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A New Model of Sheep Ig Diversification: Shifting the Emphasis Toward Combinatorial Mechanisms and Away from Hypermutation

Craig N. Jenne,* Laurie J. Kennedy,* Peter McCullagh,† and John D. Reynolds2*

The current model of Ig repertoire development in sheep focuses on the rearrangement of a small number (~20) of Vλ gene segments. It is believed that this limited combinatorial repertoire is then further diversified through postarrangement somatic hypermutation. This process has been reported to introduce as many as 110 mutations/1000 nucleotides. In contrast, our data have indicated somatic hypermutation may diversify the preimmune repertoire to a much lesser extent. We have identified 64 new Vλ gene segments within the rearranged Ig repertoire. As a result, many of the unique nucleotide patterns thought to be the product of somatic hypermutation are actually hard-coded within the germline. We suggest that combinatorial rearrangement makes a much larger contribution, and somatic hypermutation makes a much smaller contribution to the generation of diversity within the sheep Ig repertoire than is currently acknowledged.


*Immunology Research Group, University of Calgary, Calgary, Canada; † Department of Molecular Medicine, John Curtin School of Medical Research, Australian National University, Canberra, Australia

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Address correspondence and reprint requests to Dr. John D. Reynolds, Department of Cell Biology and Anatomy, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1. E-mail address: reynolds@ucalgary.ca

Abbreviations used in this paper: V gene, Ig variable gene segment; CDR, complementarity-determining region; FR, framework region; λ, A L chain joining gene segment; PP, Peyer’s patch; Vλ, A L chain variable gene segment.
multiple genes would require a reassessment of those base changes that had previously been considered the result of mutations. Additionally, there appears to be an unusually high rate of successful rearrangement, and a lack of junctional diversity within the λ L chain gene. These results, taken together, have led us to propose a new model of sheep Ig gene repertoire development.

Materials and Methods

Animals

Ouftbred sheep (Sheep Advisory Service and Systems, Andlyn Farm, Innisfail, Canada) were used for 24-h and older genomic DNA sources. Fetal tissues (60–142 days gestation) were obtained from time-bred ewes (John Curtin School of Medical Research, Australian National University, Canberra, Australia).

Tissue and cell isolation

Lymphocytes were isolated from either peripheral blood or ileal PP follicles. Blood was collected into 74 mM sodium citrate, 41 mM citric acid, and 78 mM dextrose in a ratio of 1 ml/10 ml of blood. After 30 min of centrifugation (800 × g) the buffy coat was collected into calcium- and magnesium-free HBSS (Life Technologies, Grand Island, NY) and then layered over 60% isotonic Percoll (Amersham Pharmacia Biotech, Piscataway, NJ), followed by an additional centrifugation. The interface containing the mononuclear cells was collected and resuspended in cold calcium- and magnesium-free HBSS. For the collection of ileal PP tissue, animals were euthanized with an i.v. injection of sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Canada). Lymphoid follicles were released from the freshly collected ileum by separating the mucosa from the muscularis externa with a scalpel blade. The follicles were then disrupted by resuspension and filtered through 40-μm pore size nylon mesh (Small Parts). Cells were counted on a Coulter Counter ZM equipped with a Channel Letterizer (Coulter Electronics, Hialeah, FL). Aliquots of 1 × 10^7 cells were pelleted by centrifugation, supernatants were discarded, and pellets were flash-frozen in an ethanol bath surrounded by dry ice. For some experiments ileal PP and liver were cut into 0.5 × 0.5-cm pieces and flash-frozen as described above. Frozen tissue was ground into a fine powder using a pestle and mortar. For fetal tissues the distal portion of the small intestine was excised, washed in buffered physiological saline, gently blot- ted onto filter paper, and then flash-frozen as previously described.

Genomic DNA isolation

DNA was isolated from either ground tissue or cell pellets using the QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit (Qiagen, Chatworth, CA) according to the manufacturer’s instructions.

