Evolution of T-Cell Receptor Gamma and Delta Constant Region and Other T-Cell-Related Proteins in the Human-Rodent-Artiodactyl Triplet

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Manuscript received February 8, 1996
Accepted for publication October 21, 1996

ABSTRACT

In this paper we report a detailed comparative and evolutionary analysis of the sequences of constant T-cell receptor (Tcr) C$\gamma\delta$ genes of artiodactyls compared to the homologous sequences of rodents and primates. Because of the frequency and physiological distribution of $\gamma\delta$ T-cells in different animals, rodents and humans are defined as "$\gamma\delta$ low" species and ruminants as "$\gamma\delta$ high" species. Such a characteristic seems to be due to an adaptive role of $\gamma\delta$ T-cell function. By analyzing the ruminant gene phylogeny of Tcr Cγ we were able to estimate the distance between cattle and sheep at 18 million years ago, a time that is in agreement with other nonmolecular estimates. For Tcr C$\gamma\delta$ genes a peculiar phylogenetic relationship was found, with human and mouse clustering together and leaving artiodactyls apart. By using appropriate outgroups, the same phylogenetic pattern was obtained with other T-cell related sequences: namely, Tcr Cα chain, CD3 γ and δ invariant subunits, Interleukin-2, Interleukin-2 receptor α chain and Interleukin-1β with the exception of Tcr Cβ chain and Interleukin-1α. In contrast, the analysis of all other T-cell nonrelated genes available in primary databases reveals a different tree, where primates and artiodactyls are sister taxa and rodents are apart in accordance with the current view of mammalian phylogeny. These data are relevant to important evolutionary issues. They show how misleading a phylogeny based on a single or on a few homologous genes may be. In addition they demonstrate that genes with correlated functions may evolve in a lineage specific manner probably in relation to environmental conditions.

T lymphocytes play an important role in the immune system, since they recognize specific peptides within a polymorphic cleft of major histocompatibility complex (MHC) antigens (MADDEN *et al.* 1991). This recognition is possible thanks to the heterodimer glycoprotein with an immunoglobulin-like structure, known as Tcr (T-cell receptor), present on the membrane of the T lymphocytes. In the examined vertebrates, T-cells express on the cell surface, in association with a cluster of proteins termed CD3, either $\alpha\beta$ Tcr or a $\gamma\delta$ Tcr (KLAUSNER *et al.* 1990). The $\alpha\beta$ T-cells that populate the peripheral immune system of adult individuals are mostly specific to MHC complexed with peptides encoded by foreign agents or pathogens. The responsiveness to this recognition is variable, depending largely on uncharacterized factors, though the end result is that $\alpha\beta$ T-cells contribute to the eradication of foreign pathogens by direct cytotoxicity toward infected cells. In common with $\alpha\beta$ T-cells, cells expressing the $\gamma\delta$ receptor exhibit typical activities of the functional T lymphocytes such as cytolysis and lymphokine release. However, both from the functional and genetic points of view it appears evident that notable differences exist between $\alpha\beta$ and $\gamma\delta$ Tcrs. In man and mouse, only a small part of the T lymphocytes of the peripheral blood (<5%) is $\gamma\delta$ (RAULET 1989), in artiodactyls (HEIN *et al.* 1990; TAKEUCHI *et al.* 1992) instead, it has been shown that the level of $\gamma\delta$ expression is strikingly higher than that observed in human and murine species. In young sheep, $\gamma\delta$ cells make up a large proportion of T circulating cells reaching ~50–60% of the total T lymphocytes (HEIN *et al.* 1990).

In humans, two constant gene segments (Cγ1, Cγ2) of Tcr γ were found; the Cγ1 gene, like the mouse Cγ gene, has three exons, whereas the Cγ2 gene has four exons, including a duplicated second exon that would create a putative protein with an enlarged constant region. However, these two duplicated exons in Cγ2 have lost the cysteine residue that is thought to be involved in the interchain disulfide bridge (LEFRANC *et al.* 1986). Another Tcr Cγ2 allele differs even more from Tcr Cγ1 due to the presence of a triplicated second exon (BURESI *et al.* 1989). In mouse four Cγ genes were reported, one of which is a pseudogene (RAULET 1989). In artiodactyls, three cDNA sequences of pig Cγ genes (THOME *et al.* 1993, 1994) and four cDNA sequences of bovine Cγ genes were identified (TAKEUCHI *et al.* 1992; ISHIGURO *et al.* 1993), while in sheep at least five cDNA sequences for Cγ were found (HEIN and DUDLER 1993). By observing these results, HEIN suggested that the five Cγ genes in sheep are descendants of an ancestral pool

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existing before the primate-rodent-artiodactyl evolutionary pathways diverged (NOVACEK 1992); thus sheep evolved through a pathway separate from the rodent-primate stream. In the present paper, we report a series of comparative studies on nucleotide sequences of artiodactyls, human and murine constant Tcr $\gamma\delta$ genes and on the amino acid sequences of their products. We studied the phylogenetic relationships between these species for Tcr C$\gamma\delta$ sequences and compared the resulting phylogenies with those obtained from sequences of Tcr C$\alpha\beta$ genes. For the above mentioned species, we have also analyzed the CD3 complex, which is believed to transduce activation signals across the cell membrane following interactions between the Tcr chains and their ligands, particularly the CD3 $\gamma\delta$ invariant subunits. Furthermore, we show the evolutionary relationships for a series of genes involved in cell-mediated immunity, namely Interleukin-2 (IL-2), Interleukin-2 receptor α chain (IL-2Rα) and Interleukin-1 (IL-1) family in the triplet artiodactyls-humans-rodents. Our results show that a group of genes specifying T-cell-related proteins in artiodactyls evolved in a different way when compared to the homologous mouse and human genes, giving phylogenetic relationships that contrast with those of most other nuclear encoded genes in the triplet artiodactyls-humans-rodents.

