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Divergent evolution of T cell repertoires: extensive diversity and developmentally regulated expression of the sheep γδ T cell receptor

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Sheep γδ T cells express an unprecedented repertoire of antigen receptors contributed by increased diversity in both variable and constant region gene segments. Variable region diversity results mainly from the utilization of a large family of duplicated Vδ genes that have retained two distinct hypervariable segments comparable with the complementarity determining regions present in other antigen receptor V genes. This implies that sheep Vδ chains have been intensely selected during evolution, probably at sites involved in ligand recognition. The sheep γδ heterodimer occurs in at least five isotypic variants formed by the association of a single Cδ segment with one of five functional Cγ segments, each with distinctive hinge regions. Our analysis also shows that the establishment of a normal peripheral repertoire is both developmentally regulated and dependent on the continual presence of a functional thymus during ontogeny. The existence of an expanded V gene repertoire and multiple receptor isotypes together with the prominence of γδ T cells in the sheep immune system argues that this lineage of T cells has a more elaborate functional role in this evolutionary pathway.

Key words: γ/δ T cells/gene expression/ontogeny/repertoire

Introduction

Two distinct types of T cells, distinguished by the surface expression of either an αβ or γδ T cell receptor (TCR), develop independently as separate lineages in mammals (Philpott *et al.*, 1992), they account quantitatively for the total pool of peripheral T cells and are the effectors of both cell-mediated immunity and T cell help. Although both lineages use similar mechanisms to diversify their antigen receptors (reviewed by Davis and Bjorkman, 1988; Strominger, 1989), clear differences exist between the repertoire of these two populations in humans and mice. Because there are only a few functional V gene segments at the TCRγ and δ loci, the combinatorial repertoire produced by gene recombination in the thymus is much smaller than for the αβ TCR, which utilizes large pools of different gene segments (reviewed by Raulet, 1989; Bluestone *et al.*, 1991; Porcelli *et al.*, 1991). Furthermore, the repertoire that is actually available in peripheral tissues is even more severely restricted in the γδ lineage since cells localizing at specific sites preferentially use certain V gene combinations in their receptors. For example, in humans, 70–90% of the γδ T cells in blood express the Vγ9 and Vδ2 gene segments (Triebel *et al.*, 1988a,b; Casorati *et al.*, 1989), and in mice, γδ T cells at different mucosal surfaces also have distinct and limited receptor diversities (Asarnow *et al.*, 1988; Itohara *et al.*, 1990). In some cases, but not in all, this limited combinatorial repertoire has been partly offset by non-germline encoded diversification at junctional regions and/or by the usage of multiple D elements (Tonegawa, 1983; Lafaille *et al.*, 1989; reviewed by Allison and Havran, 1991). The diversity of γδ TCRs is thereby confined largely to short junctional regions whereas αβ TCRs have more widely distributed patterns of variability (Schiffer *et al.*, 1986, 1992).

In the face of such a marked disparity between the repertoire of αβ and γδ T cells in humans and mice, it is perhaps hardly surprising that their reported functional properties also differ. While αβ T cells recognize precisely an enormous range of antigens when presented as processed peptide fragments bound to polymorphic MHC molecules (Davis and Bjorkman, 1988), the potential of γδ T cells to recognize diverse antigens, and the mechanism involved, remain controversial (reviewed by Matis and Bluestone, 1991; O'Brien and Born, 1991). The effector functions of γδ T cells are also ill defined and no satisfying consensus has yet emerged about the overall role of these cells in the immune system. These factors, together with the relative scarcity of γδ T cells in rodents and primates (~5% of blood lymphocytes) have led to the prevailing view that they may have a specialized role in defence against a limited number of antigens, especially at mucosal surfaces (Janeway *et al.*, 1988).

More recent studies in artiodactyls, an order of animals that includes the ruminants and that diverged from the rodent–primate evolutionary stream around 100 million years ago (Novacek, 1992), have revealed a highly discordant representation of the two T cell lineages. In artiodactyls, γδ T cells form a much larger proportion of the peripheral T cell pool (Hein *et al.*, 1989; Mackay and Hein, 1989; Mackay *et al.*, 1989; Clevers *et al.*, 1990; Hirt *et al.*, 1990). In the blood of young lambs, for example, γδ T cells are a major lymphocyte population and account for 50–60% of T cells; they are still prominent in the blood of young adults, forming 20–30% of T cells (Hein *et al.*, 1990a). Although some γδ T cells become localized prominently at mucosal surfaces, a large mobile pool of cells recirculates between the blood, tissue and lymph and is widely disseminated throughout peripheral body compartments (Hein and Mackay, 1991). Chickens also have a high proportion of γδ T cells (reviewed by Bucy *et al.*, 1991) indicating that this might also have been the case in ancestral mammalian lineages and that during the mammalian radiation, γδ T cells have been retained as a prominent immune component in some species but lost in others.

The frequency and physiological distribution of γδ T cells in different animals therefore define a quantitative spectrum ranging between 'γδ low' species such as rodents and

humans on the one hand and 'γδ high' species such as ruminants and chickens on the other. Current perceptions about the repertoire of γδ T cells, their evolutionary development, thymic dependency and functional potential are based almost entirely on the analysis of a highly skewed sample of mammalian immune systems, the 'γδ low' species, which should raise important questions about their wider relevance. The results reported here show that in a species at the other end of this spectrum, γδ T cells have a significantly greater degree of repertoire complexity, the gene segments involved show hallmarks of ligand-mediated selection and the ontogeny of the peripheral repertoire is strictly regulated by the thymus. These striking molecular contrasts in conjunction with the quantitative differences evident between species argue that a spectrum of γδ T cell function also exists, ranging from an adaptive but still elusive role in the 'γδ high' species to probable redundancy in the 'γδ low' species.

Results

To examine repertoire diversity, an anchored PCR technique utilizing 3′ primers specific for known sheep constant region sequences was used to amplify and clone the Vγ and Vδ regions being expressed in peripheral γδ T cells. RNA was prepared directly from spleen or blood lymphocytes recovered *ex vivo*, thereby avoiding any procedural artefacts that might have been introduced during separation or enrichment of γδ T cells. A total of 48 Vγ clones and 62 Vδ clones derived in this way from four fetuses at three stages of development (gestation length = 150 days), from two normal adult animals and from two adults that had been previously thymectomized *in utero* were fully sequenced (Figure 1 and Table I). We reasoned that this strategy would allow us to obtain an unbiased representation of the diversity within the recirculating peripheral repertoire, to determine whether the available repertoire varied at different stages of ontogeny and to assess the effect of fetal thymectomy on repertoire development.

