novel types of secondary genetic rearrangements that alter the specificity of a pre-existing B cell receptor (BCR) or T cell receptor (TCR). V gene replacement is one of them and it replaces an already rearranged V gene segment with an upstream germline V gene segment. This review focuses on the molecular aspect of rearrangement. The role of this mechanism in the immune system is debated.

Key words: VDJ recombination; immunoglobulin and TCR loci; embedded heptamer, knock-in mice, gene rearrangement, receptor editing.

Introduction

The adaptive immune system uses a widely diverse repertoire of antigen (Ag) receptors to fight against environmental pathogens. In most vertebrates, this enormous diversity is generated in developing T and B cells, primarily by somatic recombination. Immunoglobulin (Ig) and T cell receptor (TCR) genes are rearranged by the process of VDJ recombination, consisting of the joining of variable (V), diversity (D) and joining (J) gene segments (for review[15, 33, 45]). The rearrangement of distinct combinations of V, D or J segments, the various possibilities of pairing of Ig heavy (H) chain (or TCRβ chain) with one of the many Ig light (L) chains (or TCRα chains), consists of combinatorial diversity. The joining of gene segments during recombination is mainly random. The numerous additions or deletions of bases at the junctions represent the junctional diversity. A supplementary mechanism contributing to immune receptor diversification is V gene replacement. A description of this mechanism and its implication in the generation of diversity in the B cell receptor (BCR) and TCR repertoires in developing lymphocytes is the focus of this review. The aspects of immune tolerance and the relation with autoimmunity are also discussed. An immune response is generally directed at non-self Ag; infrequently it may be directed against self, with the probable emergence of autoimmune disease. Regulation of this mechanism highlights the importance of the process of selection of T and B cells with clonally distinct non-autoreactive Ag receptors.

The molecular aspect of secondary rearrangements (V gene replacement and new V to J rearrangement) is described in Fig. 1. To explain the mechanism of V gene replacement, the best example is the H chain of Ig. VH replacement is a recombination event by which an already rearranged H chain can be altered. It occurs

Abbreviations used: BCR – B cell receptor, TCR – T cell receptor, Ig – immunoglobulin, RSS – recombination signal sequence, RAG – recombinase-activation gene, KI – knock-in, EH – embedded heptamer, QM mouse – quasi-monoclonal mouse, LM-PCR – linker-mediated PCR, SLE – systemic lupus erythematosus.

* Correspondence to: Rachel Golub, Ph.D., Assistant Professor, Unité du Développement des Lymphocytes, Institut Pasteur, 25 Rue du Docteur Roux, 75724 Paris Cedex 15, France, tel.: +33 1 45688766, fax: +33 1 45688921, e-mail: rgolub@pasteur.fr

by replacing an already rearranged VH gene with an upstream germline VH gene segment (Fig. 1B1). Therefore, it can result in a new VDJ combination which is either non-functional and inactivates the previous rearrangement or is functional and creates a new BCR specificity. Mechanistically, secondary rearrangements depend on the number and organization of gene segments, and the organization and type of recombination signal sequences (RSS), sequences flanking the coding region of the gene segments (Fig. 1A). V to J secondary rearrangements are common for the L chain of Ig and the α chain of TCRαβ. Owing to the organization of their locus, secondary rearrangement of the L chain (or α chain) genes is easier than for the H chain (Fig. 1B2). Moreover, in this case recombination takes place between two real RSS.

