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Comparison of Chimpanzee and Human Leukocyte Ig-Like Receptor Genes Reveals Framework and Rapidly Evolving Genes[1]

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The leukocyte receptor complex (LRC) on human chromosome 19 contains related Ig superfamily killer cell Ig-like receptor (*KIR*) and leukocyte Ig-like receptor (*LIR*) genes. Previously, we discovered much difference in the *KIR* genes between humans and chimpanzees, primate species estimated to have ~98.8% genomic sequence similarity. Here, the common chimpanzee *LIR* genes are identified, characterized, and compared with their human counterparts. From screening a chimpanzee splenocyte cDNA library, clones corresponding to nine different chimpanzee *LIR*s were isolated and sequenced. Analysis of genomic DNA from 48 unrelated chimpanzees showed 42 to have all nine *LIR* genes, and six animals to lack just one of the genes. In structural diversity and functional type, the chimpanzee *LIR*s cover the range of human *LIR*s. Although both species have the same number of inhibitory LIRs, humans have more activating receptors, a trend also seen for KIRs. Four chimpanzee *LIR*s are clearly orthologs of human *LIR*s. Five other chimpanzee *LIR*s have paralogous relationships with clusters of human *LIR*s and have undergone much recombination. Like the human genes, chimpanzee *LIR* genes appear to be organized into two duplicated blocks, each block containing two orthologous genes. This organization provides a conserved framework within which there are clusters of faster evolving genes. Human and chimpanzee *KIR* genes have an analogous arrangement. Whereas both *KIR* and *LIR* genes can exhibit greater interspecies differences than the genome average, within each species the *LIR* gene family is more conserved than the *KIR* gene family. *The Journal of Immunology,* **2001, 167: 5786–5794.**

The leukocyte receptor complex or cluster (LRC)[6] occupies about 1 Mb of human chromosome 19q13.4 (1–5). It contains families of genes that encode receptors in which the extracellular domains are made up of Ig-like domains. One family of genes is the killer cell Ig-like receptors (*KIR*s) expressed by NK cells (6, 7) and subpopulations of T cells (8, 9). Adjacent to the *KIR* genes on their centromeric side is a distinct, but related, gene family, members of which were independently discovered by different investigators and given different names*:* leukocyte Ig-like receptors (*LIR*) (10, 11), Ig-like transcripts (*ILT*s) (12), and monocyte Ig-like receptors (*MIR*s; Table I) (13). Members of this second gene family encode receptors with two or four C2-type Ig domains; most are expressed by monocytes, macrophages, and dendritic cells, but some are also found on NK, B, and T cells (14, 15). In the absence of a single, agreed nomenclature, we shall use *LIR* here as a general descriptor for the gene family and the receptors they encode as it more accurately describes the proteins and their tissue distributions than does *ILT* or *MIR*.

Characterization of cDNA sequences combined with genomic analysis has shown the human *LIR* gene family consists of 11 expressed genes and two pseudogenes (4, 5). They form two homologous blocks of genes that are in opposite orientations and separated by ~200 kb (4). In accordance with the convention adopted in the human genomic analyses, 11 of the genes are named *ILT1–11*, the other two are named *LIR6* and *LIR8* (4, 5). A minor *LIR* gene haplotype is found lacking the expression of *ILT6* (5, 16).

Within both the LIR and KIR families are subsets of receptors that have the potential for either activating or inhibitory function. The long cytoplasmic tails of inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that interact with the tyrosine phosphatase SH2-containing phosphatase-1 to inhibit cell activation (17, 18). Activating receptors have short cytoplasmic tails that lack ITIMs, with their activating function being determined by the presence of a charged residue in the transmembrane domain. Such residues allow association with signaling adaptor molecules that possess immunoreceptor tyrosine-based activation motifs. For activating KIR the adaptor molecule is DAP-12 (19), whereas for activating LIR it is FcϵRIγ (20).

All known ligands for KIR and LIR are MHC class I or class I-like molecules (7, 14). Different *KIR* genes have been shown to encode receptors for HLA-A, -B, -C, and -G, and they account for approximately half of the *KIR* gene family (7, 14). By contrast,

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[6] Abbreviations used in this paper: LRC, leukocyte receptor complex or cluster; ILT, Ig-like transcript; ITIM, immunoreceptor tyrosine-based inhibition motif; KIR, killer cell Ig-like receptor; LIR, leukocyte Ig-like receptor; MIR, monocyte Ig-like receptor; SSP, sequence-specific primers; SSPE, standard saline sodium phosphate-EDTA buffer.

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Table I. *Nomenclature and the GenBank accession numbers for common chimpanzee sequences*[a]

LIR	*ILT*	*MIR*	Others	HGMW	*Pt-LIR*	GenBank Accession No.
LIR1	*ILT2*	*MIR7*	*CD85*	*LILRB1*		
LIR2	*ILT4*	*MIR10*		*LILRB2*		
LIR3	*ILT5*		*HL9*	*LILRB3*		
LIR4	*ILT6*		*HM31, HM43*	*LILRA3*		
LIR5	*ILT3*		*HM18*	*LILRB4*	*Pt-ILT3*	AF383162
LIR6				*LILRA1*		
LIR7	*ILT1*			*LILRA2*	*Pt-ILT1*	AF383161
LIR8				*LILRB5*	*Pt-LIR8*	AF383164
	ILT7				*Pt-ILT7*	AF383163
	ILT8					
	ILT9					
	ILT10					
	ILT11					
					Pt-LIRa	AF383165
					Pt-LIRb	AF383166
					Pt-LIRc	AF383167
					Pt-LIRd	AF383168
					Pt-LIRe	AF383169

[a] Human Gene Mapping Workshop (HGMW)-approved symbols; an "A" preceding the numeral designates a "short-tailed" activating receptor, whereas a "B" designates a "long-tailed" inhibitory receptor.

only LIR1 and LIR2 are known to bind MHC class I, which they do with a broad specificity, encompassing products of both classical and nonclassical HLA class I genes (17, 21–24). For other members of the LIR family, the functions and ligand specificities remain unknown.

Population studies and species comparisons have shown that MHC class I genes can be highly polymorphic and rapidly evolving. Analogous studies of the *KIR* gene family have revealed similar characteristics. Within the human population, *KIR* haplotypes vary in the number and functional type of *KIR* genes (25, 26), and certain of the genes are also highly polymorphic (27–29). However, the *LIR* gene cluster appears more conserved in its organization (30). A comparison of common chimpanzee (*Pan troglodytes*) and human *KIRs* revealed that although the two species had similar numbers of *KIR* genes, a minority of them appeared orthologous (31). To examine the recent evolution of the *LIR* gene family, we have characterized common chimpanzee *LIR* genes and compared them to their human counterparts.

