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Exon-intron organization of *TRGC* genes in sheep

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Abstract A series of genomic clones derived from a sheep library were used to determine the germline configuration and the exon-intron organization of *TRGC2*, *TRGC3*, and *TRGC4* genes. Based on the outcomes of molecular analysis, we compared and aligned the genomic sequences with the known complete cDNA sequences of sheep and deduced the exon-intron organization of *TRGC* genes in this ruminant animal, EX1, corresponding to the disulfide-linked constant domain, and EX3, corresponding to the transmembrane and cytoplasmatic domains, are similar in length in all genes. Conversely, the hinge-encoding EX2A, EX2B, and EX2C exons differ in number and length between genes, and EX2A contains the TTKPP motif irrespective of whether it occurs in single or triplicate form. The molecular data also indicate that at least one additional gene is present in sheep. Phylogenetic analysis grouped the ruminant *TRGC* genes in two clusters that could have emerged from two ancestral forms that underwent a series of duplications giving rise to the new sequences that were selected and then fixed in the ruminant lineages. A correlation between the cluster distribution in the phylogenetic tree of *TRGC* genes and their expression during fetal development is discussed.

Keywords T cell receptor · *TRGC* genes · Sheep · Cattle · Exon-intron organization

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The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers AF234763–AF234764, AF241307–AF241309, AF312555–AF312558, and AF312559–AF312561

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Introduction

One of the two types of clonotypic heterodimers may be expressed on the surface of T lymphocytes to confer antigen specificity. The four T-cell antigen receptor (TCR) chains (α, β, γ, δ) are similar to immunoglobulin (Ig) molecules in both gene organization and protein structure. The proteins have variable (V) and constant (C) Ig-like domains. The variable domains of the β and δ chain are encoded by variable (*V*), diversity (*D*), and joining (*J*) genes juxtaposed by somatic rearrangement during T-cell differentiation; the α and γ variable domains are generated from *V* and *J* genes. The heterodimer consisting of an α and β chain is used predominantly in humans and rodents, while the alternative type of TCR comprising a γ and δ chain is used sparingly (Strominger 1989).

Recent studies in ruminants have revealed that the above αβ and γδ TCR rule does not apply to all mammals, since a large and sometimes predominant fraction of T cells in sheep and cattle expresses the γδ TCR (Hein and Mackay 1991). Moreover, in addition to this striking difference between the relative usage of the two types of TCR in these divergent species, the γδ chain complex differs at the structural level. Sheep (Hein and Dudler 1993) and cattle (Hein and Dudler 1997; Ishiguro et al. 1993; Takeuchi et al. 1992) contain five functional *TRGC* genes, isolated as cDNA, all of which are related to each other by sequence and, to a lesser extent, to their human and mouse homologues. In contrast, humans contain two *TRGC* genes and mouse three *TRGC* genes, respectively (Lefranc and Rabbitts 1985; Raulet 1989).

TRGC1 in humans and *TRGC1* and *TRGC2* in mouse have a short hinge domain, while the human *TRGC2* and the mouse *TRGC4* are polymorphic for a longer hinge region (Buresi et al. 1989; Ghanem et al. 1991; Lefranc and Rabbitts 1985; Raulet 1989). Study of the genomic organization of these genes has shown that the human *TRGC1*, like the three *TRGC* genes of mouse, comprises three exons (Lefranc et al. 1986; Raulet 1989). Human *TRGC2* contains two or three homologous tandem copies of exon II that does not encode the cysteine residue.

Therefore, the human *TRGC2* gene displays an allelic polymorphism resulting in either four or five distinct exons (Lefranc et al. 1986; Littman et al. 1987). In mouse, the *TRGC4* hinge region is partially encoded by two small exons with 18 and 15 amino acids, respectively. Some mouse strains express a longer Cγ4 chain than that of BALB/c mice; thus the number of exons that encode the hinge region of *TRGC4* is possibly polymorphic in mice, similar to the human *TRGC2* chain (Vernooij et al. 1993).

