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Platypus *TCRμ* Provides Insight into the Origins and Evolution of a Uniquely Mammalian TCR Locus

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TCRμ is an unconventional TCR that was first discovered in marsupials and appears to be absent from placental mammals and nonmammals. In this study, we show that TCRμ is also present in the duckbill platypus, an egg-laying monotreme, consistent with TCRμ being ancient and present in the last common ancestor of all extant mammals. As in marsupials, platypus TCRμ is expressed in a form containing double V domains. These V domains more closely resemble Ab V than that of conventional TCR. Platypus TCRμ differs from its marsupial homolog by requiring two rounds of somatic DNA recombination to assemble both V exons and has a genomic organization resembling the likely ancestral form of the receptor genes. These results demonstrate that the ancestors of placental mammals would have had TCRμ but it has been lost from this lineage. ***The Journal of Immunology*****, 2011, 187: 5246–5254.**

Conventional T cells exist in two distinct lineages based on the composition of their TCR heteroduplex: αβ T cells use a TCR composed of α- and β-chains, whereas γδ T cells use γ- and δ-chains. Like Ig, the Ag-binding V domains of the TCR chains are encoded by exons that are assembled from gene segments by somatic DNA recombination. All jawed vertebrates have both αβ and γδ T cells, and the genes encoding these four TCR chains are highly conserved both in sequence and organization (1–3). Recently, a fifth locus encoding TCR chains, named *TCRμ*, was found in marsupial mammals (4). *TCR*μ contains C regions related to TCRδ but is transcribed in a form that would include double V domains that are more related to Ig H chain V region (VH) than to TCR V genes (2, 4, 5). TCRμ does not substitute for TCRδ in marsupials because the genes encoding conventional TCRδ-chains are highly conserved and expressed (2, 6).

TCRμ genes are distinct and unlinked to those that encode conventional TCR chains and have atypical gene organization. The N-terminal V of TCRμ (Vμ) is encoded by somatically recombined genes (V, D, and J), with the recombination taking place in thymocytes, resulting in clonal diversity (4). The second, C-proximal V domain (Vμj) is encoded by an exon in which the V, D, and J genes are already prejoined in the germline DNA and are relatively invariant (4). This is the only known example of germline-joined V genes being used in a TCR. The *TCRμ* locus is also organized in tandem clusters, which is also atypical of TCR genes (2, 4).

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Abbreviations used in this article: CP, connecting peptide; CT, cytoplasmic; FR, framework region; L, leader; MYA, million years ago; NAR, new Ag receptor; RSS, recombination signal sequences; TM, transmembrane; UTR, untranslated regions; VH, Ig H chain V region.

Searching the available placental mammal, avian, and amphibian genomes failed to uncover TCRμ orthologs (2). However, in this study, we show that TCRμ is present in a monotreme, the duckbill platypus *Ornithorhyncus anatinus*. The monotremes are oviparous mammals that last shared a common ancestor with marsupials and placentals at least 165 million years ago (MYA) (7). The genomic organization of the platypus *TCRμ* locus reveals insight into the evolution of this uniquely mammalian TCR locus and supports its ancient presence in mammals.

Materials and Methods

Whole genome analysis and annotation

Analyses were performed using the platypus genome assembly version 5.0.1 available at GenBank (http://www.ncbi.nlm.nih.gov/genome/guide/platypus/). Marsupial Cμ sequences were used to search based on homology using the BLAST algorithm (4, 5, 8). Scaffolds containing Cμ sequences were retrieved, and exon boundaries were determined by the presence of canonical mRNA splice sites. Platypus cDNA sequences were used to search against the *O. anatinus* genome project to identify the genomic V, D, and J gene segments. The beginning and end of each coding exon of V, D, and J gene segments were identified by the presence of mRNA splice sites or flanking recombination signal sequences (RSS). Supplemental Fig. 1 shows the location of each TCRμ V, D, J, and C segments on the scaffolds. Platypus TCRδ-chain C region sequence (GenBank accession number XM_001516959) was used to identify the single-copy platypus Cδ on scaffold 588, which is separate from any of the scaffolds containing the putative platypus TCRμ sequences.

PCR and cDNA analyses

A spleen cDNA library constructed from tissue from a Tasmanian platypus was screened by PCR (9). All PCR primer sequences used in this study are presented in Table I. PCR amplification was performed using Advantage-HF 2 PCR (BD Biosciences, Clontech Laboratories, Palo Alto, CA) with the following conditions: denaturation at 94˚C for 1 min for 1 cycle, followed by 34 cycles of 94˚C for 30 s, annealing/extension at 62˚C for 4 min, and a final extension period of 68˚C for 5 min. Forward and reverse primers complementary to sequence internal to the platypus Cμ exon were paired with primers in the λgt10 vector used to construct the library to amplify clones containing the 5′ and 3′ untranslated regions (UTR) (10). This approach generated the partial cDNA sequences analyzed. Full-length platypus TCRμ cDNA sequences were isolated by PCR using primers complementary to 5′ and 3′ UTR. PCR products were cloned using TOPO TA cloning Kit (Invitrogen, Carlsbad, CA) and sequenced using the Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The GenBank accession numbers of the cDNA sequences described in this study are: clone 21, GU458338; clone 26, GU458339; clone 2.22, GU458341; clone 3815, GU475137; clone 1951, GU475138; clone 1953,

Table I. Sequences and description of oligonucleotide primers used

Sequence (5′- to -3′)	Orientation	Region
CCTGGGCAGTGGGGGCCATGGCCTG	R	Cμ
GGGATAGTAATCTTTCACCAGGCAAG	R	Cμ
AGCAAGTTCAGCCTGGTTAAG	F/R	λ gt10 vector
ATTATGAGTATTTCTTCCAGGGTA	F/R	λ gt10 vector
CCCAACCCATGGTCTTTGTCATG	F	Cμ
GGAACCAGAGCTTCGCTGCTTGCC	F	Cμ
AACCATGCTGGTCCAGGTC	F	5′ UTR
CAGGAGGGAAATGATTCAGG	R	3′ UTR
CGGAAACAAAAGAAGGCAGA	R	3′ UTR
CGTGAAATACTCGGGGGAAT	F	Vμ1
AGGCTCTGCATTGATCTTCG	F	Vμ2

F, forward; R, reverse.

GU475139; clone 1954, GU475140; clone 1955, GU475141; clone 4951, GU475142; clone 4942, GU475143; clone 786, GU475144; clone 6, GU458343; clone 17, GU475135; clone 2.34, GU458340; clone 10 GU264000; clone 36, GU475136; clone 4966, GU475145; and clone 1.22, GU458342.

Phylogenetic analysis

Phylogenetic analyses were performed on nucleotide alignments using the MEGA4 program (11) with unweighted pair group with arithmetic mean, maximum parsimony, neighbor-joining, and minimum evolution methods. Amino acid translations were first aligned to establish gapping and then converted back to nucleotide using the BioEdit program (12).

