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V(D)J recombination in zebrafish: normal joining products with accumulation of unresolved coding ends and deleted signal ends

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Abstract

V(D)J recombination proceeds from a site-specific cleavage to an imprecise end joining, *via* generation and resolution of recombination ends. Although rearranged antigen receptor genes isolated from zebrafish (*Danio rerio*) resemble those made in mammals, differences may arise during evolution from lower to higher vertebrates, in regard to efficiency, fidelity and regulation of this recombination. To elucidate the V(D)J recombination reaction in zebrafish, we characterized recombination ends transiently produced by zebrafish lymphocytes, as well as joining products. Similar to their mammalian counterpart, zebrafish lymphocytes make perfect signal joints and normal coding joints, indicating their competent end resolution machinery. However, recombination ends recovered from the same zebrafish lymphoid tissues exhibit some features that are not readily seen in normal mammalian counterpart: deleted signal ends and accumulation of opened coding ends. These results indicate that the recombination reaction in zebrafish lymphocytes is inefficient and less stringently regulated, which may result from unstable post-cleavage complexes, and/or slow transition from cleavage to resolution. Our data suggests that the V(D)J recombination machinery may have undergone evolution selection to become more efficient in higher jawed vertebrates.

Keywords

non-homologous end joining; recombination ends; zebrafish

1. Introduction

The V(D)J recombination reaction consists of two steps, a site-specific cleavage and an imprecise end joining. The cleavage reaction is catalyzed by recombination-activating gene 1 and 2 (RAG1/2) proteins, which are expressed in developing lymphocytes (Gellert, 2002; Jung and Alt, 2004; Schatz, 2004). RAG1/2 proteins initiate recombination by recognizing recombination signal sequences (RSSs), making excision at the border between the RSSs and the coding segments, generating blunt signal ends (SEs) and hairpin coding ends (CEs), which are likely held in a complex containing RAG1/2 proteins (Fugmann et al., 2000). Subsequently, recombination ends are resolved into signal joints (SJs) and coding joints (CJs) *via* ubiquitously expressed non-homologous end joining (NHEJ) proteins, including Ku70, Ku80, XRCC4 and DNA ligase IV, as well as DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Artemis for processing hairpin CEs before their final joining (Schatz, 2004).

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The zebrafish has emerged as a useful vertebrate genetic model for the study of the immune system due to its rapid growth, high genetic conservation with mammals, and most importantly its feasibility for experimental manipulation at earlier developmental stages (Traver et al., 2003). The V(D)J recombination process appears to be highly conserved among all jawed vertebrates, as inferred from the structural similarity of their RAG1 and RAG2 proteins, and from the characteristics of rearranged antigen receptor genes, especially in the CDR3 region (Hansen and McBlane, 2000). Although zebrafish RAG1/2 genes are orthologous with mouse and human in terms of general gene organization, lineage-dependent expression, and the conserved structure of the catalytic domain, zebrafish recombinases are phylogenetically diverged from mammals. There are 60% and 53% amino acids identical for RAG1 and RAG2, respectively, between zebrafish and mammals (Willett et al., 1997). Furthermore, some differences also exist in Ku70 and Ku80 proteins between zebrafish and mammals although many NHEJ genes from zebrafish have not been identified or fully characterized (Bladen et al., 2005) (unpublished analyses). It would be interesting to determine whether these structural differences have any functional significance on recombination activity. Specifically, does the V(D)J recombination reaction in zebrafish proceed differently from that in mammals? To address this question, we set out to analyze recombination intermediates to get a view of ongoing recombination in zebrafish.

In this study, we found that the joining products formed by zebrafish lymphoid cells resemble those made in mammals, i.e., containing junctional diversity at CJs and possessing perfect junction at SJs. However, zebrafish lymphoid tissues exhibit some unusual features in their end resolution: accumulation of opened CEs and nucleotide deletions at SEs. These findings point to an inefficient transition of recombination ends from cleavage to resolution in zebrafish lymphocytes, which is in striking contrast to the one adopted by mammalian lymphocytes, efficient and seamless. Our findings reveal phylogenetic changes in the V(D)J recombination reaction from lower to higher jawed vertebrates.

Methods

2.1. Animals and DNA preparation

Adult zebrafish (*Danio rerio*) were bred and maintained at 28.5°C with a photoperiod of 14 hrs light and 10 hrs dark, according to the established guidelines in the Zebrafish Facility set up at the Biodesign Institute of Arizona State University (Westerfield, 1995). All animal protocols were conducted under the supervision and approval of the ASU IACUC committee. Kidneys and spleens were taken from 10-15 adult zebrafish after they were euthanized by tricaine medium (Sigma). In some cases, aurintricarboxylic acid (Sigma), a nuclease inhibitor, was included in the buffer (at 1mM) for preparing cells from zebrafish kidney. Bone marrow cells were isolated from adult Balb/c mice. To obtain intact chromosomal DNA, single cell suspensions were prepared from these tissues, embedded in agarose plugs and deproteinized with proteinase K (Roche).

