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The locus encoding an oligomorphic family of *MHC-A* alleles (*Mane-A**06/*Mamu-A**05) is present at high frequency in several macaque species

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Abstract Several macaques species are used for HIV pathogenesis and vaccine studies, and the characterization of their major histocompatibility complex (MHC) class I genes is required to rigorously evaluate the cellular immune responses induced after immunization and/or infection. In this study, we demonstrate that the gene expressing the *Mane-A**06 allele of pig-tailed macaques is an orthologue of the locus encoding the *Mamu-A**05 allele family in rhesus macaques. Analysis of the distribution of this locus in a cohort of 63 pig-tailed macaques revealed that it encodes an oligomorphic family of alleles, highly prevalent (90%) in the pig-tailed macaque population. Similarly, this locus was very frequently found (62%) in a cohort of 80 Indian rhesus macaques. An orthologous gene was also detected in cynomolgus monkeys originating from four different geographical locations, but was absent in two African monkey species. Expression analysis in pig-tailed macaques revealed that the *Mane-A**06 alleles encoded by this locus are transcribed at 10- to 20-fold lower levels than other *MHC-A* alleles (*Mane-A**03 or *Mane-A**10). Despite their conservation and high prevalence among Asian macaque species, the alleles of the *Mane-A**06 family and, by extension their orthologues in rhesus and cynomolgus monkeys, may only modestly contribute to cellular immune responses in macaques because of their low level of expression.

Keywords Pig-tailed macaques · MHC class I · Locus · Site specific primer-PCR (SSP-PCR)

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Introduction

Macaques are Asian non-human primates frequently used in biomedical research to study human pathogens or organ transplantation because of their genetic and immunologic similarities to humans. In this regard, infections of macaques with simian immunodeficiency viruses (SIV) or chimeric simian human immunodeficiency viruses (SHIV) have been extensively used as animal models to study HIV pathogenesis and vaccine development. Three species are commonly used for these experiments: the rhesus macaque (*Macaca mulatta*), the pig-tailed macaque (*M. nemestrina*), and the cynomolgus or crab-eating macaque (*M. fascicularis*). Although rhesus monkeys are currently the principal non-human primate species used, the differential susceptibility of Indian and Chinese rhesus macaque sub-species to SIV-induced disease and the small number of animals having particular immunologic characteristics (e.g., the *Mamu-A**01 allele) can profoundly influence the outcome and the interpretation of logistically demanding experiments. The two other macaque species therefore represent valid alternatives as animal models. Furthermore, the relative higher susceptibility of pig-tailed macaques to a variety of SIV strains (SIVmac, SIVagm, and SIVsun)

compared to rhesus and cynomolgus macaques, offers an opportunity to evaluate host factors affecting primate lentivirus induced disease and to study the role of immune responses in controlling these infections (Beer et al. 2005; Hirsch et al. 1995; Reimann et al. 2005).