The GenBank accession numbers of the sequences used in the phylogenetic analyses of TCRμ C and V region sequences were: Cβ sequences are echidna, AY423735; platypus, XM_001509180; opossum, AY014507; human, AF043178; mouse, FJ188408. Cγ sequences are opossum, DQ499632; platypus, DQ011295; human, X15019; and mouse, X03802. Cα sequences are echidna, DQ011301; platypus, XM_001507799; opossum, AY014504; human, FJ79357; and mouse, DQ186679. Cδ sequences are platypus, XM_001516959; human, M21624; mouse, M37694; bandicoot, AY955295; opossum, XP_001379771; wallaby, AY238447; frog, GQ262033 and GQ262033; and chicken, XM_423780. Cμ sequences are wallaby, AY956350; bandicoot, AY955293; and opossum Cμ sequences are from MonDom5 scaffold 3.430000001-435000000 (13). The sequences of platypus Cμ used in the alignment are from platypus assembly version 5.0.1, and scaffold locations are presented in Supplemental Fig. 1: wallaby Vδ48, AY238448; wallaby Vδ51, AY238451; bandicoot Vδ46, DQ076246; cattle Vδ13, D16113; human Vα96, Z14996; human Vα34, AB360834; human Vβ04, M27904; mouse Vβ16 M15616; cattle Vβ19, D90129; rabbit Vβ19, D17419; sheep Vβ11, AF030011; human Vγ29, M13429; mouse Vγ38, M13338; cattle Vγ 88, U73188; sheep Vγ98, Z12998; platypus Vγ95, DQ011295; platypus Vγ19, DQ011319; shark NAR62, AY114762; shark NAR78, AY114978; shark NAR82, AY261682; shark NAR60, EU213060; shark TNAR05, DQ022705; shark TNAR88, DQ022688; shark TNAR10, DQ022710; and opossum Vμ sequences are DQ979402, DQ979398, EF503722, EF5037719, DQ979397, DQ979396, EF503721, and EF503718. The sequences of platypus Vμ used in the alignment are from platypus assembly version 5.0.1, and locations are presented in Supplemental Fig. 1: frog VHδ sequences are GQ262028, GQ262032, and GQ232013. IgVH sequences are: possum VH50, AAL87470; possum VH1, AAL87474; bandicoot VH5.1, AY586158; opossum VH sequences are from MonDom5 scaffold 1.295000001-300000000 (12); mouse VH3660, K01569; mouse VH3609N, X55935; mouse VHDNA4, M20829; mouse VHJ588, Z37145; mouse VHJ606, X03398; mouseVHQ52, M27021; mouse VHS107, J00538; mouse VHSM7 M31285; mouse VH11, Y00743; and mouse VH98, AJ851868. Human VH sequences were obtained from the VBASE database. Other sequences are: pig VH3, U15194; cattle VH, AF015505; sheep VH, Z49180; echidna VH7g, AY101438; echidna VH8g, AY101439; echidna VH51g, AY101442; platypus VH29, AF381294; platypus VH26, AF381293; platypus VH3, AF381314; and platypus VH53, AF381304.

Results

Identification of a platypus TCRμ homolog

Fifteen gene sequences with similarity to opossum Cμ were identified in the platypus whole genome assembly (14). Searching the unassembled, raw trace sequences from the platypus whole-genome shotgun sequence did not uncover any additional genes with homology to opossum Cμ. Six of these contained complete open reading frames and were used in all subsequent analyses (Supplemental Figs. 1, 2). When compared with opossum Cμ and conventional TCR C genes from a variety of mammals, the platypus sequences had greatest nucleotide identity to opossum Cμ (Supplemental Fig. 2, Table II). Included in these analyses was the single-copy conventional platypus TCRδ C gene, which is located on scaffold 588 in the genome assembly, separate from any of the TCRμ-related genes (Fig. 1, Table II, and data not shown). Phylogenetic analyses using several models for tree reconstruction result in the platypus and marsupial Cμ together forming a well-supported monophyletic clade consistent with having identified the platypus TCRμ homolog (Fig. 1).

Platypus TCRμ is transcribed in a double V form

To investigate the structure of expressed platypus TCRμ, full-length transcripts were isolated from a spleen cDNA library. Transcripts averaged 1300 bp in length, which is longer than a conventional TCR transcript and more similar to the double V encoding opossum TCRμ (Fig. 2, Table I). Each encoded a leader (L) peptide followed by two complete V domains, designated V1 and V2 for the 5′ (N-terminal) and 3′ (C-proximal) domains, respectively. They also contained one C domain along with sequences corresponding to the connecting peptide (CP), transmembrane (TM), and cytoplasmic (CT) regions typical of *trans*-membrane TCR chains (Fig. 2). The clones encoded conserved residues found in conventional TCR including cysteines forming intrachain disulfide bonds in the V and C domains as well as interchain disulfide bond in the CP (Fig. 2). The framework region (FR) 4 of V1 and V2 contain the sequence YGXG and FXXG, respectively, similar to the conserved FGXG motif in conventional TCR and marsupial TCRμ (4, 15, 16) (Fig. 2). Also present are two positively charged amino acids (arginine and lysine) in the TM region that, in conventional TCR chains, participate in association with the CD3 signaling complex (17). Comparison to the genomic sequence revealed that the CP is unusual in platypus TCRμ in that it is encoded on two exons, designated CP1 and CP2 with the conserved cysteine in CP2 (Fig. 2). This is unlike the opossum TCRμ and most conventional TCR in which the CP is encoded by a single exon (4).

Both V1 and V2 are encoded by somatically recombined genes

The germline genes encoding the V1 and V2 domains were identified by comparing 18 unique V1 and 16 V2 sequences from both partial and full-length platypus splenic cDNA clones to the genome assembly. V1 and V2 domains share <65% nucleotide identity to each other and, by convention, are encoded by different V gene subgroups designated Vμ1 and Vμ2, respectively. Nine Vμ1 and six Vμ2 genes were identified in the germline sequence (Supplemental Fig. 1). All nine of the Vμ1 genes contained upstream exons encoding a conserved L sequence; however, none of the Vμ2 germline genes had an L exon (not shown). The sequences corresponding to FR4 in V1 and V2 were also used to identify 8 Jμ1 and 12 Jμ2 genes, respectively. Jμ1 and Jμ2 are easily distinguished by length and sequence, with Jμ1 being shorter and sharing <50% nucleotide identity with Jμ2 genes (Fig. 3). All Vμ and Jμ genes were flanked by conserved RSS, the recognition substrates for the RAG product (18). The RSS flanking the Vμ and Jμ genes contained 23- and 12-bp spacers, respectively, typical of TCR genes (Fig. 3). In all cDNA sequences analyzed, Vμ1 were recombined to Jμ1 and Vμ2 to Jμ2. These results support that both the V1 and V2 domains in platypus

Table II. Comparison of platypus Cμ with opossum Cμ and conventional mammalian TCR C regions