The sheep and cattle TCR γ chains contain the longest hinge region of any TCR γ chain described so far. In addition, the sheep hinge regions are notably heterogeneous, ranging in length from 24 (Cγ5) to 75 (Cγ2) amino acids. Some residues are conserved among all sequences, including the four most membrane proximal ones (SAYY) and a cysteine residue. In the extreme distal region of the connecting peptide, each Cγ1, Cγ2, and Cγ4 chain contains two additional cysteine residues at conserved positions. In addition, a five-amino-acid motif consensus sequence TTE(K)PP, with one exception only (sheep Cγ5), is constantly present; five sequences show the motif only once (Cγ3 and Cγ4 in cattle; Cγ1, Cγ3, and Cγ4 in sheep), whereas cattle Cγ2, sheep Cγ2, and cattle Cγ1 sequences have this motif repeated two, three, and four times, respectively. Thus, although the essential structure of the γ chain appears to be well conserved through evolution, the marker of heterogeneity, evident in the hinge region of the chain both within and between species, may be of structural and functional importance. To study the genomic organization of the *TRGC* genes in animal ruminants, we isolated and characterized genomic clones of *TRGC* genes in sheep. Based on this molecular analysis, we deduced the exon-intron organization of all *TRGC* genes in sheep and cattle. The data presented here provide a basis for understanding the physiological significance of the different usage of Cγ isotypes during development.

Materials and methods

Genomic library screening

The genomic DNA library was constructed in λDASHII (Stratagene) using high-molecular-weight DNA obtained from sheep (Altamurana breed) lung. The library was screened with a 380-bp *Sac*I–*Pst*I clone fragment corresponding to cDNA for the *TRGC2* gene (C2 probe), with a 600-bp *Sac*I clone fragment corresponding to cDNA for the *TRGC3* gene (C3 probe), and with a 690-bp *Hin*cII fragment corresponding to cDNA for the *TRGC4* gene (C4 probe) under normal stringency conditions. The cDNA clones were a gift from Dr. W.R. Hein (Basel Institute for Immunology, Basel, Switzerland).

Isolation and sequencing of genomic clones

A total of 16 genomic clones were recovered and characterized by Southern blot analysis. Using primers deduced from the corresponding cDNA (Hein and Dudler 1993), sequencing analysis was performed on both fragments of DNA amplification and genomic subclone and phage DNA. The sequences of the primers were as follows (shown 5′ to 3′). The *TRGC2* primers were: γ1 CCTCAGCTGTCACTACTACT, γ1R AGTAGTAGTGACAGCTGAGG, γ5RGACAAGCTGTTTTAGAACCA, γ6 CAAAGGCATGTCAGGAAGGT, γ6R ACCTTCCTGACATGCCTTTG, γ10 AAACCTTGCTGCAGACACTT, γ11 TCTTCCCGTCACAGCAGTT. The *TRGC3* primers were: 3A TCCCAAGCCCACTATGTTTC, 3B CATCATTTGGAGGTTTAGT, 3C AGATGATGAAGGAACGTTGA, 3E AAGTCCCTGTTGCAAATTCT. The *TRGC4* primers were: 3M AACAATCATCCGGAGGCTCA, 4M TCACCCGATTCTACAAAAGCA, 6M ATAACCCAAACCCAGAAAG, 7M TCATGGGGAAACCCACAA, 8M CCTCCGGATGATTGTTTG. Amplified DNA fragments were gel purified, eluted using the Gene Clean Kit (BIO101), digested with the appropriate restriction enzymes, and cloned into a compatible Bluescript plasmid vector. Nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al. 1977) using the deaza-dGTP reaction of the Sequenase kit (U.S. Biochemicals).

