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Efficient antibody diversification by gene conversion *in vivo* in the absence of selection for V(D)J-encoded determinants

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Antibody diversification in the bursa of Fabricius occurs by gene conversion: pseudogene-derived sequences replace homologous sequences in rearranged immunoglobulin genes. Bursal cells expressing a truncated immunoglobulin μ heavy chain, introduced by retroviral gene transfer, bypass normal requirements for endogenous surface immunoglobulin expression. Immunoglobulin light chain rearrangements in such cells undergo gene conversion under conditions where the products are not selected based on their ability to encode a functional protein. The efficiency with which gene conversion maintains a productive reading frame exceeds 97% under such non-selective conditions. By analysis of donor pseudogene usage we demonstrate that bursal cell development is not driven by a restricted set of antigenic specificities. We further demonstrate that gene conversion can restore a productive reading frame to out-of-frame VJ_L junctions, providing a rationale for the elimination of cells containing non-productive VJ_L rearrangements prior to the onset of gene conversion in normal bursal cell development.

Keywords: B cell-development/bursa of Fabricius/gene conversion/immunoglobulin light chain/V(D)J recombination

Introduction

The rearrangement of immunoglobulin (Ig) gene segments is required for the production of antibody molecules by B cells (Tonegawa, 1981; Alt *et al.*, 1987). At the heavy chain (H) locus, variable (V_H), diversity (D_H) and joining (J_H) segments undergo rearrangement to form the VDJ_H complex, which encodes the V_H domain of the IgH protein. At the light chain (L) locus, V_L genes undergo rearrangement to J_L segments to form the VJ_L complex, which encodes the V_L domain of the IgL protein. In many species, notably mouse and human, the presence in the genome of multiple functional V, D and J segments ensures that the process of V(D)J recombination itself results in the generation of antibody diversity as a consequence of the random assortment of V, D and J elements in the V(D)J complex.

In addition to the 'multi-V(D)J' model of antibody diversification seen in mouse and human, other mechanisms that generate a primary repertoire of B cell-specificities have been demonstrated. For example in sheep, limited numbers of different VJ_L rearrangements are diversified by a hypermutational process in which multiple point mutations are introduced into the functionally rearranged VJ_L complex (Reynaud *et al.*, 1991a, 1995). It remains unclear whether the somatic hypermutation seen in sheep IgL diversification occurs by the same mechanism as that occurring in germinal center V gene hypermutation during antibody responses (Jacob *et al.*, 1991). Nonetheless, at present, mutations introduced into sheep IgV_L genes appear to be non-templated in that no donor sequences for the mutations have been identified.

In contrast, chickens (Reynaud *et al.*, 1987, 1989; Thompson and Neiman, 1987) and rabbits (Becker and Knight, 1990; Knight, 1992) generate primary antibody repertoires by somatic gene conversion. The most striking example of this mechanism occurs in the chicken. The chicken IgL locus contains a single functional V_L gene (V_L1), which undergoes rearrangement in all B cells to a unique J_L sequence. Upstream of the VJ_L complex is a family of ~25 pseudo-V region genes (ΨV_L) that act as donors for the replacement of homologous sequence within the rearranged VJ_L complex (Reynaud *et al.*, 1987). In this process, the major role of V(D)J recombination appears not to be the generation of diversity but rather the formation of a rearranged complex that can function as a 'cassette' into which donor pseudogene derived sequences can be introduced. The molecular mechanism of gene conversion is unclear, although there is strong evidence that it is unidirectional because the donor ΨV_L sequence remains unchanged in the genome (Carlson *et al.*, 1990).

The bursa of Fabricius plays a central role in avian B cell-development; deletion of the bursa profoundly inhibits normal B cell-development (Jalkanen *et al.*, 1984; Weill and Reynaud, 1987; Ratcliffe and Paramithiotis, 1990). During embryonic development, B cell-precursors that express a functional sIg complex as a consequence of productive V(D)J rearrangement undergo clonal expansion and V gene diversification by gene conversion within the bursa (McCormack *et al.*, 1989a). Cells that contain non-productive V_H or V_L rearrangements are eliminated prior to the onset of gene conversion (McCormack *et al.*, 1989a; Reynaud *et al.*, 1991b). It has been assumed that this elimination is to prevent gene conversion restoring a productive reading frame to out-of-frame VJ_L junctions in cells that also contain an in-frame VJ_L junction, thereby breaking allelic exclusion (Reynaud *et al.*, 1989; Langman and Cohn, 1993). However, experimental evidence in support of this assumption is lacking.

In addition to its role in supporting gene conversion, the bursa is a site of extensive B cell death (Motyka and Reynolds, 1991; Paramithiotis *et al.*, 1995). We have

shown previously that those B cells that lose the expression of sIg are rapidly eliminated by apoptosis (Paramithiotis *et al.*, 1995). Non-productive gene conversion events, resulting in frame shifts within the VJ_L gene, have been observed in a cell line that undergoes gene conversion *in vitro* (Buerstedde *et al.*, 1990). *In vivo*, however, non-productive gene conversion events that disrupt the expression of bursal sIg would be rapidly lost from the B cell-pool within the bursa. Therefore it is currently unclear whether non-productive gene conversion events occur with a significant frequency under normal circumstances *in vivo*.

To address this issue we have developed a model in which a truncated surface μ receptor (Tμ) lacking the V_H and Cμ1 domains is expressed following retroviral gene transfer *in vivo* (Sayegh *et al.*, 1999). The Tμ molecule is expressed on the surface of B cell precursors in the absence of IgL expression and is sufficient to promote clonal expansion of B cells within the bursa independent of endogenous Ig gene expression. A population of bursal cells (10–20%) expressing Tμ contain VJ_L rearrangements, providing a unique opportunity to assess VJ_L gene rearrangement and gene conversion *in vivo* in the absence of any selective pressures that might be imposed within the bursal microenvironment on the resulting protein products. The results presented here demonstrate that the efficiency with which gene conversion maintains the correct reading frame exceeds 97%. The relative usage of different donor pseudo-V_L sequences demonstrates that B cell-development within the bursa is not driven by a restricted set of B cell specificities. We further demonstrate that gene conversion can restore the reading frame of non-productively rearranged VJ_L junctions.

Results

VJ_L gene conversion in the absence of selection for endogenous sIgM expression

RCAS-Tμ is a productive avian retrovirus that contains the sequence encoding a truncated sIgμ heavy chain protein in which the VDJ_H and Cμ1 domains have been deleted, allowing Tμ expression in the absence of light chain expression. Transcripts encoding Tμ are initiated in the 5′ long terminal repeat of the RCAS virus and Tμ mRNA is produced by splicing between a splice donor at the 5′ end of the *gag* gene and a splice acceptor 5′ to the Tμ sequence. Transcription termination and polyadenylation signals are provided by the 3′ long terminal repeat of the RCAS virus.