MATERIALS AND METHODS

The phylogenetic analyses were carried out at the EMBnet node in Bari (Italy). In this study, DNA and amino acid sequences from the following members of T-cell related genes were analyzed: Tcr Cγ of human (LEFRANC *et al.* 1986; BURESI *et al.* 1989), mouse (HAYDAY *et al.* 1985; GARMAN *et al.* 1986; IWAMOTO *et al.* 1986), sheep (HEIN and DUDLER 1993), cow (TAKEUCHI *et al.* 1992; ISHIGURO *et al.* 1993) and pig (THOME *et al.* 1993, 1994); Tcr Cδ of human (LOH *et al.* 1987), mouse (CHIEN *et al.* 1987), sheep (HEIN *et al.* 1990), cow (TAKEUCHI *et al.* 1992) and pig (THOME *et al.* 1993); Tcr Cβ of human (YANAGI *et al.* 1984; JONES *et al.* 1985), mouse (HEDRICK *et al.* 1984; MALISSEN *et al.* 1984), sheep (GROSSBERGER *et al.* 1993), cow (TANAKA *et al.* 1990), pig (THOME *et al.* 1993) and chicken (TJOELKER *et al.* 1990); Tcr Cα of human (SIM *et al.* 1984), mouse (CHIEN *et al.* 1984), sheep (HEIN *et al.* 1991), cow (ISHIGURO *et al.* 1990), pig (THOME *et al.* 1993) and chicken (GOBEL *et al.* 1994); CD3 γ of human (KRISSANSEN *et al.* 1986), mouse (SAITO *et al.* 1987), sheep (HEIN and TUNNACLIFFE 1990), cow (CLEVERS *et al.* 1990) and chicken (BERNOT and AUFFRAY 1991); CD3 δ of human (KRISSANSEN *et al.* 1986), mouse (SAITO *et al.* 1987), sheep (HEIN and TUNNACLIFFE 1990), cow (CLEVERS *et al.* 1990) and chicken (BERNOT and AUFFRAY 1991); IL-2 of human (HOLBROOK *et al.* 1984), mouse (KASHIMA *et al.* 1985), sheep (GOODALL *et al.* 1990), cow (CERRETTI *et al.* 1986) and pig (GOODALL *et al.* 1991); IL-2R α chain of human (COSMAN *et al.* 1984), mouse (MILLER *et al.* 1985), sheep (BUJDOSO *et al.* 1992) and cow (WEINBERG *et al.* 1988); IL-1α of human (GUBLER *et al.* 1986), mouse (LOMEDICO *et al.* 1984), sheep (ANDREWS *et al.* 1991), cow (MALISZEWSKI *et al.* 1988) and pig (MALISZEWSKI *et al.* 1990); IL-1β of human (BENSI *et al.* 1987), mouse (GRAY *et al.* 1986), sheep (FISKERSTRAND and SARGAN 1990), cow (MALISZEWSKI *et al.* 1988) and pig (VANDENBROECK *et al.* 1993).

The following members of T-cell nonrelated genes were analyzed: alpha-globin (V00488, V00714, X70214, M14567), beta-globin (V00497, V00722, M63453, Y00501), tryptophan-tRNA ligase (M61715, X69656, X52113, M12081), serum albumin (L00132, M16111, X17055, X58989, 60688), glutamine synthetase II (M59834, M60803, Z29636, M29076), alpha-lact-albumin (J00270, M87863, X06367, X06366, M80520, X15421), alpha-1-antitrypsin (M11465, M33567, X15555, X63129, Z18906), IL-3 (M20137, K03233, Z18291, L31893), atrial natriuretic factor (M30262, K02781, M54669, D01043). In the list of T-cell-related genes, author's references are reported; T-cell-nonrelated genes are followed by their accession numbers in EMBL or GenBank databases.

The sequences were multialigned on the basis of amino acid alignment by using the PILEUP program of the GCG package and by optimizing the alignment by hand. The sequences were analyzed by a stochastic model of gene evolution, the "Stationary Markov Clock" (SMC) (LANAVE *et al.* 1984; SACCONE *et al.* 1990), a method devised in our laboratory that allows a very accurate measurement of the evolutionary distances between sequences. The phylogenetic trees were calculated with the UPGMA method and the neighbor-joining method, both included in the PHYLIP package (FELSENTEIN 1993). For this analysis, nonsynonymous positions were used (see the Figure 2 legend). For the sequences under examination, only the aligned sites with no insertions or deletions were considered in the evolutionary analyses. For each group of sequences, a rooted tree was constructed by analyzing the sequences in the presence of an outgroup species. When an outgroup was not available, as for the Tcr C$\gamma\delta$ family, the different subgroups of Tcr C$\gamma\delta$ genes served as outgroups to one another, as it is also the practice of other authors (HUGHES 1993). Thus, within each cluster (*i.e.*, Tcr Cγ, Tcr Cδ) a rooted tree is available. Parsimony trees on amino acid sequences were constructed with PAUP 3.1.1 (SWOFFORD 1993). All the above described methods are reported only in the figure for Tcr Cγ phylogenies. The trees describing all other examined sequences are based on the UPGMA method.

RESULTS

Evolution of T-cell receptor constant $\gamma\delta$ genes: The amino acid sequences obtained from the Cγ cDNA of artiodactyls are aligned with their homologous sequences of Cγ genes from human and mouse (Figure 1A). As regards the nomenclature of artiodactyl cDNA clones, for which no information is available at the genomic level so far, we renamed them in alphabetical order (Figures 1A and 2A). The alignment shows strong conservation along the immunoglobulin domain of the extracellular section, transmembrane and cytoplasmic regions. Instead, considerable differences are present in the connecting peptide of the extracellular section. While human and murine Cγ genes lack the TTE(K)PP motif, in the 12 artiodactyls sequences this motif is constantly present with three exceptions only (sheep L and pig A, C); six sequences show the motif only once (pig B, cow G, sheep H, cow F, sheep J and sheep K), whereas cow E, sheep I and cow D sequences have this motif repeated two, three and four times, respectively (Figure 2A). Five cysteine residues at alignment positions 32, 88, 173, 212 and 220 (Figure 1A) are highly conserved, and two cysteine residues at positions 140 and 156 in the connecting peptide are unique for ruminant Tcr Cγ