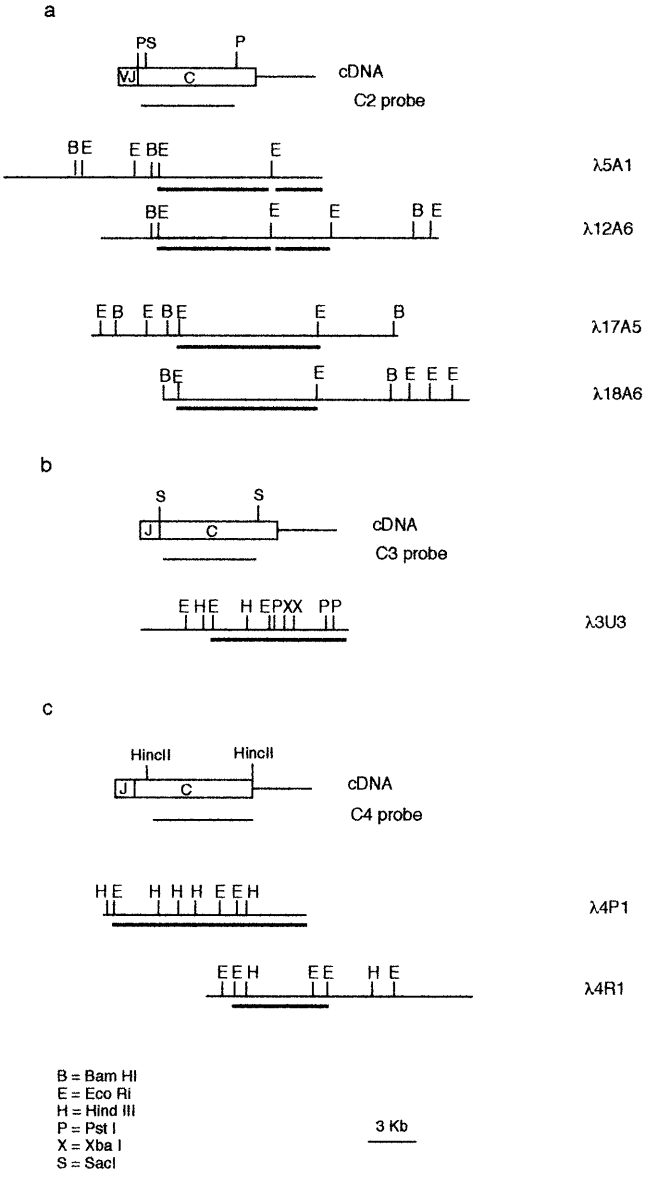

Fig. 1 Restriction maps of λ5A1, λ12A6, λ17A5, and λ18A6 (C2 probe) (**a**), λ3U3 (C3 probe) (**b**), and λ4P1 and λ4R1 (C4 probe) (**c**) phage clones. The probes used for each screening were obtained from the corresponding cDNAs. cDNA clones were a kind gift from W.R. Hein (Hein and Dudler 1993)

Construction of the phylogenetic tree

DNA sequence data were processed and analyzed using the BLASTA program of the GCG software package available at the Italian EMBNET node (http://area.ba.cnr.it). The sequences were multialigned on the basis of amino acid alignment using the PILEUP program of the GCG package, with optimization of alignment by hand. Evolutionary distances were computed using various stochastic methods: the "Stationary Markov clock" (Lanave et al. 1984; Saccone et al. 1990) and the DNADIST programme of the PHYLIP package with the maximum-likelihood method. The phylogenetic tree was created with the UPGMA and neighbor-joining methods, both included in the PHYLIP package. For this analysis, nonsynonymous positions were used. For the sequences under examination, only the aligned sites with no insertions or deletions were considered in the evolutionary analyses.

Results

Exon-intron organization of sheep *TRGC2*, *TRGC3*, and *TRGC4* genes

To study the germline configuration and to determine the exon-intron organization of *TRGC* genes in sheep, we screened a genomic sheep phage DNA library with different cDNA probes for the *TRGC* genes. Initially, the library was screened with the 380-bp *Sac*I-*Pst*I fragment of a clone corresponding to cDNA for the *TRGC2* gene (C2 probe; Fig. 1a). Four genomic clones were isolated and named λ5A1, λ12A6, λ17A5, and λ18A6. Restriction maps of the phage clones indicated that λ5A1 and λ12A6 are overlapping clones; phages λ17A5 and λ18A6 also overlap. Southern blot analysis of the DNA clones digested with *Eco*RI demonstrated that the C2 probe detected two hybridizing *Eco*RI fragments of 6 and 3 kb in λ5A1, and 6 and 3.5 kb in λ12A6. λ17A5 and λ18A6 contained a single C2-hybridizing *Eco*RI fragment of 7.5 kb conserved in both clones. The *Eco*RI fragments of λ12A6, λ17A5, and λ18A6 showing hybridization to the probe were subcloned and characterized further.

The phage library was subsequently screened with the 600-bp *Sac*I clone fragment corresponding to the cDNA for the *TRGC3* gene (C3 probe; Fig. 1b), and eight positive clones were isolated. Southern blot analysis on phage DNAs with the C3 probe showed that the entire gene is contained in one phage clone, λ3U3. Screening with the 690-bp *Hinc*II fragment (C4 probe; Fig. 1c) gave rise to four positive clones that were isolated and analyzed. In this case, the entire gene is contained in two overlapping clones (λ4P1 and λ4R1).