Bursal cells isolated from chicks that had been inoculated as day 3 embryos with RCAS-Tμ infected fibroblasts were stained with anti-μ and anti-L antibodies (Figure 1A). In addition to B cells expressing endogenously encoded sIgM, detected as cells co-expressing μ and L, we detect a population of μ^+L^- cells. We have demonstrated previously that this population represents B lineage cells that have productively colonized the bursa and undergone clonal expansion in bursal follicles as a consequence of expressing the Tμ protein on their surface in the absence of endogenous sIgM expression (Sayegh *et al.*, 1999). A population of these cells (10–20%) contains endogenous VDJ_H and VJ_L rearrangements and based on an analysis of complementarity determining region 3 (CDR3) length,

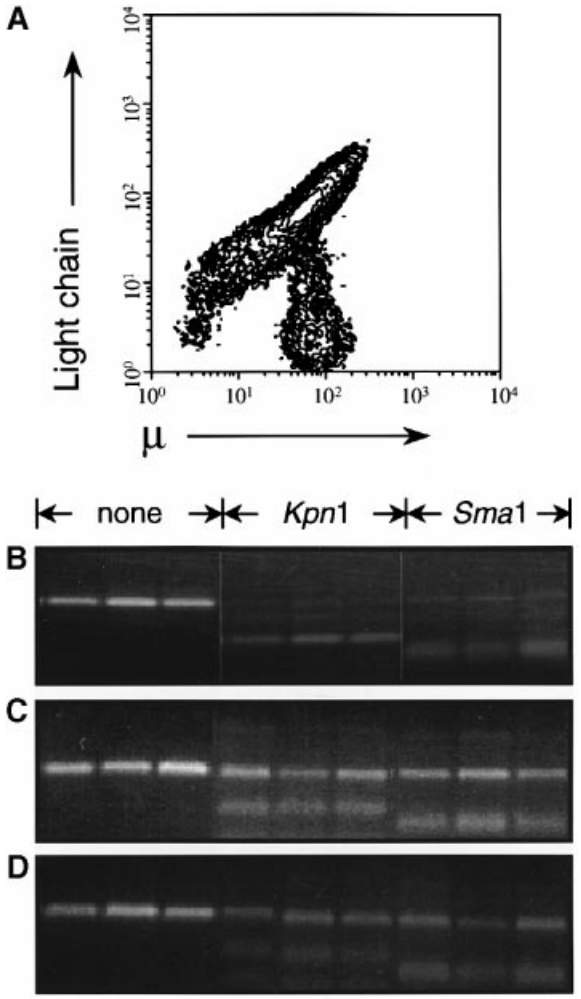

Fig. 1. Rearranged VJ_L genes from cells expressing Tμ have lost restriction enzyme sites. (**A**) Contour plot of 100 000 bursal cells from a neonatal chick, infected as a day 3 embryo with RCAS-Tμ, stained for μ and light chain expression. (**B**) Germline V_L1 sequences were PCR-amplified from FACS sorted neonatal μ^+L^- bursal cells of three individual chicks infected as day 3 embryos with RCAS-Tμ. VJ_L rearrangements were also amplified from μ^+L^+ (**C**) or μ^+L^- (**D**) bursal cells of the same chicks. Amplified sequences were undigested (none) or digested with *Kpn*I or *Sma*I prior to electrophoresis.

VJ_L rearrangements in neonatal μ^+L^- cells have not been selected for in-frame VJ_L junctions (Sayegh *et al.*, 1999).

Cleavage sites for the restriction endonucleases *Sma*I, *Sca*I and *Kpn*I are present in the unmodified V_L1 gene segment (Reynaud *et al.*, 1987; Thompson and Neiman, 1987), either before (Figure 1B) or after VJ_L rearrangement. Sequence modification at these sites by gene conversion typically results in a loss of these restriction enzyme recognition sequences and consequently resistance of the rearranged VJ_L segments to cleavage by these enzymes. PCR-amplified rearranged VJ_L fragments from both μ^+L^+ (Figure 1C) and μ^+L^- (Figure 1D) populations of neonatal RCAS-Tμ infected chicks showed comparable resistance to cleavage with *Kpn*I and *Sma*I. Equivalent results were obtained with *Sca*I (data not shown). To obviate the possibility that resistance to cleavage was simply due to limiting restriction enzyme, the plasmid pUC18 was added to all digests and was fully linearized in the reaction (data not shown). Thus V_L gene diversification occurs among

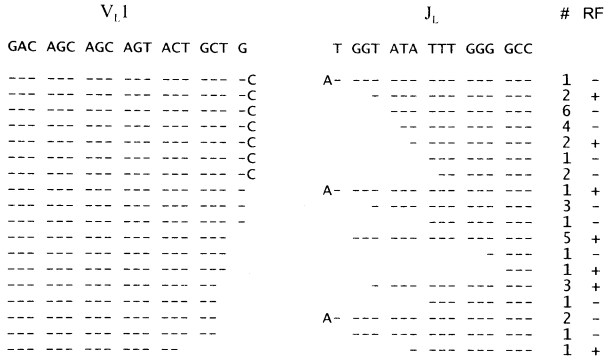

Fig. 2. VJ_L junctions from μ^+L^- bursal cells. VJ_L genes were PCR-amplified from FACS sorted μ^+L^- bursal cells. Shown are VJ_L junctions from those sequences which had not been modified at the VJ_L junction by gene conversion. '#': the number of occurrences of each junction in independent sequences. RF: '+' and '–' reading frames correspond to in-frame and out-of-frame rearrangements respectively.

μ^+L^- cells at levels indistinguishable from those seen in μ^+L^+ bursal cells expressing endogenous sIg receptors.

We therefore cloned and sequenced representative examples of diversified VJ_L sequences from μ^+L^- cells. Eighty independent sequences were isolated from FACS-sorted μ^+L^- bursal cells from four RCAS-Tμ infected SC line chicks at day E21, the day of hatch or 1 day after hatch. The SC line was selected for analysis because polymorphisms in the V_L1 gene as well as the donor ΨV_L sequences (McCormack *et al.*, 1993 and W.T.McCormack, personal communication) facilitated assignment of V_L allele and donor pseudogene usage. Each of the 80 sequences reported here represent independent VJ_L sequences based on differences in the VJ_L junction and/or the VJ_L sequence itself.

