A Novel, Nonclassical MHC Class I Molecule Specific to the Common Chimpanzee

Erin J. Adams, Stewart Cooper and Peter Parham

*J Immunol* 2001; 167:3858-3869; doi: 10.4049/jimmunol.167.7.3858

http://www.jimmunol.org/content/167/7/3858

**References**

This article cites 63 articles, 28 of which you can access for free at:

http://www.jimmunol.org/content/167/7/3858.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
A Novel, Nonclassical MHC Class I Molecule Specific to the Common Chimpanzee

Erin J. Adams, Stewart Cooper, and Peter Parham

All expressed human MHC class I genes (HLA-A, -B, -C, -E, -F, and -G) have functional orthologues in the MHC of the common chimpanzee (Pan troglodytes). In contrast, a nonclassical MHC class I gene discovered in the chimpanzee is not present in humans or the other African ape species. In exons and more so in introns, this Patr-AL gene is similar to the expressed A locus in the orangutan, Popy-A, suggesting they are orthologous. Patr-AL/Popy-A last shared a common ancestor with the classical MHC-A locus >20 million years ago. Population analysis revealed little Patr-AL polymorphism: just three allotypes differing only at residues 52 and 91. Patr-AL is expressed in PBMC and B cell lines, but at low level compared with classical MHC class I. The Patr-AL polypeptide is unusually basic, but its glycosylation, association with β₂-microglobulin, and antigenicity at the cell surface are like other MHC class I. No Patr-AL-mediated inhibition of polyclonal chimpanzee NK cells was detected. The Patr-AL gene is present in 50% of chimpanzee MHC haplotypes, correlating with presence of a 9.8-kb band in Southern blots. The flanking regions of Patr-AL contain repetitive/repetitive elements not flanking other class I genes. In sequenced HLA class I haplotypes, a similar element is present in the A*2901 haplotype but not the A*0201 or A*0301 haplotypes. This element, 6 kb downstream of A*2901, appears to be the relic of a human gene related to Patr-AL. Patr-AL has characteristics of a class I molecule of innate immunity with potential to provide common chimpanzees with responses unavailable to humans. The Journal of Immunology, 2001, 167: 3858–3869.

To recognize infection by pathogens, the immune system uses families of genes and gene segments to make Ag-binding proteins. In addition to providing a diversity of Ag specificities, gene families facilitate rapid evolution of novel specificities through recombination between the genes. For Ig and TCRs, change takes place within the lifetime of an individual organism, whereas for MHC class I and other families of nonrearranging immune system genes, such evolution occurs over the lifetimes of populations and species.

As a consequence of ~70 million years of divergence, the complement of class I genes in the MHC of human and mouse are now different to the point where no orthologous relationships are discernable. In contrast, the MHC of the common chimpanzee (Pan troglodytes) contains genes orthologous to all the expressed class I genes of the human MHC: HLA-A, -B, -C, -E, -F, and -G (1–6). In both humans and chimpanzees, the classical MHC-A, -B, and -C genes are highly polymorphic, and their products bind to cytotoxic CD8⁺ T cells (1, 7, 8). However, no allotype is held in common, showing that ~5 million years has been sufficient to modify all the alleles once present in the common ancestral population (3). In both chimpanzees and humans, some MHC-A and B allotypes are ligands for killer cell Ig-like receptor (KIR) 3D of NK cells, but the specificities differ (9–13). In contrast, the human and chimpanzee KIR that engage MHC-C determinants have identical specificity but substantial structural differences (11, 14–16).

The nonclassical MHC-E and G genes have low polymorphism, and their proteins are implicated in the NK cell response. In humans and chimpanzees, MHC-E binds peptides derived from MHC-A, -B, and -C leader sequences and in this manner forms ligands for the inhibitory CD94:NKG2A receptor of NK cells (17). In structure and specificity, this ligand-receptor pair is highly conserved (1, 11, 18). By contrast, NKG2C, which forms with CD94 an activating receptor for MHC-E in humans (17, 19), is less highly conserved in chimpanzees (11). Human MHC-G binds KIR2DL4 (20) and because both these components are highly conserved in chimpanzees (11, 21), they are likely to perform similar functions in the two species. MHC-F has been shown to bind the inhibitory receptors immunoglobulin-like transcript-2 and immunoglobulin-like transcript-4; however, cell surface expression of this particular class I molecule has not been demonstrated (22).

Although human and chimpanzee genomes are estimated to be ~98.6% identical in nucleotide sequence (23) the two species have striking differences in response to certain pathogenic microorganisms. A striking example is HIV infection, which is controlled by chimpanzees and does not progress to AIDS as in most infected humans (24). Chimpanzees are also relatively resistant to malaria (25, 26), and the incidence of certain cancers is lower (27). In this context, any immune system gene that is present in chimpanzees...
and absent from humans is a potential contributor to disease resistance. Here, we describe a novel MHC class I gene, *Patr-AL*, that fulfills these criteria and has characteristics of a nonclassical class I gene. Not only is *Patr-AL* present in common chimpanzees and absent from humans, but it is also undetectable in the other African ape species.