The identification and characterization of genes involved in cellular immune responses, particularly the major histocompatibility complex (MHC) class I genes, is currently a major goal for understanding how non-human primates control lentivirus replication. A better definition of macaque immunogenetics would allow the selection of more homogeneous groups of animals for pathogenesis and vaccine studies, and possibly reduce the variable clinical courses frequently observed in SIV- or SHIV-infected animals due to their non-inbred status. Improved characterization of macaque genetic make-up would also contribute to a deeper understanding of the kinetics of immune responses induced after immunization and/or infection. Current knowledge of MHC organization in macaques accrues primarily from studies conducted in rhesus macaques (Adams and Parham 2001; Bontrop 2006). Initial experiments identified MHC class I alleles expressed by individual animals and demonstrated that rhesus macaques possess *MHC-A* (*Mamu-A*) and *MHC-B* (*Mamu-B*) genes but no *MHC-C* locus (Boyson et al. 1996). Since that initial report, several rhesus MHC class I alleles have been shown to present SIV-derived peptides, and detailed peptide-binding properties have been described for several of them (*Mamu-A**01, *Mamu-A**02, *Mamu-A**08, *Mamu-A**11, *Mamu-B**01, *Mamu-B**03, *Mamu-B**04, *Mamu-B**12, *Mamu-B**17; Allen et al. 2001; Dzuris et al. 2000; Hickman-Miller et al. 2005; Loffredo et al. 2004, 2005; Mothe et al. 2002; Sette et al. 2005; Sidney et al. 2000). Based on the number of *MHC-A* and *MHC-B* alleles detected per animal, initial studies also suggested that the loci encoding *MHC-A* and *MHC-B* alleles were duplicated at least once on the rhesus chromosome. This observation has been recently confirmed by sequencing the MHC region of two different haplotypes (Daza-Vamenta et al. 2004; Kulski et al. 2004). Two functional *Mamu-A* loci were detected for each sequenced haplotype, whereas up to 15 *MHC-B* loci were identified on the rhesus chromosome. Some of these *Mamu-B* loci appeared to be functional, whereas others were shown to contain incapacitating mutations. A recent study involving a large rhesus macaque cohort reported that several haplotypes could be identified based on the number of *MHC-A* loci carried per chromosome (Otting et al. 2005). The presence of specific haplotypes for rhesus macaque *MHC-B* genes has been difficult to demonstrate because of the large number of loci and their highly variable copy number in different animals.

All macaque species have evolved from a common African ancestor. It is therefore expected that their genomes exhibit some strong organization similarities in their MHC class I region. The 20 recognized macaque species (Brandon-Jones et al. 2004; Sinha et al. 2005) have been separated into several "species groups" based on morphologic (Fooden 1976) and, more recently, genetic differences (Morales and Melnick 1998; Tosi et al. 2000, 2003). Cynomolgus and rhesus macaques are included in the Fascicularis group, whereas pig-tailed macaques are members of the Silenus group. These two groups evolved from the common ancestor of all Asian macaques that colonized Asia about 5.5 million year ago. The Silenus group, including pig-tailed macaques, diverged from the ancestor of the Fascicularis group 4.9 million years ago, and the rhesus and cynomolgus macaques speciation occurred 2.4 million years later. Whereas a similar genetic organization is expected between rhesus and cynomolgus macaques, pig-tailed macaques could have accumulated significant differences compared to the two other taxons during their 2.4 million year of separate evolution.

In contrast to the knowledge accumulated about the organization of the rhesus macaque MHC, investigations of the MHC class I genes of other macaque species have only recently begun. MHC class I alleles have been described for pig-tailed macaques (Lafont et al. 2003; Smith et al. 2005) and cynomolgus monkeys (Krebs et al. 2005; Uda et al. 2004, 2005). By microsatellite analysis, the MHC region in Mauritian cynomolgus macaques appears to have a general organization similar to that of rhesus macaques (Daza-Vamenta et al. 2004; Wiseman et al. 2007), but the number of MHC class I loci per haplotype remains unknown. Nothing is currently known about the organization of the MHC class I region in pig-tailed macaques nor the similarities or orthology of their loci with the rhesus and cynomolgus macaque loci. It is therefore important to investigate the orthology and paralogy of MHC class I loci between these three macaque species. Orthologues are genes, present in different species, which have been inherited from their common ancestor, whereas paralogs are homologous genes that derived from one another by gene duplication. The presence of an orthologue between pig-tailed macaques and rhesus or cynomolgus monkeys would indicate that other macaque species likely possess this orthologue. As orthologous genes may frequently exhibit similar functions, their identification may prove useful in assessing their role in immune responses of macaques to infectious agents such as SIV.

In this study, we have focused on *MHC-A* alleles of pig-tailed macaques and show that the gene expressing the *Mane-A**06 allele (Lafont et al. 2003) is in fact an orthologue of the locus encoding the *Mamu-A**05 allele family in rhesus macaques. An analysis of the distribution of this locus in a cohort of pig-tailed macaques demonstrated that it encodes an oligomorphic family of alleles,

highly prevalent within the pig-tailed macaque population. An orthologous gene was also detected in cynomolgus monkeys but not in two African monkey species. Despite their conservation and high prevalence among Asian macaque species, the *MHC-A* alleles present at this locus appear to be expressed at very low levels, suggesting that *MHC-A* alleles of the *Mane-A**06 family and, by extension, their orthologues in rhesus and cynomolgus monkeys, are very likely minor MHC class I alleles.