	Platypus Cμ (n = 6)	Platypus Cδ (n = 1)	Opossum Cμ (n = 8)	Cδ[a] (n = 5)	Cα[b] (n = 5)	Cβ[c] (n = 5)	Cγ[d] (n = 4)
Platypus Cμ (n = 6)[e]	80–98 (84)	43–47 (44)	50–56 (52)	41–54 (50)	21–26 (24)	25–32 (29)	27–33 (31)
Platypus Cδ (n = 1)	43–47 (44)	100	43–47 (45)	46–53 (50)	26–30 (28)	29–32 (30)	29–33 (32)
Opossum Cμ (n = 8)	50–56 (52)	43–47 (45)	75–96 (83)	41–54 (48)	21–30 (25)	26–34 (30)	26–33 (29)
Cδ (n = 5)	41–54 (50)	46–53 (50)	41–54 (48)	55–83 (67)	21–30 (25)	24–31 (27)	28–34 (31)
Cα (n = 5)	21–26 (24)	26–30 (28)	21–30 (25)	21–30 (25)	45–87 (54)	22–33 (27)	24–33 (27)
Cβ (n = 5)	25–32 (29)	29–32 (30)	26–34 (30)	24–31 (27)	22–33 (27)	63–93 (72)	28–36 (31)
Cγ (n = 4)	27–33 (31)	29–33 (32)	26–33 (29)	28–34 (31)	24–33 (27)	28–36 (31)	48–76 (54)

Values are range of percent nucleotide identity (mean percent nucleotide identity).
[a]Cδ sequences of human, mouse, opossum, bandicoot, and wallaby.
[b]Cα sequences of human, mouse, opossum, echidna, and platypus.
[c]Cβ sequences of human, mouse, opossum, echidna, and platypus.
[d]Cγ sequences of human, mouse, opossum, and platypus.
[e]Number of sequences included in the comparison.

TCRμ are encoded by exons that are fragmented in the germline DNA and undergo RAG-mediated V(D)J recombination.

The sequences corresponding to CDR3 differed both in length and diversity between the V1 and V2 domains (Fig. 2). The V1 CDR3 are longer and up to 22 codons in length, whereas none of the V2 CDR3 exceeded 12 codons. Using the V1 CDR3 sequences identified 35 putative Dμ genes in the platypus genome assembly, all of which were asymmetrically flanked by RSS containing a 12-bp spacer on the 5′ side and 23-bp spacer on the 3′ side, as is typical of TCR D genes (Supplemental Fig. 3). Based on length and nucleotide identity, the D genes fell into two groups designated Dμ1 and -2. Dμ1 (n = 20) contained coding regions 10–13 nucleotides in length, whereas Dμ2 (n = 15) were 18 to 19 nucleotides (Supplemental Fig. 3). There was >75% nucleotide identity within each group but <40% nucleotide identity between Dμ1 and Dμ2 genes. Although Dμ genes could be distinguished in the genomic sequence, individual contributions to the V1 junctions were difficult to establish due to their similarity and short length. Nonetheless, it was possible to determine that the Vμ1–Jμ1 junctions contained two, three, or four Dμ genes, in an ~1:2:1 ratio, similar to the multiple D genes found in opossum TCRμ rearrangements (Fig. 4, Supplemental Table I). Typical of D gene segments, the Dμ present in V1 junctions were used in multiple reading frames (Supplemental Fig. 3). The gene segments encoding the V1 domains demonstrated extensive trimming and no evidence of P nucleotide additions, although N nucleotide additions were common (Fig. 4).

In contrast to V1, the CDR3 of 14 of the 16 V2 cDNA sequences could be accounted for entirely by recombination between germline Vμ2 and Jμ2 genes, with evidence for P and N nucleotide

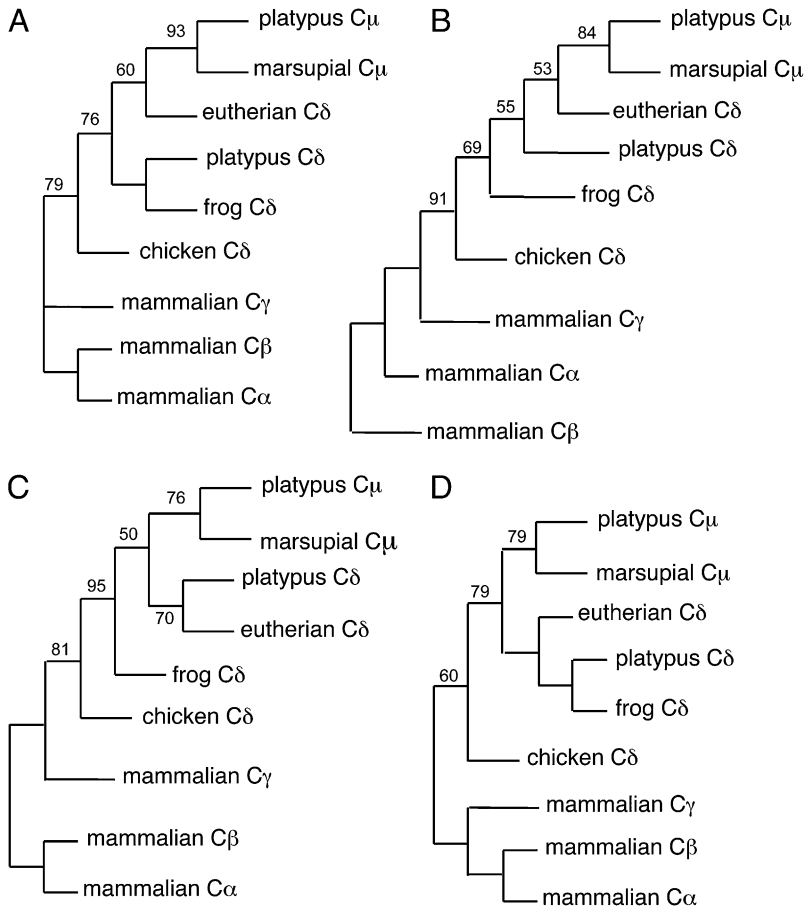

FIGURE 1. Phylogenetic analyses of platypus and marsupial Cμ and C regions from conventional TCR chains. Phylogenetic relationship between Cμ and other conventional TCRs are simplified according to the phylogenetic trees constructed using different methods: neighbor-joining (*A*); maximum parsimony (*B*); unweighted pair group method with arithmetic mean (*C*); and minimum evolution (*D*). All phylogenetic analyses are based on nucleotide alignments, and branch support is indicated as the percentage out of 1000 bootstrap replicates.