**Materials and Methods**

**Establishment of cell lines**

Blood samples were obtained from common chimpanzees housed at the following institutions: Yerkes Regional Primate Center (Atlanta, GA); White Sands Research Center (Las Cruces, NM); and Laboratory for Experimental Medicine and Surgery in Primates, New York University Medical Center (Tuxedo, NY). PMBCs isolated by Ficoll gradient separation were used to establish Epstein-Barr virus-transformed B cell lines by the method described by Lawlor et al. (5).

**Isolation of *Patr-AL* sequences from cDNA and genomic DNA (gDNA)**

Total RNA and high molecular mass gDNA was isolated from EBV-transformed B-lymphoblastoid cell line (BLCL) using the reagents RNAzol (Teltest, Friendswood, TX) and DNAzol (Molecular Research Center, Cincinnati, OH) respectively. cDNA was made from mRNA using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) following the manufacturer’s protocol. gDNA from the animals listed in Table I and cDNA from the animals listed in Tables I and II were used as template for locus-specific amplification following the protocols of Donema et al. (28) with the following primers. 

**Common chimpanzee subspecies determination**

Nucleotide sequences for −360 bp of the mitochondrial D loop were determined according to the protocol and database of Morin et al. (29). Alignments of chimpanzee subspecies were made on the basis of pedigree and mitochondrial D loop sequence.

**Patr-AL typing system**

To detect the presence of *Patr-AL* within common chimpanzees and other species, a specific typing system for gDNA was established. The *Patr-AL*-specific primer set (AL4S (5'-GGGCTGCAGCTGAACTCGAACTCCCGAGCGCGAG-3') and 3'-UTA (5'-CCCGAATCTTGGGGAGGAGGAACAGTGCAGGTGGAAGAC-3') for *Patr-AL*, 5'-UT (see above) and SCAL-3'-UT2 (5'-TACAGGAGTGGTTGCTCTCTTAC-3') products were cloned into either the pBluescript vector or into the TOPO-TA subcloning system (Invitrogen, Carlsbad, CA). A minimum of three clones was sequenced bidirectionally to construct a consensus sequence for each *Patr-A* and *Patr-AL* allele isolated in this study.

**Evolutionary analysis**

Alignment of sequences was performed by inspection using the Genetics Computer Group (GCG) software program Sequlab (GCG, Madison, WI) with the MacX interface (Apple Computer, Cupertino, CA). Pairwise differences were calculated as uncorrected p distance using PAUP 4.0 (31). Pairwise difference values were also calculated using the Kimura two-parameter distance method, correcting for differences in transition and transversion rates; however, significant differences between these results and the uncorrected p distance. Neighbor-joining phylogenetic trees (32) were constructed using the computer program PAUP 4.0. The bootstrap method (33) of 500 replicates was used to assess the confidence in tree nodes. Dotplots were constructed using the GCG programs Compare and Dotplot, to graphically plot the results.

**Patr-AL transfection and expression studies**

To test for cell surface expression of *Patr-AL*, two *Patr-AL* expression constructs were made. The first construct consisted of a wild-type *Patr-AL* error-free cDNA clone isolated from the common chimpanzee Miss-Eve, inserted into Invitrogen’s pcDNA3.1 expression vector. The second construct was identical with the first except for the leader peptide, which was modified from VMPPRTLLL to VTPPRTLL so that it would not bind HLA-E. Both constructs were independently transfected into the 721.22I cell line, which is deficient in expression of the classical class I molecules, HLA-A, HLA-B, and HLA-C, but does express HLA-E. The transfection conditions were as described by Cooper et al. (7). Cell surface expression was assayed by FACS analysis on a FACScan (Becton Dickinson, Franklin Lakes, NJ) using the class I-specific mAb W6/32 and the β2-microglobulin (β2m)-specific mAb BRM7. To establish stably expressing populations, both transfecants were sorted on a Becton Dickinson FACStar, based on reactivity with W6/32.