Materials and Methods

Isolation of RNA and DNA samples

A frozen spleen sample from the common chimpanzee (*Pan troglodytes verus*) Amanda was macerated with scalpels, strained through a 70-μm pore size cell strainer, and resuspended in RNAzol B (Tel-Test, Friendswood, TX). Total RNA was isolated from the lysate following the manufacturer's recommended protocols. mRNA was purified using a poly(dT) cellulose column from the Poly(A) Quik mRNA Isolation kit following the manufacturer's instructions (Stratagene, La Jolla, CA). Genomic DNA samples were prepared from 48 B lymphoblastoid cell lines of unrelated chimpanzees (31–33) following standard protocols (34). Samples of chimpanzee spleen and peripheral blood were purchased from the Yerkes Regional Primate Center at Emory University (Atlanta, GA).

cDNA library construction

A common chimpanzee spleen cDNA library was constructed with the ZAP Express Gigapack III Gold kit (Stratagene) according to the manufacturer's instructions and screened with a human *ILT2* cDNA probe following standard procedures (34). Briefly, cDNA was synthesized from 5 μg mRNA, cloned into the ZAP Express λ phage vector, packaged, and used to infect the XL1-Blue strain *Escherichia coli* (Stratagene). For screening, 2×10^6 PFU of the primary cDNA library were plated at a density of $\sim 1 \times 10^5$ plaques/150-mm petri dish and transferred in duplicate onto Colony/Plaque Screen nylon membranes (NEN Life Science Products, Boston, MA). The membranes were hybridized under low stringency conditions (in 30% formamide, 5× standard saline sodium phosphate-EDTA (SSPE) buffer (pH 7.4), 5× Denhardt's reagent, 5% dextran sulfate, 1% SDS, and 1 mg/ml fragmented and denatured salmon sperm DNA at 42°C for 16 h) with a human *ILT2* cDNA probe, washed four times with 2× SSPE/0.5% SDS at 42°C, and autoradiographed. Positive phage were plaque-purified and subjected to an in vivo excision procedure according to a Stratagene protocol; the resulting phagemids contained chimpanzee cDNA within the pBK-CMV vector.

Nucleotide sequencing and alignment

Nucleotide sequences were determined on both DNA strands using the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 377 DNA sequencer (Applied Biosystems). Complete DNA sequences of the plasmid inserts were obtained by using T3 and T7 universal oligonucleotide primers and internal primers based on sequences of chimpanzee *LIR*s. DNA sequences were assembled and analyzed using the computer program AutoAssembler (version 2.1; Applied Biosystems) and the Wisconsin Package sequence analysis software (version 10.1; Genetics Computer Group, Madison, WI) (35).

LIR *gene nomenclature*

Individual cDNA sequences in the human *LIR* family have been named, mainly using two different nomenclatures: either *ILT* (12) or *LIR* (10, 11), followed by a number in series (Table I). These alternative and competing nomenclatures have yet to be reconciled into a single system. In human genomic analyses a hybrid nomenclature is being used (4, 5) in which the preference is for the *ILT* nomenclature (11 of the genes are named *ILT1–11*), but *LIR* nomenclature is retained for two genes (*LIR6* and *LIR8*, first described by Cosman et al., who introduced the *LIR* nomenclature) (10, 11). Common chimpanzee *LIR* genes that are clearly orthologous to a human *LIR* gene have been given the same names as the human genes with the additional prefix *Pt-* for *Pan troglodytes*. Chimpanzee *LIR* genes that are paralogous to human *LIR* genes or for which orthology is less certain have been given provisional designations using lowercase letters: *Pt-LIRa-e*. The chimpanzee cDNA sequences reported here were deposited into the GenBank database (Table I).

5′ RACE

We performed 5′ RACE analysis to obtain the 5′ coding region sequences of certain incomplete chimpanzee *LIR* cDNAs. First-strand cDNA was synthesized using 1 μg mRNA isolated from the chimpanzee Amanda with the SMART RACE kit according to the manufacturer's instructions (Clontech, Palo Alto, CA). Two specific reverse oligonucleotide primers were designed for each chimpanzee *LIR* gene based on sequences determined for partial cDNAs isolated from library screening. These oligonucleotide primers were used for amplifying the 5′ cDNA of desired genes in two rounds of PCR amplifications. The PCR conditions were 94°C for 30 s; 25 cycles of 94°C for 5 s, 66°C for 10 s, and 72°C for 3 min; and 72°C for 3 min for

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the initial reactions and 94ºC for 30 s; 15 cycles of 94ºC for 5 s, 65ºC for 10 s, 72ºC for 3 min; and 72ºC for 3 min for the second-round nested amplifications. Antisense oligonucleotide primers used in the initial amplification were 5′-GGG CTC AGA TCA CAG GAC TCA CG-3′ (*Pt-LIRc*), 5′-CGG GCA TGG GAA TGG GAG TTC AGA C-3′ (*Pt-LIRd*), and 5′-CTG TCG GTC AGG GCG CTG GGC G-3′ (*Pt-ILT7*). Antisense primers used in the second-round nested PCR were 5′-CCA CAC CTG GTC GTT GTA AGT AT-3′ (*Pt-LIRc*), 5′-CTT GCG TGT TCC CAG GTG ATG GAC G-3′ (*Pt-LIRd*), and 5′-GTC AGA TTC TCT CCG GGG GTC ACA A-3′ (*Pt-ILT7*). Amplified cDNA fragments were separated by electrophoresis in 1% agarose gel, purified using the QIAEX II gel extraction kit (Qiagen, Chatsworth, CA), cloned into the pCR-TOPO plasmid vector (Invitrogen, Carlsbad, CA), and sequenced.

Phylogenetic and recombination analyses

Phylogenetic analyses were performed separately with nucleotide sequences encoding for the entire mature proteins and for only the Ig-like domains, using the PAUP 4.0 (Phylogenetic Analysis Using Parsimony) software package (version 4.0b4a, Sinauer Associates, Sutherland, MA) and other methods. Phylogenetic trees were constructed using the neighbor-joining method (36), and confidence values for each tree branch were estimated from 1000 replicates using the bootstrap technique (37). Gaps were counted as single mutational events in pairwise comparisons. Initial analysis to assess recombination between *LIR* genes was performed using the Partimatrix program (38), including all chimpanzee and human *LIR* gene sequences. Further analyses were made with groups of four sequences (two each from chimpanzee and human) and also with clusters of *LIR* sequences identified from phylogenetic analyses.

Analysis of nonsynonymous and synonymous nucleotide substitutions

Rates of d_N (nonsynonymous) and d_S (synonymous) substitutions were determined using the synonymous/non-synonymous analysis program (SNAP, available at http://hiv-web.lanl.gov/SNAP/WEBSNAP/SNAP.html) based on the method of Nei and Gojobori (39) and incorporating a statistic developed by Ota and Nei (40). This analysis was performed separately on complete *LIR* mature protein-coding sequences, on sequences encoding all Ig-like domains, on individual Ig-like domains, or on contiguous small regions (60 nt) of each Ig-like domain. Each chimpanzee *LIR* gene was compared with the most closely related human *LIR* sequence.