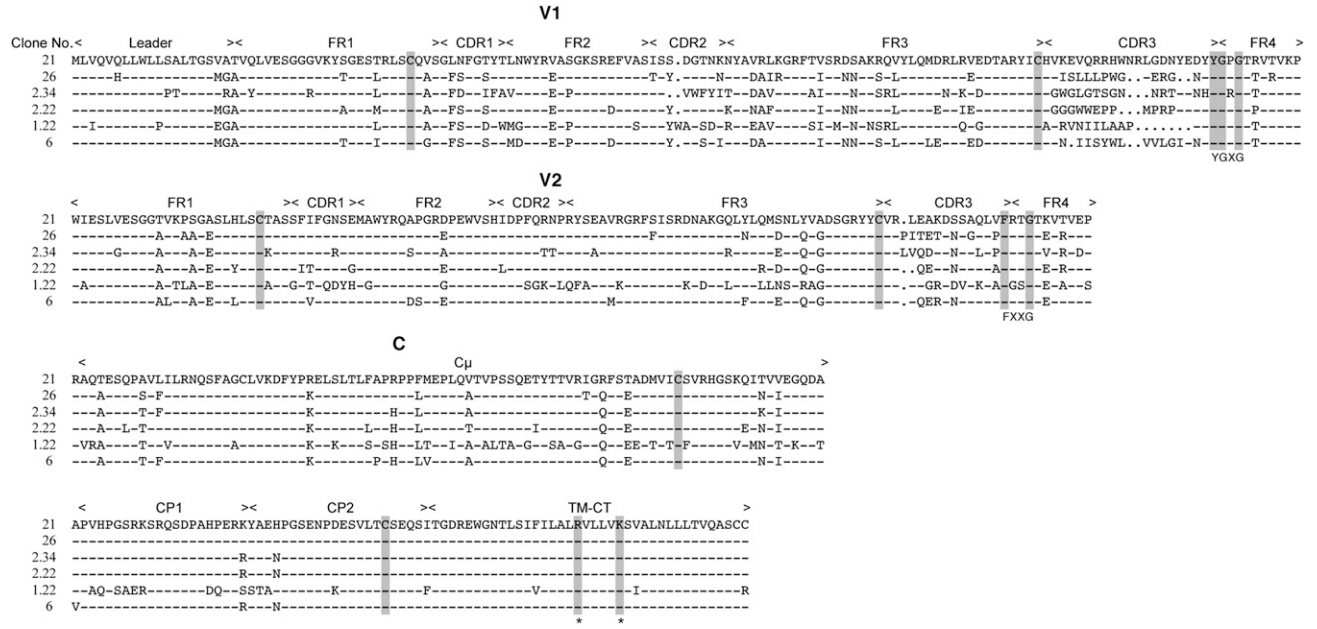

FIGURE 2. Predicted amino acid alignment of full-length platypus TCRμ cDNA clones. Dashes indicate identity, and gaps introduced to the alignment are shown as dots. The sequences were divided into the leader, V1, V2, and C domains. The FR and CDR of the V domains along with the Cμ, CP, and TM-CT of C domain are shown above the sequence alignment. Conserved cysteines are shaded in gray. Conserved lysines and arginines are shaded and indicated with an *. Conserved residues YGXG and FXXG in FR4 of the V1 and V2 domains, respectively, are noted. The borders of CDR and FR are indicated above the sequences.

additions but no Dμ genes being incorporated (Fig. 4, Supplemental Table I). The remaining two clones contained a short stretch of four or five nucleotides that matches Dμ2.8 and cannot be ruled out as being from a D segment. Whether this is coincidence or evidence of a D segment is not clear and is not evident from the genomics in which no Dμ has been found between Vμ2 and Jμ2 gene segments (see below). These results are consistent with the longer CDR3 in V1 domains being due to incorporation of multiple D segments and the shorter V2 CDR3 being the result of direct V to J recombination in most, if not all, junctions.

Platypus TCRμ V genes are related to clan III VH genes

The relationship Vμ genes have to each other and with V genes from Ig and conventional TCR was investigated by phylogenetic analyses. These analyses included VH from the platypus *IgH* locus (19). The results of these analyses support Vμ1 and Vμ2 each forming their own distinct clades with strong bootstrap support (99 to 100%), consistent with their designation as separate subgroups (Fig. 5). Furthermore, the platypus Vμ subgroups together form a single clade nested within mammalian clan III VH genes. This is in contrast to the marsupial Vμ (Vμ and Vμj), which are not monophyletic but are closely related to VH (Fig. 5, Table III) (4).

Platypus TCRμ genomic organization

The *TCRμ* locus is not fully assembled in the current version of the platypus genome, but rather was scattered on 55 separate scaffolds ranging in length from <1 kb up to 64.8 kb (Supplemental Fig. 1). Seventeen of the 35 Dμ segments were on scaffolds also containing Vμ, Jμ, and/or Cμ sequences, supporting their being part of a larger *TCRμ* locus (Supplemental Fig. 1). Combining the scaffold analyses with the cDNA sequences reveals a minimal model for the organization of the platypus *TCRμ* locus. Three scaffolds contain multiple Dμ either transcriptionally downstream of Vμ1 genes (scaffold 3930) or upstream of a Jμ1 gene (scaffolds Ultra190 and 19044) consistent with the evidence from cDNA sequences having multiple Dμ in the junctions between Vμ1 and Jμ1 genes (Fig. 6*A*, Supplemental Fig. 1). One scaffold (28416) contains single Vμ2 and Jμ2 genes that correspond to those used in expressed recombinations (Fig. 6*A*, Supplemental Fig. 1, Supplemental Table I). However, no Dμ genes were found on this scaffold consistent with the lack of D segments in the majority of Vμ2–Jμ2 junctions (Figs. 4, 6, Supplemental Fig. 1, Supplemental Table I).

Full-length cDNA clones containing similar or identical Vμ1 sequence also had similar or identical Jμ1, Vμ2, Jμ2, and Cμ (Supplemental Table I). The most parsimonious explanation for these observations is a cluster organization of platypus TCRμ genes, similar to that found in marsupials (4). In other words, the V, D, and J genes encoding V1 domains are upstream of the V and J gene segments encoding V2, followed by Cμ (Fig. 6*B*). Consistent with this prediction, three scaffolds (19044, 26255, and 33931) contain Jμ1 genes upstream of Vμ2 genes, and many of the scaffolds containing Cμ genes also contained an upstream Jμ2 (Fig. 6*A*, Supplemental Fig. 1). A conservative model for the organization of the platypus TCRμ genes is presented in Fig. 6*B*. The model may be overly conservative because two cDNA clones appeared to use different Vμ1 but the same Jμ1, whereas two others appeared to use the same Vμ1 recombined to two different Jμ1 (compare clones 2.34, 10, and 17 in Supplemental Table I). These results imply there may be multiple Vμ1 and Jμ1 in some clusters or alternatively may be due to *trans*-cluster recombination, as has been found for both opossum TCRμ and shark TCRδ genes (4, 20).

To estimate the possible number of TCRμ clusters, the number of unique Cμ sequences that could be isolated from an individual platypus was determined. PCR was performed on genomic DNA from a single platypus using primers designed to amplify all 15 Cμ identified in the genome assembly. Twenty individual clones were sequenced and yielded nine distinct C sequences consistent