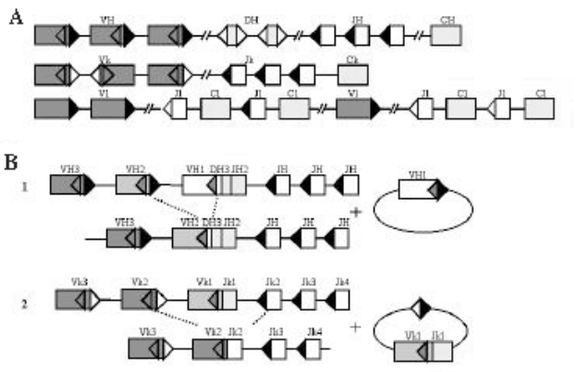

Fig. 1. Schematic diagram of the gene organization of the IgH, Igκ and Igλ loci. **A.** Simplified gene organization of the mammal IgH, Igκ and Igλ loci before recombination. V, D, J coding segments are flanked by recombination signal sequences (RSS) carrying a either 12 bp spacer (open triangles) or a 23 bp spacer (black triangles). Embedded heptamers in the V segment are indicated by open triangles inside the coding region. **B.** Overall description of the possible secondary rearrangement at the IgH and Igλ loci. 1 – V gene replacement takes place between the embedded heptamer of the VDJ rearrangement and the RSS of the upstream VH segment. In this case, the "old" VH segment is cut at the embedded heptamer and eliminated as a signal joint; 2 – V to J leapfrogging rearrangement takes place between the upstream VH RSS and the downstream JH RSS. The "old" VJ rearrangement is eliminated as a signal joint

In addition to having a role in the generation of repertoire diversity, secondary rearrangements could be implicated in various pathways depending on where it occurs. The two main concepts described in the literature are receptor editing/replacement and receptor revision. Receptor editing is related to *de novo* rearrangements that occur in immature cells in primary lymphoid organs. Receptor editing is tightly linked to the regulation of immune central tolerance. Receptor revision takes place in the secondary lymphoid organs and somehow implies an unusual activation of the recombination activating genes (RAG).

Molecular Events during V Gene Replacement: the Importance of the Embedded Heptamer

As V gene replacement is directly linked to VDJ recombination process, few features should be recalled. VDJ recombination allows the assembly of the variable portion of Ig and TCR genes (for review[15, 33, 45]). To undergo VDJ recombination, it is essential that the RAG1 and RAG2 proteins are expressed. These lymphoid-specific proteins are involved in the cleavage of blunt double-stranded (ds) DNA at the RSS. RSS consists of a highly conserved heptamer and less conserved A-T-rich nonamer elements that are separated by 12 and 23 nucleotide spacer sequences. As a rule, a 12 RSS can only recombine with a 23 RSS *in vivo*. As every gene segment of a particular type is associated with an RSS of a particular spacer length, joining of elements of the same type is prohibited (Fig. 1A). For example, the arrangement of the RSS at the H chain locus avoids upstream VH genes recombining with downstream JH genes. The process of rearrangement is temporally ordered as RAG1 and RAG2 gene expression. In B and T cells, D to J rearrangements preceed V to DJ rearrangements. In B cells, VDJ recombination is initiated at the Ig H chain locus. The H chain is then expressed in combination with the pseudo L chain to produce a pre-BCR. Then, RAG1 and RAG2 expression is down-regulated and the maturation of B cells is induced. The second wave of the RAG expression is then activated to allow the rearrangement and expression of the L chain. In the developing thymocytes, the pattern of recombination is similar. Recombination starts at the TCRβ locus, and then a pre-TCR, formed of β chain and pre-Tα chain, is expressed. The maturation of T cells is accompanied by the down regulation of the RAG genes. The second wave of recombination is settled to permit the expression of the α chain. Recombination comprises the steps of cleavage first and then the steps of joining. The signal ends are precisely fused to form the signal joint (deleted gene segments and their RSS) and the hairpin coding ends, which are nicked, repaired and fused in an imprecise coding joint.

Embedded heptamer (EH)-mediated V gene replacement was first described at the Ig H chain locus[2, 8, 10, 22, 29, 46, 51]. Located at the 3' end of most VH genes, it allows a secondary type of rearrangement. As shown in Fig. 1B, the EH encoded by the rearranged VH gene

could be "re-rearranged" with the RSS of an upstream germline VH gene segment. Since no embedded spacer or nonamer could be found upstream in the VH segment, this secondary rearrangement could be classified as an illegitimate RAG-mediated rearrangement. Studies on artificial recombination substrates have shown that only heptamers can serve as recombination targets[29]. In the "quasi-monoclonal" (QM) RAG-deficient mouse, no VH gene replacement was detected[6] while the QM mouse frequently uses this mechanism to diversify the Ag receptor repertoire[2, 5, 7, 34].