Using primers deduced from the cDNA sequences (Hein and Dudler 1993), sequencing analysis was performed on both fragments of DNA amplification and genomic subclone and phage DNA (Fig. 2).

Analysis of the genomic sequence in λ12A6 (*TRGC2*), λ3U3 (*TRGC3*), and λ4P1 and λ4R1(*TRGC4*) phage clones revealed that the first Ig-like domain of 331 bp (110 amino acids) is well conserved in all three genes along with EX3 which encodes 47 amino acids (Fig. 2). The partial sequence of exons 3 (557 for *TRGC2*, 492 for *TRGC3*, and 322 for *TRGC4*) depends exclusively on the cloning site of the different isolated phages. The complete length of EX3 exons should com-

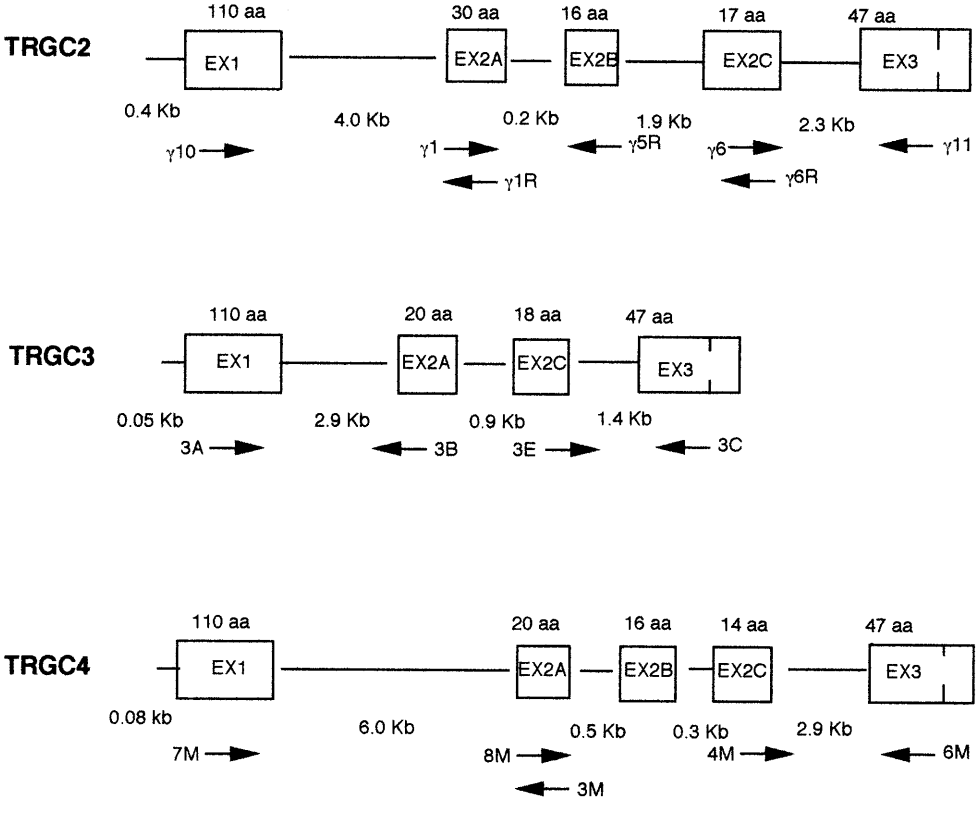

Fig. 2 Exon-intron organization of sheep *TRGC2*, *TRGC3*, and *TRGC4* genomic genes. Exon lengths (in amino acids) are shown at the *top*, and intron lengths (in kilobases) *below*. The primers used for amplification and sequencing analysis are shown. The sequences of each primer were deduced from the corresponding cDNA. The accession numbers of the genomic sequences are: AF312555–AF312558 (*TRGC2*), AF234763–AF234764 (*TRGC3*), AF241307–AF241309 (*TRGC4*)

prise a 3′ untranslated region of over 800 bp. The *TRGC2* and *TRGC4* genes have a similar exon-intron organization, with five exons and four introns (Fig. 2). *TRGC2* contains introns of 4, 0.2, 1.9, and 2.3 kb, while the *TRGC4* gene includes introns of 6, 0.5, 0.3, and 2.9 kb. The hinge region is encoded by three exons, EX2A, EX2B, and EX2C, with 30, 16, and 17 amino acids for *TRGC2*, and 20, 16, and 14 amino acids, respectively, for *TRGC4*. The hinge region of *TRGC3*, in contrast, includes only two EX2 exons, encoding 20 and 18 amino acids. Overall, the length of the *TRGC3* gene, with smaller introns (2.9, 0.9, and 1.4 kb), seems to be reduced.