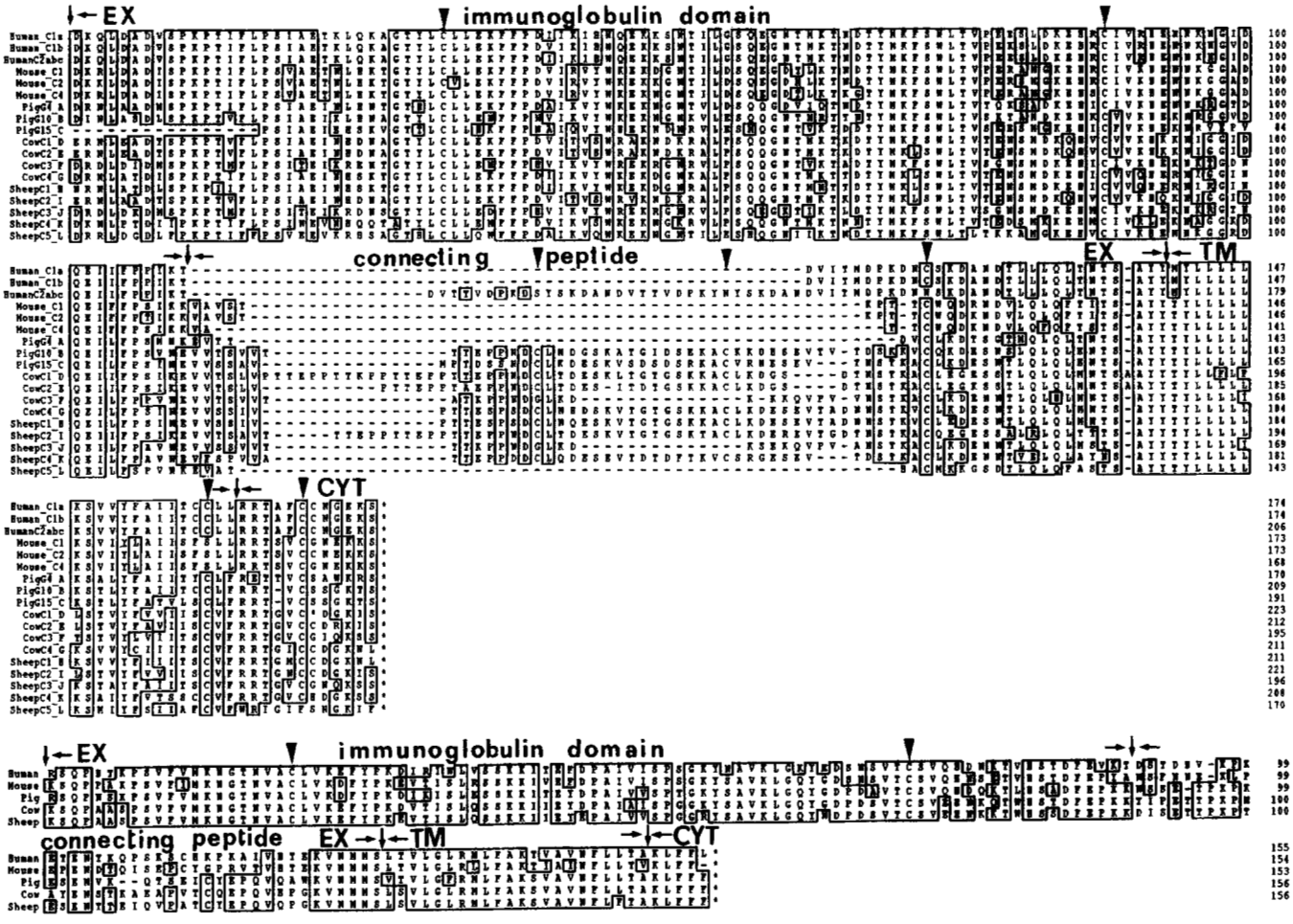

FIGURE 1.—Comparison of the amino acid sequences of Tcr C$\gamma\delta$ regions from human, mouse, pig, cow and sheep. Amino acid similarities are boxed, conserved cysteine residues are marked with ▼. The predicted extracellular (Ex) immunoglobulin domain and connecting peptide, transmembrane (Tm), and cytoplasmic (Cyt) sections of the constant region are indicated. The numbers on the right show protein sequence length. (A) Alignment of Tcr Cγ amino acid sequences. Tcr Cγ Human C1a_1b and C2a_2b are from LEFRANC and coworkers (1986); Tcr Cγ Human C2c is from BURESI and coworkers (1989); Tcr Cγ Mouse C1_C2_C4 are from GARMAN and coworkers (1986), HAYDAY and coworkers (1985), IWAMOTO and coworkers (1986). As regards artiodactyl sequences, for which no information at the genomic level is available, we have renamed them in alphabetical order: A, B, C etc. and we report also the original nomenclature given by the authors as follows: PigG4_A, PigG10_B, PigG15_C, CowC1_D etc. Tcr Cγ PigG4_A and PigG10_B are from THOME and coworkers (1993); PigG15_C is from THOME and coworkers (1994); Tcr Cγ CowC1_D, CowC2_E, CowC3_F are from TAKEUCHI and coworkers (1992); CowC4_G is from ISHIGURO and coworkers (1993); Tcr Cγ SheepC1_H, SheepC2_I, SheepC3_J, SheepC4_K, SheepC5_L are from HEIN and coworkers (1993). (B) Alignment of Tcr Cδ aminoacid sequences. Tcr Cδ Human is from LOH and coworkers (1987); Tcr Cδ Mouse is from CHIEN and coworkers (1987); Tcr Cδ Pig is from THOME and coworkers (1993); Tcr Cδ Cow is from TAKEUCHI and coworkers (1992); Tcr Cδ Sheep is from HEIN and coworkers (1993).

gene segments. Figure 1B shows the alignment between human, mouse and artiodactyl amino acid sequences of Cδ, which demonstrates that these genes are extremely conserved along all protein domains except a small intraconnecting peptide segment where, at position 114, a conserved cysteine residue is present responsible for the inter chain disulphide bond. Phylogenetic trees were constructed for DNA sequences encoding the constant part of γ chain for Tcr $\gamma\delta$ from human, mouse and artiodactyls. The UPGMA method, which assumes a molecular clock, (Figure 2Aa), and the neighbor-joining method, which is insensitive to rate variations among lineages, (Figure 2Ab) were used for nonsynonymous positions. Besides, the amino acid sequences were analyzed by the parsimony method (Figure 2Ac). In all these trees (Figure 2A), two different clusters are observed: the one grouping human and mouse Cγ genes with significant values of bootstrap replicates (55–81%) and the other grouping artiodactyl Cγ cDNA sequences. The results will be herewith discussed considering the trees obtained by the UPGMA method.

The human genes C1 and C2, probably originated through a duplication event, have allelic forms designated $C1^a$, $C1^b$ and $C2^a$, $C2^b$, $C2^c$, respectively (LEFRANC *et al.* 1986; BURESI *et al.* 1989). The allelic forms display a peculiar clustering, namely $C2^{abc}$ and $C1^a$ are indistinguishable and $C1^b$ is significantly more divergent (Figure 2Aa). One suggestion is that the human $C2^{abc}$ gene