Primary structure of Vγ chains

Ten distinct V region segments which could be divided into six families were identified in the 48 Vγ clones sequenced (Figure 2). Two families, Vγ2 and Vγ5, containing four and two members respectively, probably arose by recent gene duplication since the V segments within each were highly homologous (~81–97% DNA identity). All other families were represented by single gene segments and the level of DNA identity between families ranged from ~40 to 75%. There were small differences in the lengths of individual Vγ segments although all showed conserved codons indicative of immunoglobulin domains. The Vγ6 sequence is somewhat atypical in that the position of the initiation codon (ATG) preceding a long open reading frame predicts an unusually long signal peptide (Figure 2).

With rare exceptions, each Vγ segment was rearranged specifically to one of five Jγ elements that contain a conserved Gly codon (GGA) and vary slightly in length as shown (see Figure 2 and Table III). The Jγ2, Jγ3 and Jγ5 sequences share 71–79% DNA identity while Jγ1 and Jγ4 are more divergent both from the other J segments and from each other (DNA identity 47–59%).

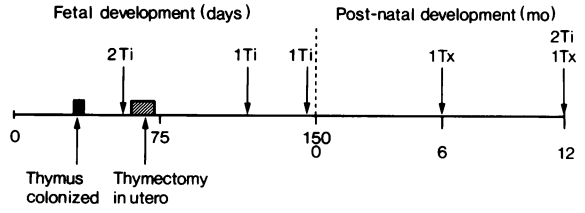


Fig. 1. Schematic diagram showing the number of thymus-intact (Ti) and thymectomized (Tx) animals examined at different stages in development.

Table I. Number of peripheral Vγ and Vδ clones sequenced from different stages of development

Developmental stage	Fetal (days)				Adult		
	61	117	146	(Total fetal)	Ti[a]	Tx[b]	Total all
No. animals	2	1	1	(4)	2	2	8
No. cDNA clones Vγ	8	5	5	(18)	17	13	48
Vδ	5	7	9	(21)	23	18	62

[a]Ti, thymus-intact.
[b]Tx, thymectomized.

Utilization of five diverse Cγ segments

By screening cDNA libraries, we have previously identified the sheep Cγ1 and Cγ2 gene segments. Southern blot analysis using these probes showed the likely presence of additional Cγ genes (Hein *et al.*, 1990b). In the present experiments, each amplified Vγ clone contained ~200 bp of C region sequence from which we identified three new Cγ segments. The five Cγ segments were expressed in a developmentally regulated way and each was specifically spliced to distinct sets of rearranged Vγ and Jγ segments (see below). The full coding region sequence of each new Cγ segment was then amplified from cDNA using a strategy of nested priming with appropriate Vγ, Jγ and 3′UT region primers.

Over the full coding sequence, the five Cγ segments show 73–84% nucleotide identity and the immunoglobulin-like, transmembrane and cytoplasmic domains generally display well conserved features although the cytoplasmic region of Cγ5 is more divergent (Figure 3). However, the connecting peptides between the immunoglobulin-like and transmembrane domains are notably heterogeneous, ranging in length from 24 (Cγ5) to 75 (Cγ2) codons (Figure 3A and B; Hein *et al.*, 1990b). Some residues are conserved between all sequences, including the four most membrane-proximal ones (SAYY) and a cysteine residue likely to form a disulphide bridge with the TCRδ chain (Figure 3B). In the more distal region of the connecting peptide, Cγ1, Cγ2 and Cγ4 each contain two additional cysteine residues at conserved positions and there is a five amino acid motif (consensus sequence TTEPP) in four Cγ regions (Cγ1–Cγ4) (Figure 3B; Hein *et al.*, 1990b).

A large family of Vδ genes

The 62 Vδ clones sequenced contained 28 distinct V regions forming four families. Twenty-five of the V regions have patterns of sequence homology indicating that they are members of a recently duplicated family, Vδ1 (Figure 4A). The level of nucleotide identity between members of Vδ1 ranged from 79 to 97%. The other families represented by