The embedded heptamer (TACTGTG) is similar to the heptamer of a canonical RSS. The GTG motif is the critical motif for the mechanism of rearrangement. RSS that differ from consensus in any positions other than the three nucleotides of the heptamer mediate VDJ recombination at lower frequencies[1, 23, 49]. This GTG motif is conserved in members of the Ig family. Concerning the mouse TCR loci, it was observed that the majority of Vα and Vβ germline segments encode an embedded heptamer at the end of the coding region[16]. The conservation of GT (positions 2 and 3 of the codon cysteine (Cys) from the βF strand of the Ig domain) reveals the importance of the Cys in the folding of the Ig domain. However, the codon usage of Cys is biased at this position in V α gene segments. Nearly 80% of these Cys residues are encoded by TGT[13], although codon usage of Cys in the mouse genome is about identical for TGT and TGC[42]. The striking conservation of this base implies a strong selective pressure to preserve a functional EH and, therefore, the capacity to undergo V gene replacement. The EH could be found frequently in L chain V genes also. In contrast to Vκ gene segments, all mouse Vλ genes possess an EH. Conservation of the EH is common in different species of vertebrates: all Vλ genes of the chicken and the rabbit, and 80% of the VH segments from other vertebrate species (e.g. sharks, amphibians, sheep).

To demonstrate the direct implication of the EH in the process of V gene replacement, ligation-mediated PCR (LM-PCR) was used. This technique amplifies the signal joint formed during a VDJ recombination event (Fig. 2). The experiments were mainly done using knock-in (KI) mice, in which the sequence of the potentially eliminated V gene is known. All KI mice were constructed on a similar pattern, where the rearranged VDJ (or VJ) transgene was inserted into the right locus. Therefore, the eventual partners for secondary rearrangement were present. In case of an IgH KI, the VDJ transgene was inserted into the JH region of the endogenous locus, and analysis of VH replacement was easy, considering that the VH sequence is known. Furthermore, the VH partners were present upstream and the specificity of the KI B cells is known. Another example is the KI α chain of the TCRαβ mouse. The VαJα rearrangement was inserted into the Jα region. Endogenous Vα and Jα germline segments were present and Vα to Jα rearrangement as well as Vα replacement could be studied.

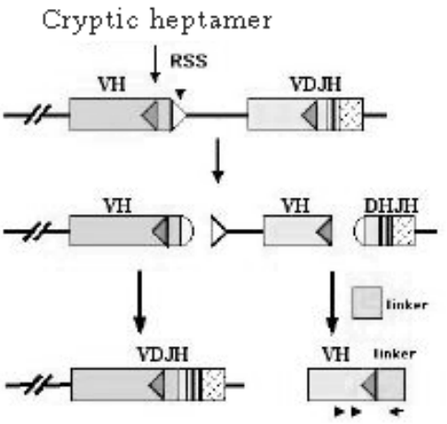

Fig. 2. Ligation-mediated PCR. This technique is used to detect the signal end intermediates during V gene replacement. A linker is added to the cell lysate and then ligated to all fragments possessing double-strand breaks. The product formed by ligation of the linker to the VH gene being replaced could be amplified using nested primers specific for the VH segment and a primer complementary to the linker

The sequences of the LM-PCR products from QM mice have directly proved that the rearranged VH gene is eliminated via the use of the EH[2]. The LM-PCR assays were successively done on VDJH KI mice, and VαJα KI mice to look at VH as well as Vα gene replacement intermediates[16].

Evidence for V Gene Replacement in Mice and Humans

Since the EH is close to the end of the coding region of the VH gene, it is difficult to identify such events *in vivo* in a normal mouse. VH gene replacement was first identified in two cell lines: an Abelson[50] and a B cell lymphoma[29] line. The generation of KI mice gave more definitive analyses of V gene replacement, where secondary rearrangements mediated by the EH were examined in a context close to that in a genome of normal mouse rearrangements[5, 8, 47, 52, 53]. In the case of the Ig H chain, the rearranged VDJ transgene is positioned

between the upstream endogenous VH gene segments and the downstream JH region; the only difference concerns the presence of the D elements that are deleted in the normal mouse during VDJ recombination (Fig. 3). This precise genomic structure explains the presence of numerous new VDDJ rearrangements in these mice[5, 8, 53]. They result from a rearrangement between the RSS of a D element and the EH of the rearranged VH gene, followed by a normal V-D rearrangement using two normal RSS. Intermediate DDJ products found in KI mice support this mechanism (Fig. 3)[8].