Comparison of amino acid sequences of cDNAs and genomic DNAs indicates no differences in the genomic sequence of *TRGC3* and *TRGC4* with respect to their corresponding cDNA. Comparison between the *TRGC2* genomic gene and the corresponding cDNA sequence showed the presence of three nucleotide differences in exon 3 leading to three amino acid substitutions (Q/R[4], M/T[8], and I/N[40]). We designated these two allelic forms as *TRGC2*^{a} corresponding to the cDNA (Hein et al. 1990) and *TRGC2*^{b}, corresponding to our genomic sequence.

Identification of an additional *TRGC* gene, *TRGC6*, in the sheep germline configuration

Analysis of λ17A5 indicated the presence of another *TRGC* gene that we designated *TRGC6*. Figure 3 shows the alignment of the resulting sequence data for λ12A6 (*TRGC2*^{b}) and λ17A5 (*TRGC6*) phages. The major difference between the *C2*^{b} and *C6* genes is a deletion in *C6* that starts from the 5′ end of EX2B and terminates at the beginning of the fourth intron. The consequences of this deletion lead to an absence of EX2B and EX2C exons. Another striking difference concerns the EX2A of *C6* which is only 20 amino acids long compared with the 30 of *C2*^{b}. Finally, it is essential to note that in EX3, nine nucleotide substitutions leading to seven amino acid substitutions were observed. Sequence analysis revealed that clone phage λ18A6 was absolutely identical to λ17A5 phage (Fig. 1a), in organization and sequence, with the exception of the presence of two *Pst*I polymorphisms in the 4.0-kb and 2.3-kb intronic regions (data not shown). The data indicate that at least one additional gene, *TRGC6*, is present in the sheep germline configuration. Moreover, work in process on *V-J-C* genomic organization of the *TRG* locus seems to indicate that this *TRGC6* gene may represent a new isotype.

Inferred exon-intron organization of *TRGC* genes in cattle and sheep

Based on the outcomes of molecular analysis of the loci of *TRGC2*, *TRGC3*, and *TRGC4* genes (Fig. 2), we compared and aligned all the known complete sequences of