2.2. Examination of recombination intermediates by LM-PCR

Immunoglobulin heavy (H) and light (L) chain loci were analyzed in this study (Fig. 1A). Ligation-mediated PCR (LM-PCR) was employed to examine double strand DNA breaks (DSBs) generated from the initiation of V(D)J recombination, as illustrated in Figure 1B. Ligated DNA samples were amplified to detect J_H- or J_L-SEs, while genomic PCR was applied to amplify VDJ_H-, DJ_H- or VJ_L-CJs and SJs. Opened J_H- or J_L-CEs were detected by a modified LM-PCR procedure, in which DNA were pre-treated with T4-DNA polymerase (T4-DNA-POL) before their amplification by LM-PCR (Schlissel, 1998; Zhu and Roth, 1995). Details on primers for relevant PCR reactions are presented in Figure 1A, Table 1 and Table 2. While the perfect SJs contain an ApaL1 restriction site (GTGCAC), ligation of intact SEs (i.e. GTG) to

an artificial linker that is ended with CAC also creates an ApaL1 site (Fig. 2A). Thus, the integrity of SJs and SEs can be verified by ApaL1 digestion of their PCR products. PCR products were separated on 1.5% agarose gels. In addition, some gel-purified PCR products (i.e., CJs and SEs) were subcloned into the pCR 2.1-TOPO vector (Invitrogen) for further sequencing analyses.

For semi-quantitative PCR, the ligated DNA-plugs were amplified for 31 cycles to reveal coding and signal ends. Given their greater abundance in the cells, CJs and SJs were amplified for 28 cycles whereas input control DNA, germline heavy chain (G_H), or germline light chain (G_L), was amplified for 23 cycles. Several dilutions of ligated DNA (1, 1:3) were subjected to PCR to ensure reliability of PCR amplification (Fig. 3 and data not shown). All these PCR products were separated by electrophoresis and analyzed by Southern blot.

3.Results

3.1 Aberrant signal ends and precise signal joints in zebrafish kidney

Examination of the quantity and quality of recombination intermediates offers a means to evaluate the functional status of the recombination machinery at both cleavage and end resolution (Roth et al., 1993; Schlissel, 1998). We performed semi-nested ligation-mediated PCR (LM-PCR) or PCR reactions to amplify recombination intermediates and products made at the immunoglobulin heavy chain (IgH- μ) locus. As shown in Figure 1C, SEs could be detected in kidney, but not in spleen, which represent primary and secondary lymphoid organs in adult zebrafish, respectively. Similarly, SJs were present in kidney but not spleen. Thus, the presence of SEs and SJs indicates ongoing recombination initiated in developing B cells, as expected. On the other hand, CJs were found in both kidney and spleen (Fig. 1C), reflecting the accumulation of these joints in both tissues. Thus, by analyzing recombination intermediates and transient signal joint formation, we have demonstrated that ongoing recombination is initiated at the IgH locus in the kidney of adult zebrafish.

In mammalian lymphocytes, recombination SEs are stable and intact in general (Chang and Brown, 1999; Nishihara et al., 2004; Roth et al., 1993; Schlissel, 1998), presumably due to their protection by a post-cleavage complex, composed of RAG1/2 and HMG1/2 (Jones and Gellert, 2001; Leu et al., 1997). These ends are then subsequently joined head-to-head to form perfect SJs in normal mammalian cells. The LM-PCR was performed with a linker that can produce an ApaL1 site upon its ligation to an intact SEs (Fig. 2A). In addition, the precisely fused SJs create an ApaL1 site (Fig. 2A). Thus, to assess the fidelity of SEs and SJs derived from zebrafish lymphocytes, we performed ApaL1 digestion of amplified PCR products. As controls, we included the analyses of SEs and SJs made from recombination-active murine cell lines, Sp9 and S9, a wild type and a DNA-PKcs-deficient mutant, respectively (Chang and Brown, 1999). As shown in Figure 2B, both SEs and SJs made in Sp9 are sensitive to ApaL1 digestion, indicating the presence of intact SEs and precise joining of these ends in wild-type cells. On the other hand, S9 cells produce intact SEs but imperfect SJs (because of incomplete ApaL1 digestion), which results from their partial abnormality in SJ formation, as reported previously (Lew et al., 2000). In contrast, the integrity of SEs and SJs recovered from zebrafish samples was found inverse to those made in S9 cells, i.e. imperfect SEs but precise junctions in SJs. As shown in Figure 2B, SEs derived from zebrafish immunoglobulin light chain locus (IgL) are resistant to ApaL1 (i.e., imperfect ends) whereas their SJs are sensitive to ApaL1 digestion (i.e., perfect junctions). Thus, in adult zebrafish lymphocytes, while some SEs can be joined accurately, the unresolved ends are susceptible to modification. To further characterize zebrafish SEs, we cloned the LM-PCR products and performed sequence analyses. As summarized in Table 3, the majority of recovered SEs contain deletions ranging from 1 bp to >200 bps.