Genomic typing for common chimpanzee LIR genes

To type for the presence of *LIR* genes in a panel of 48 unrelated chimpanzees, we designed two pairs of gene-specific PCR oligonucleotide primers for each of the nine chimpanzee *LIR*s that anneal to exon sequences encoding the first and second Ig-like domains (Table II). PCRs were conducted with ~100–200 ng genomic DNA, 25 pmol of each primer, 2.5 mM of each dNTP, 1.5 mM $MgCl_2$, 1× AmpliTaq buffer (Applied Biosystems), and 1 U AmpliTaq DNA polymerase (Applied Biosystems). The PCR parameters were 94ºC for 10 s; 30 cycles of 94ºC for 40 s, 68ºC for 30 s, and 72ºC for 30 s; and 72ºC for 10 min. Amplified genomic DNA fragments were analyzed on ethidium bromide-stained 1% agarose gels. Typing reactions were tested on genomic DNA of the common chimpanzee Amanda from whom the spleen cDNA library was constructed; the PCR-amplified DNA were directly sequenced to confirm specificities. Subsequently, genomic DNA from a panel of 48 unrelated chimpanzees were typed, including individuals from three common chimpanzee subspecies: *P. troglodytes schweinfurthii*, *P. troglodytes troglodytes*, and *P. troglodytes verus*.

Results

Isolation and characterization of cDNAs encoding nine chimpanzee LIR *genes*

A cDNA library was made from spleen cells of an individual chimpanzee and screened with a human *ILT2* cDNA probe. The screen yielded 83 hybridizing cDNA clones, which were further characterized by DNA sequencing using the T3 and T7 phagemid-based oligonucleotide primers. On the basis of partial sequences in the 5′ and 3′ ends of the cDNAs, the clones were sorted into nine groups, of which four were well represented. The other five groups were represented by only one clone each, three of these being incomplete clones lacking the 5′ end. Of the 83 clones 41 appeared to be full-length cDNA clones; the remaining 42 clones appeared either as partial clones lacking 5′ regions or products of alternatively spliced variants. A total of 44 cDNA clones representing all nine groups were completely sequenced. For the three groups represented by only single partial clones, complete coding region sequences were characterized using 5′ RACE analysis. All nine sequences were novel, but clearly belong to the gene family defined by the human *LIR* sequences.

Comparison of chimpanzee and human LIR

Eight of the nine chimpanzee *LIR* genes encode proteins with four extracellular Ig-like domains (D1–D4); the ninth encodes a protein with two Ig-like domains. This strong bias toward receptors with four Ig-like domains parallels that seen in the human LIR family (14). The positions of cysteine residues that make up the intradomain disulfide bonds of the Ig-like domains are conserved in chimpanzee and human. Aligning and comparing the chimpanzee and human LIR as a group reveals sequence variability of ~78% in D1, ~72% in D2, ~49% in D3, and ~64% in D4. Variability is well spread within all Ig-like domains, although D3 appears to the most conserved (data not shown).

To identify affinities between individual chimpanzee and human LIR, we performed a systematic pairwise comparison of amino acid sequences. Four of the chimpanzee sequences had particular affinity with one human sequence, consistent with them being pairs of orthologous genes (Fig. 1 and identified by arrows). Within each of the four orthologous pairs, the amino acid sequences were <5% different, whereas differences between the four chimpanzee orthologs and other human paralogs were >17%. The human *LIR* shown to have chimpanzee orthologs were ILT1, ILT3, ILT7, and LIR8; we therefore gave these chimpanzee LIR the same name as their human orthologs and also the prefix Pt- for *Pan troglodytes*. For the other five chimpanzee LIRs, the patterns of pairwise difference are distinct from those seen for the four with obvious human orthologs (Fig. 1). There is a more continuous range of differences, in which no single human LIR is as closely related as seen with the orthologous pairs. To indicate this difference, these chimpanzee LIRs have been given names that do not correspond to human LIRs: Pt-LIRa, -b, -c, -d, and -e. These five chimpanzee LIRs can be further divided into two groups according to the groups of human LIR to which they are more closely related. Thus, Pt-LIRa, -b, and -c are more closely related to human ILT1, -2, -4, and -6 and LIR6, whereas Pt-LIRd and -e are closer to human ILT5, -8, and -9 (Figs. 1 and 2). These affinities could reflect orthologous or paralogous relationships. None of the chimpanzee LIRs shows particular affinity with ILT11 or the pseudogene ILT10 (Fig. 2). Within the population of cDNA clones we sequenced, the relative abundance of chimpanzee *LIR*s were: 32% for *Pt-LIRa*, 32% for *Pt-LIRb*, 14% for *Pt-LIR8*, 12% for *Pt-ILT1*, and 2% each for *Pt-ILT3*, *ILT7*, *LIRc*, *LIRd*, and *LIRe*.

The similarities and differences in the chimpanzee and human LIR proteins are depicted in the schematic comparison of Fig. 2. The orthologous LIRs include two with four Ig-like domains and short cytoplasmic tails (Pt-ILT1, Pt-ILT7), one with four Ig-like domains and a long cytoplasmic tail (Pt-LIR8), and one with two Ig-like domains and a long cytoplasmic tail (Pt-ILT3). Each group of paralogous LIR contains members with long and short cytoplasmic tails (Fig. 2, *B* and *C*). All short-tailed chimpanzee LIR contains an arginine residue in the transmembrane region homologous to those found in human short-tailed LIRs and which is implicated in association with an activating adaptor molecule. The three or four ITIMs present in the long-tailed chimpanzee LIR could be important for inhibitory function. The chimpanzee LIRs with three ITIMs (Pt-ILT3, Pt-LIR8, Pt-LIRb) lack either the first or the second ITIM compared with human and chimpanzee LIRs that have four ITIMs. With one exception, the first pair of ITIMs

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Table II. *Oligonucleotide primers used for typing chimpanzee genomic DNA*[a]