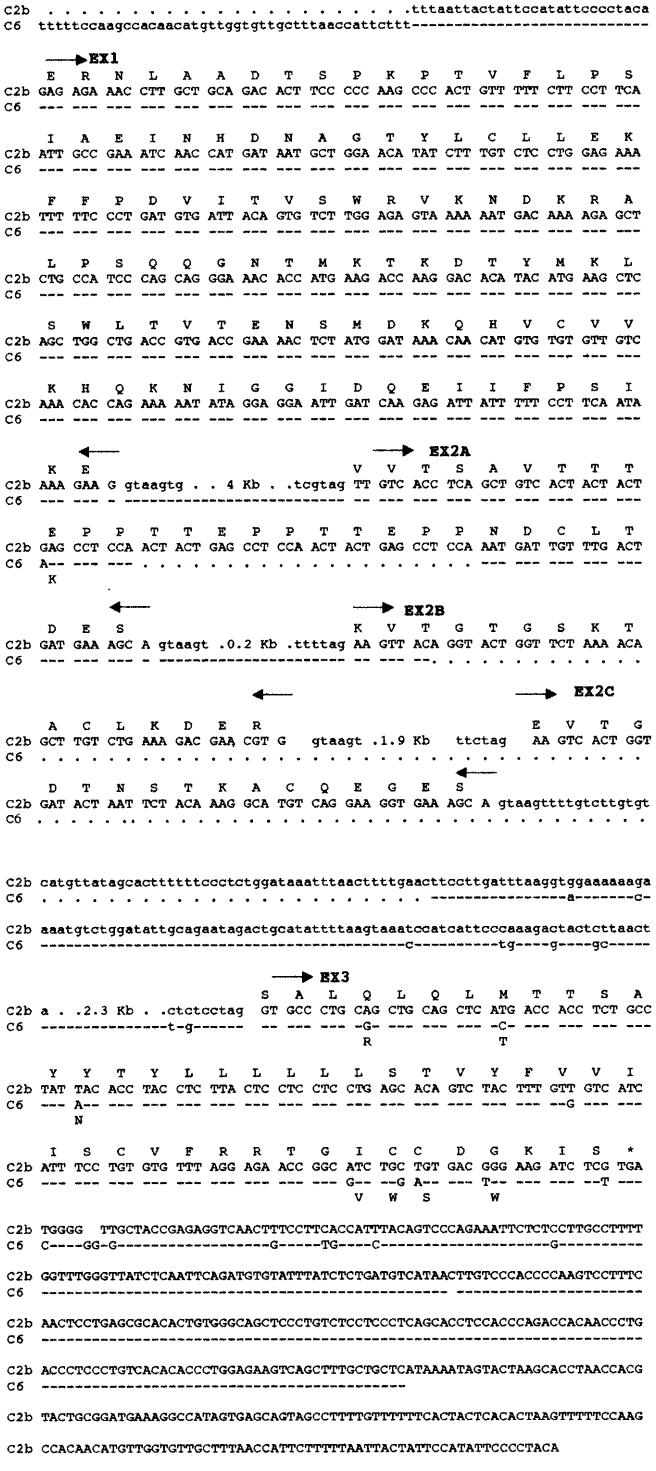

Fig. 3 Alignment of *TRGC2*^{b} and *TRGC6* genes. The potential protein-coding regions (*uppercase*) are indicated in triplet form. Agreement of *C6* with *C2*^{b} is indicated by *dashes*; disagreements are clearly denoted by *dotted lines* that indicate the deleted regions of the *C6* gene compared to *C2*^{b}. Accession numbers of the *TRGC6* genomic sequences are AF312559–AF312561

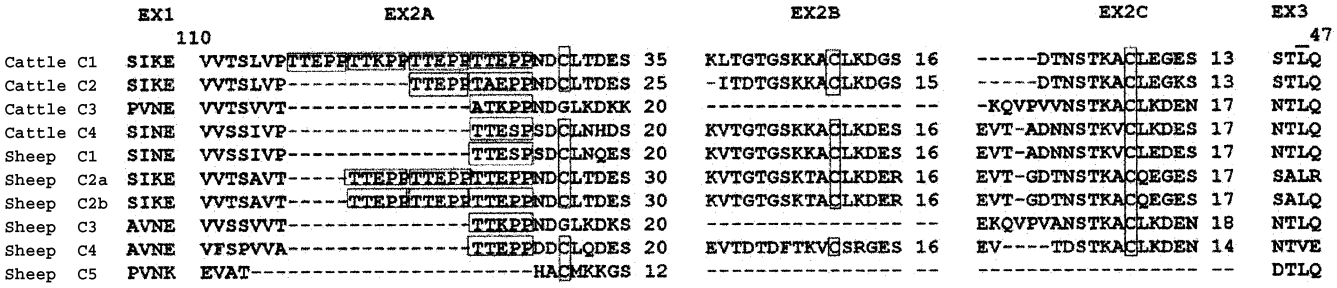

Fig. 4 Comparison of all the known complete amino acid sequences deduced from *TRGC* cDNAs and the inferred exon-intron organization of EX2A, EX2B, and EX2C covering the connecting region of the *TRGC* genes in cattle and sheep. Numbers *110* and *_47* indicate the EX1 and EX3 length in amino acids, respectively. Numbers next to each EX2A, EX2B, and EX2C indicate the length in amino acids. The repeated TTE(K)PP motif and conserved cysteine residues are *boxed*

ruminant cDNA, i.e., from cattle and sheep. The comparison of exons EX1 (110 amino acids) and EX3 (47 amino acids) is reported in a previous paper (Ciccarese et al. 1997). Here we present the results on the inferred exon-intron organization of the *TRGC* gene connecting region covering EX2A, EX2B, and EX2C in cattle and sheep (Fig. 4). Of particular interest is the fact that the TTE(K)PP motif (boxed in the figure) is contained in a single exon (EX2A) irrespective of whether it occurs in single or quadruplicate form.