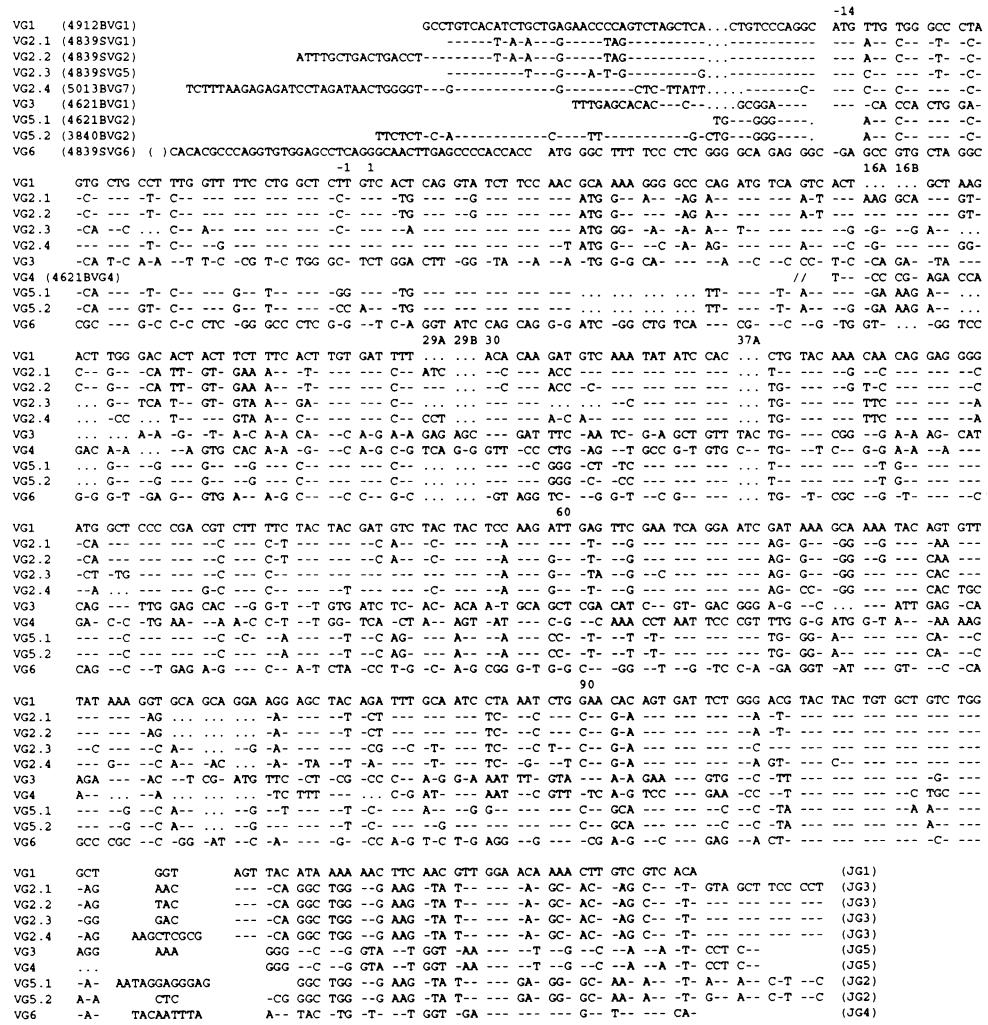


Fig. 2. Nucleotide sequences of sheep Vγ−Jγ regions. Dashes indicate nucleotide identity and gaps introduced to maximize alignment are shown as dots. Additional nucleotides at the start of the 5′UT region of Vγ6 are not shown in the alignment but are included in the database entry.

single V segments, Vδ2, Vδ3 and Vδ4, diverge greatly from the Vδ1 group and from each other (DNA identity 42−67%). The Vδ1 coding regions are remarkably uniform in length and contain long stretches of relatively invariant sequence with two main regions of variability around codons 29−31 and 54−57. In Southern blot analysis of genomic DNA digested with several enzymes, a Vδ1.1 probe hybridized to 8−15 bands confirming the presence of a large family of related genes (data not shown).

As in the human and mouse homologue, the sheep TCRδ locus is located on the chromosome within the TCRα locus (Hein *et al.*, 1991). Some V genes are shared between these two loci in humans and mice and may contribute to either an αβ or γδ TCR (Takihara *et al.*, 1989; Miossec *et al.*, 1990). We therefore tested whether this might occur with the sheep Vδ1 family by PCR amplification using a primer for the conserved 5′UT region of Vδ1 members (sequence 5′-TCTCAGCTTGAGGCAG-3′) in combination with a Cα specific primer. Under these conditions, we were unable to amplify any product from peripheral lymphocyte cDNA of two normal 1 year old animals, as assessed by ethidium bromide staining of agarose gels. Control PCR experiments using a combination of the 5′UT Vδ1 and Cδ primers gave a prominent amplified band as expected (data not shown).

Comparative alignments of sheep TCRδ clones allowed us to identify three related Jδ elements (Figure 4B). In contrast to the Vγ−Jγ junctions, there appeared to be no particular bias in terms of Vδ−Jδ combinations.

Homology of mammalian Vγ and Vδ chains

To assess the evolutionary relatedness of TCR γδ V regions, amino acid sequences representative of the major functional Vγ and Vδ families of sheep, humans and mice were compared (Table II). The different Cγ sequences of these three species show 51−63% amino acid identity. We therefore chose an arbitrary value of 60% identity as being an indicator of likely pairs of homologous sequences. By this criterion, four sheep V regions have clear homologues in either humans or mice. The most striking example is the sheep Vγ4 sequence, which has 74.7% protein identity to mouse Vγ3. Other examples, and the human or mouse homologue, include Vγ3 (human Vγ10, 60.4%), Vδ1.1 (human Vδ1, 69.5%) and Vδ4 (mouse Vδ5, 61.8%; human Vδ3, 65.3%). A number of Vδ regions showed 50−60% identity, in some cases to more than one sequence, while the majority of Vγ regions had no clear homologue in other species. Among the sheep sequences, there are no homologues of the two most frequently expressed human genes, Vγ9 and Vδ2 (see Table II).