Materials and methods

Animals

Pig-tailed macaques (*M. nemestrina*; $n=63$) were obtained from New Iberia and Osage Research Centers. Most of the animals were born in the US from parents having Indonesian ancestors. A minority were feral animals imported directly from Indonesia. Rhesus macaques (*M. mulatta*; $n=80$) were all of Indian origin, whereas cynomolgus macaques (*M. fascicularis*) were imported from the Philippines ($n=5$), Mauritius ($n=2$), Vietnam ($n=1$), China ($n=3$) or were of unknown origin ($n=2$). The vervet African green monkeys (*Chlorocebus pygerythrus*; $n=2$) were imported from Tanzania and the Patas monkeys (*Erythrocebus patas*; $n=4$) were born in the US from Nigerian ancestors. All animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (Committee on Care and Use of Laboratory Animals 1985). The animals were housed in a biosafety level 2 facility; biosafety level 3 practices were followed. Animals were anesthetized with intramuscular injections of ketamine hydrochloride (Ketaject; Phoenix Pharmaceutical, St Joseph, MO, USA) and acepromazine acetate (Fermenta Animal Health, Kansas City, MO, USA) during phlebotomies.

Cell lines

Pig-tailed macaque B lymphocyte cell lines (B-LCL) were established by infecting peripheral blood mononuclear cells, isolated from whole blood using Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gradient centrifugation, with *Herpesvirus papio*, present in the supernatant of the 594S cell line (Rabin et al. 1977), kindly provided by Dr. Jeffrey I. Cohen (Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA). The stable B cell lines were maintained in RPMI 1640 medium (Cambrex Bio Science Walkersville, Walkersville, MD, USA) supplemented with 20% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin-G, and 100 U/ml streptomycin (R20 medium).

Genomic DNA preparation

Genomic DNA from pig-tailed macaques, rhesus macaques, cynomolgus macaques, African Green monkeys, and Patas monkeys was extracted from peripheral blood leukocytes using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, 5 ml of ethylenediamine tetraacetic acid (EDTA)-treated whole blood were centrifuged 10 min at 2,500 rpm. After removing the plasma, red blood cells were lysed and the leukocytes were recovered after centrifugation at 2,500 rpm for 10 min, lysed with nuclei lysis solution, and incubated with RNase A (20 μg/ml) for 1 h at 37°C degrees. Cellular proteins were precipitated with protein precipitation solution and the genomic DNA was precipitated in 5 ml isopropanol. After a wash in 70% ethanol, the genomic DNA pellet was air dried and solubilized in 300–400 μl of water by incubating for 1 h at 65°C.

*Mane-A**06/*Mamu-A**05 genotyping by SSP-PCR

Genotyping for the presence of *Mane-A**06/*Mamu-A**05 alleles was performed on genomic DNA. Briefly, a sequence specific amplification, site specific primer-PCR (SSP-PCR), was performed using Mane-A6S sense (CAAGGCCGACACACAGACTCT) and Mane-A6R reverse (CTCTCCACTGCTCCGCCA) primers. An internal control of amplification was obtained using Mane-DRB S4 sense (TAAGTCTGAGTGTCATTTCTTCAATGGGA) and Mane-DRB R2 reverse (CCTCGCCGCTGCACTGT) primers. The PCR mixture contained 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 0.2 mM each dATP, dTTP, dCTP, and dGTP, 20 pmol of each *Mane-A**06 specific primers, 25 pmol of each *Mane-DRB* specific primers, and 5 U of Ampli Taq DNA polymerase (Perkin Elmer, Wellesley, MA, USA) in a final volume of 50 μl. The reactions were heated at 94°C for 5 min, and amplification was conducted for 25 cycles as follows: denatured for 30 s at 94°C, annealed for 30 s at 65.5°C, and extended for 30 s at 72°C. A final extension was then conducted for 7 min at 72°C. Fifteen microliters of PCR reactions were loaded onto a 1.5% agarose Tris-acetate-EDTA gel and separated by electrophoresis. A 246 bp *Mane DRB* specific product was amplified in all animals, whereas an additional 679 bp product was generated with *Mane-A**06 positive animals. For sequence analysis, PCR was performed without the *Mane DRB* primers and using the SuperTaq plus DNA polymerase (Ambion, Austin, TX, USA). PCR amplicons were gel-purified using Qiaquick gel