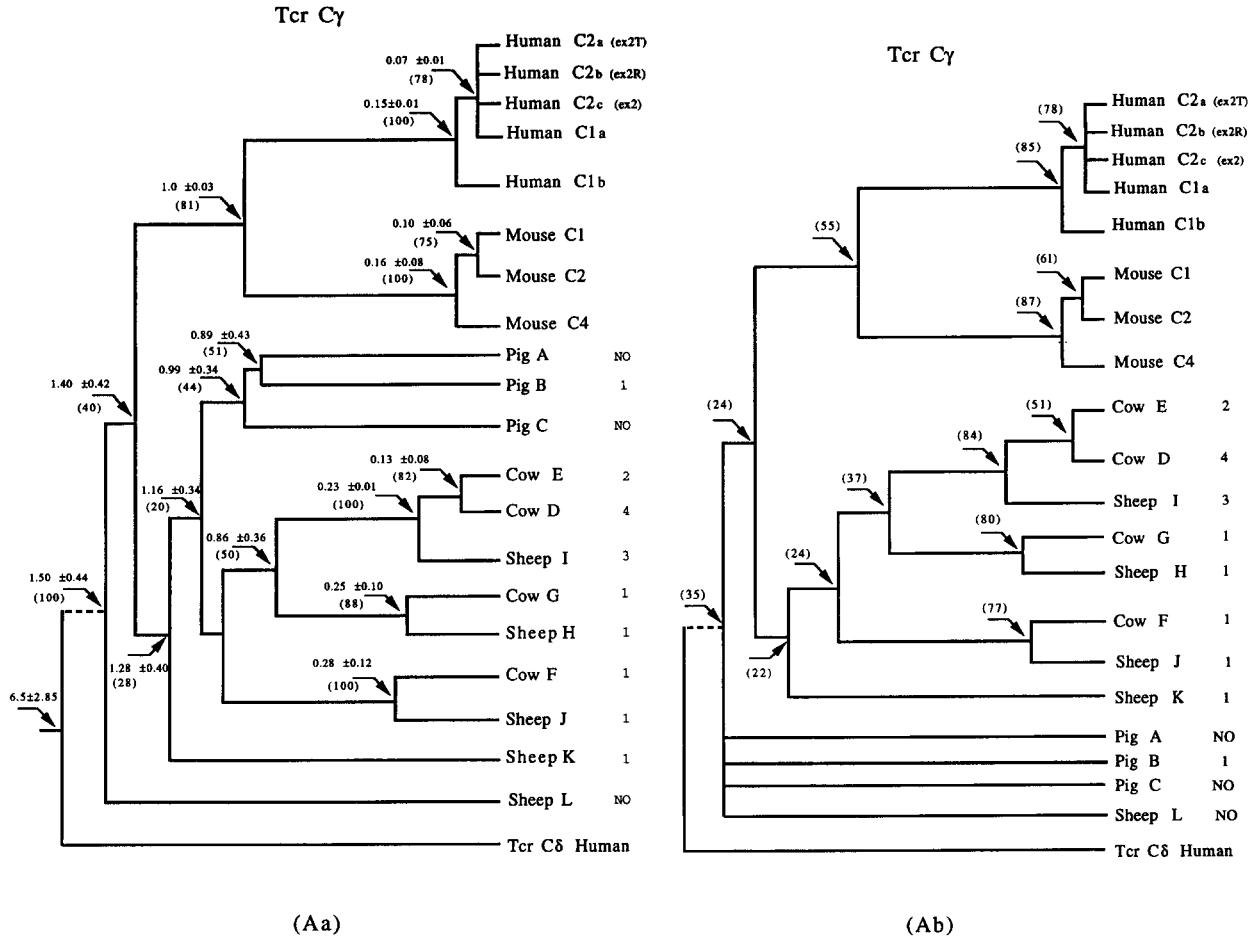

FIGURE 2.—Phylogenetic tree calculated on the nonsynonymous (first plus second) codon positions of Tcr Cγ (A) and Tcr Cδ (B) nucleotide sequences available so far from human, mouse, pig, cow and sheep by using the Stationary Markov Clock (SMC), UPGMA method (Aa, B), neighbor-joining method (Ab) or by parsimony analysis of amino acid sequences (Ac). On each node, the relative time of divergence, with standard error and bootstrap values out of 100 replicates in brackets, are shown. Numbers on the right of the artiodactyl sequences indicate how many times the TTE(K)PP motif is present (A). For other details, see the legend of Figure 1A. UPGMA and neighbor-joining phylogenetic trees of Tcr Cγ and Cδ genes have been rooted using human Cδ and human Cγ sequences, respectively. The parsimony tree (Ac) reports phylogenetic relationships of both Cγ and Cδ clusters.

is the modern form of a sequence that originated by duplication of the $C1^a$ gene by means of an unequal crossing-over event. In mouse, the three genes seem to have originated through two duplication events. It is striking to note that the first duplication event in mouse and the only duplication event in human occurred apparently at the same divergence time, which we calculated to be, within statistical fluctuations, ~12 million years ago (MYa), once the divergence time between human and mouse is fixed at 75 MYa (DAYHOFF 1978). The second duplication event in mouse occurred at ~8 MYa, giving rise to two functional Cγ segments, namely C1 and C2 (Figure 2Aa).

In artiodactyls, where no information is available at the genomic level, the phylogenetic tree of Tcr Cγ nucleotide sequences (Figure 2Aa) shows a situation suggesting we are dealing with genes that underwent a great number of duplication events. Sheep L, occurring ~112.5 ± 33.0 MYa, behaves as the ancestral gene existing before the primate-rodent-artiodactyl evolutionary divergence. Sheep K belongs to the same ancestral pool as sheep L and is more closely related to other artiodactyl genes, supported only by 28% of the bootstrap replicates. The other available sequences of ovine and bovine Tcr Cγ cDNAs show that three nodes, the one related to the cow_F-sheep_J split, the second related to the cow_G-sheep_H split and the third D-E_cow-I_sheep split, correspond, within statistical fluctuations, to a divergence time cow/sheep of ~18 MYa (Figure 2Aa).

It is worthwhile noting that the split between D and E cow sequences could correspond to an intraspecies gene duplication event whose dating (~9 MYa) roughly coincides with that of mouse and human. Moreover the results obtained on cDNA clones (S. CICCARESE, C. LANAVE and C. SACCONE, unpublished results) seem to confirm that the branch representing sheep I sequence probably split in the same way as the cow D-E sequences.