	Set	Primers	
		Forward Primers	Reverse Primers
Pt-ILT3	1st	GGG AGT TAC CGC TGT TAC TAT CAC	ACG CTC TTT CCT GAG GTC ACA A
	2nd	CTG TAG CCA GGT CAC AGC CCA GCA	GGA CTG CCC GCT CCT TGA T
Pt-ILT1	1st	ACA AGA GCC TGG GAA GAA GGG TC	CCT TTG AGC CAC ACT GGA GGG TT
	2nd	CAC CAT CTG TGC TGA GCC AGG TT	CGG GTC CCA CGG AGA AGA C
Pt-LIR8	1st	AGG GAC TCT CAT GGG CCT GGG AG	AGC ATC TGA ACC TCC ACC TGA AG
	2nd	GCC AAG ACC AAG TTC CAC ATT CT	CCA CAG GAC TCG GCA GGG CTA AG
Pt-ILT7	1st	TGG AGT CTG AAA ACA AGG TCA AAT	GGG TCC TGG AGA GCC TGT GGT A
	2nd	GGG AAC ATG CAG GGC GAT ACC A	ACT GGG GTC CGA CCA CAC GTA C
Pt-LIRa	1st	AAG CGC CAG TTC CCC ATC CCA C	CGA GAT GAC CCA CGG GCA TGG T
	2nd	ACA CTA CAG GCC GGT CAG AGA A	CAC AGT ATG AAG CCG TCA AAT T
Pt-LIRb	1st	CTG GAT TAA ACG GAT ACA ACC AC	TGG GAC TGG GAG TTC ACG
	2nd	CTC AGT GGT CAG AGC CCA GTG AT	GAA GAT GGA CCA GGA CCA CCC AA
Pt-LIRc	1st	CAC AGA GCC CTG GGA CAA AAC G	GAG CAC ACC TGG TCG TTG TAA GT
	2nd	TTC TCC ATC CCA TCC ATG ACA CA	GGC TGG GAG CAC ACC TGG TCG
Pt-LIRd	1st	ACA CTT CAG GCC GGT CAG AGA G	GGC GTG GGA ATG GGA GTT CAG A
	2nd	CAA ACA CAC CCT CTG GGC TGA A	TAC CTT ACA CAG AAT GAA GCC AT
Pt-LIRe	1st	AAA GAG GGA AGC CGA GAA CCA C	GGC AGG GCT GAG AGG GTG GGT G
	2nd	GAA GCC GAG AAC CAC GCG ACA C	TGG AAC CCC CCA CTG TGG AGG T

[a] Eighteen sets of oligonucleotide primers were used, two for each *LIR* gene found in chimpanzee.

has YxxV motifs, and the second pair has YxxL motifs; the exception is the last ITIM of the chimpanzee Pt-ILT3, which has a YxxV motif. Pt-ILT3 also has a deletion in the cytoplasmic tail that is not present in its human ortholog, but is identical with that present in human ILT4 and LIR8. Sequence analysis by PCR amplification of this region of the *Pt-ILT3* gene showed the presence of a pseudoexon that encodes the missing part of the cytoplasmic tail (data not shown). That the pseudoexon is not incorporated into mRNA was confirmed by RT-PCR analysis of *Pt-ILT3* from several individual chimpanzees (data not shown).

Phylogenetic trees were constructed from the human and chimpanzee *LIR* nucleotide sequences. Shown in Fig. 3 are two trees, one made from nucleotide sequences encoding the mature proteins, and the other made only from sequences encoding the extracellular Ig-like domains. Both trees have a similar topology consisting of five main clusters of *LIR*, each containing both chimpanzee and human *LIR*, and two additional branches represented by single human sequences (*ILT10* and *ILT11*). Comparison of the amino acid sequences also gave trees with similar topology (data not shown). Three of the clusters (III, IV, and V in Fig. 3) contain only a pair of orthologous *LIR*s: *ILT3*/*Pt-ILT3*, *LIR8*/*Pt-LIR8*, and *ILT7*/*Pt-ILT7*. Cluster I in Fig. 3 involves four chimpanzee and five human *LIR*s, including the orthologs *ILT1*/*Pt-ILT1*. Cluster II of two chimpanzee and three human sequences contains no orthologous *LIR*s. In both trees *Pt-LIRb* and *ILT4* (*LIR2*) form a separate branch, raising the possibility that they are orthologs. However, they are more diverged from each other than is the case for the other orthologous pairs, and in both trees the confidence in the *ILT4*/*Pt-LIRb* branch (86 and 83%) is considerably less than for *ILT1*/*Pt-ILT1*, *ILT3*/*Pt-ILT3*, *ILT7*/*Pt-ILT7*, and *LIR8*/*Pt-LIR8* (100%).

To assess the role of recombination in generating *LIR* gene diversity, we used the Partimatrix program described by Jakobsen et al. (38). The extent to which individual *LIR* genes have been involved in recombination varies. Those genes for which human/chimpanzee orthologies were readily identified (Fig. 1) and which

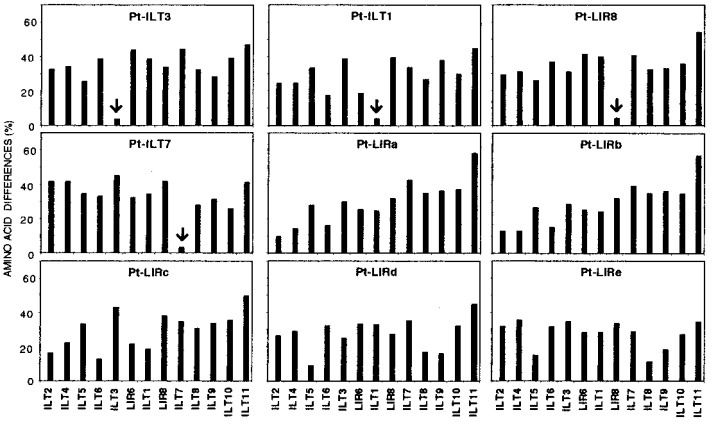

FIGURE 1. Pairwise comparison of amino acid sequences of common chimpanzee and human LIRs identifies four pairs of orthologs. Pairwise differences were calculated from amino acid sequences of the mature proteins and were plotted as percentages. Vertical arrows mark comparisons of orthologs.

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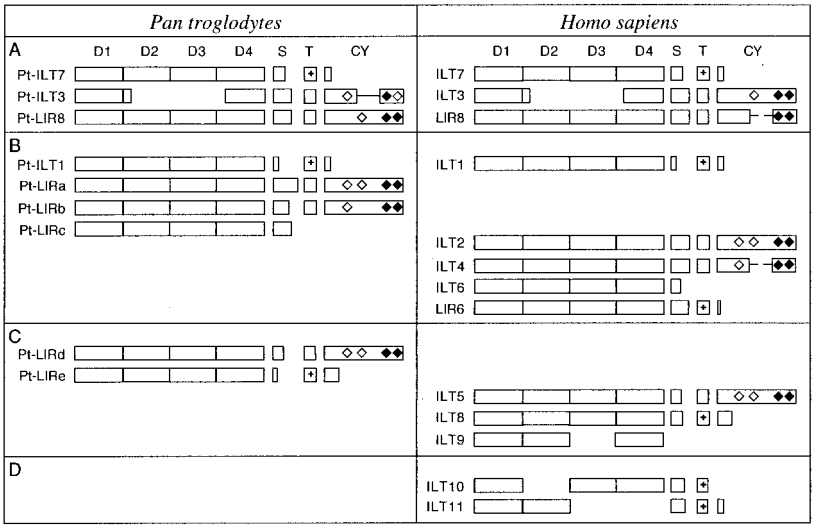