PCR amplification

Rearranged V genes were amplified from DNA isolated from lymphocytes using a sense primer specific for the common leader sequence of all known VA gene segments (5’-TGGCTCTGGTCCTTCTGCT-T3’- 3 and an antisense primer specific for the λ L chain joining gene segment 1 (the most frequently rearranged L chain joining gene segment;5’-GGTGCTCGCC CGTGCAGAAA-3’). The final product of ~480 bp included the leader, intron, VA, and 26 bp of the Ja1 gene. Three separate PCR reactions were set up for each DNA sample. Germline VA were amplified from liver DNA using the following primer pairs: VA 3.0 x sense (5’-TGGCTGAT CATGTGAGAGAC-3’) and VA 3.0 x antisense (5’-GCTGATTCGACTTCCTC-3’), VA 5.1 x sense (5’-GCTGATTCGACTTCCTC-3’) and VA 5.1 x antisense (5’-GCTGATTCGACTTCCTC-3’), VA 5.3, VA 5.4, and VA 4.1 PCR reactions used the following primer pairs: Vα 3.0 x sense (5’-GAGGTTGGGGCTGATC-3’) and Vα 4.5 x sense (5’-GCTC TCTCCACATATC-3’) and Vα 4.5 x antisense (5’-GCTTCCACATATC-3’) and Vα 5.1 or Vα 5.4) represented >70% of all VA rearrangement events (our unpublished observations). It is believed that this limited combinatorial repertoire is further diversified through the process of somatic hypermutation (17). To study the impact of hypermutation on the Ig repertoire, sequences originating from the frequently rearranged VA were aligned against each other and compared with the published sequences. The alignment of these rearranged sequences yielded several surprising observations.

Cloning and sequencing

Three separate VA PCR products were pooled and served as the template for ligation reactions that were cloned into JM-109 competent cells using either the TOPO TA Cloning kit (Invitrogen, San Diego, CA) or the pGEM-T Easy Vector System (Promega, Madison, WI). For each ligation reaction 30 clones were screened for positive inserts. Plasmid DNA was isolated using a QIAprep Miniprep (Qiagen) and served as the template for sequencing. Sequencing reactions used the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) along with 3.2 pmol of either M13 forward or M13 reverse primers (Invitrogen). Automated sequencing was performed by the University Core DNA Services (University of Calgary, Calgary, Canada).

Southern blotting

Ovine genomic DNA was prepared by standard extraction procedures (26). Restriction endonuclease-digested ovine genomic DNAs (10 μg) were electrophoresed on 1% agarose gels in Tris acetate buffer. DNA was transferred to Hybond XL nylon membranes (Amersham Pharmacia Biotech) under vacuum using 0.4 N NaOH as a transfer solution. For dot-blot hybridization, genomic and plasmid DNAs were sheared in an ultrasonicator (Branson Ultrasonics, Danbury, CT) to an average length of 1 kb. Serial dilutions with known concentrations of DNA were applied to Hybond XL nylon membranes (Amersham Pharmacia Biotech), denatured, and bound to the membrane by incubation on pads of filter paper soaked in 0.4 M NaOH before hybridization. Radioactive probes were prepared from plasmids containing either VA 5.1 or VA 12.2 using the Random Prime-II kit (Stratagene, La Jolla, CA) and [32P]dCTP (Amersham Pharmacia Biotech). Membranes were hybridized to probes in Expresshyb (Clontech, BD Biosciences, Mountain View, CA) according to the manufacturer’s protocol. Final washes were performed at 65°C for 15 min in 0.1× SSC/0.1% SDS (high stringency). For quantification purposes, known amounts of a plasmid containing a single copy VA gene segment were also spotted adjacent to the test samples on each membrane. Membranes were exposed to phosphor screens (Eastman Kodak, Rochester, NY) and analyzed using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Eugene, OR) to compare test samples to a curve generated from the known standards.