Among these sequences, 38 did not contain sequence modification between codon 87 and the VJ_L junction and we considered that these represented sequences where the VJ_L junction sequence had not been modified by gene conversion. Out of 38 such sequences, 23 (60%) contained an out-of-frame VJ_L junction (Figure 2). The non-random nucleotide additions (C and/or A) in some VJ_L junctions are consistent with the presence of palindromic (P) nucleotides generated as a consequence of the mechanism of V(D)J recombination together with an absence of non-templated (N) nucleotide additions introduced by TdT (McCormack *et al.*, 1989b; Roth *et al.*, 1992). The junctional diversity, proportion of out-of-frame junctions and presence of P nucleotides is consistent with VJ_L junctions observed elsewhere prior to selection for the formation of a productive protein product (McCormack *et al.*, 1989a; Reynaud *et al.*, 1991b). Thus, VJ_L sequences isolated from μ^+L^- bursal cells have not undergone selection based on their ability to form a productive protein product.

All 80 VJ_L sequences isolated contained at least one gene conversion event, consistent with the results from Figure 1. Examples selected from among the 38 VJ_L sequences that have not undergone sequence modification at the VJ_L junction are shown in Figure 3. Since these sequences come from μ^+L^- cells that do not express light chains as part of the endogenous sIgM receptor, the gene conversion events contained within these sequences cannot have been selected based on their ability to form part of a productive sIgM complex.

The efficiency of gene conversion in the absence of B cell-selection

We have identified a total of 269 gene conversion events in this population of 80 VJ_L sequences which result in 1317 nucleotide changes; individual VJ_L sequences containing between one and seven gene conversion events. This represents a minimum estimate of the absolute number of gene conversion events in this population. Due to homologies between the different pseudogenes, sequence modifications that can be accounted for by a single gene conversion event may have occurred as a consequence of two gene conversion events involving different donor sequences. For example, codons 22–30 of clone 6.29 (Figure 3) can be uniquely ascribed to ΨV5, whereas codons 31–38 have several potential donors (ΨV4, 10, 14, 16, 18 or 23) in addition to ΨV5. In this instance we have ascribed the sequence modification between codons 22 and 38 to a single gene conversion event utilizing ΨV5 as donor sequence. Gene conversion events can overlap and be overwritten by subsequent gene conversion events (Kim *et al.*, 1990). For example, we have ascribed codons 15–60 of clone 6.78 (Figure 3) to overlapping gene conversion events involving ΨV12 and ΨV10, and any prior gene conversion events that might have modified this region would not be detected. In addition, given the extensive homology between the ΨV_L genes and the V_L1 sequence, gene conversion events may not always result in V_L1 sequence modification and would not be detected.

The minimal frequency of detectable gene conversion events among the 80 VJ_L sequences, 3.4 ± 1.7 SD gene conversion events per sequence, is consistent with data reported elsewhere for sequences derived from neonatal bursal cells of normal chicks (Reynaud *et al.*, 1987; McCormack and Thompson, 1990). Thus normal levels of gene conversion have occurred in the rearranged VJ_L genes from μ^+L^- cells.

Gene conversion events can result in templated nucleotide insertions. For example ΨV14 contains a CAG insertion between codons 4 and 5, which appears in several VJ_L sequences (e.g. clone 6.29, Figure 3). Similarly, several ΨV_L genes contain insertions in CDR1, which spans codons 21–28. These insertions are also observed in the VJ_L sequences from μ^+L^- cells as exemplified by the ΨV5 derived sequence in clone 6.29 and ΨV12 derived sequence in clone 6.78 (Figure 3).

Apart from such insertions, which can be accounted for by gene conversion events bounded by regions of homology at both the 5′ and 3′ flanks, we have found examples of insertions that typically occur at the borders of gene conversion events, most easily interpreted as a result of misalignment of the 3′ border between the ΨV_L donor sequence and the V_L1 target. In clone 6.115 the 3′ end of codon 91 of ΨV7 is contiguous with the 5′ end of V_L1 codon 90, resulting in a two codon insertion in CDR3 (Figure 4A). In total, from our pool of 269 gene conversion events, 14 contain flanking insertions.

We have also observed nucleotide deletions that cannot be accounted for by corresponding deletions contained within donor ΨV_L sequences. As with the nucleotide