extraction kit (Qiagen, Valencia, CA, USA) and were directly sequenced using an Applied Biosystems 3130XL genetic analyzer.

MHC cDNA cloning and sequencing

Cloning of *Mane-A**06 allele cDNA was performed by reverse transcription (RT)-PCR amplification in two steps. Briefly, total cellular RNA was extracted from 5×10^6 cells from individual pig-tailed macaque B cell lines using Tri reagent (Molecular Research Center, Cincinnati, OH, USA). To generate the complete 5′ extremity of *Mane-A**06 allele cDNA, a RNA ligase mediated rapid amplification of cDNA extremities (RLM-RACE) was performed using the First Choice RLM RACE kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The first PCR amplification was performed using the 5′ RACE outer primer from the kit and ManeA7ex (GCACTGTCACTGCTTGCAG), an *MHC-A* specific primer annealing to the junction between exons 6 and 7. The PCR reactions contained 50 mM KAc, 1.5 mM $MgSO_4$, 10 mM Tris-HCl, pH 9.0, 0.2 mM each dATP, dTTP, dCTP, and dGTP, 20 pmol of each primer, and 5 U of SuperTaq Plus DNA polymerase (Ambion). The reactions were heated at 94°C for 5 min, and amplification was conducted for 30 cycles as follows: denatured for 30 s at 94°C, annealed for 30 s at 63.5°C, and extended for 90 s at 72°C. A nested PCR was then performed using the 5′ RACE inner primer provided in the kit and the ManeA6R reverse primer. Almost full-length *Mane-A**06 allele cDNAs were generated using the 3′ RACE adapter from the First Choice RLM RACE kit (Ambion) as primer in a reverse transcription reaction following the manufacturer's instructions. PCR amplification was performed using Mane-A6S and the 3′ RACE outer primer from the kit. The PCR reactions contained 50 mM KAc, 1.5 mM $MgSO_4$, 10 mM Tris-HCl, pH 9.0, 0.2 mM each dATP, dTTP, dCTP, and dGTP, 20 pmol of each primer and 5 U of SuperTaq Plus DNA polymerase (Ambion). Each reaction contained 2 μl of cDNA in a final volume of 50 μl. A single PCR was sufficient to amplify enough products in 3′ RACE reactions. The reactions were heated at 94°C for 5 min, and amplification was conducted for 25 cycles as follows: denatured for 30 s at 94°C, annealed for 30 s at 63.5°C, and extended for 1 min and 30 s at 72°C. A final extension was conducted for 7 min at 72°C. PCR products were gel-purified using a Qiaquick gel extraction kit and then cloned into the pCR2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA, USA). Individual clones were sequenced using an Applied Biosystems 3130XL genetic analyzer. Following recommendations to avoid PCR-generated mutations for *HLA* alleles (Ennis et al. 1990; Marsh et al. 2002), individual alleles were defined by the presence of an identical sequence in a minimum of three independent clones.