The kidney has been considered as the major primary lymphoid organ in teleost fish. However, unlike mammalian bone marrow, the teleost kidney also contains structural cells associated with renal function, such as nephrons, glomeruli and podocytes (Westerfield, 1995). This raises a question as to whether the different end integrity observed between mammals and zebrafish is attributed to the presence of these non-hematopoietic cells in zebrafish kidney. For example, during sample preparation, renal cells may release a high level of nuclease to surrounding cells and degrade SEs that are newly generated from recombination-active cells. To test this possibility, we mixed the zebrafish kidney tissues (K) with a wild type murine cell line (Sp9, used in Fig. 2B) that was induced to undergo IgL rearrangement. The mixed cell populations were prepared for DNA plugs that were subsequently analyzed by LM-PCR for both mouse and zebrafish light chain SEs (IgL-SEs). Based on the sensitivity to ApaL1 digestion, it is clear that mouse IgL-SEs are intact whereas zebrafish IgL-SEs are not (Fig. 2C). In addition, LM-PCR products amplified from the DNA plugs that were prepared from zebrafish kidney in the presence of nuclease inhibitor (aurintricarboxylic acid) also show resistance to ApaL1 digestion (Fig. 2D). Therefore, the deletion of SEs found in zebrafish is not likely due to an experimental artifact, but rather reflecting incomplete end-protection in zebrafish.

To determine whether the presence of abnormal SEs is unique to adult kidney, we also examined SEs and SJs from day 14 zebrafish embryos. By assessing resistance of amplified PCR products to ApaL1 digestion, we found that the SEs recovered from embryos are modified whereas their joining products contain perfect junctions (Fig. 2E), similar to what we found in adult kidney (Fig. 2B). Thus, unlike mammalian lymphocytes, recombination-active B cells of zebrafish, regardless of their age and developmental stages, fail to maintain integrity of SEs derived from immunoglobulin gene loci. Similarly, SEs derived from the T cell receptor- α (TCR- α) locus also exhibit deletions (unpublished observation).

3.2. Accumulation of opened coding ends

The lack of SE integrity in zebrafish is in striking contrast to the intact signal ends in mouse lymphoid cells. This raises a question as to whether zebrafish and mammals handle recombination intermediates differently. To directly address this issue, we compared the levels of recombination signal and opened coding ends between zebrafish kidney and wild-type mouse bone marrow cells. For coding end detection, DNA plugs were pre-treated with T4-DNA polymerase (T4-DNA-POL) to make the ends blunt before subjecting them to LM-PCR, as CEs were reported to be staggered with a 3'-overhang in mammals (Schlissel, 1998). To make a semi-quantitative comparison of recombination ends made between zebrafish and mouse, we used primary LM-PCR followed by Southern blot analyses.

In consistence with known features of recombination ends in mammalian lymphoid tissues: 1) blunt-ended SEs; 2) very few unresolved opened CEs because of rapid CE resolution (also very few hairpin CEs) (Livak and Schatz, 1996; Roth et al., 1993; Schlissel et al., 1993; Schlissel, 1998; Zhu and Roth, 1995), our data showed that the level of SEs and CEs in the mouse bone marrow could not be altered by the pre-treatment of DNA samples with T4-DNA-POL, as demonstrated by comparable levels of SEs, and a minute amount of CEs present in samples treated with or without T4-DNA-POL (Fig. 3A, left panel). In the same experiment, opened CEs were detected in S9 (Fig. 3A, right panel), a DNA-PKcs deficient murine scid cell line that was known to contain unresolved CEs (Brown et al., 2000), and used as a control. In contrast to mouse bone marrow, the pattern of recombination ends in zebrafish kidney is quite different. As shown in Figure 3B and 3C, a significant elevation of both SEs and CEs was found at both IgH and IgL gene loci in the zebrafish DNA samples that were pre-treated with T4-DNA-POL in comparison to those without T4-DNA-POL treatment. The increased detection in SEs upon T4-DNA-POL treatment reflects modification of the ends, which is consistent with our data on deleted SEs (Table 3). Although the elevation of CEs after T4-

DNA-POL treatment is expected given their staggered end structure, the increase is much more apparent in zebrafish than in mouse (Fig. 3B and 3C to Fig. 3A).