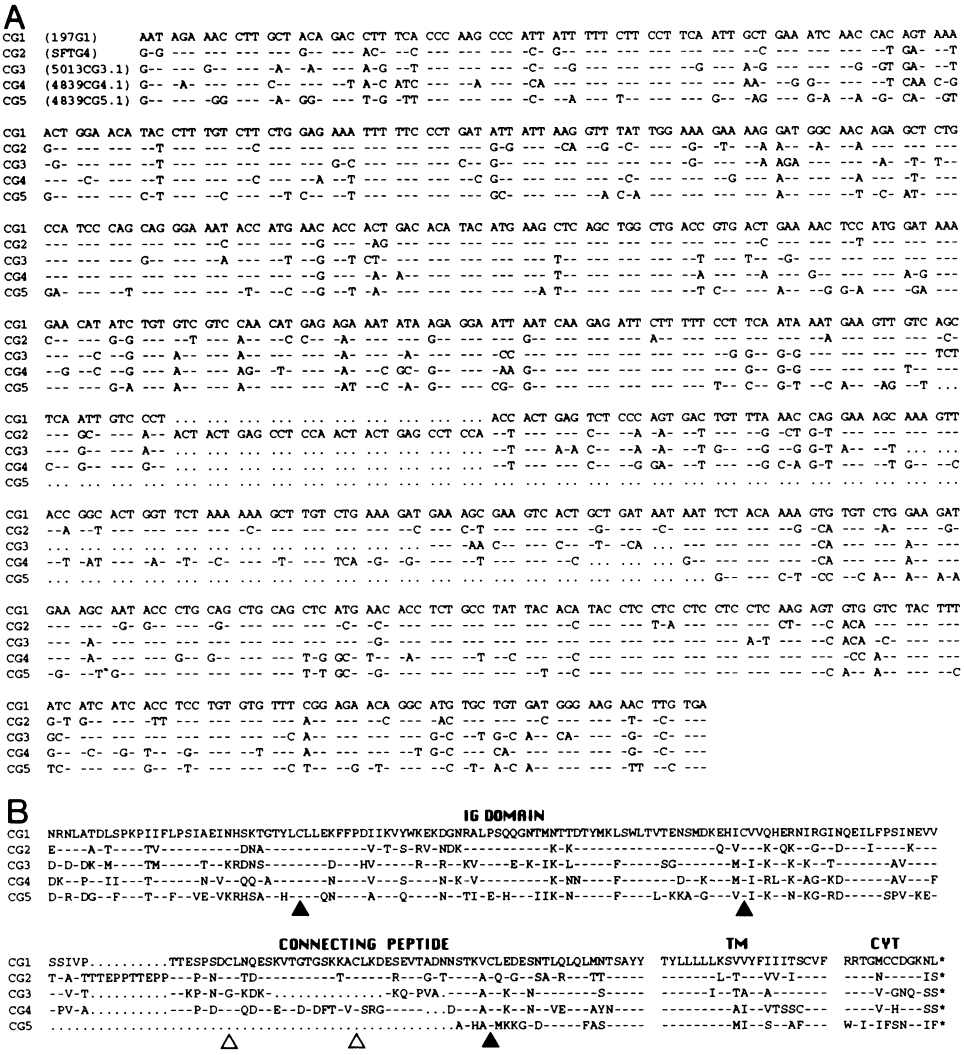
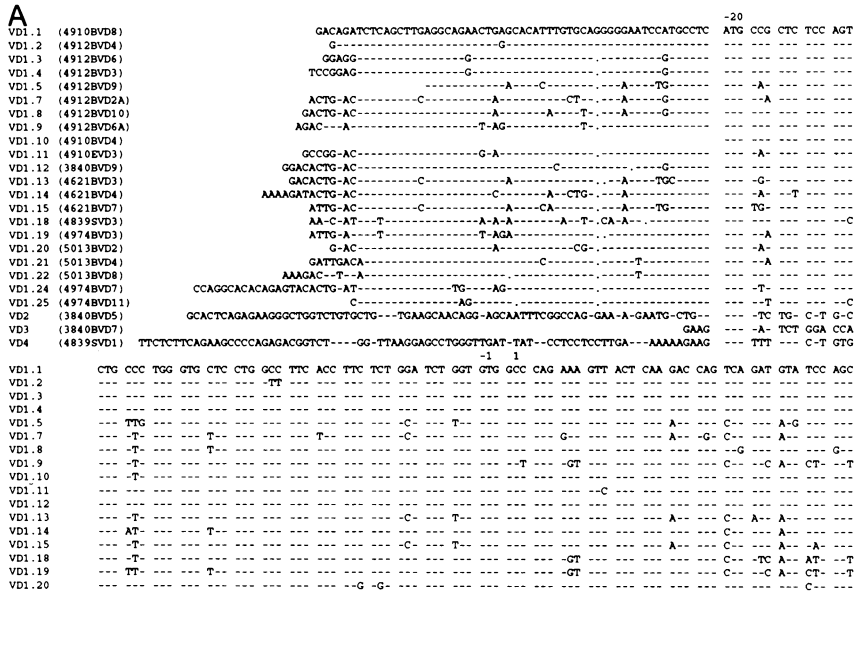


Fig. 3. (**A**) Nucleotide and (**B**) amino acid sequences of three new sheep Cγ segments. Sequences are shown from the first residue of the constant region up to the stop codon. The immunoglobulin domain, connecting peptide, transmembrane and cytoplasmic regions are indicated above the amino acid sequences. The positions of conserved cysteine residues found in all chains (▲) and additional cysteine residues located in the connecting peptide of the Cγ1, Cγ2 and Cγ4 chains (△) are indicated. The Cγ1 and Cγ2 sequences were determined previously (Hein *et al.*, 1990b) and are shown for comparison.



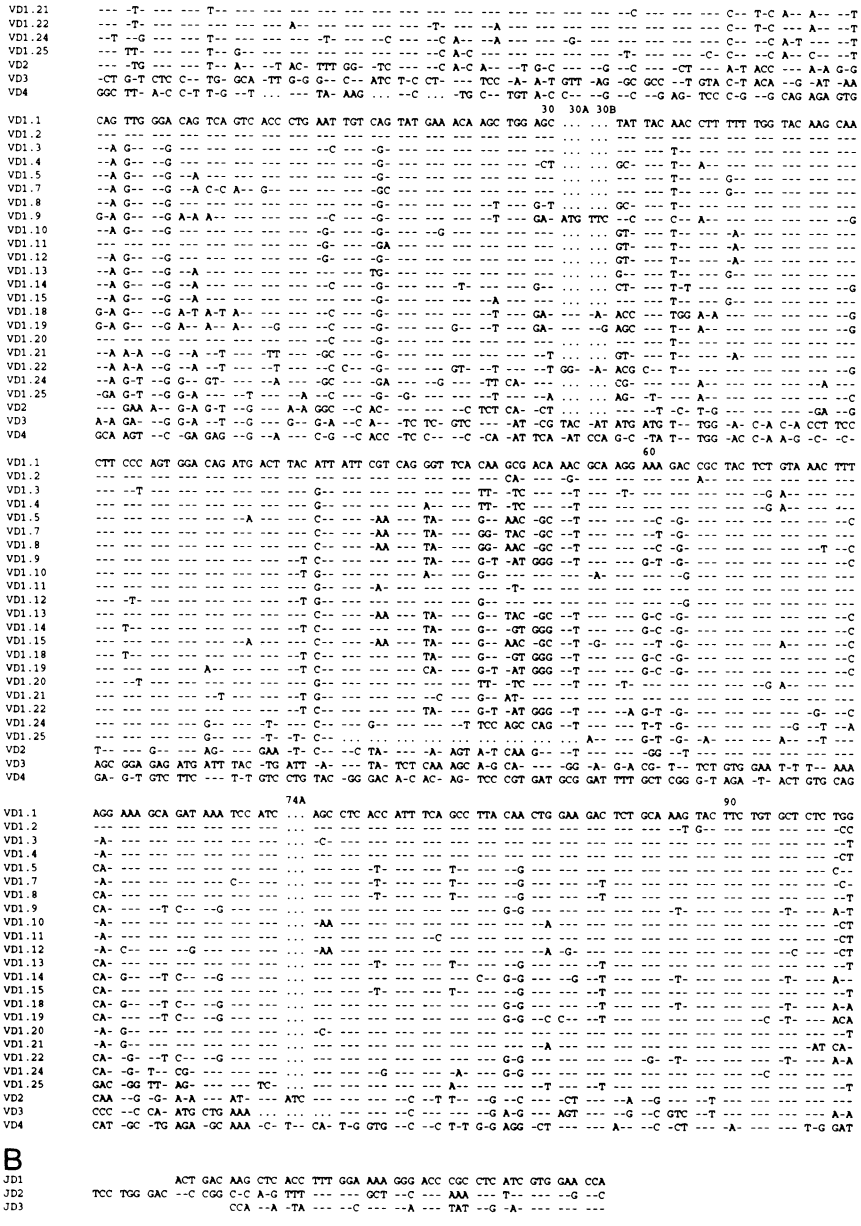


Fig. 4. Nucleotide sequence of sheep (**A**) Vδ and (**B**) Jδ segments. Dashes indicate nucleotide identity and gaps introduced to maximize alignment are shown as dots. Only those sequences obtained as full-length V regions are shown. A further four truncated V region sequences have been included in the database entry.