*Mane-A**06 expression analysis

RT-PCR amplifications were performed on total RNA extracted from pig-tailed macaque B-LCL to analyze *Mane-A* allele expression. Complete *Mane-A* cDNAs were generated using the 3′ RACE adapter from the First Choice RLM RACE kit (Ambion) as primer in a reverse transcription reaction. The PCR amplification was performed using Mane5UA sense primer (GATTCTCCGCAGACGCCCA), an oligonucleotide annealing in the 5′UTR of Mane-A cDNA, and the 3′ RACE outer reverse primer. The PCR reactions contained 50 mM KAc, 1.5 mM $MgSO_4$, 10 mM Tris-HCl, pH 9.0, 0.2 mM each dATP, dTTP, dCTP, and dGTP, 20 pmol of each primer and 5 U of SuperTaq Plus DNA polymerase (Ambion). Each reaction contained 2 μl of cDNA in a final volume of 50 μl. The reactions were heated at 94°C for 5 min, and then amplification was conducted for 25 cycles as follows: denatured for 30 s at 94°C, annealed for 30 s at 63.5°C, and extended for 90 s at 72°C. A final extension was conducted for 7 min at 72°C. PCR products were gel-purified using Qiaquick gel extraction kit and then cloned into the pCR2.1 TOPO cloning vector (Invitrogen). Thirty clones were randomly chosen for miniprep, and clones containing an insert were identified by restriction analysis using *Eco*RI. *Mane-A* alleles of each clone were identified by sequencing and/or allele specific SSP-PCR.

Sequence analysis and phylogeny

Sequences were aligned using the Clustal W program of MacVector 8.1.2 software (Accelrys, San Diego, CA, USA) with minor manual adjustments. Phylogenetic trees were constructed based on the alignment using the neighbor-joining method of the same software (Saitou and Nei 1987). Genetic distances were estimated using Kimura's two-parameter method (Kimura 1980). Bootstrap analysis was performed (1,000 replicates) to assign confidence to tree nodes (Felsenstein 1985). Values higher than 70% are shown on the tree.

GenBank accession numbers of MHC sequences

The GenBank accession numbers of sequences used in Fig. 1 are the following: *Mane-E**0101 (AY204714), *Mane-A**01 (AY204715), *Mane-A**02 (AY204723), *Mane-A**03 (AY204724), *Mane-A**0302 (DQ097863), *Mane-A**04 (AY204725), *Mane-A**05 (AY204726), *Mane-A**06 (AY204727), *Mane-A**07 (AY204728), *Mane-A**08 (AY204729), *Mane-A**09 (AY204730), *Mane-A**10