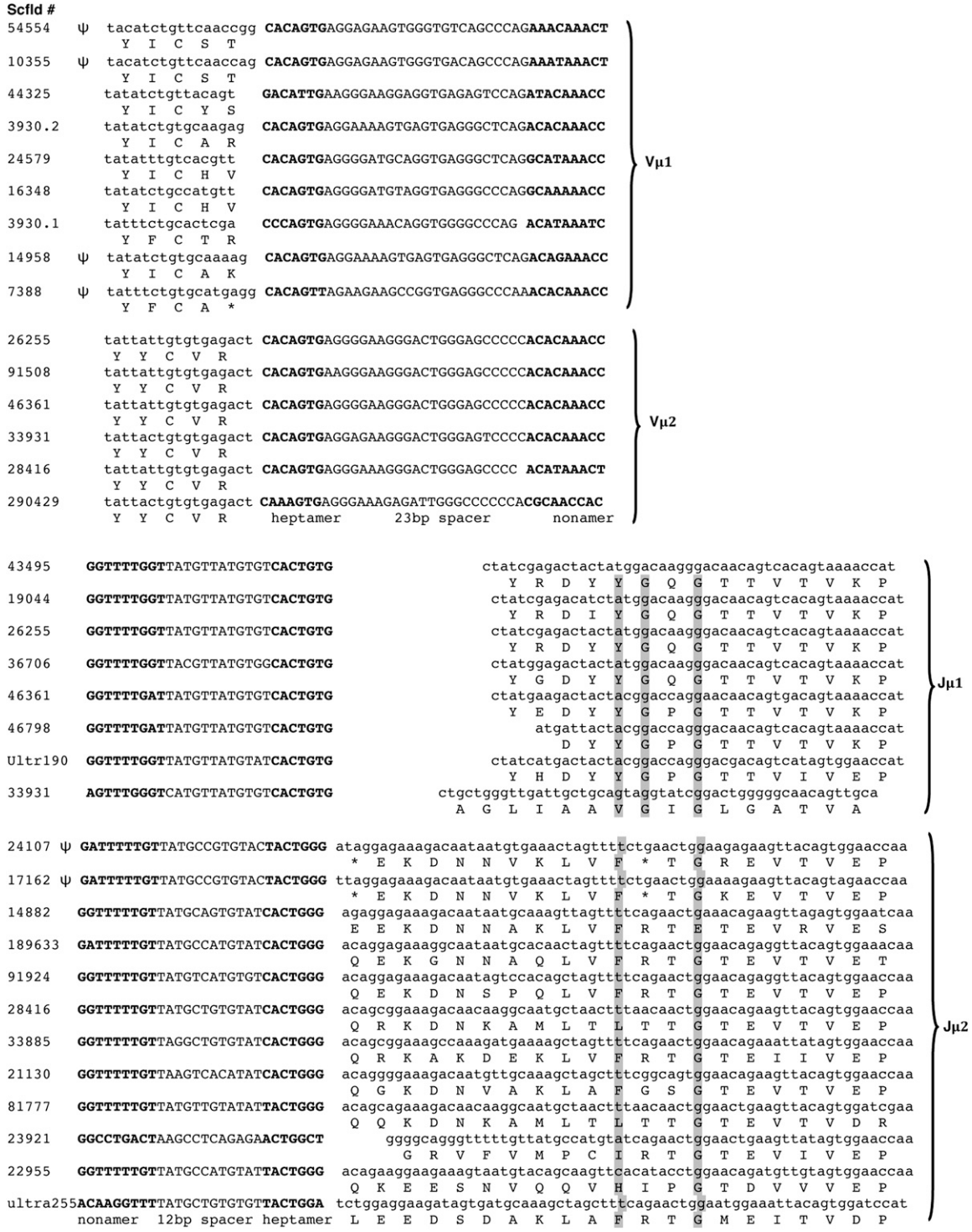

FIGURE 3. Nucleotide sequence and translations of the 3′ end of Vμ1 and Vμ2 gene segments and complete Jμ1 and Jμ2 gene segments. RSS flanking V and J gene segments in platypus genome are indicated. The scaffolds on which V and J sequences were identified are shown on the left. Pseudogenes are indicated by ψ. Stop codons are indicated with an *. Nucleotide sequences of V and J genes are shown in lowercase with amino acid sequences underneath, whereas the RSS sequences are shown in uppercase. Heptamers and nonamers are in bold, and 12-bp or 23-bp spacers are indicated. The YGXG and FXXG conserved motifs corresponding to FR4 are shaded.

with at least five Cμ exons per haploid platypus genome (not shown). This number is slightly lower but not significantly different from what would be predicted from the platypus whole genome sequence in which 15 different Cμ were identified or a minimum of eight per haploid genome. Whether this is an artifact of the assembly or normal platypus variation remains to be determined.

Discussion

The discovery of a platypus TCRμ homolog confirms that this unconventional TCR locus is not unique to marsupials but rather it is ancient in the mammalian lineage and appeared prior to the divergence of the prototherian (monotremes) and therian (marsupial and placental) mammals >165 MYA (7). TCRμ was clearly retained in the marsupial lineage and, therefore, would have been

A

Clones No.	V1 3′ end	N	D	N	D	N	D	N	D	N	J1 5′ end
21	CGGTACATCTGCCACGTT	A	AGGAGG	TCCAACGTCGGC	ACTGGA		ATAGG	TTGGG	GGACAA	C	TATGAAGACTACTATGGACAGGAACAAGA
26	CGGTATATCTGCCACGTT	ATATCGCT	ACTAC	TCCCTTGGGGTT	TGGAAC	G	TGGG		GGAAC		GTGGGAACTACTACGGACCAGGAACAACA
2.22	CGGTATATCTGCCAC	GGGGG	AGGATGG		TGGG		GGAAC	CTCCT	ATGCCT	CGCCCC	TATGAAGACTACTACGGACCAGGAACACCA
3815	CGGTATATCTGCCACGTT		AGACTG	AAGTTGATTTTC	CATAG	TTATGA	GAAC				ACTACTACGGACCAGGAACACCA
1915	CGTTATATCTGCCAC	GTGTT	TGGAGC	ATATGTG	GGAAC	GGGT	TGGG	GCCAT			TATGAAAACTACTACGGACCAGGAACAACG
1953	CGGTATATCTGCCACGTT	AGAGTTCTTT	TGGA		AGACTGGT		TGGG	ATCCTATTAT	GAGGA	C	TACTACGGACCAGGAGCAACA
1954	CGGTATGTCTGTCACGTT	CG	TGGAGC	TTGG	CTTA	GTTAT	GAGGA	C			TATGAGGACTACTACGGACCAGGAACAACA
1955	CGGTATATCTGT	CCTCGA	AGAGGAC	CT	ACTGGA		AGGGGG	CTGGT	TGGG	TCTTGGAGACTC	TATGAAAACCACTACGGACCAGGAACAACA
4951	CGGTATATCTGCCACGTT	AACGT	AATATA	TGTCA	ACTGGT		TGGGC	TCTCT	TGGAT	CT	GAAAACTACTACGGACCAGGAACAACA
4942	CGTTATATGTGCCAC	ACTCCCCCACCGTATATTTAAAC	TGGCC	GTCTTTCGCTGGT	ATGAC	ACC					AGGAACAACA
786	GTATATCTGCCACGTC	GTCATTATA	TATAGGAGAGGAC	AGGGCT	ATGAC	GTTCTAGGTATA					CTACTACGGACCAGGAACAACA
6	CGGTACATCTGCCACGTT	AACATTATATCATA	TTGG	CT	GGTC		TGGG	G			ACTACTACGGACCAGGAACAACA
17	GGTACATTTGCCAC	CCACGGCTTTCGGT	TGGG	GCGCCGT	GTCTGGCTTT		GGAAC	AATCGGACC			ACTTACTACGGACCAGGAACAACA
2.34	CGTACATTTGCCAC	GCT	TGG	GCT	TGGGT	ACCTCTG	GGAAC	ACTAACACAT			CTATGAAAACTACTACGGACCAGGAACAACA
10	CGGTATATCTGTCACGTC	GGTGTCTACAGGA	ACTGGA	ATGGCGTGGGAG	AGGAGA	ACGAGGTTCCAATCACAT	GGATGGAG	ACTAACACAT			GACCAGGGACGACAGTCAGAGTGCGACCAT
36	CGGTATATCTGT	GCAAAA	TGGG	TTAAGAGACGGT	GAAC	TGGCAATACCTGG	GGTC	CTATATTGACGTC			TACGGACAGGGACAACC
4966	CGATACATCTGTGCAAGA	CAGG	ATGACTGGAG	TC	CTTA	TGCT					ACTACTACGGACCGGGGACTACAGTCACAGT
1.22	CGGTACATCTGTGC		GGTCAGG	GTCAATATTATTTTGGCGGC	GCCT						GACTACTACGGACCAGGGACAACA