**Immunoprecipitation and isolectric focusing (IEF)**

BLCL or PBMC (5 × 10⁶) were incubated in methionine-free RPMI 1640 supplemented with 10% FCS, glutamine, penicillin, and streptomycin for 45 min at 37°C. To radiolabel newly synthesized proteins, we added 50 μCi ¹¹C and incubated at 37°C for 4 h for the BLCL (transfected and native cell line) and 10 h for the PBMC. After lysis of the cells, normal rabbit serum was used to preclar nonspecific binding to Ab; immunoprecipitations were performed with *Staphylococcus aureus*. The class I-specific mAb W6/32 was used for specific immunoprecipitation of class I molecules. Immunoprecipitates were analyzed on IEF gels (1.5% ura, 7% acrylamide-bisacrylamide (29.1), 3% Nonidet P-40, 7.7% Ammonium, pH 3–10) for 7 h to increase resolution of basic proteins. To remove sialic acid residues, immunoprecipitates were incubated overnight at 37°C with either 0.2 or 0.4 U neuraminidase type VIII (Sigma, St. Louis, MO). The IEF gels were acid fixed and soaked in the fluorographic reagent,
P. troglodytes troglodytes). The P. troglodytes verus heterozygous: Fred Astaire carries the number of nucleotide differences. Miss Eve is homozygous for the based on sequence alignment, phylogenetic analysis, and the number of allelic differences identiﬁed by pairwisedifferences between a panel of class I transfectants. Two Patr-AL transfecants were tested, one expressing the Patr-AL wild-type protein (Patr-ALwt) and the other expressing the Patr-AL protein with modiﬁed leader peptide (Patr-AL-Eko). This comparison was done to assess whether reactivity against the Patr-AL transfectant was due to Patr-AL-induced up-regulation of HLA-E on the cell surface. Transfectants expressing the common chimpanzee class I alleles Patr-A*0402, Patr-B*1601, Patr-C*0501, and Patr-C*1201 were used as positive controls; each has been shown previously to inhibit NK cell lysis (11). The untransfected 721.221 cell line was used as a control to establish maximum NK cell killing. Target cells were labeled with 51Cr for 1 h at 37°C. Each killing assay was performed in duplicate; values shown are averages. An equal number of wells were used to determine spontaneous release (target cells in medium alone) and maximum release (target cells in medium containing 0.5% Triton X-100) release. Four E:T ratios were used for each polyclonal culture; the maximum E:T used varied with the availability of NK cells, other E:T ratios used were sequential 1/3 dilutions of the maximum value. Speciﬁc lysis was calculated as the ratio of the mean chromium release (MR) minus the spontaneous release (SR) and the total release (TR) minus the spontaneous release (SR); speciﬁc lysis = ([MR − SR]/[TR − SR]).

Results
Transcripts from a novel class I gene

The orthology of human and common chimpanzee (P. troglodytes) expressed MHC class I genes (MHC-A, -B, -C, -E, -F, and -G), has been well established through sequencing, Southern blot analysis and functional studies (1, 3, 5–7, 34). In this investigation, we describe characterization of a novel expressed MHC class I gene in the common chimpanzee, Patr-AL. We ﬁrst characterized this gene by PCR ampliﬁcation of cDNA from BLCLs from three common chimpanzees, each representing one of three common chimpanzee subspecies: Miss Eve (P. troglodytes schweinfurthii), Fred Astaire (P. troglodytes troglodytes), and Ericka (P. troglodytes verus). The ampliﬁcations were aimed at the chimpanzee MHC class I A locus (Patr-A), yet produced two distinct groups of cDNA. Sequences of the majority group corresponded to alleles of the chimpanzee MHC class I A locus (Patr-AL), whereas the other two chimpanzees are Patr-A heterozygous: Fred Astaire carries the Patr-A*1502 and Patr-A*1801 alleles; Ericka carries the Patr-A*0601 and Patr-A*0901 alleles.

A minority of the cDNA clones isolated from the Patr-A-speﬁc ampliﬁcation, typically represented by one clone for each individual, were similar to each other and distinct from Patr-A by several unique nucleotide substitutions. Pairwise comparisons of these cDNA sequences with all human and chimpanzee MHC-A, -B, and -C alleles revealed that they were mostly closely related to the alleles of the A locus (Fig. 1). On this basis, we have named this locus A-like or Patr-AL. That the Patr-AL sequence could be isolated from individuals that express two Patr-A alleles conﬁrms that this is an independent locus from Patr-A. Patr-AL clones were not isolated from all the common chimpanzees from which we characterized Patr-A, raising the possibility that Patr-AL was either polymorphic and not recognized by our PCR or absent from the class I haplotypes of these individuals. The isolation of Patr-AL cDNA establishes that this locus is transcribed, and because all class I residues conserved throughout vertebrate phylogeny (35) are also present in Patr-AL, it is thus likely to be functional. We therefore pursued further characterization of Patr-AL.

Patr-AL has low polymorphism characteristic of a nonclassical class I gene

To assess diversity in the Patr-AL gene, we characterized gDNA and cDNA Patr-AL sequences using a Patr-AL-speciﬁc oligonucleotide primer, SCAL3'-UT2, designed from a consensus of the Patr-AL clones already characterized. The SCAL3'-UT2 primer was paired with a 5’ class I-speciﬁc primer, 5'-UT, in PCR ampliﬁcation of cDNA and gDNA. This primer pair gave efﬁcient PCR ampliﬁcation of Patr-AL. PCR products were subcloned, and three or more clones for each Patr-AL allele were sequenced to establish the correct sequence for each allele. The alleles charac¬terized from each individual and the nucleotide differences between them are described in Table I.

Four Patr-AL alleles were deﬁned from the three chimpanzees from which cDNA clones were ﬁrst isolated: two were identiﬁed in Ericka (EAL-1 and EAL-2), whereas single alleles were identiﬁed in Miss Eve (MEAL-1) and Fred Astaire (FRAL-1). All the corresponding allelic differences identiﬁed in genomic DNA sequences were also seen in cDNA sequences, demonstrating that all the Patr-AL alleles are transcribed. Although each individual possessed unique Patr-AL alleles, few substitutions discriminated the alleles in either the coding or noncoding regions. The two alleles isolated from Ericka, EAL-1 and EAL-2, are the two most diver¬gent, differing by six substitutions: one in exon 3 and in the noncoding regions of introns 5, 6, and the 3’-UT region (Table I). MEAL-1 and FRAL-1 were most closely related to EAL-1; MEAL-1 differs by three substitutions located in intron 1, exon 3, and exon 4, and FRAL-1 differs from EAL-1 by one substitution in exon 2.