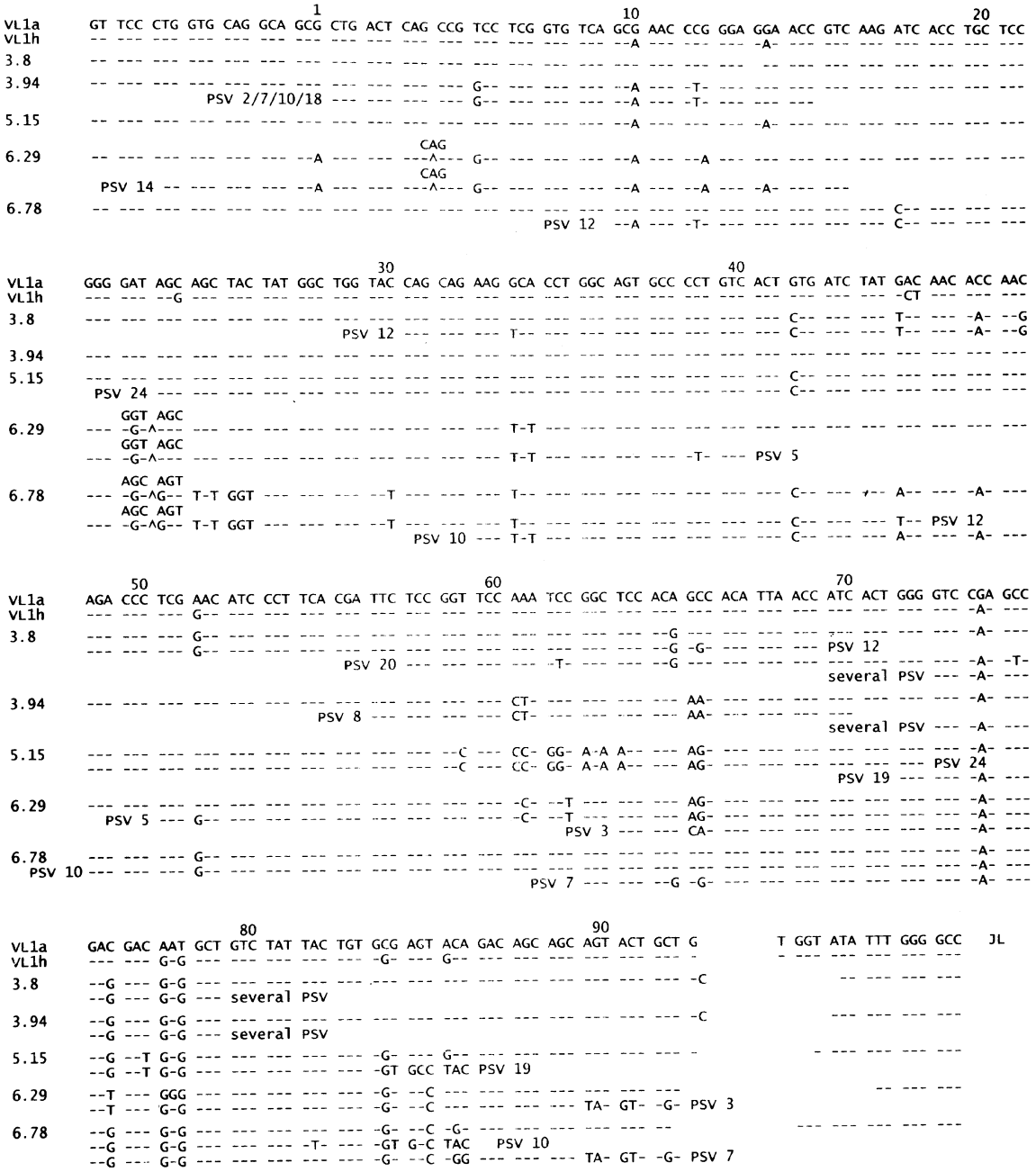

Fig. 3. Representative VJ_L sequences from μ^+L^- bursal cells. Shown are five examples (clones 3.8, 3.94, 5.15, 6.29 and 6.78) of VJ_L sequences PCR-amplified from FACS sorted μ^+L^- bursal cells. Each of these clones has undergone out-of-frame VJ_L rearrangement and gene conversion. Dashes indicate identity with the V_L1a allele sequence. SC line chicks are heterozygous at the IgL locus, V_L1a/V_L1h, (Benatar and Ratcliffe, 1993) and the sequence of the V_L1h allele is shown. The sequence and identity of pseudo-V_L (PSV) donors for gene conversion events are indicated below the cloned VJ_L sequence. Donor sequences designated 'several PSV' occur frequently in the pseudo-V_L gene family. '^' indicates nucleotide insertions.

insertions described above, these frequently occur at the border of gene conversion events. Clone 3.54 (Figure 4B) contains a deletion of codon 25 in a region of CDR1 that has been subject to two gene conversion events that utilize ΨV5 and ΨV10 as sequence donors. Not all deletions, however, occur in regions that have undergone unequivocal gene conversion events. Clones 7.18 and 1.81 (Figure 4C) are examples in CDR3 of such deletions that may have occurred in the absence of gene conversion. Alternatively, it is possible that codons 84–88 may be derived from ΨV6 or ΨV11, which have the same sequence in this region as the unmodified V_L1h allele used by clones 7.18 and 1.81. We have observed a total of 11 examples of nucleotide deletions in 80 VJ_L sequences.

A total of 69 nucleotide changes in the 80 VJ_L sequences could not be accounted for be gene conversion events. For example, codon 78 (GGG) of clone 6.29 (Figure 3) has no potential donor among the pseudogene family and is likely to represent a GAG→GGG mutation. While many of these mutations occur at or close to the borders of

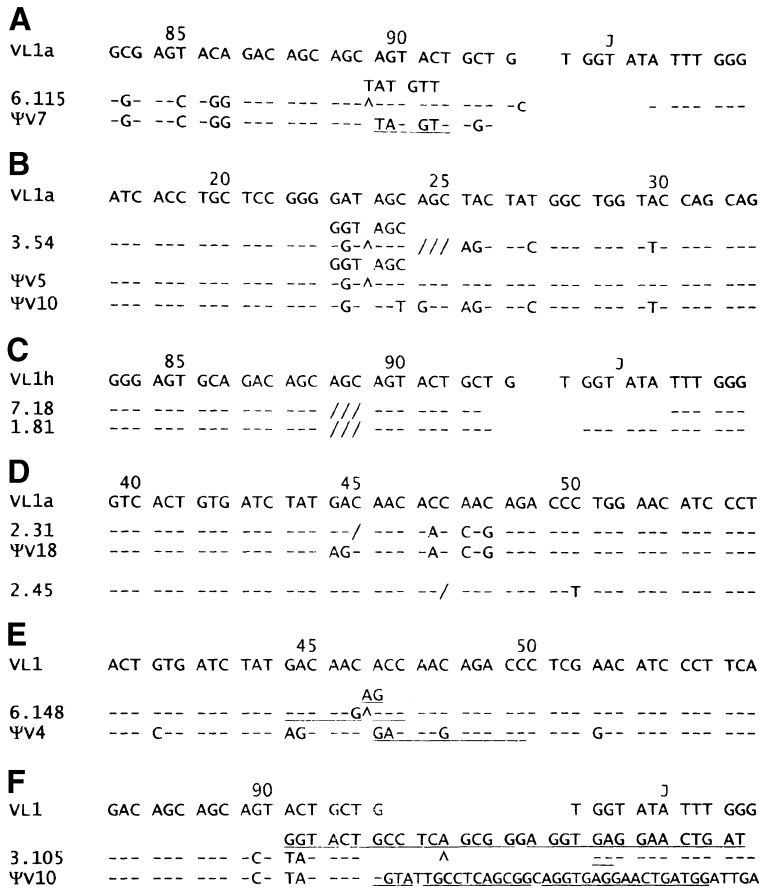

Fig. 4. Examples of atypical gene conversion events from μ^+L^- bursal cells. Shown are partial sequences of eight VJ_L PCR-amplified clones from FACS sorted μ^+L^- bursal cells. Dashes indicate identity with the V_L1a or V_L1h allele used by that clone. '^' indicates nucleotide insertions, '/' indicates nucleotide deletions. (**A**) The sequence underlined is identical to the insert in clone 6.115. (**B**) Clone 3.54 contains a three nucleotide deletion at codon 25. (**C**) Clones 7.18 and 1.81 each contain a deletion of codon 88 or 89 (both AGC) but different VJ_L junctions. (**D**) Clones 2.31 and 2.45 each contain single nucleotide deletions in CDR2. (**E**) The sequence underlined in ΨV4 is identical to the sequence underlined in clone 6.148. (**F**) The sequence underlined in ΨV10 is identical to the sequence underlined in clone 3.105.

gene conversion events, as has been discussed elsewhere (Buerstedde *et al.*, 1990; Kim *et al.*, 1990; McCormack and Thompson, 1990), this was not always the case.

Among 25 insertions or deletions we have only identified four examples that disrupt the reading frame of the resulting VJ_L sequence. Three such instances occur in CDR2, of which two are single nucleotide deletions (Figure 4D). This is a region in which both the V_L1 sequence as well as many members of the ΨV_L family contain a series of codons that include the AC dinucleotide in all three reading frames and may therefore increase the likelihood of out-of-frame imprecision in the resolution of gene conversion events.