B

Clones No.	V2 3′ end	P	N	D	P	J2 5′ end
21	GGCCGCTATTATTGTGTGAGACT	C	TG	GATTA	C	AGGCGAAAGACAGTAGTGCACAGTTAGTTTTCAGAACT
26	GGCCGGTATTATTGTGTGAGAC	C	GCCC			GGAGACAGACAATAGTGCACAGTTAGTTTTCAGAACT
2.22	GGCCGGTATTATTGTGTGAGAC	C	GCAC	GATTAG		ACAGGAGAAGACAATAGTGCACAGTTAGTTTTCAGAACT
3815	GGCCGGTATTACTGTGTG					GGAGACAGACAACAGTGCACAGCTACCCTTCAGAACT
1951	GGCCGCTATTATTGTGTGAGACT				C	GAAAGACAATAGTGCGCAGGTGGCTTTCAGAAGT
1953	GGCCGGTATTATTGTGTGAGACT	AG	GA			AGGAGAAAGACAATAGTGCCACAGCTAGCTTTCAGAACT
1954	GGCCGGTATTATTGTGTGCGACT					ACAGGAGAAAGACAATAGTGCCACGGCTATCTTTCAGAAGT
1955	GGCCGGTATTATTGTGTGAG		GGA			ACAGGAGGAAGACAATACTGCACAGGCTACCTTTCAGAACT
4942	GGCCGGTATTATTGTGTGCGACT		GGG			ACAGGAGAAAGACAATAGTGCACGGCTATCTTTCAGAAGT
4951	GGCCGGTATTATTGTGTGAGACT					ACAGGAGAAAGACAGTACTGCACAGGTAGTTTTCAGAACT
6	GGCCGGTATTATTGTGTGAGACT					ACAGGAGAGAGACAATAGTGCACAGCTAGTTTTCAGAACT
17	GGCCGGTATTATTGTGTG	C	GA		GT	ACAGGATAAAGACAATAGTGCGCAGTTAGTTTTCAGAACT
4966	GGCCGGTACTACTGTGTGAGAC					ACAGGGGGCGGACAATGTTGCAGAGGTACCTTTCGGCAGT
1.22	GGCCGGTATTACTGTGTGAGACT	AG				AGGGGAGAGACGATGTTGCAAAGCTAGCTTCGGCAGT
2.34	GGCCGGTATTATTGTGTGAGACT		TG		TG	CAGGACAAAGACAACAGCGCACTGCTACCTTTCAGAACT
10	GGCCAGTATTATTGTGTGAGAC					ACAGCGGAAAGACACCAAGGCAATGCTGACTTTAACAACT

FIGURE 4. Sequences corresponding to the CDR3 of V1 (*A*) and V2 (*B*) domains from full-length and partial platypus splenic TCRμ cDNAs.

present in the last common ancestor of marsupials and placental mammals. However, no TCRμ homolog has been identified in placental mammals, consistent with gene loss in this lineage (2). Furthermore, a TCRμ homolog has yet to be found in the available avian, reptilian, and amphibian genomes, consistent with its appearance in the synapsids (mammals and their extinct relatives) after their divergence from the diapsids (birds and reptiles) 310 MYA (2, 21). This conclusion is also consistent with phylogenetic analyses of TCRμ C region genes published previously, in which marsupial Cμ appears to diverge from Cδ after the split between mammals and birds (4).

The most distinctive feature common to both marsupial and platypus TCRμ is their transcription in a form predicted to encode three extracellular Ig domains (V-V-C) instead of the conventional two domains (V-C). TCR with this characteristic have only been described in one other vertebrate lineage, the cartilaginous fish. Both the elasmobranchs (sharks, rays, and skates) and the holocephalins (ratfish) use an isoform of TCRδ, called new Ag receptor (NAR)-TCR, that also has a double V expressed with a conventional Cδ (22).

There are a number of common characteristics shared between mammalian TCRμ and shark NAR-TCR, as well as distinctive differences (Table III). In both platypus TCRμ and NAR-TCR, the exons encoding both V domains require somatic DNA recombination to be assembled (22). The supporting or V2 domains in NAR-TCR are encoded by a dedicated subset of Vδ gene segments that, like the platypus Vμ2, lack L sequences and would be unable to encode the N terminus of an extracellular protein (22). This is different, however, in marsupials in which the exon encoding the V2 domain, called Vμj, is preassembled as a germline-joined gene and contains an L sequence that is contiguous with the exon encoding the extracellular V domain (Fig. 6*C*) (4). In the case of marsupial TCRμ, this L sequence is left out of the Vμj exon in the mature mRNA due to a canonical RNA splice site at the junctions between the L and V sequences (2, 4). This arrangement makes it possible to transcribe a two-domain form of marsupial TCRμ that contains only the Vμj and C regions. Indeed, such transcripts are found in the opossum thymus; however, they are rare in peripheral lymphoid tissues, leading to the current working hypothesis that it is the double-V form that is the mature, functional chain (4). Furthermore, in the opossum, *Monodelphis domestica*, there are eight tandem clusters of TCRμ genes, and in six of these, the Vμj L sequences contain mutations rendering them nonfunctional (2, 4). Therefore, whereas the shark and platypus have fully deleted the L sequence of the supporting V, the L sequences in marsupials are apparently degenerating due to lack of use.

Both TCRμ and NAR-TCR use V domains more similar to Ab V genes than conventional TCR V genes. The N-terminal V domains in NAR-TCR are related to V used in IgNAR, which are L chainless Abs unique to cartilaginous fishes (22, 23). As already described, the second V in NAR-TCR is a Vδ gene, making the NAR-TCR appear to be a hybrid between IgNAR and TCRδ (22). In contrast, the genes used to encode both V1 and V2 domains in platypus TCRμ are indistinguishable from mammalian clan III Ig VH genes and unrelated to NAR V genes. Marsupial Vμ and Vμj, in contrast, are somewhat intermediary. Vμj are more similar to Ig VH, but do not fall within the three traditional mammalian VH clans, and Vμ appears to be more related to NAR V genes, although this latter relationship is only weakly supported in phylogenetic analyses (Fig. 5).