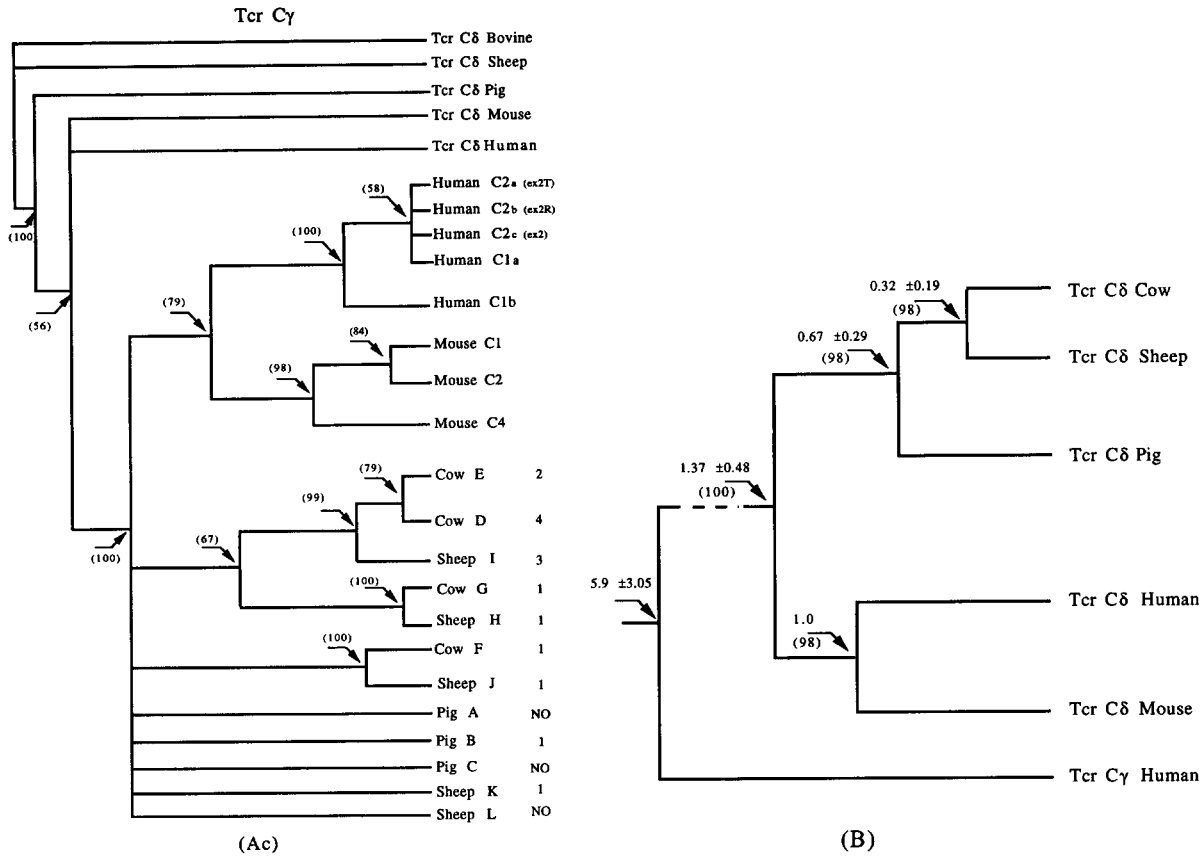

FIGURE 2.—*Continued*

The divergence between the three pig Cγ cDNA sequences, with Pig_A-B more closely related with respect to Pig_C, is supported by 44% of bootstrap replicates, suggesting a very ancient intraspecies diversification. Figure 2B reports the result of the UPGMA method and represents the phylogenetic tree for Tcr Cδ genes, where human and mouse cluster together, leaving artiodactyls apart as an outgroup. This clustering pattern is statistically significant (from 90% to 98% of bootstrap replicates) when the UPGMA, the neighbor-joining or the parsimony methods are used. In addition, this cluster fully agrees with the phylogenetic description we derived from the analysis of Cγ genes. Phylogenetic trees of Tcr Cγ and Cδ genes were rooted using, respectively, human Cδ and human Cγ sequences.

Evolution of other T-cell-related genes: In Table 1 we report the T-cell-related genes under examination for the triplet human-mouse-artiodactyl. In each column is listed the first author and year of the reference for each gene.

The evolutionary behavior of the Tcr Cαβ heterodimer and the CD3 complex has been considered. The behavior of other gene sequences that are related to cell-mediated immune function and are available for the triplet human-rodent-artiodactyl in the databases, namely, Interleukin-2 (IL-2), Interleukin-2 receptor α chain (IL-2Rα), Interleukin-1α (IL-1α) and Interleukin-1β (IL-1β) has been also evaluated.

αβ or γδ Tcr heterodimers are known to be associated noncovalently on the surface of mature T lymphocytes with, so far as is known, five invariant proteins, three of which (CD3 γ, δ and ε) are designated as the CD3 complex. CD3 complex is believed to transduce activation signals across the cell membrane following interactions between the Tcr chains and their ligands (ALEXANDER *et al.* 1989).

Phylogenetic results of human, mouse and artiodactyls Tcr Cα and Cβ indicate two different topologies. In the case of Cα genes, the tree (Figure 3A) agrees with that of γδ Tcr C genes in which human and rodent genes group together (83% of bootstrap value). Tcr Cβ genes, instead, give a phylogenetic tree showing artiodactyls more closely related to human (80% of bootstraps) than to mouse (Figure 3B). Chicken Tcr Cα (GOBEL *et al.* 1994) and Cβ (TJOELKER *et al.* 1990) sequences were used to root phylogenies. Phylogenetic relationships were constructed for human, mouse, cow and sheep CD3 γδ invariant genes rooted by the chicken CD3 sequence (BERNOT and AUFFRAY 1991). UPGMA trees in Figure 4 show that human and mouse are closely related with bootstrap values 66% for CD3γ (Figure 4A) and 62% for CD3δ (Figure 4B).

IL-2, formerly known as T-cell growth factor (GILLIS *et al.* 1978), causes maturation of cytotoxic T-cells, and IL-2Rα is a transmembrane protein expressed by activated T-cells during an immune response (WALDMAN

TABLE 1

T-cell-related genes and T-cell-nonrelated genes analyzed for phylogenetic relationship between the species of human, mouse, pig, cow and sheep

T-cell-related gene products	Human	Mouse	Sheep	Cow	Pig	Chicken
Tcr Cγ	*C1, C2*	*C1, C2, C4*	*C1, C2, C3, C4, C5*	*C1, C2, C3, C4*	*G4, G10, G15*	
	LEFRANC *et al.* (1986), BURESI *et al.* (1989)	GARMAN *et al.* (1986), HAYDAY *et al.* (1985), IWAMOTO *et al.* (1986)	HEIN and DUDLER (1993)	TAKEUCHI *et al.* (1992), ISHIGURO *et al.* (1993)	THOME *et al.* (1993), THOME *et al.* (1994)	
Tcr Cδ	LOH *et al.* (1987)	CHIEN *et al.* (1987)	HEIN *et al.* (1990)	TAKEUCHI *et al.* (1992)	THOME *et al.* (1993)	
Tcr Cβ	*Cb1, Cb2*	*Cb1, Cb2*	*Cb1, Cb2*	*Cb1, Cb2*	*Cb1*	*Cb1*
	YANGI *et al.* (1984); JONES *et al.* (1985)	HEDRICK *et al.* (1984), MALISSEN *et al.* (1984)	GROSSBERGER *et al.* (1993)	TANAKA *et al.* (1990)	THOME *et al.* (1993)	TJOELKER *et al.* (1990)
Tcr Cα	SIM *et al.* (1984)	CHIEN *et al.* (1984)	HEIN *et al.* (1991)	ISHIGURO *et al.* (1990)	THOME *et al.* (1993)	GOBEL *et al.* (1994)
CD3 γ	KRISSANSEN *et al.* (1986)	SAITO *et al.* (1987)	HEIN and TUNNACLIFF (1990)	CLEVERS *et al.* (1990)		BERNOT and AUFFRAY (1991)
CD3 δ	KRISSANSEN *et al.* (1986)	SAITO *et al.* (1987)	HEIN and TUNNACLIFFE (1990)	CLEVERS *et al.* (1990)		BERNOT and AUFFRAY (1991)
IL-2	HOLBROOK *et al.* (1984)	KASHIMA *et al.* (1985)	GOODALL *et al.* (1990)	CERRETTI *et al.* (1986)	GOODALL *et al.* (1991)	
IL-2Rα	COSMAN *et al.* (1984)	MILLER *et al.* (1985)	BUJDOSO *et al.* (1992)	WEINBERG *et al.* (1988)		
IL-1α	GUBLER *et al.* (1986)	LOMEDICO *et al.* (1984)	ANDREWS *et al.* (1991)	MALISZEWSKI *et al.* (1988)	MALISZEWSKI *et al.* (1990)	
IL-1β	BENSI *et al.* (1987)	GRAY *et al.* (1986)	FISKERSTRAND and SARGAN (1990)	MALISZEWSKI *et al.* (1988)	VANDENBROECK *et al.* (1993)	