The 5′ border of gene conversion events invariably maps to a precise positional homology between donor and recipient sequences. Clone 6.148 represents an exception to this rule (Figure 4E). Within the donor ΨV_L family there are no examples of codons 44–46 having the sequence TATGACAAG, the codons immediately 5′ to the AG insertion in clone 6.148. However this AG insertion can be accounted for by a gene conversion event in which the 5′ border of the ΨV4 donor sequence is displaced by two codons with respect to the V_L1 sequence. This displacement results in sequence homology at the 5′ border of the gene conversion event. Overall with respect to 238 gene conversion events that do not impinge upon the VJ_L junction, 235 (>98%) retain a productive VJ_L reading frame.

Clone 3.105 contains an insert of 32 nucleotides at the VJ_L junction (Figure 4F). This clone contains a single large gene conversion event, which utilizes ΨV10 with the 5′ border being 5′ to CDR1. The insert at the VJ_L junction represents the sequence immediately 3′ to the V_L1 homologous sequence of ΨV10. This demonstrates that sequences from the ΨV_L locus outside the V_L1 homologous sequence can be involved in gene conversion.

ΨV_L gene usage in the absence of bursal cell selection for V(D)J encoded determinants

The gene conversion events identified here have occurred in the absence of selection for a functional product and therefore cannot be biased by possible selection for or against particular antigen binding specificities in the bursa. From 269 gene conversion events, we have identified 204 gene conversions that have one or two possible ΨV_L donors. The remaining gene conversion events have three or more potential donors. Figure 5 shows the frequency with which the various ΨV_L genes are used as sequence donors; for gene conversion events with two potential ΨV_L donors each donor was assigned 0.5 event.

An analysis of ΨV_L usage based on ~160 gene conversion events derived from normal bursal cells, i.e. cells

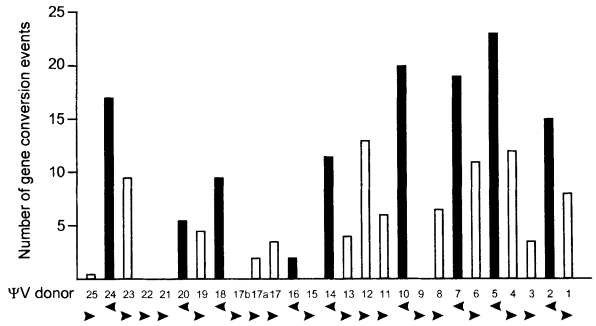

Fig. 5. ΨV_L gene usage in the absence of bursal cell selection. Frequency of ΨV_L gene usage among 204 gene conversion events from VJ_L PCR-amplified clones from FACS sorted μ^+L^- bursal cells. Included are those gene conversion events that can be ascribed to one or two donor ΨV_L genes. For gene conversion events that can be ascribed to two possible donor genes, each donor gene was assigned 0.5 event. Numbering of the ΨV_L family is according to Reynaud *et al.* (1987), modified in the case of ΨV17 cluster by McCormack *et al.* (1993). Arrowheads represent the polarity of the ΨV_L gene according to Reynaud *et al.* (1987) with the V_L1 gene proximal to ΨV1. Filled bars are used for ΨV_L genes of opposing polarity to V_L1, open bars are used for ΨV_L genes with the same polarity as V_L1.

that have been subject to selection based on expression of endogenous sIgM in the bursa, demonstrated that ΨV_L gene usage was influenced by ΨV_L proximity to the V_L1 gene, ΨV_L polarity and ΨV_L homology to the target sequence (McCormack and Thompson, 1990). Our results support this conclusion. ΨV_L donors from the 3′ (V_L1 proximal) region of the ΨV_L locus are used more frequently than those from the 5′ region of the locus. ΨV_L donors that have an inverted polarity with respect to the V_L1 gene (Figure 5, solid bars) are used more frequently than adjacent ΨV_L donors having the same polarity as V_L1 (open bars). Many ΨV_L that are used rarely have extensive 5′ (ΨV3, 22, 25) and/or 3′ (ΨV15, 16, 21, 22, 25) truncations. Critically, however, our analysis is based on sequences that have not been selected either for their ability to form a functional protein product on the bursal cell surface or by any antigen-based selection in the bursa. The striking similarity between the ΨV_L gene usage presented here (Figure 5) and that reported elsewhere (McCormack and Thompson, 1990) provides strong support for the contention that B cell-expansion in the embryo bursa is not driven by a restricted set of antigens.

Gene conversion can modify the reading frame at the VJ_L junction

Unlike mammalian B cells, in which the non-expressed Ig light chain allele is frequently non-productively rearranged, chicken B cells typically retain the non-expressed light chain allele in germline configuration (Reynaud *et al.*, 1985; Weill *et al.*, 1986; Thompson and Neiman, 1987). It has been assumed that this is required to prevent the possibility of gene conversion correcting the reading frame of non-productive VJ_L junctions, resulting in B cells containing two functional VJ_L sequences (Reynaud *et al.*, 1989; Langman and Cohn, 1993). However, in normal bursal cells, elimination of cells containing non-productive VJ_L rearrangements precedes the onset of gene conversion (McCormack *et al.*, 1989a; Reynaud *et al.*, 1991b) and consequently it has not been possible to determine whether gene conversion can indeed restore a productive reading frame to out-of-frame VJ_L junctions.

The sequences reported here have not been selected for productivity. Thirty-seven percent of VJ_L junctions in sequences that have not undergone gene conversion in this region are in-frame (Figure 6A). In contrast, sequences that have undergone gene conversion at the VJ_L junction show a substantially increased proportion, 74%, of in-frame junctions (Figure 6B). This increase cannot be accounted for by gene conversion selectively modifying VJ_L junctions that have undergone productive rearrangement. This would result in selective depletion of productive rearrangements from the pool of VJ_L rearrangements that have not undergone gene conversion at the VJ_L junction, a depletion that is not consistent with the low frequency of productive rearrangements seen in Figure 6A.

Among the VJ_L junctions which have undergone gene conversion, six involve ΨV10. Four of these have generated a VJ_L junction that is in-frame and has maximized the 3′ homology between the VJ_L sequence and the ΨV10 3′ flanking sequence (Figure 6C). Conversely, the two events that have left an out-of-frame VJ_L junction are likely to have 3′ borders within the V_L sequence itself and have therefore probably not modified the pre-existing VJ_L junction. Similarly, among five gene conversion events that use the closely related ΨV3, ΨV7 or ΨV19 donors, four are in-frame and generate VJ_L junctions with the GGTATx nucleotides contained in the ΨV_L sequence (Figure 6D).