Data analysis

Sequences were compared with the published genes in GenBank with BLAST and to each other using Multalin software (27, 28). Rearranged sequences were classified based on the gene to which they shared the highest homology. In some instances a consistent nucleotide difference from the published gene was observed in rearranged sequences. To account for these common differences, rearranged genes were instead compared with a consensus sequence generated from the alignment of all sequences corresponding to a single published gene. Nucleotide differences between an individual rearranged sequence and the consensus sequence of a gene were tallied for each region (leader, intron, FRs, and CDRs). These totals were standardized as a value per 1000 bases.

Results

Biased gene usage

Our initial analysis was of >800 sequences that had been generated from the rearranged VA of several animals of varying ages. The results supported the previous conclusion that there is a biased VA usage in the sheep (15). Only 15 of the 26 published VA (17) were identified, and of these, five gene segments (VA 3.0, VA 4.1, VA 5.1, VA 5.3, and VA 5.4) represented >70% of all VA rearrangement events (our unpublished observations). It is believed that this limited combinatorial repertoire is further diversified through the process of somatic hypermutation (17). To study the impact of hypermutation on the Ig repertoire, sequences originating from the frequently rearranged VA were aligned against each other and compared with the published sequences. The alignment of these rearranged sequences yielded several surprising observations.

Identification of novel VA

Following nucleotide alignment, identical sequences were found in multiple animals of various ages originating from different continents. These sequences, although identical with each other, shared common motifs that were not observed within the published gene
segments (Fig. 1). It seemed unlikely that identical sequences could arise in different individuals as the product of an untemplated somatic hypermutation process. Rather, it is more probable that these sequences originated from new, previously unidentified, germline V<sub>9261</sub>. To determine whether the nucleotide patterns observed in rearranged sequences originated from either somatic hypermutation or new germline V<sub>9261</sub> a set of criteria was established (Fig. 2). First, if a unique sequence seen in rearranged DNA could also be amplified from germline DNA and was capable of a functional (in-frame) rearrangement, the sequence was confirmed to be a new V<sub>9261</sub>. Second, if the rearranged sequence had not yet been identified in germline DNA (due to the difficulty in designing gene-specific primers) it was considered to be a new V<sub>9261</sub> if it had two or more bases that differed from the published gene, it was found in at least two animals, and it was capable of a functional rearrangement. Finally, if a sequence had only one base that differed from the published gene, but it was found in more than three animals and it was capable of a functional rearrangement, it was also considered to be a new V<sub>9261</sub>. Although the second and third criteria do not necessarily provide conclusive evidence for the existence of the proposed V<sub>9261</sub> in germline DNA, they greatly reduced the possibility that the common motifs observed between sequences are the result of an untemplated, postrearrangement, hypermutation process.

Applying these criteria to the sequences of the five most frequently rearranged V<sub>9261</sub> (V<sub>9261</sub> 3.0, V<sub>9261</sub> 4.1, V<sub>9261</sub> 5.1, V<sub>9261</sub> 5.3, and V<sub>9261</sub> 5.4) resulted in the identification of an additional 64 potential V<sub>9261</sub> (GenBank accession numbers AY158468–AY158531). These newly identified sequences were named by decimalizing the published V<sub>9261</sub> to which they most closely matched. The new genes were sequentially numbered based on decreasing homology (i.e., V<sub>9261</sub> 5.1.1–5.1.19). Each of these newly proposed genes have an open reading frame, code for a unique protein, and are capable of functional, in-frame rearrangements (Fig. 4 and data not shown). The identification of 64 novel V<sub>9261</sub> suggests that combinatorial rearrangement is not as limited as previously reported. The existence of this larger number of V<sub>9261</sub> was further supported by the results of genomic DNA analysis. Southern and dot blots using a probe specific for a V<sub>9261</sub> have identified at least 150 germline copies (Fig. 5 and data not shown), a substantially higher estimate than previously reported (15, 17). Additionally, eight
FIGURE 3. Nucleotide alignment of newly proposed VA vs the published VA. A, Nucleotide alignment of newly proposed VA 5.1 genes (5.1.1–5.1.19) vs the published VA 5.1 (r5.1) (15). B, Nucleotide alignment of newly proposed VA 3.0 genes (3.0.1–3.1.16) vs the published VA 3.0 (r3.0) (15). C, Nucleotide alignment of newly proposed VA 5.3 genes (5.3.1–5.3.9) vs the published VA 5.3 (r5.3) (17). D, Nucleotide alignment of newly proposed VA 4.1 genes (4.1.1–4.1.8) vs the published VA 4.1 (r4.1) (15). E, Nucleotide alignment of newly proposed VA 5.4 genes (5.4.1–5.4.12) vs the published VA 5.4 (r5.4) (17). Nucleotide identity between sequences is indicated by a dot, differences are shown by letter code of the new base, and gaps are indicated by a dash. Leader, intron, FRs, and CDRs are as indicated. The GenBank accession numbers of the new sequences are AY158468 and AY158531.
(~12%) of the proposed 64 $\lambda$ (VA 3.0.12, VA 3.0.14, VA 5.1.1, VA 5.1.2, VA 5.1.14, VA 5.3.2, VA 5.4.2, VA 5.4.7) have been identified in unarranged, germline DNA using gene-specific primers (Fig. 6 and data not shown).