FIGURE 2. A schematic comparison of the proteins encoded by 9 chimpanzee and 13 human *LIR* genes. Orthologs are depicted on the same line. Protein domains are shown as rectangles, with D1, D2, D3, and D4 corresponding to the Ig-like domains and S, T, and CY corresponding to the stem, transmembrane, and cytoplasmic regions, respectively. +, a conserved arginine residue in the transmembrane domain of short-tailed LIRs. ◇ and ◆, Positions of ITIMs; ◇, YxxV motif; ◆, YxxL motif. The pseudoexon in the region encoding the cytoplasmic tail of Pt-ILT3 is represented by a solid horizontal line. Dashed lines in the homologous regions of human ILT4 and LIR8 indicate unknown data. *A*, The three orthologous pairs that are more divergent from the other LIRs (see also Fig. 3). *B*, The orthologous Pt-ILT1/ILT1 and related LIRs that form a separate cluster (I) in Fig. 3. *C*, Another group of paralogous chimpanzee and human LIRs, including the pseudogene ILT9, that form a distinguishable cluster (II) in Fig. 3. *D*, The two divergent human LIR (ILT11 and the pseudogene ILT10) that are not represented in the chimpanzee LIRs here defined. Putative domain designation of the chimpanzee LIRs, human ILT11, and pseudogenes ILT9 and ILT10 were obtained from comparison with other human LIRs.

were defined with deeper branches and high confidence in the phylogenetic analyses (Fig. 3) have not been involved in recombination: these comprise *ILT3*/*Pt-ILT3*, *ILT7*/*Pt-ILT7*, and *LIR8*/*Pt-LIR8*. An example of output given for such genes by the Partimatrix program is shown for *ILT3*/*Pt-ILT3* in Fig. 4*A*. It shows that there has been little recombination between genes in two major clusters of the phylogenetic trees (*ILT5*/*Pt-LIRd* vs *ILT3*/*Pt-ILT3*; Figs. 3 and 4*A*).

Partition matrix analysis of all five *LIR*s in cluster II (Fig. 3), the cluster containing no obviously orthologous genes, shows that recombination has played a considerable role in the diversification of these five *LIR*s (Fig. 4*B*). For example, *ILT5* and *ILT8* are very similar in the region encoding the extracellular part of the molecule (column 3 in Fig. 4*B*), but they diverge at the 3′ exons encoding the transmembrane and cytoplasmic domains. Here, *ILT5* groups with *Pt-LIRd* (column 2 in Fig. 4*B*), and *ILT8* with *Pt-LIRe* (column 1 in Fig. 4*B*), reflecting the divergence of sequences encoding the long-tail and short-tail LIRs. That recombination between exons encoding the Ig-like domains and the 3′ exons has been instrumental in diversifying cluster II *LIR*s is further supported by differences in the fine structures of this cluster in phylogenetic trees made from sequences encoding mature proteins

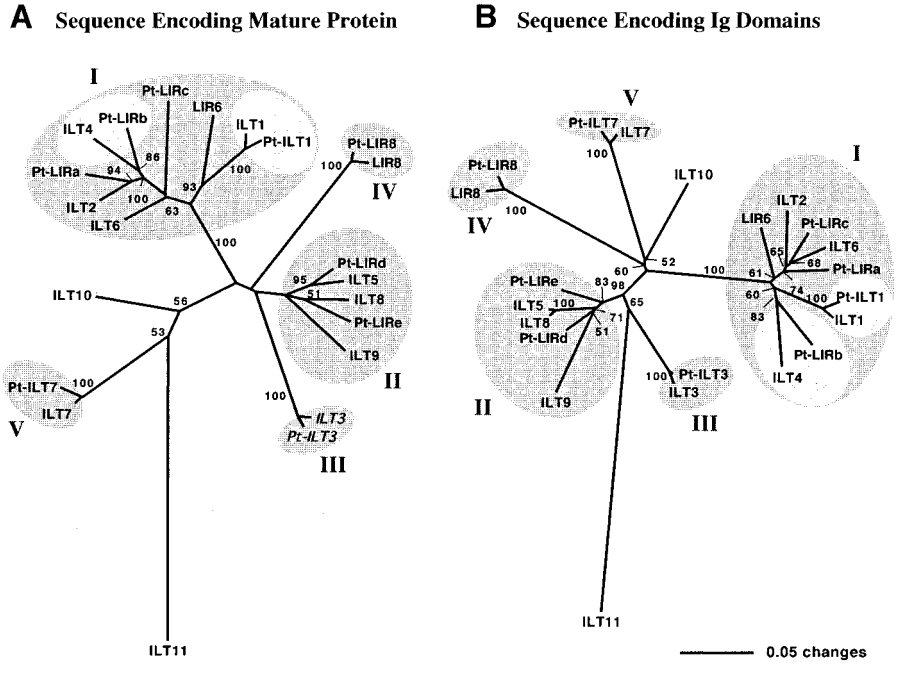

FIGURE 3. Phylogenetic analyses of chimpanzee and human *LIR*. Consensus trees were obtained using the neighbor-joining method (36) with the Kimura two-parameter distance correction (46). The tree in *A* was generated from nucleotide sequences encoding the mature protein, and the tree in *B* was generated from sequences encoding only the Ig-like domains. Numbers on nodes are percent bootstrap values calculated from 1000 replications using the heuristic search parameters of the PAUP program (Sinauer Associates). Clusters shaded in a darker gray are major groupings containing both human and chimpanzee sequences and are represented in both trees; these clusters are also noted by Roman numerals. The lighter gray shading indicates potential orthologs found within cluster I.

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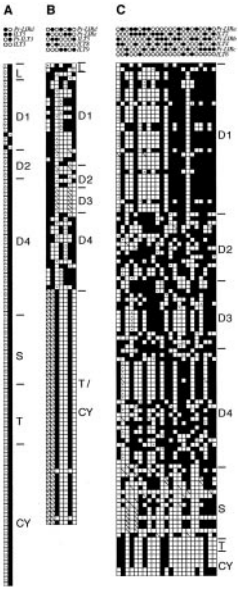

FIGURE 4. Recombination analyses of chimpanzee and human LIR. Outputs from the program Partimatrix (38) are shown. Shown above each matrix are the partitions into which the data were grouped (each column of circles represents an individual partition); filled circles indicate the group being considered for each partition. Each row of boxes corresponds to an informative nucleotide position along the cDNAs; the protein domains to which they contribute are noted with L for the leader peptide, D1–4 for Ig-like domains 1–4, S for the stem region, T for transmembrane domain, and CY for the cytoplasmic domain. Open boxes show partitions in agreement at the given nucleotide position; furthermore, a dot in the box indicates perfect agreement with the partition, and a slash indicates positions for which two or more partitions are in perfect agreement. Filled boxes show positions that disagree with the partition shown at the head of the column. *A,* An example for which no recombination was detected ,as can be seen by the fact that only two sites disagree with grouping of *ILT3* and *Pt-ILT3*. *B,* Potential recombinations within the group comprised of *Pt-LIRd*, *Pt-LIRe*, *ILT5*, *ILT8*, and *ILT9* (cluster II in Fig. 3). *C,* More extensive recombination within the group comprised of *Pt-LIRa*, *Pt-LIRb*, *Pt-LIRc*, *ILT2*, *ILT4*, and *ILT6* (from cluster I in Fig. 3).