Clone 1.45 contains a VJ_L junction that has no corresponding donor sequence in the ΨV_L family without invoking a minimum of three point mutations. However, the AGTGATGTGGGG sequence, which spans the VJ_L junction of this clone, is found within the 3′ flanking sequence of ΨV20 (Figure 6E). This suggests the possibility that sequences flanking the V_L1 homologous region of the ΨV_L genes can be used as sequence donors in gene conversion events, albeit with low efficiency.

Discussion

The VJ_L sequences reported here are derived from neonatal bursal cells that express a truncated μ protein on the cell surface introduced by retroviral gene transfer into the developing embryo. We have demonstrated that the viability of these cells *in vivo* is maintained by the presence of the Tμ protein and not by endogenous Ig gene expression. Fewer than 20% of $T\mu^+$ cells contain either VDJ_H or VJ_L rearrangements as judged by semi-quantitative PCR (Sayegh *et al.*, 1999). In addition, an analysis of gene rearrangement in v-*rel* transformed clones of $T\mu^+$ cells from RCAS-Tμ infected chicks demonstrated that one out of 16 clones contained a VDJ_H rearrangement and four out of 16 clones contained a VJ_L rearrangement (Sayegh *et al.*, 1998). The presence of VJ_L rearrangements in cells that do not contain VDJ_H rearrangements is consistent with the stochastic rearrangement of chicken Ig V genes demonstrated elsewhere (Benatar *et al.*, 1992). Thus the majority of $T\mu^+$ (μ^+L^-) cells do not contain either VDJ_H or VJ_L rearrangements.

Mammalian B cells frequently contain more than one rearranged light chain gene (Yancopoulos and Alt, 1986). Under these circumstances, one allele is rearranged productively leading to sIg expression, the other allele is typically rearranged non-productively. In chickens, how-

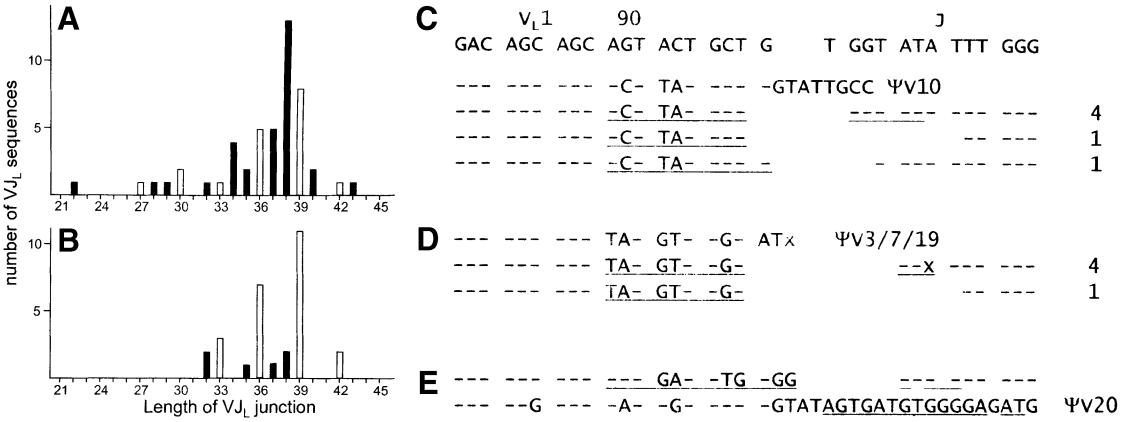

Fig. 6. Increased frequency of in-frame VJ_L junctions following gene conversion. VJ_L junction length from 49 clones which had not undergone gene conversion at the VJ_L junction (**A**) or 29 clones which contain gene conversion events at or after codon 87 (**B**). VJ_L junction length is from codon 84 of the V_L1 sequence (see Figure 3) to the GGG codon of J_L inclusive. Filled bars correspond to out-of-frame VJ_L junctions, open bars correspond to in-frame VJ_L junctions. (**C**) Examples of six clones that use ΨV10 sequence at the VJ_L junction, numbers to the right of the panel refer to the number of independent clones containing this sequence. The sequence underlined is identical to the ΨV10 sequence. (**D**) Examples of five clones that use ΨV3, 7 or 19 as sequence donors at the VJ_L junction, numbers to the right of the panel refer to the number of independent clones containing this sequence. In this case the nucleotide designated 'x' differs between the three ΨV_L genes and the corresponding nucleotide is found in the J_L homologous region of the VJ_L sequence. The sequence underlined is identical to the ΨV_L donor sequence. (**E**) Example of a gene conversion event at the VJ_L junction of clone 1.45 where maximal homology aligns with sequences proximal to the V_L1 homologous region of ΨV20 (underlined sequences).

ever, the vast majority of B cells (>95%) contain one productively rearranged light chain, the other light chain allele remaining in germline configuration (Reynaud *et al.*, 1985; Weill *et al.*, 1986; Thompson and Neiman, 1987). Since sIg expression is required to maintain the viability of B cells in the bursa, essentially all rearranged VJ_L loci in the normal chicken are subject to the constraints imposed by the requirement for functional protein expression. The sequences described here represent the first analysis of diversified chicken VJ_L sequences obtained under conditions where such constraints have been bypassed. The demonstration that a high proportion of these sequences retain an out-of-frame VJ_L junction (Figure 2), at a time when normal B cells containing out-of-frame junctions have been largely eliminated (McCormack *et al.*, 1989a; Reynaud *et al.*, 1991b) supports this conclusion.

The frequency of gene conversion events in this population of VJ_L sequences, 3.4 ± 1.7 SD, is entirely consistent with the levels of gene conversion observed elsewhere for VJ_L sequences from bursal cells around the time of hatching (Reynaud *et al.*, 1987; McCormack and Thompson, 1990). Therefore, the molecular processes involved in gene conversion have been induced to normal levels in μ^+L^- cells developing in the bursa and the expression of the Tμ protein has not disrupted the gene conversion events that diversify endogenous VJ_L rearranged loci.

Prior to the onset of gene conversion, the diversity of bursal sIg receptors is extremely limited. It has been proposed that ligand(s) recognized by pre-diversified bursal sIg may be present within the bursal stroma and that recognition of these ligand(s) by pre-diversified bursal sIg may drive the induction of B cell-development and the initiation of gene conversion (Reynaud *et al.*, 1989, 1991b; Mansikka *et al.*, 1990; McCormack *et al.*, 1991; Salmonsen *et al.*, 1991; Langman and Cohn, 1993; Pandey *et al.*, 1993). Since expression of sIg, either endogenous or Tμ is required to maintain bursal cell viability (Paramithiotis *et al.*, 1995; Sayegh *et al.*, 1999) it is possible that some component of the bursal sIg receptor complex is recognized by a ligand on the bursal stroma. However, the $T\mu^+$ cells reported here lack V(D)J encoded determinants on their surface. Nonetheless, these cells contain VJ_L loci that have undergone normal levels of diversification by gene conversion. Therefore, there is no requirement for interactions between the variable region domains of the pre-diversified bursal sIgM and ligand(s) on the bursal stroma to induce V gene diversification by gene conversion.