Hypervariable elements within Vγ and Vδ regions

The translated protein sequences of the full-length V regions obtained (nine Vγ and 24 Vδ, Figures 2 and 4) were analysed using the formula of Wu and Kabat (1970) to determine the variability of amino acid residues at each position of the two chains (Figure 5). The Vδ chains contain two clear peaks of variability around residues 29−31 and 54−57, while other parts of the chain have a relatively invariant framework character. In contrast, comparable hypervariable regions were not obvious in the Vγ chains although the three most variable residue positions were broadly clustered towards the N terminus of the mature protein (residues 19−40). The most 3′ V region residues and the V−Jγ or V−D−Jδ junctional regions are also highly variable (Figure 5 and see below).

Regulated development of the peripheral repertoire

A comparison of gene expression in peripheral γδ T cells at three different stages of fetal ontogeny and in normal adult animals revealed distinct patterns of repertoire development. With the exception of the Vγ2 family of genes, which was expressed at all developmental stages examined, discrete sets of V genes were used in the fetal and adult periphery (Table III). The relative frequency of usage of 'fetal specific' V genes (Vγ4, Vγ5, Vγ6, Vδ2, Vδ1.12−1.18, Vδ2, Vδ3 and Vδ4) also changed with advancing fetal age. V genes detected only in the periphery of normal adult animals included Vγ1, Vδ1.2, Vδ1.4−1.8, Vδ1.10 and Vδ1.11. A few members of the large Vδ1 family of genes were expressed in both fetuses and adults (Table III).

The TCRγ clones derived at all stages of development

Table II. Protein identity among families of functional mammalian Vγ and Vδ chains

	Sheep							Sheep			
	Vγ1	Vγ2.1	Vγ3	Vγ4	Vγ5.1	Vγ6		Vδ1.1	Vδ2	Vδ3	Vδ4
Mouse											
Vγ1.1	–	–	40.4	–	–	–	Vδ1	–	–	–	–
Vγ2	–	–	41.4	–	–	–	Vδ2	–	–	–	–
Vγ3	–	–	–	74.7	–	–	Vδ3	–	–	–	–
Vγ4	–	–	–	–	–	–	Vδ4	52.7	48.4	–	–
Vγ5	41.4	46.4	–	–	44.2	–	Vδ5	–	–	–	61.8
							Vδ6	57.4	53.1	40.4	–
							Vδ7	–	–	–	–
Human											
I Vγ2	44.7	50.0	–	–	49.5	–	Vδ1	69.5	57.6	41.3	–
II Vγ9	–	–	–	–	–	–	Vδ2	–	–	–	–
III Vγ10	–	–	60.4	–	–	–	Vδ3	–	–	–	65.3
IV Vγ11	–	–	–	–	–	–	Vδ4	55.1	57.4	43.4	–
							Vδ5	–	–	–	–

The percentage amino acid identity between compared sequences is shown. Identities >60%, which may indicate homologous genes, are underlined. (–) indicates identity below 40%. The nomenclature and source of human and mouse V region sequences are as follows: mouse Vγ and Vδ (Raulet, 1989), human Vγ (Lefranc and Rabbitts, 1990), human Vδ (Hata *et al.*, 1989; Takihara *et al.*, 1989).

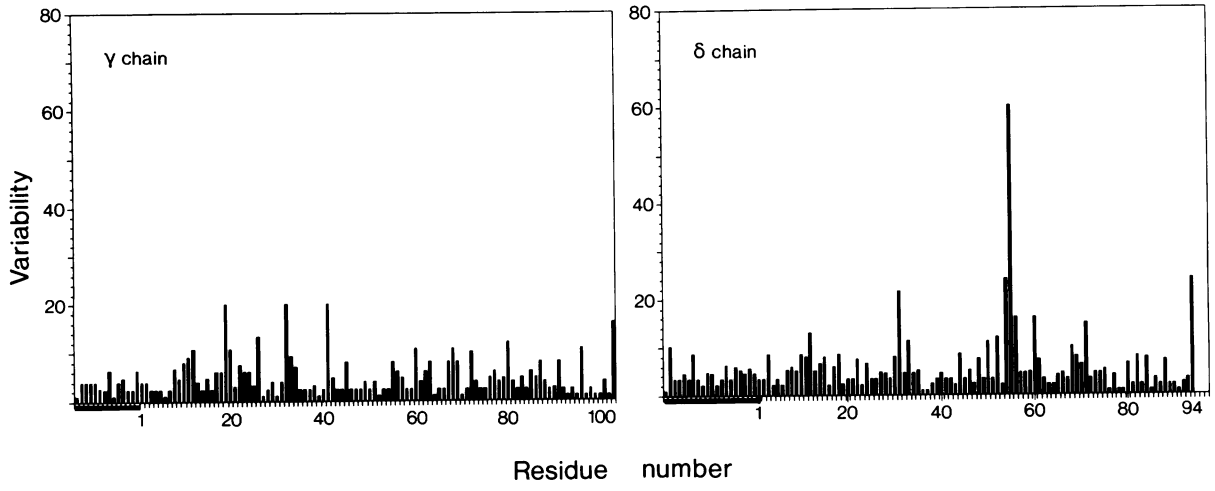


Fig. 5. Variability plot of sheep TCRγ and δ chains. The variability of amino acid residues at each position in the two chains was calculated by the method of Wu and Kabat (1970). The signal peptide sequences are underlined.

showed nearly invariant patterns of rearrangement between particular Vγ and Jγ segments and splicing to distinct Cγ regions as shown in Table III. Among the 35 clones sequenced from fetuses and normal adults, only a single exception to this rule was found whereby one adult clone contained the Vγ2.1 gene segment combined with Jγ1Cγ1 rather than Jγ3Cγ3.