In normal mouse primary lymphoid tissues, opened CEs are very low or virtually undetectable (Fig. 3A), especially in comparison to their SEs (Livak and Schatz, 1996; Roth et al., 1992; Schlissel et al., 1993; Schlissel, 1998). Here, we demonstrate that zebrafish kidney contains a substantial amount of opened CEs, at a level comparable to the recovered SEs. Despite numerous efforts and trying various experimental procedures, we fail to identify hairpin CEs in zebrafish kidney. Although our assays did reveal hairpin CEs in our scid cell line (S9), they may not be sensitive enough to capture hairpin ends from zebrafish lymphoid tissues that only contain a fraction of recombination-active cells. Nevertheless, a significant level of opened CEs present in the zebrafish kidney suggests that the end resolution machinery in zebrafish lymphocytes is incapable of promptly resolving all their opened CEs.

Taken together, our findings reveal some unexpected features unique to zebrafish recombination reaction: 1) modification of SEs; 2) accumulation of unresolved CEs, which contrast to the blunt-ended SEs and few CEs in mammalian counterpart. Our data points to important differences between these two species in handling recombination intermediates, and implies an inefficient end resolution process in the zebrafish cells.

3.3. Normal junctions of rearranged CJs

Given the accumulation of opened CEs, we wonder whether joining products in zebrafish bear some abnormalities, as observed in S9, a DNA-PKcs deficient murine scid cell line (Lew et al., 2000). To analyze all the rearranged products, including both productive (P) and non-productive (NP) joints, we use genomic PCR assay to amplify VDJ_H-CJs, derived from specific gene segments, V_H4 (FR1) and J_H4 (3' region) (Danilova et al., 2000). The sequences of cloned VDJ_H products are presented in Table 4. Owing to a recent report on the genomic structure of IgH D-loci by Danilova et al (Danilova et al., 2005), the D segments of these VDJ_H joints can be assigned. As summarized in Table 4, most joints exhibit relative normal junctions with 0-6 nucleotide (nt) deletions at V and J, and up to 9 nt deletions in the D-region, similar to what was reported in mouse lymphoid tissues (Pennycuik et al., 1993). Similar to the characteristics of rearranged immunoglobulin (Ig) genes in mammalian lymphoid tissues, these coding joints also contain nucleotide (N) addition catalyzed by terminal deoxynucleotide transferase (TdT), and palindromic (P) sequences as a result of hairpin end processed by DNA-PKcs and Artemis. These data imply that the NHEJ machinery for resolving coding ends, such as, TdT, DNA-PKcs and Artemis, is likely to be functional in zebrafish lymphocytes, and is conserved between zebrafish and mammals. Yet, compared to the mammalian counterpart, this machinery in zebrafish lymphocytes may still not be very efficient, as it left unresolved CEs behind (Fig. 3B and 3C).

All five IgH-D_μ-segments can be identified in our PCR products of rearranged VDJ_H joints although D_μ-1-3 is more frequently used than the others (Table 4). Interestingly, the D-sequence in one VDJ-CJ does not belong to D_μ, but rather resemble the recently identified D_ζ family (Danilova et al., 2005). This D_ζ element was found to join to the J_μ4 segment, as the primer used is complementary to the J_μ4 but not to the J_ζ regions (all primers used are listed in Table 1). The presence of rearranged D_ζ-to-J_μ4 joints may reflect inter-cluster rearrangement. This D_ζJ_μ4 joint is in-frame, and can potentially encode CDR3 regions. This finding indicates that in addition to D_ζ-to-J_ζ and D_μ-to-J_μ, rearrangement between D_ζ and J_μ may occur at a low frequency, which can potentially increase the combinatorial diversity of immunoglobulin genes in zebrafish.

Discussion

In this study, we provide the first evidence for the presence of aberrant SEs and unresolved CEJs without apparent abnormality in final joining products. These unusual features of the recombination process do not seem to be a locus-dependent, or stage-dependent event (Fig. 2, Fig. 3 and Table 3), rather reflecting an intrinsic nature of the V(D)J recombination reaction in zebrafish. Given the normal junctions in the rearranged products, zebrafish lymphocytes possess proficient NHEJ machinery. And yet, substantial amounts of CEJs and SEs remain unresolved and unprotected. To reconcile these seemingly conflicting data, we hypothesize that the post-cleavage complex generated in zebrafish may not be stable, or may fail to be promptly modulated and transformed for subsequent resolution. As a result, although some SEs released from the post-cleavage complex can quickly get access to NHEJ factors for normal joining, many SEs may be quite vulnerable to deletion, and not be joinable. Thus, newly generated SEs face two possible outcomes, one to be joined right away to form perfect SJs and the other being deleted. Our detected SEs may represent those that are on their way to be deleted and ultimately eliminated, rather than being true recombination intermediates. Similarly, inefficient NHEJ machinery or lack of full collaboration between RAG1/2 recombinases and NHEJ factors leads to incomplete resolution of CEJs, in which some of them can be resolved into normal joints while others may remain unresolved, and eventually be eliminated. In some aspects, this recombination process resembles the decoupled recombination reaction in DNA-PKcs deficient murine scid cells, where unresolved coding ends can be readily detected (Brown et al., 2000; Roth et al., 1992). However, the mechanism utilized by the mutant murine cells differs from the one in zebrafish since scid cells produce aberrant joining products but maintain intact signal ends. Instead, the V(D)J recombination system in zebrafish may be intrinsically less developed than the one in mammals, with a resulting impact on the efficiency but not the accuracy of end resolution.