V gene replacement was also demonstrated in normal B cells in human[56]. Hybrid VH genes were cloned in various tonsillar B cell populations from different individuals. The new VDJ rearrangements detectable were formed by the recombination of the heptamer present in the beginning of the FR3 region of the VH genes. This heptamer is in opposite orientation, and it was postulated that replacement occurs by hybrid joint formation. Since the EH in the right orientation is only 5 bp upstream of the coding end of VH genes, this precise place of secondary rearrangement is not detectable even after careful analysis of sequences. However, the hybrid junctions reveal that the rearrangement occurs at a GTG motif and is probably RAG mediated. This type of VH replacement was also observed in synovia of rheumatoid arthritis (RA)-diagnosed patients[26] and in KI mice, where the EH was not preferentially used for secondary rearrangement even if V gene replacement was also RSS mediated[5, 52, 53, 60].

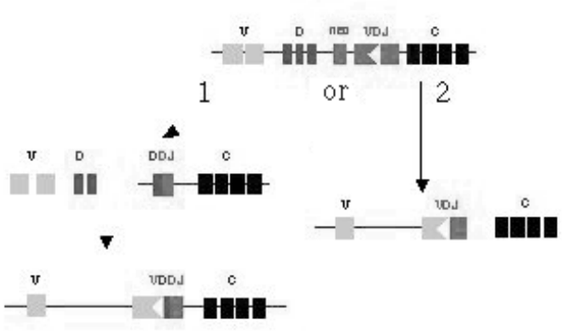

Fig. 3. Example of secondary rearrangements in knock-in (KI) mice. The KI mouse has a heavy chain transgene inserted into the JH region of one allele. The upstream VH and DH are still present. Generally, the other allele is not functional because it lacks the JH region. During secondary rearrangement, two different events could arise: 1 – a DH to VDJ rearrangement followed by a VH to DDJ, resulting in a VDDJ rearrangement; 2 – a direct V gene replacement resulting in a new VDJ rearrangement

Discussing the Roles of V Gene Replacement

Its role in generating diversity

V gene replacement is an important mechanism that generates the diversity of the BCR and TCR variable regions. This is not contradictory to a potential role in the maintenance of self-tolerance. However, it seems that V gene replacement does not need to receive a signal through the BCR to be activated[17]. The outcome, tolerance or diversity, should depend on the developmental stage and localization of the targeted cells. In some cases of KI mice, the transgene used is non-autoreactive, so the only driving force is the need to diversify the Ag receptors.

Using QM mice, it was shown that V gene replacement is an important diversifying mechanism for the pool of B cells[2, 5, 7]. More than 20% of B cells changed their specificity. Detection of replacement intermediates by LM-PCR was successful in the bone marrow and spleen of unimmunized QM mice.

In the periphery, VH gene replacement is used to generate Igs of new specificity, and the new H chains can be used in a specific response to Ag[18, 34]. This study concludes that V gene replacement could occur at least at two stages of B cell development. It takes place in bone marrow during the pro-B cell stage[2, 34] and in the periphery[34], either as a precursor B cell[58] or as a more mature B cell[41], as part of the immune response to Ag. No result seems clear concerning RAG activation in the periphery. Even with the generation of RAG-GFP mice, the controversy subsists. Secondary rearrangements of the light chain could occur in the periphery. In some studies, receptor revision was revealed by the expression of RAG genes in germinal centers along with excised products[18, 19, 24]. Some clones of B cells seem to have undergone both somatic mutation and receptor revision[4, 11, 56]. Some works tend to demonstrate that RAG genes are only expressed in immature B cells and not re-expressed in mature ones[59]. Others identify a re-expression of these proteins in cells that have a surface Ig-phenotype[41, 48]. It seems that B cells participating in germinal center reactions could have diverse phenotype and undergo receptor revision for the L and H chains of BCR.

In immunized QM mice, lots of VH replaced clones from the periphery were unmutated and IgM^+, favoring the occurrence of VH gene replacement before affinity maturation. From this study, the conclusion was more in favor of a diversifying mechanism, considering that immunization gave rise not only to higher affinity clones, but also to clones with new non-related specificities.