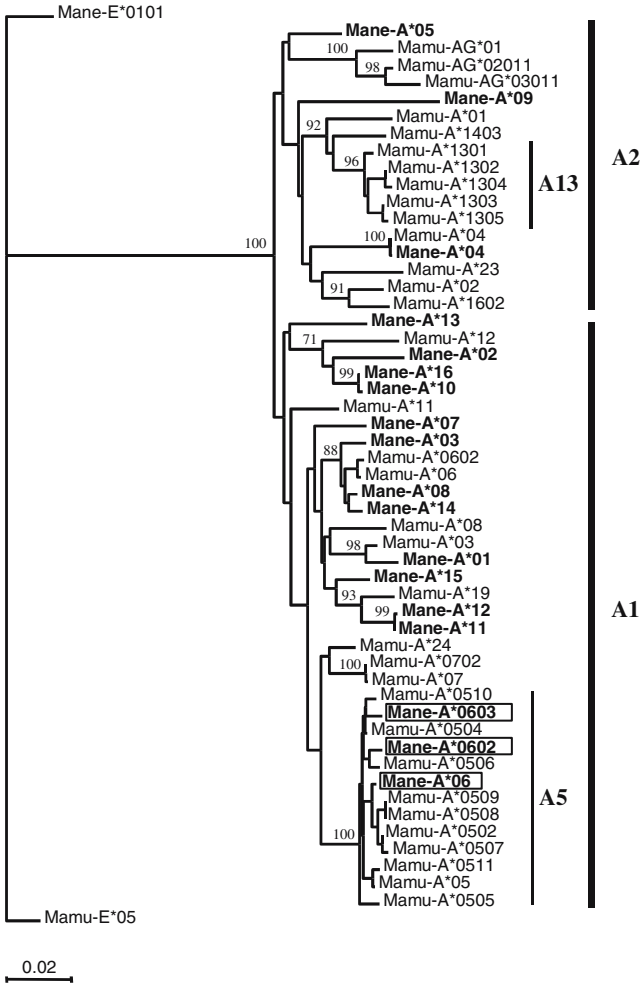

Fig. 1 Neighbor-joining tree of pig-tailed (*Mane*) and rhesus macaque (*Mamu*) MHC-A alleles. The relationship between *Mane-A* and *Mamu-A* alleles was calculated from a Kimura's two-parameter distance matrix using a common 943-bp sequence. Bootstrap analysis was performed (1,000 replicates) and values greater than 70 are indicated. The GenBank accession numbers of all sequences used in this analysis are indicated in "Materials and methods". Pig-tailed macaque MHC-A alleles are labeled in *bold* and three *Mane-A**06 alleles are *boxed*. The two major clusters of *MHC-A* alleles, designated A1 and A2, are indicated with *bold vertical lines*. Subclusters, grouping the *Mamu-A**13 (*A13*) and *Mamu-A**05 (*A5*) allele families, are indicated by *fine vertical lines*

(AY557348), *Mane-A**11 (AY557349), *Mane-A**12 (AY557350), *Mane-A**13 (AY557351), *Mane-A**14 (AY557352), *Mane-A**15 (AY557353), *Mane-A**16 (AY557354), *Mamu-E**05 (U41837), *Mamu-AG**01 (U84783), *Mamu-AG**02011 (U84784), *Mamu-AG**03011 (U84787), *Mamu-A**01 (U50836), *Mamu-A**02 (U50837), *Mamu-A**03 (U41379), *Mamu-A**04 (U41380), *Mamu-A**05 (U41831), *Mamu-A**0502 (AF157394), *Mamu-A**0504 (AF157396), *Mamu-A**0505 (AJ551315), *Mamu-A**0506 (AJ551316), *Mamu-A**0507 (AJ551317), *Mamu-A**0508 (AJ551318), *Mamu-A**0509 (AJ551318), *Mamu-A**0510 (AJ551319), *Mamu-A**0511 (AJ551320), *Mamu-A**06 (U41834), *Mamu-A**0602 (AJ542567), *Mamu-A**07 (U41832), *Mamu-A**0702 (AF157397), *Mamu-A**08 (AF243179), *Mamu-A**11 (AF199357), *Mamu-A**12 (AF157398), *Mamu-A**1301 (AF157399), *Mamu-A**1302 (AJ542569), *Mamu-A**1303 (AF157401), *Mamu-A**1304 (AY707076), *Mamu-A**1305 (AJ551321), *Mamu-A**1403 (AY707077), *Mamu-A**1602 (AJ542572), *Mamu-A**19 (AJ542573), *Mamu-A**23 (AJ542575), and *Mamu-A**24 (AJ542576).

The intron 2 sequences of *Mane-A* alleles described in Fig. 3 have been deposited in GenBank under accession numbers DQ916060–DQ916065. GenBank accession numbers for intron 2 sequence of *Mamu-A**01 and *Mamu-A**05 are AF161322 and AF161323, respectively. The sequence of the new *Mane-A* alleles described in Figs. 5 and 6 were deposited to GenBank under accession numbers EF010510–EF010521.

Results

*Mane-A**06 is phylogenetically related to *Mamu-A**05 alleles from rhesus macaques