NHEJ is an ancient mechanism for repairing DSBs. It is present in all eukaryotes, and is highly conserved from yeast to mammals (Daley et al., 2005; Lieber et al., 1988). In contrast, the RAG1/2 recombinases are relatively new, presumably acquired by horizontal transfer of a insect transposon into primitive jawed vertebrates or earlier species because of recent demonstration of RAG1/2 homolog in sea urchin (Fugmann et al., 2006). In addition to generating recombination ends, RAG1/2 recombinases also participate in the joining of these ends by interacting with the NHEJ machinery (Leu et al., 1997; Qiu et al., 2001; Ramsden et al., 1997; Yarnell Schultz et al., 2001). Thus, unlike other DSBs, an efficient resolution of recombination ends depends on close interaction between the RAG1/2 recombinase and the NHEJ machinery. It is conceivable that since the acquisition of RAG1/2 in primitive species, the V(D)J recombination machinery may have undergone phylogenetic adaptive evolution to make the RAG-mediated excision apparatus more compatible and integrated with NHEJ for a rapid transition from cleavage to resolution, thereby ensuring genome stability during recombination. Our findings of inefficient end resolution in zebrafish lymphocytes support this argument.

Recently, it has been postulated that NHEJ can adopt multiple biochemical reactions to process and resolve diverse structures of DSBs, exhibiting high flexibility in terms of efficiency and junction variability (Ma et al., 2005). In addition to core NHEJ factors (i.e., Ku70, Ku80, DNA-PKcs, Artemis, XRCC4 and ligase IV), several members of polymerase X family have also been reported in DSB repair and V(D)J recombination (Bertocci et al., 2003; Nick McElhinny et al., 2005; Nick McElhinny and Ramsden, 2003; Nick McElhinny and Ramsden, 2004). Interestingly, while the yeast transformed with mouse RAG1/2 and recombination substrates do form SJs with precise junction they make very few SJs, fewer than transposition products, implicating inefficient resolution of recombination ends (Clatworthy et al., 2003). In this case, both core NHEJ factors and Mre11/Rad50/Xrs1 (MRX) complexes were found essential for

SJ formation in yeast although both are required for their general DSB repair (Clatworthy et al., 2005). It has been controversial whether the MRX homolog, Mre11/Rad50/Nbs1 (MRN), is essential in non-homologous end joining and V(D)J recombination in mammals. Although *in vitro* studies with purified proteins indicate the involvement of MRN in end processing and resolution (Huang and Dynan, 2002; Paull and Gellert, 1998; Paull and Gellert, 1999), no deficiency in V(D)J recombination was identified in human or mouse cells harboring hypomorphic mutations in the Mre11, or NBS1 gene (Bender et al., 2002; Carney et al., 1998; Stewart et al., 1999; Theunissen et al., 2003). Recently, NHEJ-mediated DSB repair has been demonstrated independent of Mre11 (Di Virgilio and Gautier, 2005). Nevertheless, given its interaction with DSBs and its involvement in the induction of cellular response to DNA damage, the MRN complex may still be able to influence the efficiency of recombination reaction by acting as a default end-processing enzyme, recruiting other factors or regulating the fate of recombination-active cells through cell cycle control and apoptosis induction. Thus, depending on how NHEJ reaction or possible other pathways are coupled to the RAG1/2-mediated post-cleavage complex, the efficiency of recombination end resolution may differ among various species.

Although we have gained significant understanding on how recombination ends are generated and how they are eventually joined, little is known about the transition from cleavage to resolution. The *in vitro* reconstitution of recombinases with various components of NHEJ may help elucidate the biochemical nature of this transition. Alternatively, phylogenetic comparison of the recombination process among various species may also provide clues for identifying molecules important in the transition. The different recombination efficiencies observed between zebrafish and mouse may reside in the structural divergences of RAG1/2 recombinases, NHEJ components, MRN or other factors between these two species. Future studies will be directed in identification of the factors that control the transition from cleavage to resolution.