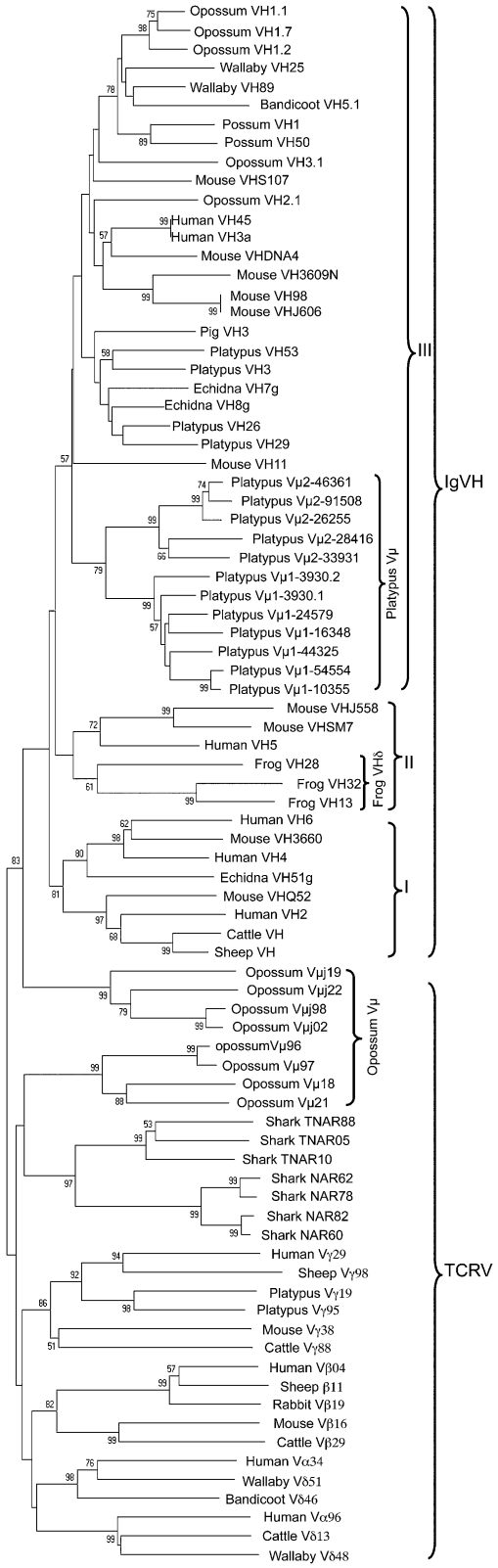

FIGURE 5. Phylogenetic analysis of platypus and marsupial Vμ including V genes from conventional TCR, shark NAR and NAR-TCR, and Ig VH. This neighbor-joining tree is based on nucleotide alignments, and branch support is indicated as the percentage out of 1000 bootstrap replicates. Only those nodes with >50% support are indicated. The three major clans of vertebrate VH are indicated by Roman numerals.

The current model for the structure of NAR-TCR is an unpaired N-terminal domain, much like the V-NAR domain in IgNAR, binding Ags as a single domain (22, 23). This Ag binding is similar to that that has been described for single V domain IgNAR Abs in sharks and L chainless IgG in camels (24, 25). It seems likely that TCRμ is structured similarly to NAR-TCR, with a single, unpaired N-terminal V domain capable of binding Ag directly. Based on conserved residues, including cysteines, TCRμ is predicted to form a heterodimer with another TCR chain (4). However, because no other TCR-related genes encoding a three-domain chain have been found in the marsupial genome, it is predicted that the partner is a conventional two-domain TCR chain, likely TCRγ, leaving the N-terminal domain unpaired (2).

The common characteristics found in mammalian TCRμ and shark NAR-TCR raise the question of whether these features are due to homology by descent or convergent evolution. An argument could be made that the evolutionary distance between sharks and mammals is sufficiently vast, and the differences between TCRμ and NAR-TCR extensive enough that each evolved independently and appear analogous due to convergence on a common structure and function. This could imply a common evolutionary pressure shared between cartilaginous fish and early mammals to have T cells capable of binding Ag directly using single domain binding sites.

Phylogenetic analyses of platypus and marsupial TCRμ C region support that they are orthologous genes that would have been found in a last common ancestor of the three living mammalian lineages. However, following the divergence of the oviparous monotremes from the viviparous marsupials and placental mammals, TCRμ appears to have followed different evolutionary paths. In the placental mammals, it was lost altogether (2). As discussed earlier, in the marsupials, the genes encoding the V2 domain appear to have been replaced in the germline by a prejoined V gene, most likely via retrotransposition (4). This novel marsupial adaptation is consistent with the V2 domains serving strictly supporting roles rather than being Ag binding and, therefore, requiring little or no clonal variation. In the platypus, the TCRμ V2 domain is encoded by somatically recombined genes, but variation remains restricted through limited junctional diversity, with no D segments and few N or P additions in the V-J junctions. Comparisons of the length of the CDR3 region in the platypus and marsupial V2 domains, where they are both relatively short, suggests that D segments, if they were ever present, were deleted early in the evolution of TCRμ prior to the divergence of prototherians and therians (4). The mean codon length of the platypus V2 CDR3 is the same (n = 11) as that found in the germline-joined marsupial Vμj genes (Table III). In contrast, the V1 domains of both platypus and opossum TCRμ have comparatively longer and more diverse CDR3 due to the incorporation of multiple D segments during V(D)J recombination in both species (4, 26).

The lack of an intron separating the L from the V in the Vμj exon is evidence of retrotransposition in the evolution of TCRμ in marsupials (4). In other words, Vμj is a functional, partially processed gene. The insertion of joined V genes into the germline by retrotransposition would require coexisting retroelements in the genome, and one noteworthy distinction between the opossum and the platypus genomes is the abundance of retroelements. The opossum has among the highest percentage of retroelements of any vertebrate genome sequenced (27). In contrast, monotremes are relatively devoid of retroelements (14, 28). Whether this extreme difference contributed to the evolution of opossum and platypus TCRμ is not known. Furthermore, this explanation is not fully satisfying because processed pseudogenes have been found