To expand the analysis of Patr-AL diversity, we further charac¬terized Patr-AL from the cDNA of 11 additional unrelated common chimpanzees (Table II). In 8 of the animals, Patr-AL alleles were already deﬁned from the analysis of Ericka, Fred Astaire, and Miss Eve. Three animals expressed novel alleles: Jacqueline and Marilyn expressed a novel allele (JAL-1) in which substitutions in previously characterized alleles are recombined; the allele from Beleka (BAL-1) differs from EAL-2 by a novel silent substitution in exon 3 at position 447. In this survey, the EAL-2 allele was most common, being present in 7 of the 14 animals studied. The overall

FIGURE 1. Pairwise differences between Patr-AL and alleles of the three classical class I loci in humans and chimpanzees. Pairwise distribution of differences between Patr-AL and full length alleles of HLA-A and Patr-A (246 pairs) are shown in black, full length alleles of HLA-B and Patr-B (441 pairs) in dark gray, and full length alleles of HLA-C and Patr-C (111 pairs) in light gray. To facilitate comparison between the loci, the distributions are normal¬ized as percent of total pairs on the y-axis.

Patr-AL and cDNA sequences from two chimpanzees (Miss Eve and Fred Astaire) were also seen in cDNA sequences, demonstrating that all the Patr-AL alleles were deﬁned from the analysis of Ericka, Fred Astaire, and Miss Eve. Three animals expressed novel alleles: Jacqueline and Marilyn expressed a novel allele (JAL-1) in which substitutions in previously characterized alleles are recombined; the allele from Beleka (BAL-1) differs from EAL-2 by a novel silent substitution in exon 3 at position 447. In this survey, the EAL-2 allele was most common, being present in 7 of the 14 animals studied. The overall
variability within the coding region of this gene is therefore low, and only two amino acid-altering substitutions exist in all alleles characterized. These replacement substitutions are at positions 228 and 344 (Tables I and II), which translate into residues 52 and 91 of the mature protein; neither are involved in the peptide-binding environment or are contact residues for αβ TCR (36, 37), nor are these positions implicated in binding to NK cell receptors (10, 38, 39). The low level of polymorphism and conservation of structure of Patr-AL is reminiscent of the nonclassical MHC class I genes in humans and chimpanzees such as E and G, which are essentially nonpolymorphic and have specialized functions in the immune response (40).

Patr-AL predates divergence of humans, chimpanzees, and gorillas

To define further the relationship of Patr-AL with Patr-A, HLA-A, and related genes in humans, apes, and old world monkeys, we constructed neighbor-joining phylogenetic trees using coding region sequences (Fig. 2A). In the phylogenetic tree, HLA-A (human), Patr-A (chimpanzee), Papa-A (bonobo), and Gogo-A (gorilla) group together in a clade with strong bootstrap support (91%). For simplicity, we will refer to this clade henceforth as the orthologous A locus. Within this clade, there is further definition of two main lineages of A locus alleles, A2 and A3, with strong bootstrap support of these groupings (82 and 79%, respectively). HLA-A locus alleles segregate into both the A2 and A3 lineages, whereas Gogo-A alleles segregate only into the A2 lineage (41) and chimpanzee Patr-A alleles only into the A3 lineage (4, 42, 43). Patr-AL groups outside of both the A2 and A3 lineages and appears as divergent from the orthologous A locus as from the orangutan expressed A locus (Popy-A).

Table II. Patr-AL alleles from 11 chimpanzees

Further evidence that Patr-AL is not closer to either the A2 or A3 lineage of the classical A locus comes from examination of the nucleotide positions that define these allelic lineages and their subfamilies within the HLA-A locus (41). At these positions, Patr-AL shares substitutions with both the A2 and A3 lineages and their allelic subfamilies and is more closely related to the A locus consensus than to any particular allelic group (Table III). Whereas Patr-AL differs from the HLA-A consensus at only 4 of these positions, the differences between Patr-AL and each individual family range from 9 to 15. These results support the hypothesis that Patr-AL predates divergence of the two lineages and thus of the human-chimpanzee divergence.

To investigate the relationship of Patr-AL with A-related loci from more divergent species, we examined the locus-specific sites that exclusively discriminate Patr-AL from both HLA-A and Patr-A. Thirty-one substitutions present as fixed differences between Patr-AL and all known sequences of HLA-A and Patr-A (Table IV). Their uniform distribution throughout the coding region argues against their having been introduced into Patr-AL through recombination events with other class I genes. Many of the polymorphisms defining Patr-AL are shared with A-related loci from more distantly related species (orangutan, gibbon, and macaque). Indeed, the more distant the species, the greater the sharing of such Patr-AL-defining sites. This comparison shows that Patr-AL is not the result of a recent duplication of a Patr-A allele but is of more ancient origin and has evolved alongside the classical A locus.