**Reduced postrearrangement diversification**

Somatic hypermutation has been proposed to be the major mechanism for generating diversity in the sheep Ig repertoire (15). This process, also seen in rabbits and mice, introduces untemplated mutations into rearranged Ig gene segments (29–32). Although this process is untemplated, it is not random, as the mutations occur more frequently in the CDRs than in the FRs, and transition mutations are more common than transversion mutations (15, 19).

The initial comparison of our sequences to the published $\lambda$ (before the identification of the new gene segments) produced results similar to what had previously been reported in sheep (15, 16, 25).
The number of nucleotide differences in the CDRs of V\textalpha{} 5.1 ranged from 79–102 nucleotide differences per kilobase, whereas these values were much lower in the FRs, from 14–19 nucleotide differences per kilobase (Fig. 7C). This pattern was also observed in the other four genes analyzed (V\textalpha{} 3.0, V\textalpha{} 4.1, V\textalpha{} 5.3, V\textalpha{} 5.4; Fig. 7 A, B, D, and E). These values are consistent with previously published data (15, 16). However, considering the evidence presented here of the existence of a larger number of V\textalpha{}, many of the nucleotide differences previously thought to result from somatic hypermutation, may, in fact, be hard-coded in the germline sequence. We have, therefore, reassessed the potential contribution of hypermutation. Rearranged sequences were classified according to either the newly proposed V\textalpha{} or the published V\textalpha{} for which they shared highest homology. This reclassification of rearranged sequences, now including 64 newly proposed V\textalpha{}, resulted in a dramatic decrease in the total number of nucleotide differences in each region of the gene (Fig. 7, A–E). In some cases the number of nucleotide differences dropped by >90%. Although the total number of nucleotide differences is much lower after reclassification, their pattern remains similar, with a greater number occurring in the CDRs and fewer in the FRs.

**Accumulation of nucleotide differences with age**

After reclassification, sequences were sorted into two groups based on the age of the animal from which they originated. The first age group (F + 24 h) included fetal and 24-h-old lambs (low exposure to exogenous Ag), whereas the second group (older) consisted of animals ranging in age from 48 h to 2 years (continual exposure to exogenous Ag). In all genes examined, the younger group had considerably fewer nucleotide differences than those of the older group (Fig. 7, F–J); however, the distribution of mutations in sequences from fetal and 24-h-old lambs was similar to that observed in the sequences of older animals with more mutations occurring in the CDRs (ranging from 3.6–14.6 mutations/kb) and fewer in the FRs (ranging from 2.9–4.2 mutations/kb). From these observations we suggest that a postrearrangement diversification mechanism, such as hypermutation, contributes to an accumulation of nucleotide differences as the animal ages.