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Abbreviation

CEs, coding ends; CJs, coding joints; DSBs, double strand DNA breaks; LM-PCR, Ligation-mediated PCR; NHEJ, Non-homologous end joining; SEs, signal ends; SJs, signal joints.

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Fig. 1.
Rearrangement of immunoglobulin (Ig) genes in zebrafish lymphoid tissues. **A.** Outline of zebrafish IgH and IgL gene loci, as well as location of primers that are listed in Table 1. These primers, denoted as small arrows, are utilized in PCR amplification of recombination ends and joints, as shown in Table 2. **B.** Diagram illustrating the production of signal ends (SEs), coding ends (CEs), coding joints (CJs) and signal joints (SJs), as well as their detection by genomic PCR (for CJs and SJs) and ligation-mediated PCR (LM-PCR for SEs and CEs). The V, D or J gene segments (rectangular boxes) are flanked by recombination signal sequences (RSSs, triangles). **C.** Analysis of IgH recombination status. Genomic DNA was prepared from spleen (S) and kidney (K), J_H-SEs were examined by a semi-nested LM-PCR (see Table 2 for primer selection). Rearranged VDJ_H- and DJ_H-CJs or DJ_H-SJs were amplified by genomic PCR. Germline heavy chain (G_H) was included as a control for DNA input.

Fig. 2.

Examination of fidelity of SEs and SJs by ApaL1 digestion. **A.** Illustration of the susceptibility of SE LM-PCR (left panel) and SJ PCR (right panel) products to ApaL1 restriction enzyme. **B.** ApaL1 digestion of SE and SJ PCR products. Genomic DNA was prepared from recombinase-active murine cell lines (Sp9 and S9), and zebrafish kidney. The PCR products of SEs and SJs, derived from IgL gene loci (Danilova et al., 2000; Haire et al., 2000) were incubated overnight with or without restriction endonuclease ApaL1, and analyzed by gel electrophoresis. The down-shift of PCR products indicates a digestion by ApaL1, reflecting the presence of the perfect junction of SJs or full-length SEs. Arrow indicates expected sized of zebrafish J_L-SEs (~280bps). **C.** Effect of zebrafish kidney tissues on the integrity of SEs made from a recombination-active murine cell line (SP9). Genomic DNA prepared from zebrafish kidney, K(z), a wild-type recombination-active murine cell line, Sp9 (m), and the mixture of zebrafish kidney and murine cells, Mix, were examined for mouse IgL-SEs (V_L) or zebrafish IgL-SEs (J_L) by LM-PCR. The PCR products were treated with (+) or without (-) restriction endonuclease ApaL1 and analyzed by gel electrophoresis. The down-shift of PCR products indicates digestion by ApaL1, reflecting full-length SEs. **D.** Inability of nucleic acid inhibitor (aurintricarboxylic acid) to rescue the integrity of zebrafish J_L-SEs. LM-PCR was performed on the DNA plugs prepared from zebrafish kidney cells in the presence of aurintricarboxylic acid. The PCR products were treated with or without ApaL1 endonuclease, and separated on 1.2% agarose gel (lane 1-2). As a positive control for ApaL1 digestion, LM-PCR from mouse IgL-SE was included (lane 3-4), in which a down-shift of the band was noticed. **E.** IgL rearrangement in day 14 zebrafish embryos. Genomic DNA was

prepared from zebrafish embryos. The PCR products amplified from VJ_L-SJs, J_L-SEs were treated with or without restriction endonuclease ApaL1.

Fig. 3.
Southern blot analysis of PCR products amplified from recombination ends and joints made in zebrafish kidney and mouse bone marrow. **A.** Analysis of recombination ends and joints present in total mouse bone marrow cells. Genomic DNA prepared in DNA plugs was treated with or without T4 DNA polymerase before being analyzed by LM-PCR. Semi-quantitative LM-PCR (two different dilutions: 1 and 1:3) followed by Southern blot analysis was performed to detect $J\kappa$ -SEs and $J\kappa$ -CEs, $VJ\kappa$ -CJs and germline (G_L) controls. S9, a ts-Ab-MLV pre-B cell line, derived from DNA-PKcs deficient scid mice (Chang and Brown, 1999) serves as a positive control for the detection of unresolved opened CE. **B-C.** Analysis of recombination ends and joints in zebrafish kidney. PCR and LM-PCR followed by Southern

blot was performed on zebrafish DNA samples to amplify J_H-SEs, J_H-CEs, DJ_H-SJs, DJ_H-CJs and G_H of the IgH-locus (B) and J_L-SEs, J_L-CEs VJ_L-SJs, VJ_L-CJs and G_L of the IgL-locus (C).