To examine further the phylogenetic relationship of Patr-AL with A-related class I genes, we compared Patr-AL intron sequences with those available from human, chimpanzee, and orangutan genes and pseudogenes (Fig. 2B). In the introns, Patr-AL has close homology with the orangutan expressed A locus, Popy-A, with strong bootstrap support (95%) (Fig. 2B), a relationship hinted at in analysis of exonic sequences in Fig. 2A. It is therefore likely that Patr-AL represents a chimpanzee orthologue of Popy-A. This Patr-AL/Popy-A clade also includes an orangutan A-related pseudogene, Popy-Ap. These sequences are separated from the orthologous A loci, HLA-A and Patr-A, with strong bootstrap support (92%). Grouping outside of these two clades are the intron sequences of the more distantly related human pseudogene, HLA-BEL (44), and the A-related pseudogenes H (45) and J (46), and nonclassical genes F (47) and G (48), all of which are found in humans and chimpanzees (1).

Patr-AL is present on only certain common chimpanzee haplotypes

PCR with primers 5′-UT and SCAL3′-UT2 failed to amplify Patr-AL from some common chimpanzees. To investigate whether this was due to polymorphism in the priming sites or absence of
the Patr-AL gene, we developed a Patr-AL-specific primer set for typing of both cDNA and gDNA. The design of these primers was based on the Patr-AL cDNA sequences presented in Table II; they...
Table IV. Sites distinguishing Patr-AL from the classical A locus

<table>
<thead>
<tr>
<th>Position</th>
<th>Patr-AL Identities</th>
<th>HLA-A/Patr-A</th>
<th>Gogo-Oko/A3</th>
<th>Popy-A</th>
<th>Mamu-A</th>
<th>Hyla-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>C</td>
<td>C</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>39</td>
<td>C</td>
<td>G</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>199</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>266</td>
<td>C</td>
<td>G</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>269</td>
<td>C</td>
<td>C</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>271</td>
<td>G</td>
<td>G</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>273</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>276</td>
<td>G</td>
<td>G</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>278</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>290</td>
<td>G</td>
<td>C</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>318</td>
<td>C</td>
<td>G</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>387</td>
<td>G</td>
<td>C</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>393</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>402</td>
<td>G</td>
<td>C</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Note: Identical to Patr-AL; information missing (position 18) or nucleotide deletion (position 402).

Patr-AL and classical A flanking regions are distinguished by repetitive elements.

Dotplot analysis was used to compare the sequence of the Patr-AL gene with the sequence of Patr-A*0901 and also with extended haplotypic sequences containing HLA-A*0201, HLA-A*0301, or
HLA-A*2902 (Fig. 4). Comparison of Patr-AL with Patr-A gives an essentially straight diagonal line of dots showing the similarity of the two genes (Fig. 4A). In comparison with HLA-A alleles, the patterns also show strong homology, but there are two regions where Patr-AL differs significantly from the three HLA-A fragments (Fig. 4, B–D). These regions are denoted by discontinuities in the diagonal line and are due to insertions in Patr-AL that comprise positions 408-1283 (875 bp, ~4 kb upstream from the Patr-AL start codon) and positions 8432–end (1381 bp) of the Patr-AL fragment.

The insertion at positions 408-1283 is a retroviral long terminal repeat, the insertion at positions 8432–end is a tandemly duplicated long interspersed nucleotide element (LINE). None of the class I genes in the three genomic sequences of HLA class I haplotypes has similar elements in its flanking regions. However, a LINE element with 95.3% sequence identity with that found in Patr-AL is placed ~6 kb downstream of HLA-A*2902 in a region located between HLA-A and HLA-80 (Fig. 4B). This element is absent from both the HLA-A*0301 and HLA-A*0201 haplotypes (Fig. 4, C and D). Whether this region has been deleted in the Patr-A sequence is unknown, because sequence information is limited to ~2 kb on the 3’ side of the Patr-A stop codon. These results suggest that the LINE element in HLA-A*2902 is a relic fragment of a former AL gene that has largely been deleted.

AL and A ancestors diverged >20 million years ago (mya)

Comparison of the flanking regions of Patr-AL (2154 bp) with those of Patr-A, HLA-A*0301, HLA-A*0201, and HLA-A*2902 allowed us to estimate the time of divergence of AL and orthologous A. Using a mutation rate calculated by Nachman and Crowell (50) of 2.5 × 10^-8 per site per generation and a generation time of 20 years, the estimated time of divergence is 26.3 ± 0.3 million years. The values do not change significantly when 1834 bp introns (23.6 ± 0.1 million years) or 1098 bp exons (26.8 ± 1.6 million years) are analyzed. We applied variations on the mutation rates and generation times to test whether this would significantly change our estimation; a mutation rate of 3.4 × 10^-8 per site per generation (the upper limit estimated by Nachman and Crowell) and a generation time of 25 years/generation gave an estimation of 24.2 ± 0.3 million years for the flanking region, whereas a mutation rate of 1.3 × 10^-8 per site per generation and a generation time of 20 years/generation gave a divergence time of 50.6 ± 0.6 million years. Taking the most conservative estimation of 24 million years, we can conclude that ancestors of AL and orthologous A diverged before the split of the hominidae, ~15–20 mya (51–54).