T-cell-nonrelated gene products	Human	Mouse	Sheep	Cow	Pig	Outgroup
Alpha-globin	V00488	V00714	X70214			M14567
Beta-globin	V00497	V00722		M63453		Y00501
Tryptophan-tRNA ligase	M61715	X69656		X52113		M12081
Serum albumin	L00132	M16111	X17055	X58989		X60688
Glutamine synthetase II	M59834	M60803			Z29636	M29076
Alpha-lactalbumin	J00270	M87863	X06367	X06366	M80520	X15421
Alpha-1-antitrypsin	M11465	M33567	X15555	X63129		Z18906
IL-3	M20137	K03233	Z18291	L31893		
Atrial natriuretic factor	M30262	K02781			M54669	D01043

In T-cell-related genes column the author's references are reported. In T-cell-nonrelated genes the accession numbers from EMBL or GenBank databases are reported.

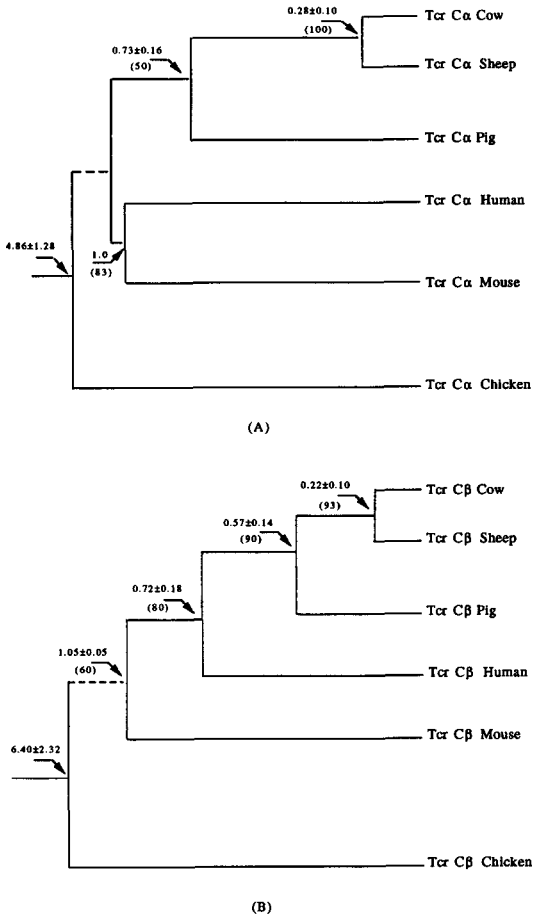

FIGURE 3.—Phylogenetic tree calculated on the nonsynonymous (first plus second) codon positions of Tcr Cα (A) and Tcr Cβ (B) nucleotide sequences available so far from human, mouse, pig, cow, sheep and chicken by using the Stationary Markov Clock and UPGMA method. Chicken Tcr Cα and Cβ sequences were used to root phylogenies. The bootstrap value out of 100 replicates supporting each branch in the consensus tree is indicated in brackets and the relative time of divergence, with standard error, is shown.

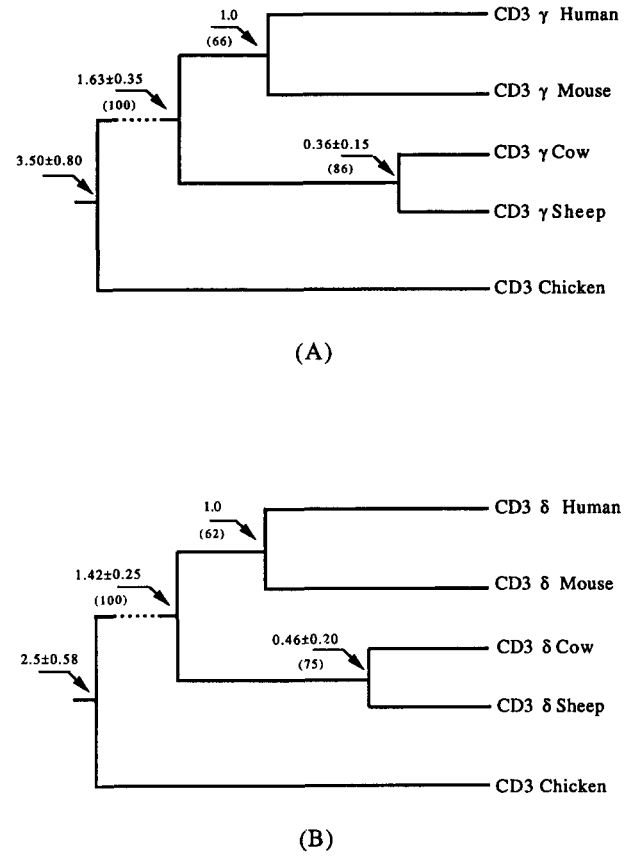

FIGURE 4.—Phylogenetic tree calculated on the nonsynonymous (first plus second) codon positions of CD3 γ (A) and CD3 δ (B) nucleotide sequences available so far from human, mouse, cow, sheep and chicken by using the Stationary Markov Clock (SMC) and UPGMA method. Chicken CD3 sequence has been used to root phylogenies. On each node, the relative time of divergence, with standard error and bootstrap values out of 100 replicates in brackets, evaluated by SMC, are shown.