At this point it is interesting to note the comments of Takeuchi and co-workers (1992) concerning the cattle *C1* and *C2* genes. First, they explain the difference in the number of repeated motifs [two TTE(K)PP for cattle *C2* and four TTE(K)PP for cattle *C1*] as due to alternative splicing of a small exon containing such motifs. In addition, they state that the nucleotide sequences in the 3′ untranslated regions of these genes are 98% identical, strongly indicating that cattle *C1* and *C2* genes are transcribed from a single *TRGC* locus. The results of the molecular analysis of exon-intron organization presented in Fig. 4 clearly indicate that the repeated motif is inserted inside the EX2A exon, and thus that cattle *C1* and *C2* genes are transcribed from two different loci. Moreover, comparison of the alignment results in the hinge region (Fig. 4) indicates that cattle *C4* is closely related to sheep *C1*.

Further noteworthy results concern the presence of a conserved cysteine residue at the same position (position 6 from the end of the exon) in all EX2s, the only exception being EX2A of *TRGC3* genes (cattle and sheep), where the cysteine is replaced by a glycine.

Phylogenetic analysis of *TRGC* genes

Phylogenetic analysis was performed on *TRGC* sequences of human, rodents, and artiodactyls including the new sheep $C2^b$, rabbit *TRGC* (Isono et al. 1995), and chicken *TRGC* (Six et al. 1996), this latter used as an outgroup. (Fig. 5). The $C2^b$ sequence duplicates the sheep *C2* node, in agreement with the experimental results presented above, which have so far shown sheep *TRGC2* to have two allelic forms.

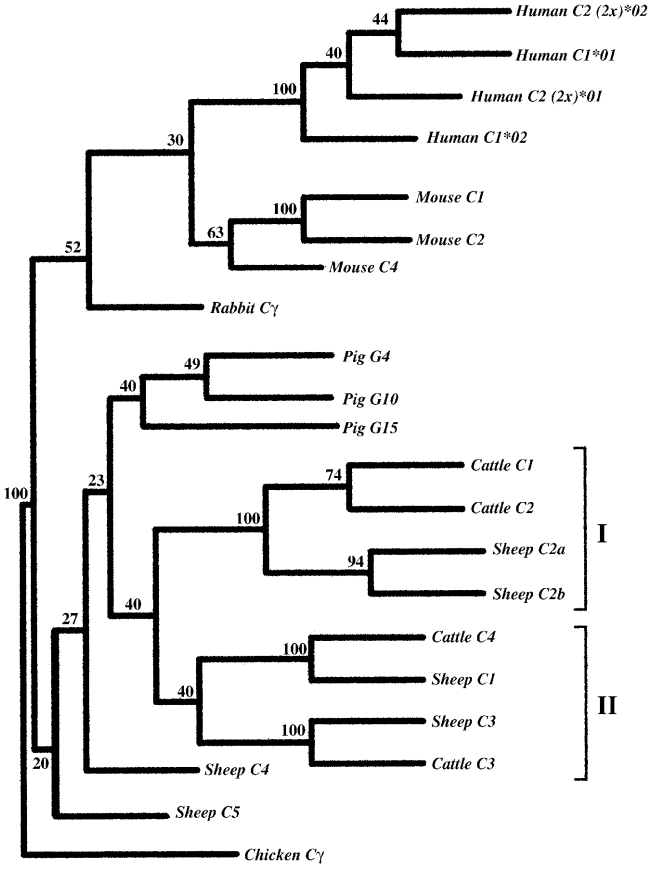

Fig. 5 Phylogenetic tree calculated on the nonsynonymous (first plus second) codon positions of *TRGC* nucleotide sequences constructed by the neighbor-joining method using the stationary Markov distances (Lanave et al. 1984). The extent of the compared sequences is 152 amino acid positions. For the analyzed sequences of human (accession numbers M14996–M14999, M15001, M15002, M15004, M15007), mouse (accession numbers X03984, X04397, X03802), pig (accession numbers L21160–L21162), cattle (accession numbers D90410–D90411, D90414, X63680), and sheep (accession numbers Z12964–Z12967, Z1386) shown here, see the references cited in a previous work (Ciccarese et al. 1997). The recommended IMGT nomenclature for the human and mouse genes is used. Sheep $C2^b$ (this paper; accession numbers AF312555–AF312558), rabbit $C\gamma$ (accession number D38134). and chicken $C\gamma$ (accession number U22666), this latter used as an outgroup, are also shown. The most significant bootstrap values out of 100 replicates are reported on nodes

In addition to grouping of the *TRGC* genes by species (human, rodents, and artiodactyls), the ruminant genes have been classified into two major clans. Thus clan I includes sheep *TRGC2*^{a} and *TRGC2*^{b} with cattle *C1* and cattle *C2*, while clan II consists of sheep *C1*, sheep *C3* with cattle *C3* and cattle *C4*. Moreover, sheep *C4* and sheep *C5* behave as ancestral genes that existed before the pig-ruminant evolutionary divergence in artiodactyls (Fig. 5).