(Fig. 3*A*) and only exons encoding the Ig-like domains (Fig. 3*B*). A more complicated pattern of recombination is revealed in the Partimatrix analysis of six members from cluster I (Fig. 3): *ILT2*, *-4*, and *-6* and *Pt-LIRa*, *-b*, and *-c* (Fig. 4*C*).

Each chimpanzee *LIR* was compared with the most closely related human *LIR*(s), and the relative frequencies of nonsynonymous (d_N, coding) and synonymous (d_S, silent) substitutions were calculated. Overall comparison of the sequences encoding the mature protein revealed an excess of synonymous substitutions for all *LIR*s, consistent with the action of purifying selection upon *LIR* genes (Table III). When sequences encoding only the Ig-like domains were compared, some ratios were higher and closer to 1.00, the value expected under neutral evolution. For the *Pt-LIRa/LIR6* and *Pt-LIR8/LIR8* comparisons the values were in slight excess of 1.00 (Table III). To refine the analysis we divided the sequence of each Ig-like domain into five similarly sized fragments of ~60 nt and computed the d_N and d_S values for these fragments. The values are consistent with purifying selection for almost all regions of the Ig-like domains of *ILT1*/*Pt-ILT1*, *ILT3*/*Pt-ILT3*, and *ILT7*/*Pt-ILT7*, with a few isolated regions containing only nonsynonymous substitutions (Fig. 5*A*). In contrast, the values for *LIR8*/*Pt-LIR8* provide evidence for positive selection throughout the fourth Ig-like domain and in the carboxyl-terminal part of the first and second Ig-like domains (Fig. 5*A*). Comparisons of *Pt-LIRd* and *Pt-LIRe*

Table III. *d_N:d_S ratios estimated for human and chimpanzee* LIR *genes*[a]

		d_N/d_S	
Chimpanzee	Human	Mature protein	Ig-like domains
Pt-ILT3	*ILT3*	0.57	0.19
Pt-ILT1	*ILT1*	0.37	0.47
Pt-LIR8	*LIR8*	0.85	1.16
Pt-ILT7	*ILT7*	0.28	0.27
Pt-LIRa	*ILT2*	0.60	0.71
Pt-LIRa	*ILT4*	0.58	0.64
Pt-LIRa	*LIR6*	0.59	1.06
Pt-LIRa	*ILT6*	0.42	0.85
Pt-LIRb	*ILT2*	0.66	0.69
Pt-LIRb	*ILT4*	0.68	0.80
Pt-LIRb	*LIR6*	0.51	0.66
Pt-LIRb	*ILT6*	0.38	0.74
Pt-LIRc	*ILT2*	0.40	0.55
Pt-LIRc	*ILT4*	0.46	0.66
Pt-LIRc	*LIR6*	0.47	0.75
Pt-LIRc	*ILT6*	0.53	0.63
Pt-LIRd	*ILT5*	0.82	0.74
Pt-LIRd	*ILT8*	0.57	0.77
Pt-LIRe	*ILT5*	0.52	0.55
Pt-LIRe	*ILT8*	0.47	0.58

[a] Either the coding regions for the mature proteins or for only the Ig-like domains were compared.

Chimpanzee sequences with no clear orthologs were tested against the closest human genes.

with human *ILT5*, *-8*, and *-9* gave no clear evidence for positive selection overall; however d_N was locally larger than d_S in segments 10 and 20 of the *Pt-LIRd* analysis and segment 9 of the *Pt-LIRe* analysis (Fig. 5*B*). Comparison of *Pt-LIRa*, *Pt-LIRb*, and *Pt-LIRc* with human *ILT2*, *ILT4*, and *LIR6* also revealed no evidence for positive selection (data not shown).

Within species LIR haplotypes are more conserved than KIR haplotypes

A characteristic of chimpanzee and human *KIR* haplotypes is variation in the number and content of genes. To determine whether this is also a feature of the chimpanzee *LIR* gene family, we developed a PCR-based system for typing the nine chimpanzee *LIR*s and used it to type genomic DNA from a panel of 48 common chimpanzees that had already been typed for *Pt-KIR* (31). Whereas 30 different *Pt-KIR* genotypes are represented in this panel, only two different *LIR* genotypes were identified (Fig. 6). The common genotype consisted of all nine *Pt-LIR*s and was observed in 42 individuals representing two subspecies, *P. troglodytes schweinfurthii* and *P. troglodytes verus*. The minority genotype was defined by negative typing for *Pt-LIRa* and was seen in six individuals representing three subspecies: *schweinfurthii*, *troglodytes*, and *verus*. Thus there is evidence for some heterogeneity in *Pt-LIR* haplotypes, but it is modest compared with the variability seen with *Pt-KIR*s.

Discussion

LIR and *KIR* are related gene families closely linked in the human genome, and both encode activating and inhibitory leukocyte receptors in which the extracellular regions are made up of Ig-like

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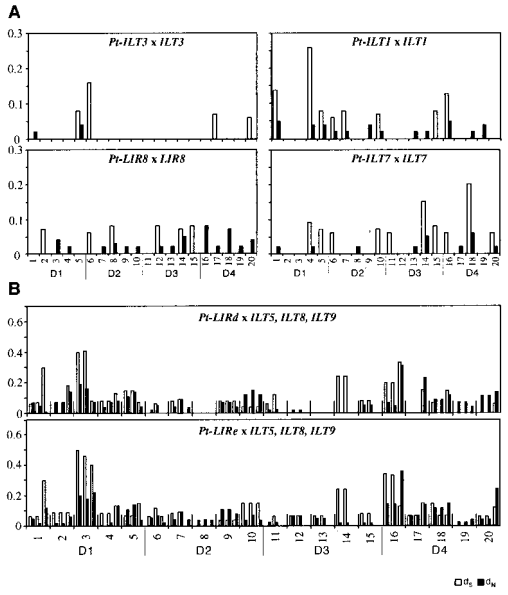

FIGURE 5. Comparison of the distribution of synonymous (d_S) and nonsynonymous (d_N) substitutions in chimpanzee and human *LIR* sequences. Each Ig-like domain-coding region was divided into small fragments of ~60 nt each. Analyses were made for each of these fragments and are plotted as a histogram. □, d_S values; ■, d_N. D1-, D2-, D3-, and D4-coding regions comprise fragments 1–5, 6–10, 11–15, and 16–20, respectively. *A*, Analysis of the four orthologous chimpanzee and human *LIR*s. *B*, comparison of the unassigned chimpanzee genes, *Pt-LIRd* and *Pt-LIRe*, when compared with the three closest human *LIR*s, i.e., ILT5, ILT8, and ILT9. In these analyses, each fragment is represented by six bars (three d_S and three d_N): the two bars on the *left* show results generated by analyses with *ILT5*, the two bars in the *middle* show results generated by analyses with *ILT8*, and the two bars on the *right* show results generated by analyses with *ILT9*.