V gene replacement was analyzed in developing T cells[16]. The model used is the KI mouse with a VαJα rearrangement inserted in the TCRα locus. LM-PCR assays have shown that Vα gene replacement occurs in T cells during the period of rearrangement of the α chain, at the double positive $CD4^+CD8^+$ stage, just before the positive selection. Vα gene replacement could not be found in the periphery, but clones that had "replaced" in the thymus were positively selected and amplified from lymph node. In this mouse, secondary Vα to Jα rearrangements are also common[55] and the notion of receptor editing in developing T cells is now settled[36, 43]. In T cells, V gene replacement is used to shape the repertoire of TCR. For the α chain, the frequent secondary V to J rearrangement also reinforces diversity. Allelic inclusion reveals that rearrangements take place on the two alleles[3, 35]. When a functional α chain allows the expression of a TCR at the cell surface, the RAG1 and RAG2 are not downregulated and secondary rearrangements are continually tested until positive selection[27, 28]. It seems that V gene replacement occurs at the same time. In a non-finalistic point of view, it could be argued that V gene replacement is only a consequence of RAG1 and RAG2 expression. However, it was estimated that at least 65% of TCRα chains are the result of secondary rearrangement[57]. These data revealed the importance of secondary rearrangement as a diversifying mechanism of the T cell repertoire.

A role in central tolerance

As clonal deletion, receptor editing/replacement is a mechanism probably implicated in the regulation of immune tolerance. Editing was shown in transgenic mice with autoantibodies (anti-HEL, MHC class I alloantigens, ssDNA, dsDNA, the erythrocytes). Some autoreactive B cells were still present in their bone marrow. However, in the periphery, none of these autoreactive B cells were observed in the presence of self-Ag[10, 14, 20, 44, 54]. The detection of excision products from secondary rearrangements of the L chain and the increased levels of RAG mRNA in bone marrow B cells were strong proof of the occurrence of receptor editing. Numerous experiments carried out with transgenic models underlines the direct link between the regulation of immune tolerance and receptor editing. A multiple round of rearrangements could be attempted on a single chromosome in order to recombine a "correct non self-reactive" chain. In fact, to escape death, autoreactive B cells could try to edit their receptor by multiple secondary rearrangements. V gene replacement has been less extensively studied than V to J rearrangement. The implication of V gene replacement in the regulation of tolerance is still a matter of debate. The model of the autoreactive KI mouse where the transgene encodes an anti-dsDNA H chain favors a role of V gene replacement in the maintenance of tolerance. Selected B cells in these mice possess functional non-autoreactive Igs resulting from VH gene replacement[9]. Consequently, it seems that B cells eliminate unwanted rearrangements via V gene replacement. However, other non-autoreactive KI mice also use VH gene replacement reinforcing, the role of this mechanism on diversification of the B cell repertoire.

V to J secondary rearrangement and V gene replacement take place at different developmental stages of the B cell. Studies have shown that L chain editing was stimulated when immature B cells were cross-linked[21, 40]. A complementary study has precisely defined the stage of IgM^{lo} IgD^- as the editing sensitive stage[39]. LM-PCR assays have shown that VH gene replacement occurs at the IgM^+IgD^+ stage (MADAN, unpublished data). It may be possible that VH gene replacement can occur earlier in B cell development when the H chain is first expressed and tested. The VH-replaced rearrangements analyzed from QM mouse splenocytes revealed the presence of an N-addition[34]. These data suggest that VH gene replacement also takes place during a pro-B stage when TdT is active. VH gene replacement and V to J rearrangements occur at distinct stages during development following the rule of temporally regulated recombination. V gene replacement probably plays a minor role in eliminating autoreactivity compared with L chain editing, and it could be stimulated only when secondary rearrangements at the L chain locus cannot be used. Finally, cells that fail to change their autoreactive receptors are then submitted to apoptosis before entering the mature B cell pool. Receptor editing of the L chain was also shown to influence the fetal B cell repertoire. The Ag receptor specificity is changed in order to escape negative selection[32]. The contribution of V gene replacement in the fetal repertoire is still unclear and not proven.

Analyses of transgenic mice that express a peptide antigen in the cortical thymic epithelial cells have shown the importance of secondary rearrangement. Conversely to what was expected, a high-affinity peptide ligand led to the internalization of the TCR and editing of the TCR specificity instead of deletion of the cell[36]. In the case of Vα gene replacement, it seems that a positively selecting background has no effect on the occurrence of the event compared with the occurrence of a leapfrog event[16].