Discussion

We investigated the exon-intron organization of *TRGC* genes in sheep. *TRGC* genes show similarities mainly in the conservation of length in EX1 (110 amino acids) and EX3 (47 amino acids), but the their hinge-encoding exons differ in number and length (Fig. 4). In fact, the hinge region is formed by three exons (EX2A, EX2B, and EX2C) for *TRGC1* (Hein and Dudler 1993), *TRGC2*, and *TRGC4*, two exons for *TRGC3*, and only one for *TRGC5* (Hein and Dudler 1993). EX2C is the most heterogeneous in length, ranging from 13 in cattle *C1* to 18 in sheep *C3*. The longest EX2A is found in cattle *C1* and includes four TTK(E)PP motifs. Sheep *C2*^{a} and *C2*^{b} have three motifs, while cattle *C2* has two motifs. EX2As of cattle *C3*, cattle *C4*, sheep *C1*, sheep *C3*, and sheep *C4* include only one TTK(E)PP motif. Hence, the length of EX2A for all the analyzed genes appears to be 15 amino acids when the motifs are excluded, the only exception being sheep *C5* which is only 12 amino acids long. Sheep *C5* also lacks EX2B and EX2C. If sheep *C4* and sheep *C5* represent ancestral genes (Fig. 5), the origin of the cattle *C3* and sheep *C3* sequences, which lack EX2B, can be explained through a model in which the *TRGC* ancestral sequences were duplicated by means of an unequal crossing-over event (Buresi et al. 1989), giving rise to a new sequence that was selected and then fixed. For a more complete picture of the *TRG* genes in ruminant animals, the unusual level of diversity in the *TRGC* genes, including both duplications and allelic forms, must first be noted. Second, the complexity of the *TRG* locus is emphasized by chromosomal mapping results that indicate that in sheep, *TRG2* maps at bands 4q1.5–2.2, whereas *TRG1* maps at 4q3.1. The same split in the *TRG* locus was observed in the homologous Chromosome 4 in cattle (Massari et al. 1998). Our recent results (unpublished data) show that sheep *TRG4* maps to the same position as *TRG2*; chromosomal mapping of the *TRG3* and *TRG5* is still in progress, together with work on *V-J-C* genomic organization.

The phylogenetic analysis confirms our previous findings (Ciccarese et al. 1997) that humans and rodents group together, apart from the artiodactyl sequences. We hypothesize that sheep *C4* and sheep *C5* represent the ancestral sequences of the genes that are then expressed in the fetus and the adult, respectively. This hypothesis is based both on analysis of the tree and on the expression results obtained by Hein and Dudler (1993), who report how the repertoire in the periphery at different stages of development appears to be highly specialized, differing not only in terms of *V* gene usage but also in *Cγ* genes. In fact, in the stages of development between 61 and 146 fetal days, there is preferential usage of *Cγ2* and *Cγ4* genes, whose expression has never been identified in adult sheep. In contrast, the genes *Cγ1*, *Cγ3*, and *Cγ5* are preferentially expressed in the adult (Hein and Dudler 1993). In the same way, expression data for cattle *C4* indicate that the corresponding γ chain is predominantly expressed on peripheral blood lymphocytes (Ishiguro et al. 1993).

The tree also shows how the ruminant sequences are grouped in two clusters: I and II. There appears to be a correlation in ruminant animals between cluster I and the frequency of expression of Cγ2 and Cγ4 isotypes in blood-borne lymphocytes at different stages of development, and between cluster II and Cγ1, Cγ3, and Cγ5 isotypes expressed in the adult. As far as cattle *C5* is concerned, the known sequence of the first 67 codons has 71–76% identity to the three known cattle *Cγ* regions but 96% identity to sheep *C5* (Hein and Dudler 1997). We therefore hypothesize the existence of a cattle *C4* that in its phylogenetic disposition behaves like an ancestral gene and that is preferentially expressed during bovine fetal development. Clearly, more information is required from several other species for a fuller evolutionary perspective to emerge.

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