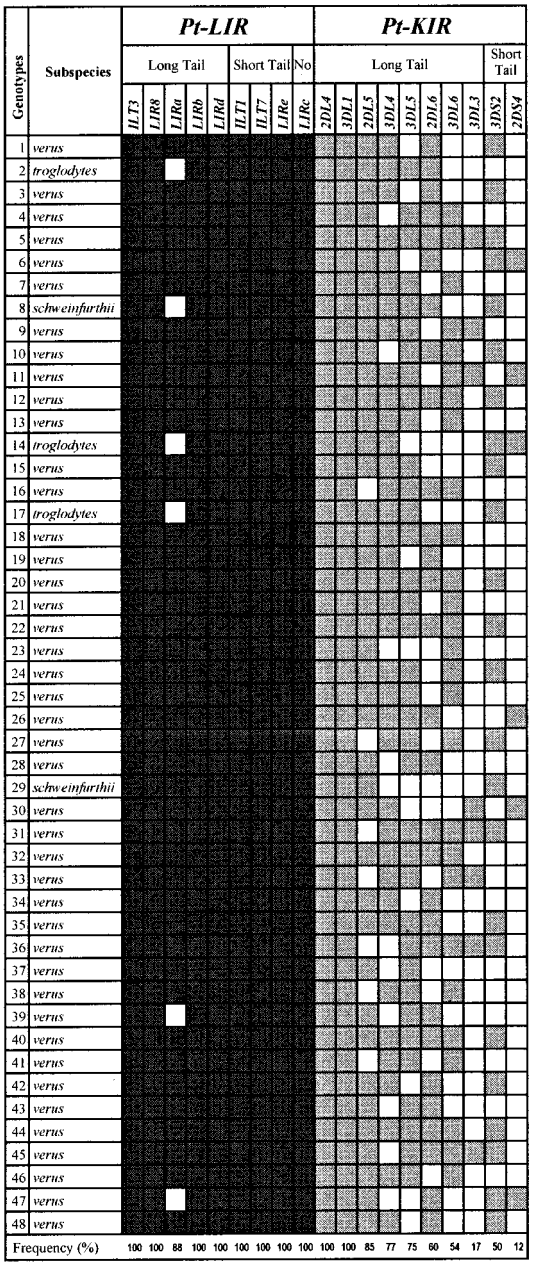

FIGURE 6. SSP-PCR typing of *LIR* and *KIR* genes from the genomic DNA of 48 unrelated common chimpanzees. Common chimpanzee *LIR* genes are divided into those with long, short, or no (for potentially soluble molecules) cytoplasmic tails, while *KIR*s are divided into those with long and short tails. *LIR* typings are shown in dark gray, and *KIR* typings are shown in light gray. The frequency of each gene in the panel is shown at the *bottom*.

domains. Neither of these gene families appears to be present in the mouse genome, at least not as a recognizable ortholog, suggesting that these receptor systems have undergone considerable change and diversification during the course of mammalian evolution (41). Investigation of this proposition requires comparison of species more closely related than mice and men; therefore, we have been studying chimpanzees, the living species most closely related to humans. This study aimed to identify the expressed *LIR* genes of the common chimpanzee (*Pan troglodytes*) and to determine their structural and evolutionary relationships with human *LIR*s.

Thirteen human *LIR* genes (*ILT1–11*, *LIR6*, and *LIR8*) have been defined, of which two appear to be pseudogenes (*ILT9* and *ILT10*) (4, 5). From the analysis of cDNA clones obtained from a splenocyte library we have characterized nine different chimpanzee *LIR*s, all expressed in the cells of one individual. The nine chimpanzee and 11 human expressed *LIR*s exhibit a similar range of diversity, as is clearly seen in phylogenetic trees (Fig. 3). In these trees are five *LIR* clusters, of which two contain only long-tailed *LIR*s (III and IV), one contains only short-tailed *LIR*s (V), and two contain both long- and short-tailed *LIR*s (I and II). All these clusters and subclusters contain both chimpanzee and human *LIR*. Thus, the set of nine chimpanzee *LIR*s defined here should provide good representation of the total set of chimpanzee *LIR* genes, although the possibility that additional family members await discovery must not be ruled out.

Genotyping 48 unrelated common chimpanzees showed 42 to have genes for all nine chimpanzee *LIR*s; the remaining six individuals had just eight of the genes. The negative typing reactions for *Pt-LIRa* could be due to absence of the gene or to *Pt-LIRa* gene polymorphism that affected the oligonucleotide priming sites used in the typing. Favoring the former interpretation is the fact that two different sets of *Pt-LIRa*-specific oligonucleotide primers gave negative results. Either way, there is clear evidence for two different chimpanzee *LIR* haplotypes with frequencies of 35% (*Pt-LIRa*$^-$) and 65% (*Pt-LIRa*$^+$) assuming Hardy-Weinberg equilibrium. Two human *LIR* haplotypes have also been defined, one containing all the genes and one containing an *ILT6* psuedogene with a ~6.7-kb deletion (5, 16); the latter haplotype appeared to be have a frequency of 18–28% in the populations examined (5, 16). The persistence of such deletion haplotypes is evident by their presence in common chimpanzee subspecies that diverged about

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1.6 million years ago (42). The modest variation in *LIR* genotypes observed within chimpanzee and human contrasts dramatically with the extensive variation in the *KIR* genotype observed in both species (Fig. 6) (25, 31).

Pairwise comparison of chimpanzee and human LIR allowed four pairs of orthologs to be assigned with confidence (Fig. 1): *ILT1*/*Pt-ILT1*, *ILT3*/*Pt-ILT3*, *ILT7*/*Pt-ILT7*, and *LIR8*/*Pt-LIR8*. These pairs of nucleotide sequences differ by about 2%, comparable to estimates of the overall genome sequence similarity of ~98.8% between human and chimpanzee (43). These genes show little evidence of having undergone recombination with other *LIR* genes, and in large part the differences between the species do not appear to be the result of natural selection; exceptional in this regard are *LIR8* and *Pt-LIR8* (Fig. 5*A*). On the basis of cDNA sequence comparison, orthologous relationships cannot be assigned between five chimpanzee and nine human *LIR*s. In the pairwise comparisons of these five chimpanzee *LIR* sequences, they differ by 9–14% in nucleotides with the closest human *LIR*s, values much greater than the genome average and which appear largely to be a consequence of recombination between *LIR* genes. Attempts to identify chimpanzee genes more closely related to human *ILT2*, *-4*, *-5*, *-8*, and *-11* were made by PCR amplification of chimpanzee genomic DNA with primers specific for these human genes. No positive reaction was obtained (data not shown) consistent with the absence of conserved, orthologs for these genes.