**Junctional diversity**

The use of primers spanning the V\textalpha{} and J\textalpha{}1 has allowed for the examination of the junctions between these elements. Analysis of 588 VJ1 junctions yielded only nine (1.5%) sequences with a non-functional rearrangement (our unpublished observations). This value is much lower than what has been reported in other mammals, where as many as two-thirds of all rearrangements are out of frame (33–35). In addition to this unusually high rate of in-frame rearrangement, it appears as though the sequences of a single V\textalpha{} share identical VJ1 junctions. These junctions demonstrate canonical rearrangement, without the random loss or addition of nucleotides (Fig. 8). Interestingly, almost all sequences corresponding to a single V\textalpha{} share a common VJ1 junction. In contrast to the junctional uniformity of the rearrangement events of a single V\textalpha{} gene, VJ junctions can differ dramatically between the various V\textalpha{} gene segments (e.g., 5.1.14 sequences share a common junction, whereas these junctions differ from the junctions of 5.1.3, 5.1.4, 5.1.5, etc.; Fig. 9).

**Discussion**

Published results have been accepted as evidence for the existence of up to 90 germline V\textalpha{} in the sheep with only a small fraction (~18 genes) of these functionally rearranging to contribute to the Ig repertoire (15, 17). Within this limited repertoire there also appeared to be biased gene usage such that two individual V\textalpha{} genes represented >50% of all rearrangement events (17, 36). It was further proposed in these studies that this limited repertoire is then further diversified by somatic hypermutation, the untemplated introduction of nucleotide changes into a rearranged V gene. Hypermutation was reported to produce >100 nucleotide changes/1000 bases in some regions of V\textalpha{} (15). The observed nucleotide differences between the rearranged V\textalpha{} and the published germline sequences seemed to fit the profile of a somatic hypermutation process: more differences in the CDRs than in the FRs, and a greater number of transition mutations than transversion mutations (15, 16). However, many of these conclusions were derived from a small number of sequences originating from a purebred population of animals.

In an attempt to better understand this biased gene usage we sequenced >800 rearranged V\textalpha{} from outbred sheep of varying ages (60 days gestation to 2 years of age). In addition to the outbred genetic nature of these sheep, the animals were obtained from different continents (Australia and North America) and thus were exposed to different antigenic environments. Alignment of these rearranged sequences revealed common motifs between sequences that were not found in the published germline genes (Fig. 1). In previous studies using only a limited number of sequences, these patterns were thought to have arisen through the process of somatic hypermutation (15, 16). Only after analysis of a large number of sequences was it noted that these patterns were common to
a number of rearranged V\(\lambda\) originating from different animals. The identification of common motifs between sequences has led us to propose that these unique patterns were not the product of untem- planted hypermutation, but rather, represented previously unidenti- fied V\(\lambda\). To date we have identified 64 potential new V\(\lambda\), all of which encode for a unique protein and are capable of functional, in-frame rearrangements (Figs. 3 and 4).

We propose that these 64 gene sequences are new, authentic V\(\lambda\). However, alternative explanations for these observations need to be considered. One possibility is that it could be the result of gene con- version, as this process has been shown to replace large sections of V\(\lambda\) genes in both rabbits and mice with sequence derived from homolo- gous pseudogenes (14, 37). In the sheep the presence of a large num- ber of pseudogenes supports the possibility of a role for gene conver- sion in the diversi- fi- cation of the Ig gene repertoire (16, 17). Since gene conversion is a homology-based process it could potentially result in similar products being generated in different animals; however, due to the random integration of the donor sequences it is highly unlikely

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Nucleotide alignment of proposed V\(\lambda\) 5.1 genes vs germline and rearranged V\(\lambda\) sequences. Proposed sequences of three new V\(\lambda\) (5.1.1, 5.1.12, and 5.1.14) were aligned against the published V\(\lambda\) 5.1 sequence (r5.1) (15), germline-derived V\(\lambda\) sequences (G5.1.1, G5.1.2, and G5.1.14), and sequences obtained from rearranged V\(\lambda\) (39C3, F7AB8, and F7LCD19). Nucleotide identity between sequences is indicated by a dot, differences are shown by letter code of the new base, and gaps are indicated by a dash. Introns and CDRs are as indicated.
that several identical products would be generated as was observed in the sheep. Additionally, of the 64 proposed V\(\lambda\), we have currently identified eight in germline liver DNA, providing evidence that the newly proposed V\(\lambda\) are not the product of a postrearrangement diversification process.