1989; BUJDOSO *et al.* 1992). IL-1 is the term for the two polypeptides, IL-1α and IL-1β, possessing a wide spectrum of inflammatory, hematopoietic and immune properties. Both forms of IL-1 recognize the same cell surface receptors and share the various biological activities (DINARELLO 1991). At present, for IL-2 and IL-2Rα, no outgroup sequences are available. In parallel for the IL-1 family as a whole, the different subfamilies can serve as outgroups to one another. The same human-mouse clustering with respect to cow and sheep was observed for IL-2 and IL-2Rα trees with bootstrap values of 68% (Figure 5A) and 97% (Figure 5B), respectively. For the Interleukin-1 family genes we found that the IL-1α tree places artiodactyls more closely related to humans (100% of bootstrap replicates) (Figure 6A). In contrast, the IL-1β tree displays human and mouse clustered together (100% of bootstraps) (Figure 6B) in accordance with the tree obtained by analyzing the Tcr Cγδ genes and the other examined genes involved in T-cell related function (Table 1).

Evolution of genes not related to the T-cell immune system: In Table 1 the T-cell-nonrelated genes under examination for the triplet human-mouse-artiodactyl are listed. In the column the accession numbers of EMBL or GenBank databases are listed for each gene.

As previously reported, eight out of 10 genes involved in the immune system, with the exception of Tcr Cβ and IL-1α, report a closer association of human and rodent sequences than of any other artiodactyl sequences. On the contrary, nine other nuclear genes examined that are not involved in the immune system, produced a phylogenetic tree supporting the closer relationship between human and artiodactyls (results not shown).

DISCUSSION

In the present paper we have analyzed a group of 10 genes (Tcr Cγδ, Tcr Cαβ, CD3 γ and CD3 δ, IL-2, IL-2Rα, IL-1α, IL-1β) apparently all with a T-cell-related

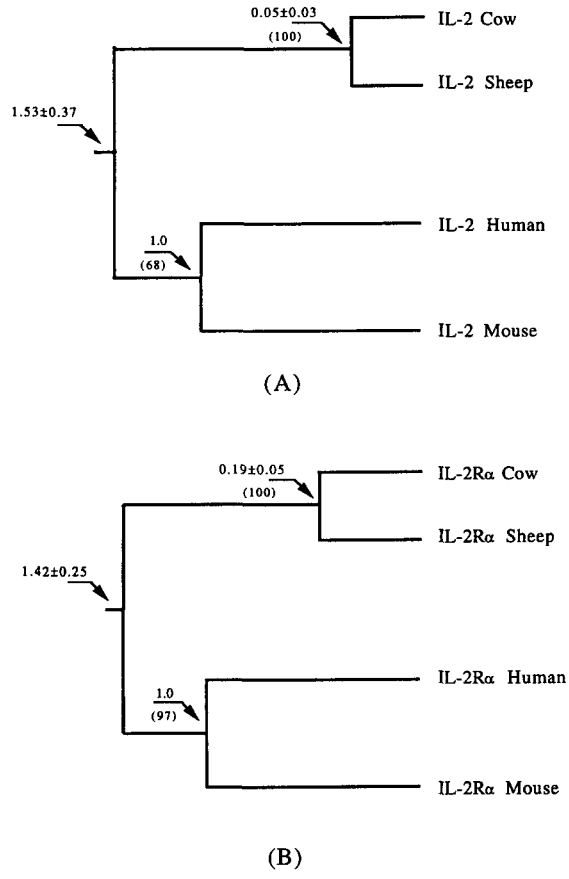

FIGURE 5.—Phylogenetic tree calculated on the nonsynonymous (first plus second) codon positions of IL-2 (A) and IL-2Rα (B) nucleotide sequences available so far from human, mouse, cow and sheep by using the Stationary Markov Clock (SMC) and UPGMA method. On each node, the relative time of divergence, with standard error and bootstrap values out of 100 replicates in brackets, evaluated by SMC, are shown.

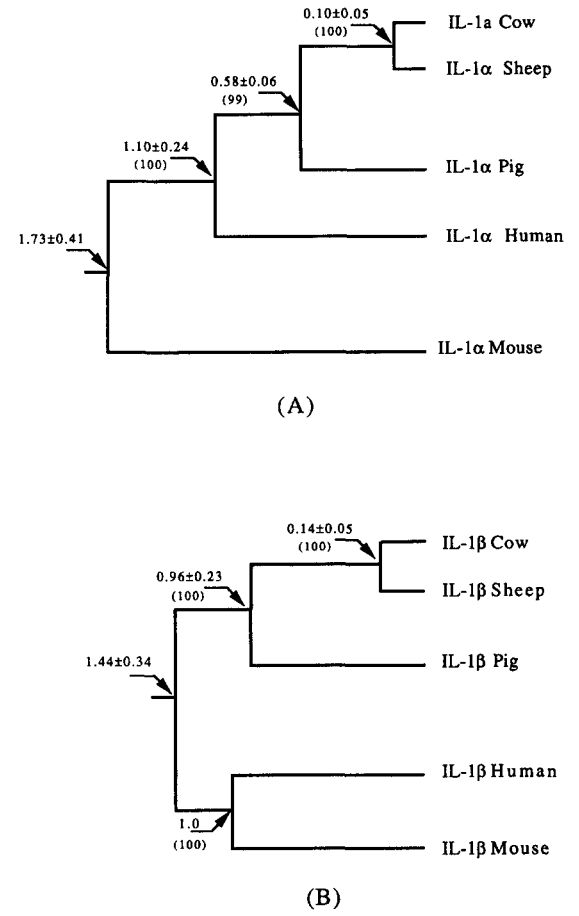

FIGURE 6.—Phylogenetic tree calculated on the nonsynonymous (first plus second) codon positions of IL-1α (A) and IL-1β (B) nucleotide sequences available so far from human, mouse, pig, cow and sheep by using the Stationary Markov Clock (SMC) and UPGMA method. On each node, the relative time of divergence, with standard error and bootstrap values out of 100 replicates in brackets, evaluated by SMC, are shown.

function, and we have always obtained for all save two genes (Tcr Cβ and IL-1α) a phylogenetic relationship that places humans and rodents closer to one another than to artiodactyls, an order of animals that includes the ruminants. The small number of γδ T-cells in rodents and primates (~5% of blood lymphocytes) is well known, as well as the specialized role these cells may have in these species for defense against a limited number of antigens, especially on the mucosal surface (JANEWAY *et al.* 1988). Because of the frequency and physiological distribution of γδ T-cells in different animals, rodents and humans can be defined as "γδ low" species and ruminants as "γδ high" species due to an adaptive role of γδ T-cell function.