Comparison of chimpanzee and human *LIR* allows us to divide the *LIR* gene family into a group of four genes that have been relatively stable during the ~5.5 million yr of evolution that separate modern chimpanzees and humans (44), and an additional group of genes that have been evolving more rapidly through intergenic recombination, gene duplication, and gene deletion. In the human *LRC*, *LIR* genes are organized as two similarly sized regions (4). These arose by en bloc duplication of an ancestral region, which gave rise to two daughter blocks in opposite transcriptional orientations and separated by an intervening region of about 200 kb (4, 16). We now see that the four human *LIR* genes having chimpanzee orthologs are evenly distributed between the two blocks, and each block contains one long-tailed and one short-tailed *LIR* (*LIR8* and *ILT7* in one block, and *ILT1* and *ILT3* in the other; Fig. 7).

In humans, two clusters of rapidly evolving genes in each duplicated block are separated from each other by the orthologous or framework genes (Fig. 7). Regarding the nonorthologous genes, *ILT4* and related members (cluster I) lie between *LIR8* and *ILT7* in one block, and between *ILT1* and *ILT3* in the other. In contrast, *ILT5* and related genes (cluster II) are downstream of *LIR8* and *ILT3*. Thus the organization of the LIR regions is such that conserved, orthologous framework genes alternate with more rapidly evolving genes. This situation is like that found in the *KIR* gene family where three framework genes define two intervals of high gene variability (5, 31, 45). Also comparable in the two gene families are the percent sequence identities between human and chimpanzee orthologs (~2–5%) and more rapidly evolving genes (>8%). Furthermore, the numbers of inhibitory, long-tailed KIR and LIR are similar in humans and chimpanzees, but humans have more activating, short-tailed KIR and LIR than chimpanzees. Thus, for both families the inhibitory receptors appear more conserved.

Fig. 7 shows a working model for the organization of the chimpanzee *LIR* complex and its comparison to human *LIR* genes. Given the good representation of human and chimpanzee *LIR* genes in all major clusters, the chimpanzee *LIR* gene family is probably organized similarly to the human *LIR* genes in two homologous blocks (Fig. 7). Chimpanzee genes have been placed in the same intervals as human genes. In this scheme one can have some confidence in the positions of the orthologous or framework genes, but the positions assigned to the faster evolving clusters of *ILT4*- and *ILT5*-related genes chimpanzee genes remain speculative. Consider first the cluster consisting of *ILT4* and related genes (cluster I in Fig. 3). As *Pt-LIRb* groups with human *ILT4* in both phylogenetic analyses (Fig. 3), and since they both encode long-tailed LIRs, we assigned them to the same interval. As *Pt-LIRc* is related to human *ILT6* (Fig. 3B), and as they are the only genes encoding soluble proteins (Fig. 2), *Pt-LIRc* could be assigned to the same interval as *ILT6*. Alternatively, *ILT6* and *Pt-LIRa* could be placed at the same interval, as they are both the missing genes on the minority haplotypes. Of the human genes in the *ILT5*-related cluster (II in Fig. 3), *ILT5* and *ILT8* are together in one gene block and represent a closely related pair encoding an inhibitory and an activating four-Ig-domain receptor. In contrast, the *ILT5*-related gene of the other block is *ILT9*, an unusual LIR with three Ig-like domains. As *Pt-LIRd* and *Pt-LIRe* encode a conventional pair of inhibitory and activating LIR, such as ILT5 and ILT8, these genes have been assigned to the same interval as *ILT5* and *ILT8*, respectively. These tentative assignments provide a working model for future direct analysis of the genomic organization of the chimpanzee *LIR* gene family.

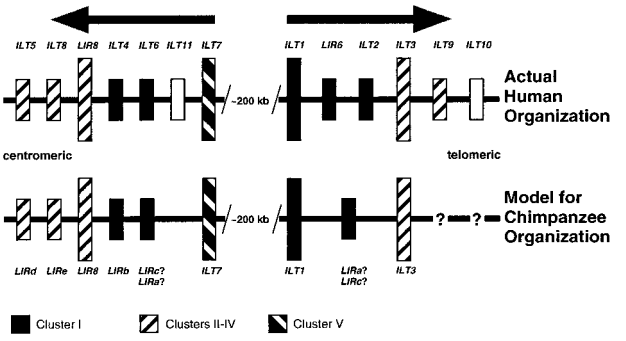

FIGURE 7. A schematic representation of the *LIR* gene cluster in human and common chimpanzee. The *upper portion* of the figure shows the genomic organization of the human *LIR* (4, 5). The arrows indicate the direction of transcription for each gene. Shown below is a model for the organization of the chimpanzee *LIR* locus as deduced from phylogenetic analysis. Orthologous genes are shown as the taller rectangles. Shading is based on the ancestral relationships previously proposed (4), which are in agreement with the analyses performed here. One ancestral group described by Wende et al. (4) corresponds to cluster I, and the other ancestral group to clusters II–IV. Finally, cluster V (*ILT7*/*Pt-ILT7*) was proposed to be a translocation/duplication that occurred subsequent to the initial inverse duplication.

In both humans and chimpanzees, their *KIR* haplotypes are much more diverse in gene number and content than are the *LIR* haplotypes. The propensity for new *KIR* haplotypes to evolve by asymmetric recombination is explained by the close proximity of the *KIR* genes and their separation by short, highly homologous intergenic sequences (5). This is not the situation for the human *LIR* gene family (4, 5). Due to this difference we anticipated that the *LIR* genes would be phylogenetically more conserved than the *KIR* genes. This assumption was proven wrong, with the interspecies variation in *KIR* and *LIR* genes being comparable. Both families contain some genes that are relatively stable and recognizable as orthologs, whereas other genes have been rapidly evolving through recombination. Thus, for some genes in both the *KIR* and *LIR* families there appears to be natural selection for new variants. The *KIR* genes known to encode receptors with specificity for classical MHC class I molecules are ones that have rapidly evolved

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since divergence of human and chimpanzee ancestors (31). Similarly, the LIRs with known specificity for MHC class I are encoded by genes, *ILT2* (*LIR1*) and *ILT4* (*LIR2*), with no clear-cut chimpanzee orthologs and that are members of a cluster of rapidly evolving genes. In conclusion, this comparison of chimpanzee and human species shows that both the *LIR* and *KIR* gene families evolve rapidly, making the *LRC* a likely hotspot of difference between chimpanzee and human genomes.

Acknowledgments

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