A role in autoimmunity

In the periphery, the high-affinity self reactivity could be enhanced after the somatic mutation process and VH replacement could be used to maintain peripheral tolerance, as it maintains tolerance in the bone marrow. Revision of the receptor would end in the loss of self-reactivity either by a non-functional rearrangement or by a new specificity. In the opposite case, receptor revision could generate a new repertoire that possesses non-negligible chances of having autoreactive specificities and then deregulating peripheral tolerance. In case of an autoimmune background, VH gene replacement results in the loss of tolerance. In an autoreactive ssDNA transgenic *lpr* mouse, many B cells undergo VH gene replacement and become reactive to various self-Ags[30]. In diagnosed RA patients, antibody H chains from synovial tissue revealed revisions resulting from VH replacements[26]. These replacements, as in normal B cells[56], take place at the EH and other "heptamer-like sequences" in the VH coding region. As for the RAG mRNA level, the frequency of replacement seems higher in the case of an autoimmune background. Receptor revision of L and H chains could be linked to the progression of the autoimmune disease. In terms of secondary rearrangements, it was already shown that in systemic lupus erythematosus patients, secondary L chain editing was largely increased[12]. The supplementary consequence could be the clonal expansion of these newly generated autoreactive cells.

For Vα gene replacement, studies have shown that the mechanism seems to be inactive in the periphery in the normal mouse. This absence would limit the occurrence of an autoimmune problem. It seems safer that secondary rearrangement are not reactivated in normal peripheral T cells, where selection could not be as drastic as it is in the thymus. Some have suggested that editing can contribute to autoimmunity by coupling receptor editing and allelic inclusion. T cells can possess two different specificities, and in some systems these T cells were correlated with RA[31]. However, these results were always related to transgenic mouse systems that are not representative of the natural occurrence of dual specificities.

In some cases, peripheral T cells edit their α and β chains. For example, in human peripheral blood, some activated T cells possess both RAG transcripts and TCRβ recombination substrates. In a β chain transgenic mouse, some peripheral $CD4^+$ T cells edit their β chain[37, 38]. In case the of viral superantigen immunization, the peripheral T cell repertoire is edited with the "reexpression" of RAG genes and the initiation of new Vα to Jα rearrangements[25]. Together, these data suggest that receptor editing of the TCR may be a common mechanism of the immune response to various stimuli. However, only leapfrog events were analyzed and no data are available concerning Vα replacement in the periphery after activation or immunization.

Conclusions

Secondary rearrangements were first seen as a dilemma when confronted with the clonal selection theory. However, these mechanisms (receptor editing/replacement/revision) act in synergy with clonal deletion and expansion to allow the generation of a diverse repertoire of antigen receptors. Secondary rearrangements could be considered as the mechanisms available for the cells to express "correct receptors". They are developmentally regulated and controlled enough to be harmless. Moreover, in case of skid, other mechanisms, such as clonal deletion, should act as guard rails. The major problem is the analysis of the extent of this mechanism. The definitive frequency of receptor revision and its impact on the Ag receptor repertoire are difficult to determine.

Receptor editing for the L chain of Igs appears to occur in at least 2/3 of autoreactive B cells in normal mice, as determined by studying the frequency of B cells with productive, inactivated κ rearrangements[39]. The secondary rearrangement of the H chain should be less frequent than for the L chain. First, L chains are encoded by two loci, and second, the organization of the κ locus favors new rearrangements (asymmetry of RSSs, consensus RSSs, orientation of Vκ gene segments). No analysis was done to reveal the presence of V gene replacement for the L chain. However, it seems probable that this mechanism can occur, as it was observed for the α chain of the TCRα.

Immunoglobulin genes, TCRα, β, γ, and δ genes, the RAG genes and MHC genes have been identified only in vertebrates and they are presumed to have evolved together in cartilaginous fishes, ancestors of modern-day skates and sharks. The development of lymphocytes may go back about 450 million years. There are fairly significant differences in the ways different species generate Ag-receptor diversity during lymphocyte development, sometimes related to the different organization of their respective loci. In any case, it seems probable to find V gene replacement as a diversifying mechanism in other species.

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