Applying similar calculations to the introns of Patr-AL and Popy-A and taking the most conservative estimation of 18.1 ± 0.4 million years places the divergence of these two loci directly within the range of the divergence of orangutans and common chimpanzees (54). That both the gene and species estimated divergence ranges overlap further support for these loci being orthologous. Estimations based on Patr-AL and Popy-A exons place the divergence date of these two loci further back in time (24.5 ± 0.7 million years), indicating that the exons have changed more rapidly than the introns.
Patr-AL is expressed at low levels on the cell surface

To test for cell surface expression of Patr-AL, a cDNA clone (MEAL-1) from the chimpanzee Miss Eve was transfected into a human class I-deficient cell line, 721.221. The transfected cells were analyzed for reactivity with the class I-specific Ab, W6/32 (Fig. 5A), and the β2m-specific Ab, BBM.1 (Fig. 5B). Specific binding of both Abs established that Patr-AL protein is made and expressed at the cell surface in association with β2m. The increased reactivity with W6/32 and BBM.1 was not due to up-regulation of HLA-E, due to binding of the Patr-AL-derived leader peptide (VMPPRTLLL), because transfection with a mutant Patr-AL cDNA encoding a leader peptide (VMPPTTLL) that does not up-regulate HLA-E gave results similar to those of the wild-type Patr-AL (data not shown).

Expression of Patr-AL was also demonstrated in the chimpanzee EBV B cell line Miss Eve from which it was originally characterized and in chimpanzee PBMCs. This was done by analyzing W6/32 immunoprecipitates made from radiolabeled cells on IEF gels (Fig. 5C). Patr-AL has an unusually basic pI (calculated from the amino acid sequence to be ~7.8), which separates it away from the other class I polypeptides on IEF gels. Bands corresponding to Patr-AL were obtained from the 721.221/Patr-AL transfectant (Fig. 5C, Patr-AL/221), and at lower levels from the BLCL and PBMCs of individuals who are Patr-AL positive (Fig. 5C, Miss Eve BLCL and Rufus PBMC). In contrast, Patr-AL was absent from immunoprecipitates made from untransfected 721.221 cells and from a BLCL that lacks the Patr-AL gene (Fig. 5C, Halpha BLCL, and Fig. 3). The lower intensity of the Patr-AL band in the BLCL and PBMC immunoprecipitates suggest the Patr-AL protein is expressed at lower levels than the classical class I molecules. Low levels of cell surface expression are characteristic of the nonclassical class I molecules MHC-E and MHC-G.

Human MHC class I molecules have one site of N-glycosylation at Asn^386. This residue is also the only possible N-glycosylation site in Patr-AL. To assess the glycosylation state of Patr-AL, immunoprecipitates were treated with neuraminidase to remove sialic acid and compared with untreated samples on IEF gels. After neuraminidase treatment, a single band at lower pl was observed (Fig. 5D). This shows that Patr-AL is glycosylated, the change in pI after neuraminidase treatment being consistent with a single site of N-linked glycosylation.

To investigate the low expression of Patr-AL, we compared the promoter region of Patr-AL with the promoters of other primate A-related class I genes (Ref. 55 and Fig. 6). Over the 223 bp compared, Patr-AL is most closely related to sequences from gorilla and orangutan. Within the regulatory elements that for human class I genes have been shown to be important for expression, enhancer A, IFN-stimulated response element, site α, enhancer B, and CCAAT and TATA elements (56), Patr-AL is identical with the orangutan (Popy-A) promoter sequence (Fig. 6, CP81A1), further supporting the model that this locus is orthologous to Popy-A. Patr-AL and CP81A1 are discriminated from the promoter elements of the classical A locus sequences by two substitutions: one in the enhancer A KB2 region (−213) and the other in the IFN-stimulated response element (−177). These changes are potential candidates for causing the low level of Patr-AL transcription; however, they do not appear to cause a low expression level for Popy-A (57).

Patr-AL is not a dominant inhibitory ligand for NK cells

The nonclassical class I molecules, HLA-E and HLA-G, are ligands for NK cell receptors. We therefore tested whether Patr-AL has similar function. Polyclonal cultures of NK cells were isolated from two Patr-AL-positive common chimpanzees and shown to

![Image](http://www.jimmunol.org/)