Table 1

Primers for PCR amplification

Primer	Primer Sequence	DNA accession No.
396	5'-ATGGAGTCRATTGAAAGCTYAGT-3'	BX649502
393	5'-GACAAGTCACTGTGACTACTAC-3	BX510335
409	5'-TGACAGTTTCCTCAGGTAAGACG-3	BX510335
262	5'-AATCCATGCAGGAGGAGCCGATT-3	BX510335
261	5'-TTCCTGGACTTTGCATTGCACAA-3	BX510335
279	5'-AGTGACATCAGGTAAGCAGTTTCCT-3'	BX510335
392	5'-CTGACGAGACGGTGACCATAGT-3	BX510335
288	5'-AGTGCAATTCATGAGCAAAATCCCA -3'	BX510335
295	5'-TGGCTTAGTTGTAAAGATCCATAGC-3'	BX510335
290	5'-TCTGCTGTCAACTATCTGGCT-3'	BX510335
410	5'-GTGAGATTGACACTAATGAAGCC-3'	AF246162
406	5'-GTCTCTACAGTCTCTCTCAGCTGC-3'	AF246162
267	5'-ACCGATCAGTCTGGATGTTCTG-3'	AF246162
273	5'-TACAATGACTTGACCAGCCCAGT-3'	UCSC Genome Browser
274	5'-TGGGGAAAACATGACTAACAAAGCA-3'	UCSC Genome Browser
404	5'-CTATGGATCTGGTTTCTCTGC-3'	AF246162
36	5'-TTCAGTGATGTCACCACCTTCC-3'	J00586
23	5'-GCTGCATACATCACAGATGC-3'	J00586
24	5'-CAATGATTCTATGTTGTGCC-3'	V00811
40	5'-CCAAGCTTTCCAGCTTGGTCCCCCTC-3'	L80040
46	5'-GGCTGCAGG(C)TTCAGTGGCAGTGGA(G)TCT(A) GGA(G)AC-3'	Cell 58, 1001-1007
224	5'- AGTGCCACTAACTGCTGAGCCACCT-3'	L80040
25	5'-GCTATGTACTACCCGGAATTTCGTG-3'	(linker-top)
116	5'-CACGAATTCCC-3	(linker-bottom)

Table 2

Targeted genes with primer pairs used for semi-nested PCR or semi-quantitative PCR

Zebrafish				
Targeted genes	1 st PCR primers	primers	2 st PCR	sizes
J _H -SE	393/25	<u>409/25</u>		263bps
J _H -CE	<u>290/25</u>	-		
DJ _H -CJ	288/290	<u>295/290</u>		334bps
VDJ _H -CJ	<u>396/392</u>	-		~350bps
DJ _H -SJ	<u>409/262</u>	409/261		473bps
G _H	<u>279/392</u>	-		
J _L -SE	410/25	<u>406/25</u>		280bps
J _L -CE	<u>273/25</u>	-		
VJ _L -SJ	410/267	<u>406/267</u>		464bps
VJ _L -CJ	<u>404/274</u>	-		
G _L	<u>404/274</u>	-		
Mouse				
Targeted genes	1 st PCR		2 st PCR	
J _λ -SE	36/25		23/25	
VJ _λ -SJ	36/24		23/24	
J _κ -SE	224/25		-	
J _κ -CE	40/25		-	
VJ _κ -CJ	46/40		-	

Underlined primer pairs were used for Southern blot analyses

Table 3
Structure of individual signal ends made at JH and JL loci

Heavy Chain (J_H-SEs)

JH-2

AAAAAGCCGAGGTAGGCTATAGCTATTACTGTGGTTATGGTATGGCTCTTATCTTAAACCTAACC**ACTGTG**

AAAAAGCCGAGGTAGGCTATAGCTATTACTGTGGTTATGGTATGGCTCTTATCTTAAACCTAACCAC -4bps
 AAAAAGCCGAGGTAGGCTATAGCTATTACTGTGGTTATGGTATGGCTCTTATCTTAAACCTAACC -6bps
 AAAAAGCCGAGGTAGGCTATAGCTATTACTGTGGTTATGGTATGGCTCTTATCTTAAACCTAAC -7bps
 AAAAAGCCGAGGTAGGCTATAGCTATTACTGTGGTTATGGTATGGCTCTTATCTTAA -14bps
 AAAAAGCCGAGGTAGGCTATAGCTATTACTGTGGTTATGGTATG -22bps
 AAAAAGCCGAGGTAGGCTATAGCTATTACTGTGGTTATG -27bps
 AAAAAGCCGAGGT -58bps
 AAAAAGCC -63bps
 -87bps
 -88bps
 -179bps

JH-3

CGTGTTTGCCAATATTAAGGCAAAATGGTTGTGTTATTGTGCACTGAATTATAAAAAAGGTTT**ACTGTG**

-224bps
 -251bps
 -239bps

Light Chain (J_L-SEs)