Changes in V gene repertoire after fetal thymectomy

To examine the role of the thymus in the development of the peripheral γδ repertoire, two fetal lambs were surgically thymectomized *in utero* on days 63 and 73 of gestation. The peripheral repertoire of these animals was then assessed by sequencing a total of 13 Vγ and 18 Vδ clones when they had reached 6 and 12 months of age respectively.

A number of clear differences were evident between the peripheral TCRγ repertoire of thymectomized (Tx) and thymus-intact (Ti) animals. First, we did not detect any usage of the Vγ1 gene in blood-borne lymphocytes of Tx animals (Table III). Secondly, within the Vγ2 family, the Vγ2.3 segment was expressed predominantly rather than Vγ2.1. Finally, there was an increased frequency (3/13 clones) of V–J–C gene combinations distinct from the rearrangement and splicing patterns seen in Ti animals and a new Jγ segment (Jγ6) joined to Cγ5 was identified in one clone (see Table III).

The differences between Vδ gene usage in Ti and Tx animals were even more striking. Apart from two gene segments, Vδ1.9 and Vδ1.10, the peripheral repertoire of Tx and Ti animals was completely distinct and several new members of the Vδ1 family (Vδ1.19–Vδ1.25) were detected. In addition, the blood of both Tx animals contained γδ T cells expressing some Vδ gene segments (Vδ1.12, Vδ1.13, Vδ3 or Vδ4) that previously had been detected only in the periphery of fetuses (Table III).

V–Jγ and V–D–Jδ junctional regions

TCRγ and δ clones showed extensive junctional diversity at all developmental stages examined. However, since the genomic sequences of sheep V, D and J gene segments are not yet available, a detailed analysis of these regions is not possible. The comparative lengths of this section of the

Table III. Frequency of expression of TCRγ and Vδ gene segments in blood-borne lymphocytes at different stages of development

Stage of development	Fetal (days)			Adult			
	61	117	146	Ti[a]		Tx[b]	
TCR-δ							
Vγ1				+++++	Jγ1Cγ1		
Vγ2.1	++	+		+++++++		++++	
2.2	+			++	Jγ1Cγ1 1/20		Jγ1Cγ1 1/12
2.3	++	+	+	+	Jγ3Cγ3 19/20	+++++++	Jγ3Cγ1 2/12
2.4		+				+	Jγ3Cγ3 9/12
Vγ3			++	+		+	Jγ6Cγ5
					Jγ5Cγ5		
Vγ4			+				
Vγ5.1			+		Jγ2Cγ2		
5.2		++					
Vγ6	+++				Jγ4Cγ4		
TCR-δ							
Vδ1.1			+	+++			
1.2				+			
1.3			++	+++			
1.4				+			
1.5				+			
1.6				++			
1.7				+			
1.8				++++			
1.9	+			++++		++	
1.10				++		+	
1.11				+			
1.12		+				+	
1.13			+++			+	
1.14			+				
1.15			+				
1.16		+					
1.17		+					
1.18	+	+					
1.19						++	
1.20						+	
1.21						+	
1.22						+	
1.23						+	
1.24						+	
1.25						+	
Vδ2		+					
Vδ3		++				++++	
Vδ4	+++		+			+	

Each (+) indicates the expression of the particular V gene segment in a cDNA clone. The Jγ and Cγ segments to which each Vγ region was rearranged and spliced is shown.
[a]Ti, thymus-intact.
[b]Tx, thymectomized.

molecule are summarized in Table IV. The junctional regions of γ chains were short, containing up to four additional codons between predicted V and J segments. In several clones, codons appeared to have been removed from the ends of participating Vγ and Jγ gene segments during rearrangement. There was no clear trend towards differences in junctional length of γ chains at different developmental stages. The junctional regions of δ chains were more variable in length and contained from one to 18 additional codons between identifiable Vδ and Jδ segments. These regions were longer in normal adult clones than in clones derived from either fetuses or thymectomized adults (Table IV).

Table IV. Average number of codons at junctional regions

	Vγ−Jγ	Vδ−(NDN)−Jδ
Fetus (days)		
61	1.7	7.6
117	1.0	9.7
146	1.4	8.0
Adult		
Ti[a]	1.5	12.0
Tx[b]	2.0	9.3

[a]Ti, thymus-intact.
[b]Tx, thymectomized.

Discussion

Our analysis of γδ TCR diversity in a mammal divergent from primates and rodents indicates that different levels of germline complexity have developed at the TCRγ and δ loci in separate evolutionary pathways. Sheep contain five functional Cγ segments, all of which are about equally related to each other by sequence, and, at a lower level, to their human and mouse homologues. Human DNA contains two Cγ genes which are nearly identical in sequence, apart from differences in length produced by a duplication or triplication of the exon encoding the connecting peptide (Lefranc and Rabbitts, 1985; Buresi *et al.*, 1989). Three of the Cγ segments in mice are also closely related and differ at only a few residues, while the fourth Cγ gene is more divergent (Raulet, 1989). These facts suggest that the five Cγ genes in sheep are descendants of an ancestral pool that existed before the primate–rodent–artiodactyl evolutionary pathways diverged about 100 million years ago (Novacek, 1992); humans and mice have retained fewer genes from this original pool, some of which have been duplicated since divergence and, in one case in the mouse, mutated to become a pseudogene. At present we do not know whether sheep DNA contains functional Cγ regions or pseudogenes in addition to the expressed segments detected in the present experiments; future genomic analyses will resolve this issue.

The likely structure of the ancestral mammalian Cγ genes is less clear. Although all mammalian Cγ segments have well conserved immunoglobulin-like, transmembrane and cytoplasmic domains, the connecting peptide regions differ markedly both within and between species, perhaps reaching an extreme example in sheep where in some chains the connecting peptide region contains extra cysteine residues and other motifs not present in human and mouse Cγ chains. These differences probably arose from the modification of ancestral genes by differential deletion, duplication or triplication of the short exon encoding this region, as occurred in the human Cγ2 gene (Lefranc and Rabbitts, 1985; Buresi *et al.*, 1989), although the likely sequence of these events remains unclear and may have differed between species. So far, four different Cγ transcripts that are similar in structure to the sheep have been detected in cattle (Takeuchi *et al.*, 1992; N.Ishiguro *et al.*, in preparation), indicating that the repertoire features we have detected are likely to be conserved in other artiodactyls.