**FIGURE 5.** Cell surface expression of Patr-AL demonstrated by transfection of Patr-AL cDNA into the class I-deficient 721.221 cell line. Positive reactivity of the Patr-AL/221 transfectant with the W6/32 class I-specific mAb (A) and BBM.1 β2m-specific mAb (B) shows that it is expressed on the cell surface and associated with β2m. Isoelectric focusing (C) of class I immunoprecipitates from the Patr-AL/221 transfectant, a Patr-AL-positive BLCL (Miss Eve BLCL) and PBMCs from a Patr-AL-positive chimpanzee (Rufus PBMC) produced a band focusing near the estimated pI of Patr-AL (~7.8); this band is absent in a Patr-AL-negative human cell line (WT49), a Patr-AL-negative chimpanzee cell line (Halpha BLCL) and in the 721.221-untransfected cell line. D, IEF gel of neuraminidase-treated and untreated immunoprecipitates from the Patr-AL/221 transfectant, confirming that Patr-AL is glycosylated.
lyse class I-deficient 721.221 cells, but be inhibited by 721.221 cells transfected with Patr-A*0402, Patr-B*1601, Patr-C*0501, and Patr-C*1201. In contrast, 721.221 cells transfected with wild-type Patr-AL, or the mutant containing a leader peptide that does not up-regulate HLA-E expression, were lysed in a manner similar to that for untransfected 721.221 cells (Fig. 7). Thus, Patr-AL appears not to be a dominant inhibitory ligand for NK cell receptors.

Alternative splicing of Patr-AL mRNA

We consistently found that a proportion of Patr-AL RNA transcripts consists of a splice variant lacking exon 5 encoding the transmembrane domain. This splice variant was encountered among cDNA from all 15 common chimpanzees studied (Table II); products from Patr-AL-specific PCR amplification consistently ran as doublets when electrophoresed on agarose gels (data not shown). The higher band corresponded to the complete Patr-AL coding region (1283 bp); the lower band was the size of transcripts lacking exon 5 (1166 bp). To demonstrate that the clones composing this lower band lacked exon 5, Patr-AL PCR products from 10 individuals were cloned and screened for inserts of size corresponding to the lower band. Sequencing of these clones showed they all corresponded to Patr-AL but lacked exon 5. Thus, expression of the Patr-AL gene may direct the synthesis of both soluble and membrane-associated Patr-AL proteins.

Discussion

In the ~5 million years since P. troglodytes and Homo sapiens shared a common ancestor, their genomic sequences have diverged by an estimated ~1.4% (23). Consistent with this close relationship is that the MHCs of the two species have a similar organization and orthologues for all expressed HLA class I genes have been found in the MHC of the common chimpanzee (1). The reverse, however, is not the case. Serendipitously we discovered, and describe here, an expressed MHC class I gene of the common chimpanzee, Patr-AL, that appears to have no counterpart in the human species or in bonobos or gorillas.

In nucleotide sequence, the coding region of the Patr-A gene is equidistant from the orthologous A locus (HLA-A in human and Patr-A in chimpanzee) and the orangutan expressed A locus (Popy-A) (Fig. 2A). It cannot represent a divergent Patr-A lineage because individual chimpanzees can express Patr-AL in addition to
two Patr-A alleles. Thus, Patr-AL and Patr-A are definitely separate genes. Whereas Patr-A is highly polymorphic, Patr-AL has the low polymorphism characteristic of a nonclassical MHC class I gene like MHC-E or MHC-G. Expression of Patr-AL was first detected in EBV-transformed B cells lines and subsequently demonstrated in transfected cells and PBMC. Patr-AL heavy chains are glycosylated, associated with β2m and expressed at the cell surface like classical class I heavy chains, but their level of expression is substantially lower, as is also true for HLA-E and HLA-G (58).

Of the African apes, the Patr-AL gene appears specific to the common chimpanzee, yet it does not have the properties expected of a young locus, for example one formed by duplication of Patr-A subsequent to the split of human and chimpanzee ancestors. That a potential remnant fragment of AL exists on at least one human haplotype also suggests the AL gene existed in the common ancestor of the two species and has been deleted in humans rather than originating in the chimpanzee line. Indeed, phylogenetic analysis shows Patr-AL to group outside of all classical A alleles of gorilla, chimpanzee, and human (Fig. 2), showing it originated before these lineages split. Consistent with this interpretation is that Patr-AL shares specific substitutions with A-related genes in more distantly related primate species and with the A-related pseudogene HLA-H/Patr-H. Estimation of divergence time points to Patr-AL and the orthologous A last having shared a common ancestor >20 mya, before the divergence of the various ape species (54).

Although MHC class I alleles resembling HLA-A have been found in all species of ape and Old World monkey examined, it is now apparent that true orthologues of HLA-A may be present only in the African apes (gorilla, chimpanzee, and bonobo). The similarity between the introns of Patr-AL and Popy-A, and the overlap of the gene and species divergence dates support the model that Patr-AL and Popy-A are orthologous and represent a second A lineage, paralogous to the orthologous A lineage found in the African apes. The common chimpanzee appears to be the only African ape to maintain both paralogues. Although humans lack Patr-AL, the HLA-A locus has diversified considerably and has almost equal representation of the two main lineages, A2 and A3, that segregate within this locus. In contrast, the common chimpanzee has only maintained one of those lineages (A3), raising the possibility that Patr-AL to some extent compensates for the lower diversity of Patr-A.