Non-productive gene conversion events, leading to out-of-frame VJ_L products have been observed in the DT40 bursal cell lymphoma (Buerstedde *et al.*, 1990), which continues to undergo gene conversion *in vitro*. However, it is currently unclear whether the rate and efficiency of gene conversion of VJ_L genes in DT40 is equivalent to that seen among bursal cells *in vivo*. Non-productive gene conversion events have also been isolated from normal bursal cells (Reynaud *et al.*, 1987; McCormack and Thompson, 1990). However, since cells that lack sIg are deleted within the bursa (Paramithiotis *et al.*, 1995) it is not been possible to estimate the efficiency with which gene conversion maintains a productive protein product from these results. We demonstrate here that, under conditions where selection is not imposed on the products of gene conversion, the efficiency with which gene conversion maintains a productive reading frame *in vivo* exceeds 97%. We have only observed four instances among 269 gene conversion events where the gene conversion event itself has disrupted the reading frame of the VJ_L sequence (Figure 4D–F).

We have observed 25 examples of nucleotide insertions or deletions among our sample of 80 VJ_L sequences from $T\mu^+$ cells. As noted elsewhere (McCormack and Thompson, 1990), most of these occur at the 3′ border of gene conversion events. Remarkably, since these sequences have not been selected for their ability to form a functional protein product the great majority involve insertions or deletions of one or more codons and do not disrupt the reading frame of the resulting VJ_L sequence. It has

been suggested that such insertions or deletions are a consequence of misalignment at the 3′ border during resolution of a gene conversion intermediate. Our results are consistent with this hypothesis since the majority of such insertions and deletions occur at regions of reduced sequence complexity; at codons 88–90 (AGCAGCAGT; Figure 4B) and codons 24–25 (AGCAGC; Figure 4A, C).

We have identified four examples of insertions or deletions that disrupt the VJ_L reading frame. Three of these examples (two single nucleotide deletions and one two nucleotide insertion; Figure 4D, E) involve a sequence in the CDR2 of V_L1 (GACAACACCAACAGACCC) where the AC dinucleotide occurs in all three reading frames. This may increase the possibility of misalignment at the 3′ border of a gene conversion intermediate.

In the vast majority of gene conversion events the 5′ border of the gene conversion event shows identity between the V_L1 sequence and the homologous sequence in the donor ΨV_L gene (positional homology). However, we have found a low frequency of exceptions to this rule. In clone 6.148 (Figure 5E) the 5′ border of the gene conversion event in CDR2 appears displaced by two codons. A second example occurs in clone 1.45 (Figure 6E), where the VJ_L junctional sequence shows homology to the sequence flanking ΨV20. In both examples, the resulting gene conversion events are flanked on both sides by short regions of homology. Thus while precise positional homology at the 5′ border of gene conversion events is the norm, the exceptions demonstrate that sequences other than those at the positionally homologous region of the donor ΨV_L can function, at low efficiency, as sequence donors for gene conversion. The proximity of such sequences to the homologous position in the donor ΨV_L sequence suggests, however, that there is a general alignment of the donor ΨV_L sequence with the target VJ_L gene during gene conversion. We have found no examples where the donor ΨV_L sequence is derived from a widely disparate region of the ΨV_L gene.

Among 80 VJ_L sequences we have identified 69 nucleotides that cannot be accounted for by unequivocal gene conversion events. Such non-templated mutations have been observed elsewhere, both in normal bursal cells and in the DT40 B cell-lymphoma (Reynaud *et al.*, 1985; Buerstedde *et al.*, 1990; Kim *et al.*, 1990; McCormack and Thompson, 1990; Parvari *et al.*, 1990). While some may be a consequence of errors introduced by PCR, mutations have been observed in sequences isolated without PCR (Parvari *et al.*, 1990). Some of these may indeed be a consequence of gene conversion events flanked by very small 5′ and 3′ borders and/or involving donor sequence from non-homologous regions of the donor ΨV_L gene as discussed in the preceding paragraph. However, at this point it seems likely that untemplated point mutations can occur during B cell-development in the bursa. We concur with conclusions elsewhere (McCormack and Thompson, 1990) that while many mutations occur around putative borders of gene conversion events, a significant proportion occur either within a gene converted sequence or within a sequence that appears unmodified by gene conversion. Thus, it remains unclear whether such mutations are a byproduct of gene conversion or whether they occur independently, analogous to the hypermutation observed during mammalian B cell-proliferation in germinal centers (Jacob *et al.*, 1991).

There is extensive cell death within the juvenile bursa (Motyka *et al.*, 1991; Paramithiotis *et al.*, 1995) and <5% of B cells generated within the bursa emigrate to the periphery (Lassila, 1989; Paramithiotis and Ratcliffe, 1993). In addition, bursal cells that lose the expression of sIg die *in situ* (Paramithiotis *et al.*, 1995). The efficiency with which gene conversion maintains a productive product, as demonstrated here, makes it unlikely that more than a small proportion of the cell death observed within the bursa is a consequence of gene conversion events leading to out-of-frame V(D)J products. Currently, however, it remains unclear whether all productive VJ_L products produced by gene conversion have the ability to pair with the diverse array of VDJ_H products that have also diversified by gene conversion. In this regard, the inability of random murine VDJ_H/VJκ combinations to form functional μκ pairs is likely to be low given the low frequency of B cells containing two productive VJκ gene rearrangements (Yancopoulos and Alt, 1986). In addition, it might be expected that any donor ΨV_L sequence that results in an inability to form a functional μL pair, would be selected against during evolution and eliminated from the ΨV_L family.

We have analyzed the usage of different members of the ΨV_L family under circumstances where no selection has been imposed on the products of the gene conversion events (Figure 5). These results complement a previous study in which ΨV_L usage was determined in normal bursal cells (McCormack and Thompson, 1990). In this latter study, bursal cells and the VJ_L sequences they contain would be subject to selective constraints imposed not just by requirements for maintained sIg expression but by any requirement for specific interaction with antigen in the bursa. We show here that the ΨV_L usage identified in $T\mu^+$ cells that do not express endogenous sIg is indistinguishable from that seen in bursal cells that express endogenous sIg receptors. This demonstrates that the diversity of VJ_L sequences potentially expressed as a consequence of gene conversion is reflected among VJ_L sequences from sIg^+ cells. Consequently, during development of the normal bursal cell repertoire there is no apparent restriction of specificities expressed that would be observed as a change in ΨV_L gene usage. This is in contrast to repertoire development in the thymus where the constraints imposed by requirements for positive and negative selection are reflected in differences between the Vβ usage observed as a consequence of V(D)J recombination and the Vβ usage observed among thymocytes subsequent to positive and negative selection (Kappler *et al.*, 1988). This observation also supports the conclusion that there are no members of the ΨV_L family that are frequently used as sequence donors which yield diversified V_L domains that are unable to pair with most diversified V_H domains.