ACTTCTGTTTGTGTCTCTTCACAGACTGAGGAGGTTTTTGTACGACTGAACATATCAAATCTTT**ACTGTG**

ACTTCTGTTTGTGTCTCTTCACAGACTGAGGAGGTTTTTGTACGACTGAACATATCAAATCTTTACTGTG
 ACTTCTGTTTGTGTCTCTTCACAGACTGAGGAGGTTTTTGTACGACTGAACATATCAAATCTTTACTGT-1bp
 ACTTCTGTTTGTGTCTCTTCACAGACTGAGGAGGTTTTTGTACGACTGAACATATCAAATCTTTACTG 2bps
 ACTTCTGTTTGTGTCTCTTCACAGACTGAGGAGGTTTTTGTACGACTGAACATATCAAATCTTTCA -5bps
 -79bps
 -56bps
 -215bps
 -216bps

Signal end sequences are aligned to the germline J gene segments (underlined). The extent of nucleotide loss is indicated by the – number. The heptamer of recombination signal sequence is highlighted as bold.

Table 4

Sequence analyses of rearranged VDJH products

	VH4	N/P	D	N/P	J μ 4
<i>Germ</i>	GTATTACTGCGCAAGACAAAT		ATAACAACCTAC (D μ 1)		ACAATGCTTTTGACTAC
<i>NP</i>	GTATTACTGCGCA	Aat	ATAACAACCTA		GCTTTTGACTAC
<i>P</i>	GTATTACTGCGCA	AGA	ATAACAA	Tt	ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGA	GAC	AACAACCT		ATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGAC	TGGG	CAACT		ATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGA	G	ACAACCTAC	T	ATGCTTTTGACTAC
<i>NP</i>	GTATTACTGTGCAAGAGAAAT		AACAACCTAC	g	ATGCTTTTGACTAC
<i>P</i>	GTATTACTGCGCAAGACAAAT		CAGTATAACAGC (D μ 2)		ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGA	G-g	CAG		TGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGA		GTA	G	ACAATGCTTTTGACTAC
<i>NP</i>	GTATTACTGTGC		TATA		ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTATGCAAGA	GG	GTATAAC	GG	TGCTTTTGACTAC
<i>NP</i>	GTATTACTGTGCAAGAGAAAT		AGTAT		ACAATGCTTTTGACTAC
<i>NP</i>	GTATTACTGTGCAAGAGAAAT	AGCGAC	CAGTATA		ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCA		AGTATA	T	ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGCGCAAGACAAAT		CATGGAGTGGC (D μ 3)		ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAG	GCCGCG	TGGAGTGG	ACAGG	GCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGA	GA	ATGGAGTGGC	gcG	GCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGAC		GGAG	GG	ACAATGCTTTTGACTAC
<i>NP</i>	GTATTACTGTGCAAGA		TGGAGTGGC	gG	GCTTTTGACTAC
<i>P</i>	GTATTACTGTGCA	AAAA	ATGGAGTGGC	g	ATGCTTTTGACTAC
<i>P</i>	GTATTACTGCGCAAGACA	CCAT	CATG		GCTTTTGACTAC
<i>P</i>	GTATTACTGCGCAAGA		GAGT	CCT	ATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGACAAAT		GGAGTGG		CAATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGA	GA	ATGGAGTGGC	C	ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGA		NGGAGT	C	ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGA		TATAGCGGGG (D μ 4)		ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGCGCAAGACAAAT	C	TAGCGGGG	GT	ACAATGCTTTTGACTAC
<i>NP</i>	GTATTACTGTGCAAGA	A	ATAGCGGGG	TCTA	ATGCTTTTGACTAC
<i>NP</i>	GTATTACTGCGCA		TATAGCGGG		AATGCTTTTGACTAC
<i>NP</i>	GTATTACTGTGCAAGA		AGCGGGG	cGG	ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGAGAAAT		TAGCGGGG		GCTTTTGACTAC
<i>P</i>	GTATTACTGCGCAAGACAAAT		ATAAATACTACGAC (D μ 5)		AATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCA	AACAT	TACGAC		ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGTGCAAGAC	TGGGC	AAT	T	ATGCTTTTGACTAC
<i>P</i>	GTATTACTGCGCA	AGC	ATA [#]		ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGCGCAAGACAAAT		CCTGGGATGG (D ζ 1)	CCGGTt	ACAATGCTTTTGACTAC
<i>P</i>	GTATTACTGCGCAAGA	GG	CTGGGATGG		TGCTTTTGACTAC

Rearranged genomic DNA sequence of CDR3 regions are compared to germline V, D, J gene segments (underlined). Junctional nucleotides are indicated in bold as N nucleotides (upper case) or putative P nucleotides (lower case). P and NP denote productive and nonproductive VDJ rearrangements, respectively.

[#]This D-element can also be assigned D μ 1, D μ 2, as well as D μ 4.