The phylogenetic results obtained on Tcr Cγδ sequences of artiodactyls, mouse and human are fully in agreement with the perspective of HEIN and coworkers, who suggest that the five Cγ genes in sheep are descendants of an ancestral pool that existed before the primate-rodent-artiodactyl evolutionary pathways diverged (HEIN and DUDLER 1993). It is relevant to note that the TTE(K)PP motif is more frequently repeated in cow and sheep sequences that experienced duplication events (Figure 2A). Thus a correlation is likely to exist between the high frequency of this motif and the duplication events.

By analyzing Tcr γ constant genes, we have estimated the distance between cattle and sheep to be 18 MYa since the time of divergence between human and mouse has been fixed at 75 MYa (Figure 2Aa). This time of divergence was also reported by ROMERO-HERRERA and coworkers (1973) and was confirmed by data from other authors who used the "Geological Society Phanerozoic time-scale" (1964) with the modification suggested in the Supplement. It is also relevant to note that the unique intraspecies divergence time between artiodactyl sequences (in the case of the split D_cow-E_cow, Figure 2Aa) coincides with that observed for the duplication events occurring in Cγ human and mouse genes that we dated at 12 MYa. This time corresponds to the last Tertiary period in the Miocene (24.6–5.1 MYa), when the mammalian radiation gave rise to a

great number of the genera that persist today. Ruminants remained small and rather rare until the Middle and Late Miocene, when the modern groups radiated.

Tcr Cα genes and genes coding for some of the invariant components of Tcr, the CD3 γ and δ chains, show the same phylogenetic relationships as Tcr C$\gamma\delta$ genes (Figures 3A and 4, Table 1). The IL-2 produced by activated T-cells (SMITH 1988), the IL-2Rα 55-kDa transmembrane protein expressed by activated T-cells and B-cells (WALDMAN 1989, DE TOTERO *et al.* 1995), and IL-1β secreted by monocytes/macrophages with the ability to stimulate T and B lymphocytes (DINARELLO 1991), show the same phylogenetic relationship as Tcr C$\gamma\delta$ genes (Figures 5 and 6B, Table 1). The Cβ Tcr gene phylogeny (Table 1 and Figure 3B) seems to be the only exception to the evolution of Tcr constant chains. It is noteworthy that GROETTRUP and coworkers (1992) put forward the hypothesis according to which the Tcr β homodimer could play a pivotal role in thymocyte maturation, allelic exclusion or induction of Tcr α chain rearrangement. The Tcr β homodimer is expressed on the surface of a pre-T-cell line and transmits a distinct signal to the cell upon triggering from the extracellular enviroment. More recently VON BOEHMER (1995) mentions the discovery of a novel pre-Tcr that promotes expansion and differentiation of thymocytes that have productive Tcr β but not Tcr α rearrangment. Moreover the frequency of productive rearrangments in developing thymocyte subsets indicates that cells with productive Tcr β rearrangments had a greater expansion potential than Tcr β chain-negative cells. For this reason, the different evolutionary relationship might be explained by the fact that Tcr β chain, acting as a starter of the T-cell maturation process, could have a peculiar role and perhaps it is not involved in the same process as all other Tcr constant chains and T-cell related elements.

As far as Interleukin-1 family genes are concerned, we obtained two different phylogenies for IL-1α (Figure 6A and Table 1) and IL-1β (Figure 6B). Remarkable differences exist in how IL-1α and IL-1β structural components are made available for binding to IL-1 receptors, since the "IL-1 membrane form" was found to be almost exclusively IL-1α (DINARELLO 1991). Considering that the amount of IL-1β mRNA found in "stimulated" human peripheral blood mononuclear cells is usually 25- to 50-fold greater than the α form and that the two forms of IL-1 appear to be under separate transcriptional control (DEMCZUK *et al.* 1987; TURNER *et al.* 1989; OHMORI *et al.* 1990), we can speculate that a different functional constraint is at the origin of the different phylogenies. HUGHES (1994), in his paper on the evolution of the Interleukin-1 gene family in mammals, produced similar phylogenetic results on both IL-1α and IL-1β genes by using the minimum evolution method (NEI *et al.* 1986), which is based on the proportion of amino acid differences among sequences.

Since the tree describing primates and artiodactyls as sister taxa and excluding rodents has the most support by both morphological and molecular data (MCKENNA 1975; SZALAY 1977; LI *et al.* 1990; EASTEAL 1992), HUGHES favored the hypothesis that the relationships among the loci encoding the available IL-1β sequences are not orthologous. This hypothesis implies a genetic divergence when the duplication of the loci, presumably by unequal crossing over, occurred independently in different lineages (HARDISON 1991). Obviously the hypothesis that we are dealing with nonorthologous genes cannot be rejected for all the eight above described genes where the phylogenetic relationship of the taxa are different from the canonical one.

In fact we show that eight genes, C segments of Tcr γ, Tcr δ, and Tcr α, CD3 γ, CD3 δ, IL-1β, IL-2 and IL-2Rα, whose products we have named "T-cell-related" proteins, behave always and significantly in the same manner, putting (in the triplet human-mouse-artiodactyl) artiodactyls as an outgroup. Thus we believe it highly improbable that all examined genes are paralogous, since it would require that duplicative events would have to have occurred several times in different loci and yet give the same phylogeny. To explain this peculiar phylogeny, we can also speculate that this group of genes involved in the cell-mediated immune system evolved much faster in artiodactyls, accumulating a great number of mutations that turned out to be advantageous for the enviromental conditions of their life and were thus naturally selected and then fixed. In his more recent paper, HEIN (1994) argues that in humans, mice and other species $\gamma\delta$ Tcr and Ig molecules represent the earliest separation between specific cell-mediated and humoral immunity, with the $\alpha\beta$ Tcr emerging later. He proposes that a set of ligands expressed on epithelia mediated the earliest selection of the progenitor $\gamma\delta$ T cells, which had a broader set of functions related mainly to the monitoring and protection of body surfaces. It seems likely that in ruminants, included in the artiodactyls order, $\gamma\delta$ T cells have acquired a T-cell mode antigen recognition involving presentation by the ligands expressed mainly on epithelia.

In any case, our results suggest that in artiodactyls, defined as "$\gamma\delta$ high" species, the majority of genes involved in T-cell-mediated function coevolve under the same evolutionary pressure.

In conclusion we believe these data are relevant to important evolutionary issues. They show how misleading a phylogeny may be when it is based on a single or on a few homologous genes, since the inferred phylogeny may not necessarily agree with the species phylogeny. In addition they demostrate that genes having a mutual functional relationship may coevolve in a lineage specific manner probably depending on environmental conditions.

We thank Dr. P. BARSANTI for his critical comments. We also thank anonymous referees for critical comments on a previous version of

this article. This work was supported by Progetto Finalizzato CNR Ingegneria Genetica, Progetto Finalizzato CNR RAISA, subproject 3, paper no. 1723 and MURST, Italy.

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Communicating editor: W-H. LI