Other possible explanations for the presence of previously unidentified V\(\lambda\) sequences are multiple alleles or PCR artifact. The question of multiple alleles is of particular concern when working with an outbred population of animals originating from different continents. Each individual could have two different alleles for any single gene (one maternal and one paternal), and thus what we have considered to be new gene segments could simply be allelic variants. Evidence against this possibility is provided by the observation of nine different V\(\lambda\) 5.1-like genes in one individual.
otide differences (Fig. 7, data not shown). By this argument there would have to be a minimum of five different Vα 5.1 like genes (four of the five each with two alleles). Continuing this line of reasoning to the other four groups of genes analyzed, we find there must be a minimum of five Vα3.0-like, three Vα 4.1-like, three Vα 5.3-like, and three Vα 5.4-like gene segments for a total of 19 Vα gene segments. This number, although less than the 64 gene segments we suggest, still represents a dramatic increase over the five previously identified gene segments. Further argument against this possibility that the newly identified Vα gene segments simply represent allelic variants is provided by the Southern and dot blot data, indicating the presence of at least 150 Vα gene segments within an individual (Fig. 5 and data not shown).

With regard to PCR artifact, previous studies have demonstrated a general TAQ polymerase error rate of ~1/1000 bases (15, 38). As a result we would expect to find <1 mutation per Vα gene (~500 bases) sequenced (38). Additionally, amplification of products from DNA containing many copies of homologous genes allows the possibility of generating chimeric sequences (39–41). These chimeric sequences are characterized as having a 5′ end corresponding to one gene fused to a 3′ end corresponding to a second, homologous gene. Our new Vα sequences do not appear to have arisen from the joining of two separate gene products. The new Vα genes have uniform homology throughout their length, with no evidence for any section of the molecule being derived from another gene.

Earlier studies indicated that only a limited number (<20) of Vα were rearranged, however, the identification of 64 new Vα, in addition to the 15 previously described Vα, has prompted a re-evaluation of the model that was originally proposed (15, 17). Further support for an increased role of combinatorial diversity has been provided by the recent identification of a second Jα (Jα2), effectively doubling the number of potential λ rearrangement products, although Jα2 appears to be used much less frequently than Jα1 (36). As a result it now appears that combinatorial rearrangement may contribute to the generation of Ig repertoire diversity to a much greater extent than previously thought. Conversely, the unique nucleotide differences in the rearranged sequences once thought to be the result of somatic hypermutation may now actually be encoded in the germline sequence of these new Vα. When the rearranged sequences were analyzed according to the new classification system we saw a dramatic drop in the number of nucleotide differences (Fig. 7, A–E). This observation makes it likely that somatic hypermutation may play a lesser role in the diversification of the Ig repertoire than previously thought. Although the impact of somatic hypermutation appears to be greatly decreased, there remains evidence of a post-rearrangement diversification mechanism. Comparison of the sequences originating from either fetal or 24-h-old animals to those of older animals (48 h to 2 yr) demonstrated that nucleotide differences accumulated with age (Fig. 7, F–J).