During normal B cell-development in the bursa, cells containing out-of-frame V(D)J rearrangements are eliminated prior to the onset of gene conversion (McCormack *et al.*, 1989a; Reynaud *et al.*, 1991b) and so it has not been possible to determine the efficiency with which such loci can undergo gene conversion. The out-of-frame VJ_L sequences reported here have undergone gene conversion

at levels indistinguishable from the levels observed in productively rearranged VJ_L genes. Thus out-of-frame VJ_L sequences are subject to gene conversion.

The efficiency with which chicken VJ_L rearrangement generates an in-frame VJ_L junction is ~35%, equivalent to the efficiency of mammalian VJ_L recombination. During mammalian B cell-development, a non-productive VJ_L rearrangement can be followed by further light chain rearrangements. Thus many mammalian sIg^+ B cells contain two κ rearrangements, one productive and one out-of-frame (Yancopoulos and Alt, 1986). In contrast, chicken bursal cells contain one productively rearranged light chain allele, the other allele remains in germline configuration. The molecular mechanism by which allelic exclusion of chicken light chain rearrangement occurs remains unclear, although there is evidence supporting a silencer/anti-silencer mechanism (Lauster *et al.*, 1993). It is likely that allelic exclusion operating at the level of gene rearrangement as opposed to protein expression is required to prevent gene conversion restoring a productive reading frame to out-of-frame V(D)J junctions (Reynaud *et al.*, 1989; Langman and Cohn, 1993). We provide here the first direct support for this proposal, demonstrating that indeed gene conversion can restore productivity to VJ_L junctions (Figure 6).

In conclusion, the sequences reported here represent the first examination of gene conversion *in vivo* under circumstances where the products of gene conversion have not been selected based on their ability to form a productive protein product. We show that, under these circumstances, the efficiency with which gene conversion generates a productive product exceeds 97%. The usage of donor ΨV_L genes, in comparison to ΨV_L usage under conditions where bursal cells are selected *in vivo*, supports the conclusion that bursal cell diversification is not driven by a limited set of B cell-specificities. We further demonstrate that gene conversion can restore a productive reading frame to non-productive VJ_L junctions, providing an experimentally determined rationale for allelic exclusion of chicken Ig occurring at the level of V gene rearrangement.

Materials and methods

Generation of RCAS-Tμ expressing chicks

Construction of the RCAS-Tμ vector and production of RCAS-Tμ transfected chick embryo fibroblasts was described previously (Sayegh *et al.*, 1999). In essence, the Tμ sequence comprised the chicken full-length transmembrane μ from which the VDJ_H and Cμ1 sequences had been deleted and was cloned into the productive chicken retroviral vector RCAS(BP)B (Hughes *et al.*, 1987). SC line embryos (Hyline International, Dallas Center, IA) were inoculated with 1×10^6 RCAS-Tμ transfected fibroblasts at day 3 of incubation by injection with a 1 inch needle through the top of the egg.

Flow cytometry and cell sorting

Bursal cells from RCAS-Tμ infected chicks were purified and stained with 11C6 (anti-chicken Ig light chain) and H-y18 (anti-chicken μ) as described previously (Paramithiotis *et al.*, 1995; Sayegh *et al.*, 1999). Binding of primary antibodies was detected using FITC or PE conjugated goat anti-mouse Ig isotype specific secondary reagents (Southern Biotechnology Associates, Birmingham, AL). Viable cells were analyzed and sorted on a FACS Vantage (Becton Dickinson Canada Inc., Mississauga, Ontario, Canada) by gating on forward and side scatter. Purity of sorted populations was >98%.

Oligonucleotides

The following oligonucleotides were synthesized by Sheldon Biolabs (McGill University, Montreal): VL5′, 5′-ACGCGTCAGGTACTCGTTGCGCCTGGTC-3′; JCI, 5′-CTCGGGCACATTTTCTGGTCAA-3′; VL3′, 5′-ACCATCAGCTGCTCCTTGCACTGGCAGG-3′; JL3′, 5′-ATCGATTCACCTAGGACGGTCAGGGTTG-3′.

Restriction analysis of VL genes

VJ_L or germline V_L1 sequences were PCR-amplified with the VL5′/JCI or VL5′/VL3′ primer combinations respectively for 30 cycles in a Hypercell Biological thermal cycler (MJ Research, Cambridge, MA) using *Taq* polymerase as described elsewhere (Benatar *et al.*, 1992). V_L1 and VJ_L PCR products generated as described above were diluted 1:60 (for V_L1 obtained from μ^+L^- cells and for VJ_L obtained from μ^+L^+ cells) or 1:3 (for VJ_L obtained from μ^+L^- cells) and 1 μl of diluted PCR product used as template for a second round PCR for 24 cycles under the conditions described above. V_L1 sequences were re-amplified using the V_L5'/V_L3' primer combination, VJ_L sequences were re-amplified with the VL5′/JL3′ primer combination. V_L1 and VJ_L PCR products from second round amplification were purified using QIAquick PCR purification kits (Qiagen Inc., Mississauga, Ontario, Canada). PCR product (0.75 μg) was combined with 0.75 μg pUC18 vector (generously provided by Dr M.S.DuBow) and digested with 10 U of the appropriate enzyme per microgram of DNA according to the manufacturer's directions (Pharmacia Biotech) for 1.5 h. Digests were analyzed by agarose gel electrophoresis in 1.5% agarose gels containing 0.5 μg/ml ethidium bromide and photographed.

PCR amplification, cloning and sequencing of VJ_L genes

Aliquots of 1500 cells were stained and sorted as described above directly into PCR tubes containing 14.5 μl deionized H_2O and 0.5 μl of 10 mg/ml proteinase K. Sorted cells were incubated at 50°C for 1 h and then 85°C for 20 min and stored at –30°C until use. VJ_L sequences were PCR-amplified with the VL5′/JCI primer combination using a 1:1 ratio of *Taq* and *Pfu* polymerases for 30 cycles according to the manufacturer's directions (Stratagene, La Jolla, CA) using the temperature conditions described above. Five microliters of the resulting PCRs were re-amplified using *Taq* polymerase for seven cycles as described above prior to cloning into pCR II (Invitrogen). Clones selected on the basis of hybridization to VJ_L specific sequences were sequenced by the Sheldon Biolabs.

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