That the orthology of Patr-AL and Popy-A is not directly evident in the exons implies that the coding region has been modified significantly since they last shared a common ancestor ~18 mya. Indeed the fate of this second A-related locus in chimpanzees and orangutans has differed considerably. In the orangutan, Popy-A has diversified to become a classical A locus with 4 to 48 nucleotide differences discriminating the 7 known Popy-A alleles (Ref. 57 and data presented in this article), only slightly less than the range of 1- to 60-nucleotide differences between 48 HLA-A alleles for which complete coding sequences are known (http://www.ebi.ac.uk/imgt/hla/). In contrast, Patr-AL resembles a nonclassical gene in its oligomorphism and low level of expressed protein.

A model consistent with these data involves a minimum of two gene duplication events in the evolution of the A, AL, and H loci from a common ancestor An1 (Fig. 8). The first duplication event likely occurred ~30 mya, producing what are now recognized to be the H pseudogene and an ancestral A locus, An2. Then, ~7 million years later, An2 duplicated to produce two paralogous A loci, now recognized to be Popy-All/Patr-AL and the orthologous A locus of the African apes (HLA-A/Patr-AL/Papa-Al/Gogo-A). Possible evidence for additional duplication and deletion of A-related loci are the unusual A-related sequences Gogo-Oko from gorilla (41) and a human pseudogene with similarity to Gogo-A (HLA-BEL), both of which are present on a subset of MHC haplotypes (44). Furthermore, an additional strongly hybridizing band in Patr-AL-positive chimpanzees at ~6.6 kb on Southern blot analysis (Fig. 3) suggests that additional A-related loci await discovery.

Although both expressed genes of the orthologous A and the AL lineage have been maintained in the lineage leading to modern chimpanzees, that does not appear to be the case in humans. Neither is the Patr-AL gene fixed in the chimpanzee population, being present at a gene frequency of ~50%. The nonclassical MHC-E and G molecules of humans and chimpanzees function as ligands for NK cell receptors: MHC-E engages the CD94/NKG2A and CD94/NKG2C receptors (17), MHC-G binds to KIR2DL4 (20). The MHC-E and G genes are both conserved and fixed, properties that also apply to those encoding their NK cell receptors. In contrast, many chimpanzee and human KIR genes, like Patr-AL, are only present on a fraction of haplotypes (11, 59). Moreover, some of these chimpanzee KIR are not found in humans, and among them are ones that from comparison with other apes appear to be specific to the common chimpanzee (11). Thus, it is possible that Patr-AL is a ligand for one or more such KIR.

Using 721.221 cells transfected with Patr-AL, we found no inhibition of cytotoxicity by polyclonal NK cells. To have produced inhibition in this experiment we would have required that Patr-AL ligate an inhibitory receptor and for that receptor to have been expressed by a substantial proportion of the NK cells clones in the polyclonal mixture. Thus, future investigation should consider the possibilities that Patr-AL is a ligand for an activating receptor of NK cells, for an inhibitory receptor expressed by a minority of chimpanzee NK cells, or for receptors expressed on other cell types.

For certain pathogens such as HIV and malaria, infected chimpanzees appear not to have the morbidity or mortality commonly seen in infected humans. Furthermore, chimpanzees have low incidences of cancers that are common in humans (reviewed in Ref. 27). The presence of a chimpanzee-specific nonclassical class I locus and several chimpanzee-specific NK receptors provides potential for chimpanzee innate immune responses being distinct from those of humans, and which could contribute to the species differences in disease outcome. The fact that Patr-AL is not fixed within the common chimpanzee population does not argue against its importance. Indeed, if an episodic sweep due to epidemic disease had caused only individuals expressing Patr-AL to survive, and the initial gene frequency of Patr-AL-containing haplotypes was low (<20%), the resulting gene frequency in the following generation would be ~50%, what we observe in the chimpanzee population studied here. Subsequent to such a selective episode, the overall gene frequency need not have changed significantly.

**FIGURE 8.** Model of A locus evolution. A, Hypothetical duplication of the ancestral An1 locus ~30 mya producing ancestor An2 and what is now recognized as the H pseudogene. A further duplication event ~23 mya of the An2 locus produced two paralogous genes, one of which is now represented by Patr-AL/Popy-A and the other by HLA-A/Patr-AL/Papa-Al/Gogo-A. B, Presence and absence of H, A, and AL in humans, bonobos, common chimpanzees, gorillas, and orangutans, based on Patr-AL typing results, Southern blotting, and phylogenetic analysis.
CHIMPANZEE-SPECIFIC NONCLASSICAL MHC CLASS I MOLECULE

over time, given the large long term effective population size of common chimpanzees (60, 61).

Acknowledgments

We thank the veterinarians and staff at the Yerkes Regional Primate Center of Emory University (in particular Rickie Bass), Drs. Jim Mahoney and Elizabeth Muchmore at Laboratory for Experimental Medicine and Surgery in Primates, Ali Javadian at White Sands, and Ann Erickson and Dr. Christopher Walker for their help in obtaining chimpanzee blood samples and cell lines; Dr. Luca Cavalli-Sforza and Dr. Ronald Bontrop for their generous donations of human and macaque gDNAs, respectively; and Professor Glensy Thomson, Dr. Lisbeth Guethlin, Dr. Salim Khakoo, Benny Shum, Diogo Meyer, and Kristie Mather for contributing their theoretical and experimental expertise and for their helpful insight and discussions.

References