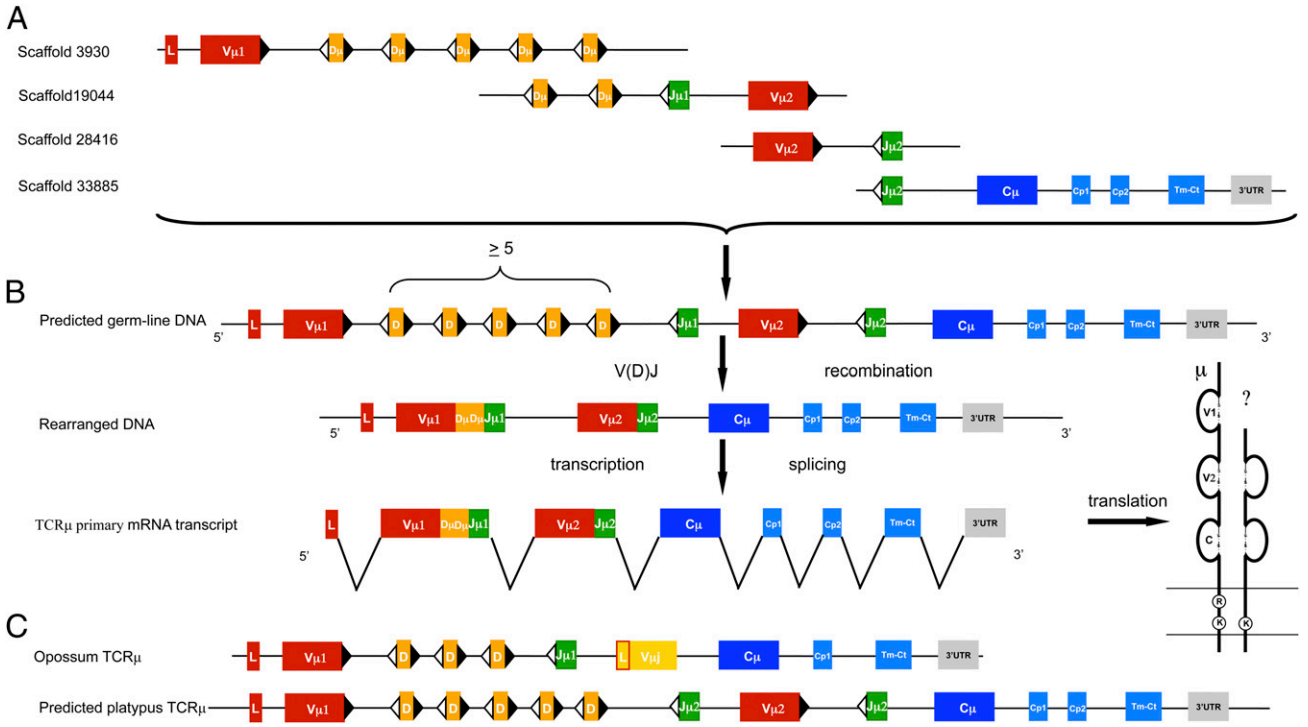

FIGURE 6. Diagrams of the predicted platypus TCRμ gene organization, transcripts, and protein structure. *A*, Representative TCRμ scaffolds containing TCRμ coding sequences. Closed or open triangles flanking the Vμ, Dμ, and Jμ gene segments indicate the presence of 23- or 12-bp spacer RSS, respectively. The L sequence, CP, TM-CT, and 3′ UTR exons are indicated. *B*, Predicted TCRμ germline DNA and rearranged DNA structure and primary TCRμ mRNA transcript structure. Conserved R and K residues in the TM region are indicated in the predicted cell surface TCR protein structure. *C*, Comparison of a representative opossum TCRμ cluster with the predicted platypus homolog.

in the platypus and echidna genomes, consistent with retrotransposition having occurred sometime in the past for some monotreme genes (10).

Phylogenetic analyses support TCRμ being related to and likely derived from a TCRδ ancestor (4, 5). As stated earlier, if TCRμ evolved from a duplication of TCRδ genes, it likely occurred after the separation of mammals from birds and reptiles (4). However, some insight into the origins of TCRμ may come from recent work on the genetics of amphibian TCRδ-chains (29). The *TCRα/δ* locus in the frog *Xenopus tropicalis* contains two Cδ genes, one of which, Cδ1, is expressed with V genes called VHδ. These frog VHδ are indistinguishable from clan II Ig VH genes, and, although the *X. tropicalis TCRα/δ* and *Igh* loci are closely linked, the VHδ genes appear to be dedicated for use in TCRδ-chains and are not used in IgH chains (29). This close linkage, however, may have facilitated insertion of VH genes among the TCRδ genes in amphibians. The region of the frog *TCRα/δ* locus containing Cδ1 and multiple VHδ genes is distinct and, in an inverted transcriptional orientation from the rest of the *TCRα/δ* genes, functioning almost as a separate minicluster (29). Amphibians, therefore, appear to be another vertebrate lineage that uses TCRδ-chains containing Ab-like V genes. Unlike TCRμ and NAR-TCR, frog TCRδ-chains are not expressed with two V domains, however. Rather, *X. tropicalis* TCRδ-chains using VHδ are structured like conventional two-domain TCR chains.

It is possible, and seems likely, that the TCRμ locus evolved from genome duplication and translocation of an ancestral region of the *TCRα/δ* locus similar to the Cδ1 region in frogs. Indeed, the discovery of VH genes in the *X. tropicalis TCRα/δ* locus is consistent with their presence in the *TCRδ* locus prior to the

Table III. Comparison of the features of TCRμ, shark NAR-TCR, and mammalian conventional TCRα/δ

				Somatic Recombination		Leader Sequence		No. of D Segments Used		CDR3 Length[a] (Mean)		V Nature		
Locus	Model Species	C Gene	Double V	N-terminal	C-Proximal	N-Terminal	C-Proximal	N-Terminal	C-Proximal	N-Terminal	C-Proximal	N-Terminal	C-Proximal	Reference No.
TCRμ	Platypus	Cμ	Yes	Yes	Yes	Yes	No	2-4	0?	9–22(14)	9–12(11)	VH clan III	VH clan III	This study
	Opossum	Cμ	Yes	Yes	No[b]	Yes	Yes[c]	1-3	NA	8–29(17)	11	VH-related	VH-related	(4)
NAR-TCR	Nurse shark	Cδ	Yes	Yes	Yes	Yes	No	1	1-2	9–25(16)	9–27(16)	V-NAR	Vδ	(22)
TCRα/δ	*Xenopus*	Cδ1	No	Yes	NA	Yes	NA	1-2	NA	7–20(13)	NA	Vα, Vδ, VHδ (VH clan II)	NA	(29)
TCRα/δ	Human	Cδ	No	Yes	NA	Yes	NA	2-3	NA	8–12(15)	NA	Vα, Vδ	NA	(26)
TCRα/δ	Mouse	Cδ	No	Yes	NA	Yes	NA	2	NA	6–19(13)	NA	Vα, Vδ	NA	(26)

[a]Range in codons.
[b]The C proximal V in marsupial TCRμ is a germline joined V.
[c]Fused to the V domain exon as the result of retrotransposition.

evolution of TCRμ. Internal duplications of clusters of V, D, and J segments within the *TCRμ* locus, as hypothesized previously, would then give rise to the double V organization in mammals (2). What remains puzzling is the variation in the source of VH genes used in each lineage. The VHδ in *X. tropicalis* are apparently derived from clan II VH, the platypus Vμ genes are clan III VH, and, although the marsupial Vμ genes are more closely related to VH than TCR V genes, they fall outside the clan I, II, and III designations. These observations suggest that the V genes used in TCRδ- or TCRμ-chains have been replaced over time with different VH lineages, even within the mammals. If the platypus *TCRμ* locus is indeed organized as tandem clusters similar to what has been shown in opossum (4), such gene clusters may facilitate gene replacement and duplication that is not easily achieved by the translocon organization of the conventional TCR genes.

The lack of TCRμ in commonly studied mammals such as humans and mice no doubt contributed to it remaining undiscovered for nearly a quarter of a century following that of the conventional TCRα, β, γ, and δ (4, 29–33). Determining why placental mammals may have lost this TCR chain will require first determining what function(s) $TCR\mu^+$ T cells perform in those species in which they are found.

Disclosures

The authors have no financial conflicts of interest.

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