Important quantitative and qualitative differences have also developed during the evolution of human, mouse and sheep γδ V gene repertoires. Although all species have a relatively small number of Vγ and Vδ gene families, there are only a few cases where putative homologous genes are shared between them and the sheep Vδ1 family is unique in that it contains an unusually large number of related members. Moreover, the Vδ1 family has evolved as a separate gene pool that is utilized predominantly, if not exclusively, in γδ TCRs as shown by the absence of these gene segments in TCRα transcripts. The pattern of variability of amino acid residues in sheep Vδ chains allowed the identification of CDR1 and CDR2 regions typical of other antigen receptor V genes. In the αβ TCR, the CDR3 regions of Vα and Vβ chains are the main sites of interaction with antigenic peptides while the CDR1 and CDR2 segments play a critical role in determining the specificity of MHC recognition (Davis and Bjorkman, 1988; Engel and Hedrick, 1988; Claverie *et al.*, 1989; Hong *et al.*, 1992). By analogy, this suggests that the sheep Vδ germline repertoire has also been shaped by selection processes operating at the level of ligand recognition. Hypervariable regions typical of CDR1 and CDR2 are not obvious in the available human or mouse TCRγ and δ chains, perhaps because there are simply too few sequences (Schiffer *et al.*, 1992), although as in sheep, there is extensive diversity at the CDR3 formed at junctional regions.

The repertoire of rearranged Vγ and Vδ gene segments expressed in peripheral γδ lymphocytes of sheep varied at different stages of fetal development and differed markedly between fetuses and adult animals. In addition, the usage of Jγ and Cγ segments varied at different developmental stages due to the nearly invariant pattern detected in Vγ–Jγ rearrangements and subsequent splicing to a particular Cγ segment. The repertoire that is available in the periphery at different stages of development therefore appears highly specialized, differing not only in terms of V gene usage but also in receptor isotype, since the five Cγ segments have distinct structural differences. As for the human and mouse homologue, sheep have a single Cδ gene (Hein *et al.*, 1990b). It remains to be established whether the different isotypic forms of the receptor associated with the usage of a particular Cγ segment are correlated with subsequent patterns of tissue localization or functional properties. A comparable pattern of development of the recirculating peripheral repertoire has not been detected in other animals, although the thymic expression of human and mouse Vγ and Vδ segments differs between fetuses and adults (Casorati *et al.*, 1989; Lafaille *et al.*, 1989; Krangel *et al.*, 1990; McVay *et al.*, 1991).

Early fetal thymectomy retarded the development of the peripheral repertoire and a number of Vδ genes normally expressed only in fetuses remained detectable when Tx animals had reached an adult age. A similar effect was observed in the case of Vγ expression; fetal thymectomy abrogated the usage of Vγ1, which was normally expressed first sometime after birth. In addition, the level of Vδ–D–Jδ junctional diversity in clones from adult Tx animals was comparable with the fetal junctional repertoire. Although these features support the notion that the few γδ T cells able to persist after thymectomy are survivors of early thymic emigrants (Hein *et al.*, 1990a), we cannot exclude the possibility that some of them developed at extrathymic sites. In this context, the increased frequency of unusual combinations of Vγ–Jγ–Cγ segments in thymectomized animals may reflect different levels of control over gene rearrangement and RNA slicing in T cells developing in the periphery as compared with the thymus. It is clear, however, that in addition to being the major source of γδ T cells in sheep (Hein *et al.*, 1990a), the thymus also profoundly influences repertoire development.

The exact processes that regulate the development of the peripheral repertoire remain unclear. In mice, there is a distinct temporal sequence in the order of V gene expression in thymus (Havran and Allison, 1988). Commencing at around day 14 of fetal development, the Vγ3 gene most 3′ to the J segment is preferentially rearranged, and more distal Vγ genes rearrange in order over the next week of fetal development. These results have been widely interpreted to imply that successive waves of γδ T cells exiting from the thymus have distinct patterns of V gene usage. Moreover, because γδ T cells resident in different body compartments

of adult mice have limited repertoires that correspond to the pattern of V gene rearrangement found in the fetal thymus at different stages, it is believed that the waves of emigrating cells migrate specifically to limited niches in the peripheral immune system (see reviews by Allison and Havran, 1991; Havran *et al.*, 1991). Although there is supporting evidence in the case of Vγ3 expressing cells in the epidermis of mice, which are derived from early fetal thymic precursors (Havran and Allison, 1990), these homing pathways have not been directly demonstrated.

If a similar programme of events occurred in sheep, this could account for both the ordered appearance during gestation of distinct γδ repertoires in the blood of fetal lambs and the later absence of some gene families in blood due to the sequestering of specific cells in tissue sites. However, it remains to be established whether position-dependent constraints to gene rearrangement persist throughout the longer periods of gestation characteristic of other mammals. Indeed, a recent study showed that at time points between 11 and 22 weeks of human fetal ontogeny, all Vγ and Vδ families (with the possible exception of Vδ4) were expressed as rearranged genes in the thymus (McVay *et al.*, 1991). Also, by sequencing 11 thymus cDNA clones derived from the two 61 day old fetal lambs included in this study, we detected different patterns of Vγ and Cγ gene expression than we did in the periphery at that time (unpublished data). Clearly, these sorts of studies need to be expanded and extended to other times in development, but they suggest that intrathymic selection is also likely to play an important role in shaping the peripheral γδ repertoire.

Our results also indicate that not all early fetal γδ T cells have an obligate tendency to home to specific tissue sites during development. When the source of new fetal emigrants was removed by thymectomy, a number of TCR specificities normally available in the periphery at that stage of development did not subsequently become sequestered in tissues but persisted in the blood for extended periods, well into post-natal life. Therefore the absence of expression of particular V genes in late fetal and adult blood does not necessarily reflect intrinsic tissue tropism by specialized subsets of cells but could also result from the continuous replacement of peripheral γδ T cells by new thymic emigrants that have a competitive advantage in terms of their ability to persist. The homing and migration patterns of γδ T cells during fetal and post-natal ontogeny, and the factors which regulate the development and distribution of peripheral repertoires, are likely to be complex and require careful study in appropriate physiological systems.

TCRγ and δ clones derived from peripheral lymphocytes at all stages of development contained extensive junctional diversity. The presence of a few additional codons in Vδ−D−Jδ regions in adult animals as compared with fetuses is consistent with similar findings in mouse and human thymus clones and may similarly reflect an increased level of N-nucleotide addition and/or the usage of multiple D elements at the adult stage (Lafaille *et al.*, 1989; McVay *et al.*, 1991). Among the clones sequenced, there was no evidence for the emigration from the fetal thymus of γδ T cells with invariant canonical junctions analogous to those found in γδ T cells in the skin and reproductive tract of mice (Asarnow *et al.*, 1988; Itohara *et al.*, 1990).