After reclassification of sequences in accordance with the new nomenclature, the percent drop in the number of mutations per 1000 bases was much greater in either the fetal or 24-h-old animals (79.6%) than in the older animals (51.5%; Table I). It had been previously reported that somatic hypermutation in the sheep ileal PP appeared to be an Ag-independent process (16). More recent studies provide evidence that Ag in gut-associated lymphoid tissues in species such as the rabbit may play a role in shaping the primary repertoire by somehow influencing somatic hypermutation (14, 42). In the sheep the placenta is essentially impermeable

![Table I. Percent drop in the number of nucleotide differences within each age group of Vα](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Vα gene</th>
<th>F+24 h (%)</th>
<th>Older (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 + 3.0.x</td>
<td>94.2</td>
<td>71.0</td>
</tr>
<tr>
<td>4.1 + 4.1.x</td>
<td>73.2</td>
<td>21.5</td>
</tr>
<tr>
<td>5.1 + 5.1.x</td>
<td>76.8</td>
<td>66.8</td>
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<tr>
<td>5.3 + 5.3.x</td>
<td>72.4</td>
<td>32.3</td>
</tr>
<tr>
<td>5.4 + 5.4.x</td>
<td>81.6</td>
<td>49.8</td>
</tr>
<tr>
<td>Average</td>
<td>79.6</td>
<td>51.5</td>
</tr>
</tbody>
</table>

*All sequences were first compared to the published gene to which they shared the highest homology and were then compared to the consensus of the proposed new Vα to which they shared homology. Values are reported as the percent reduction of nucleotide differences after reclassification. Average values represent the weighted average of the five groups of genes. F+24 h, fetal and 24-h-old sequences; Older, sequences obtained from animals 48 h to 2 yr of age.*
to most macromolecules, such that the fetus develops in an environment free of foreign Ag (43). From other studies of isolated gut loops in sheep it has been suggested that the absence of extrinsic Ag following birth results in the premature involution of ileal PP follicles, a process that could be reversed by re-establishing the normal flow of gut contents through the isolated ileum (44, 45). Our data suggest that very little postarrangement diversification occurs in the sheep before the presence of foreign Ags, and thus the development of the primary immune repertoire is much less dependent on somatic hypermutation than previously reported (16). However, once the animal is exposed to the external environment, nucleotide differences are quickly introduced into the rearranged Vα gene segments.

Finally, it appears as though the VJ joining of these sequences is of a canonical nature. Regardless of whether sequences originated from peripheral blood or the ileal PP, or whether sequences were derived from fetal vs older animals, the vast majority (98.5%) of rearrangements were in-frame and functional, with a marked absence of P or N nucleotide addition (data not shown). Within any single gene, most rearrangement events occurred in a common fashion, resulting in identical VJ joints (Fig. 8). This canonical rearrangement within the sheep is similar to what is seen in neonates of other species, but is in stark contrast to the situation in the adults of many other mammals, where most Ig VJ junctions demonstrate profound diversity (46–52). The exception to this appears to be the bovine system, where, as in the sheep, most junctions, regardless of age, demonstrate limited diversity (53–55). It has been proposed by others that this precise joining and lack of junctional diversity may be attributed to homology-based recombination (25, 46). In this model it is hypothesized that sequence elements encoded within the gene segments dictate the specific site for DNA cutting and ligation. Thus, all genes sharing the identical sequence elements should rearrange in the same manner. By the same argument, different genes would rearrange to produce diverse VJ joints. The rearrangement of many of the newly proposed Vα yielded unique VJ joining patterns, thereby providing further evidence that these sequences originated from individual germline genes (Fig. 9).

In conclusion, the identification of 64 potential new Vα in the sheep has forced a re-evaluation of the current model of Ig repertoire development in this species. Previously it was thought that a limited number of Vα were rearranged and then later diversified by somatic hypermutation. Our data indicate that combinatorial mechanisms may play a much larger role in the generation of Ig diversity. In addition, it now appears as though somatic hypermutation does not contribute as greatly to the diversification process as once believed. As a result of these findings, we now suggest a new model for the development of the Ig gene repertoire in sheep, one based heavily on combinatorial rearrangement of many VA, supplemented, to a much lesser extent, by postarrangement diversification mechanisms that become particularly evident after birth.

With these observations in the λ L chain, it will be necessary to determine whether a similar pattern of increased combinatorial diversification and reduced somatic hypermutation exists in both the H and κ L chains.

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