In summary, our results emphasize that many critical perceptions about γδ T cells need to be reappraised since features common to humans and mice cannot always be generalized to all other species. Conclusions about the role that γδ T cells play in immunity, and the way in which this has evolved, must ultimately be drawn from a far wider evolutionary perspective. To achieve this, it will be important to examine the repertoire and function of these cells in other 'γδ high' species, including phylogenetically more primitive organisms such as chickens. The present inability of immunologists to formulate a satisfying overall consensus may in no small way reflect the narrow emphasis given to those species in which the γδ T cell system is arguably devolving towards redundancy.

Materials and methods

Animals and surgical procedures

Healthy White Alpine sheep were obtained from Versuchsbetrieb Sennweid, Olsberg, Switzerland. Fetuses of known gestational age (±1 day, term = 150 days) were recovered by caesarian section on days 61, 117 and 146 of fetal development. Two fetuses were surgically thymectomized *in utero* as described (Hein *et al.*, 1990a) on days 63 and 73 of gestation and kept under normal husbandry conditions after birth.

Cells

A pooled single-cell suspension of lymphocytes was prepared by finely mincing the spleens of two 61 day old fetuses. Mononuclear cells were isolated from the peripheral blood of a 117 and 146 day old fetal lamb, from two normal adult sheep (1 year old) and from two thymectomized adult sheep (6 months and 1 year old) by centrifugation in Percoll gradients (Miyasaka and Trnka, 1985). Recovered cells were washed several times in phosphate-buffered saline, pelleted and used immediately for RNA extraction.

cDNA synthesis

Total cellular RNA was isolated by the acid phenol method and ~5 μg was used as a template for the synthesis of dT primed single-stranded cDNA using a commercial cDNA synthesis kit (Boehringer Mannheim) according to the supplier's instructions. Single-stranded cDNA was tailed with oligo(dG) using terminal deoxytransferase (Bethesda Research Laboratories). The procedures used throughout followed the detailed description given by Thiesen *et al.* (1990).

Polymerase chain reaction and cloning of V gene transcripts

An anchored PCR technique employing two oligonucleotide primers was used to amplify the expressed TCR V regions (Thiesen *et al.*, 1990). One oligonucleotide was specific for sequences contained in the constant regions of sheep γ and δ chains. A stretch of 35 nucleotides located ~200 bp from the 5′ end of the C region that is conserved between the sheep Cγ1 and Cγ2 sequences (Hein *et al.*, 1990b) was used as the Cγ primer. This region lies downstream of a conserved *Sac*I site. The Cδ primer was complementary to a stretch of sequence 40 bp downstream of a *Hinc*II site at the 5′ end of the single sheep Cδ region (Hein *et al.*, 1990b). The second oligonucleotide primer used in each PCR mixture was complementary to the poly(G) tail as described (Loh *et al.*, 1989) and contained a *Sac*II restriction site. In other experiments, an oligonucleotide primer specific for the constant region of the sheep Cα segment (Hein *et al.*, 1991) was used in combination with a primer specific for a stretch of conserved sequence found in the 5′UT regions of the sheep Vδ1 family. The sequences of these primers are as follows (shown 5′ to 3′): Cγ, TCACGGTCAGCCAGCTGAGCTTCATGTATGTGTC; Cδ, GTAGAA-CTCCTTCACCAAACAAGCGACGTTTGTC; Cα, GAGTCAAAATCG-GTCAACAGGCAGAC; 5′UT Vδ1, TCTCAGCTTGAGGCAG; Poly(G), GCATGCGCGGCCGCGGGAGGCCCCCCCCCCCCCC.

Reaction mixtures were denatured by heating to 94°C for 5 min, then subjected to 30 rounds of amplification using a thermo-cycler and commercial kit (Perkin-Elmer Cetus) under the following conditions: 94°C for 30 s, 56°C for 20 s and 72°C for 1 min. Final extension was done at 72°C for 10 min. Amplified DNA fragments were gel purified, eluted using Gene Clean (BIO 101), digested with appropriate restriction enzymes and cloned into compatible Bluescript plasmid vectors. The amplifed Vγ fragments were initially cloned into *Sac*II−*Sac*I sites. However, we found on sequencing that many of these were truncated due to the presence of a *Sac*I site in one Jγ segment. Subsequently, Vγ fragments were blunt-ended and cloned

into *Sac*II–*Eco*RV sites. The amplifed Vδ fragments were cloned into *Sac*II–*Hin*cII sites.

Cloning of new Cγ transcripts

The Vγ clones generated above contained ~200 bp of the 5′ end of the constant region and from these clones we identified three new sheep Cγ regions. Each Cγ region was spliced to distinct sets of rearranged Vγ–Jγ segments and was expressed at specific stages of ontogeny. 5′PCR primers specific for sequences in the Vγ and Jγ segments that paired to each Cγ were synthesized. Since the 3′ regions of the sequences of interest were unknown, but might be similar to related genes, we designed four primers that would hybridize to different stretches of the known sheep and bovine 3′UT regions of TCRγ transcripts (Hein *et al.*, 1990b; Takeuchi *et al.*, 1992). We then used a two-stage PCR amplification procedure on appropriate samples of cDNA to obtain the full-length coding sequence of the new Cγ regions. DNA fragments amplified first with the different Vγ–3′UT primer combinations were used as a template for a second amplification using internal Jγ–3′UT primers. In this way we were able to amplify specifically the new Cγ transcripts which were cloned into Bluescript and sequenced.

Sequencing

Miniprep plasmid DNA (Chen and Seeburg, 1985) was sequenced along both strands using terminal primers and Sequenase (United States Biochemical) according to the maker's instructions. The average length of the cloned fragments was such that the terminal runs overlapped in the central 100–150 nucleotides of the V regions. In a number of cases where ambiguities remained, and as sequence data accumulated, several additional nucleotide primers were designed from known sequences to allow directed internal sequencing runs of individual clones or of different V region families. Sequences were assembled and analysed with the University of Wisconsin Genetics Computer Group (GCG) software run on a VAX computer (Digital, Maynard, MA). Identity between two sequences was determined with the GAP program, which uses the Needleman–Wunsch algorithm.

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