To compare the *MHC-A* alleles of pig-tailed macaque (*Mane-A*) with those expressed by rhesus macaques (*Mamu-A*), phylogenetic analyses were performed using the sequences of 38 *Mamu-A* alleles and 16 *Mane-A* described previously (Boyson et al. 1996, 1997; Evans et al. 2000; Lafont et al. 2003, 2004; Miller et al. 1991; Smith et al. 2005; Urvater et al. 2000a; Voss and Letvin 1996; Watanabe et al. 1994). Three *Mamu-AG* alleles were also included because of their relationship with *Mamu-A* alleles (Boyson et al. 1997, 1999). One *MHC-E* sequence from each macaque species was used as a phylogenetic root. As shown in Fig. 1, *MHC-A* alleles did not segregate based on their species of origin but were distributed into two major clusters of alleles, designated A1 and A2. We have previously reported that this separation results from the presence of multiple polymorphic sites in exon 2 (Lafont et al. 2003). Each cluster contained *MHC-A* alleles from both species; additional sub-clusters were observed for alleles closely related to one another, in particular for alleles of the *Mamu-A**05 (A5) and *Mamu-A**13 (A13) families (Fig. 1). Among the 16 *Mane-A* alleles, two were closely related to particular *Mamu-A* alleles: *Mane-A**04 was associated with *Mamu-A**04, differing from one another by a single T to C transition at position 527 of the ORF; *Mane-A**06 was found in association with the group of *Mamu-A**05 alleles. This family of rhesus *MHC-A* alleles currently comprises 11 different members, and their sequence identity to *Mane-A**06 is very high, ranging from 98.5% (*Mamu-A**05 for 1,076 bp) to 100%

(*Mamu-A**0504 for 423 bp). By comparison, the sequence identity between *Mane-A**06 and the 15 other *Mane-A* alleles is more limited, varying from 91.7% (*Mane-A**09) to 96.3% (*Mane-A**08) over a common 943 bp fragment. Interestingly, among the five nucleotide positions found to differ between *Mane-A**06 and one or several *Mamu-A**05 alleles, four (80%) were also polymorphic within the *Mamu-A**05 family. This close identity of *Mane-A**06 with *Mamu-A**05 alleles suggested that the *MHC-A* locus expressing *Mane-A**06 in pig-tailed macaques might be orthologous to the locus expressing *Mamu-A**05 in rhesus macaques.

*Mane-A**06 possesses an insertion in intron 2 similar to *Mamu-A**05 alleles

The *MHC-A* locus encoding the *Mamu-A**05 allele family has been reported to possess an unusual 162 bp insertion at the 3′ extremity of intron 2 (Otting et al. 2005; Urvater et al. 2000b). To analyze the potential relationship between the loci encoding *Mane-A**06 in pig-tailed macaques and the *Mamu-A**05 allele family in rhesus macaques, the length of intron 2 was examined for several *Mane-A* alleles. Allele specific primers were designed within the polymorphic regions of exons 2 and 3 of *Mane-A**01, *Mane-A**03, *Mane-A**06, *Mane-A**07, *Mane-A**08 and *Mane-A**10 (Fig. 2a). Site-specific PCR amplifications were carried out for each allele on genomic DNA extracted from leukocytes of pig-tailed macaques, previously identified as carriers of these alleles (Lafont et al. 2003). Whereas the PCR for *Mane-A**01, *Mane-A**03, *Mane-A**07, *Mane-A**08, and *Mane-A**10 samples resulted in the amplification of a fragment ranging between 492 bp for *Mane-A**10 and 549 bp for *Mane-A**01, the *Mane-A**06 amplification generated a significantly larger 679 bp fragment (Fig. 2b). Sequence analysis was performed on each PCR product to verify the identity of the allele and to assess the length of intron 2 (Fig. 3). Intron 2 sequences of *Mane-A**01, *Mane-A**03, *Mane-A**08 and *Mane-A**10 alleles were all 243 bp long as previously described for *Mamu-A**01 and for *MHC-A* of human and several non-human primate species (Urvater et al. 2000b). The intron 2 of *Mane-A**07 contained a 5 bp deletion resulting in a length of only 238 bp. In contrast, intron 2 of *Mane-A**06 was 405 bp in length, possessing an additional 162 bp at its 3′ extremity, compared to all of the other intron 2 sequences of *Mane-A* alleles. The size of *Mane-A**06 intron 2 was, in fact, identical to the size of intron 2 present in *Mamu-A**05 and differed from that of *Mamu-A**05 at only 3 nucleotide positions. Taken together, these results indicate that the locus expressing *Mane-A**06 is an orthologue of the locus expressing *Mamu-A**05 alleles in rhesus macaques.

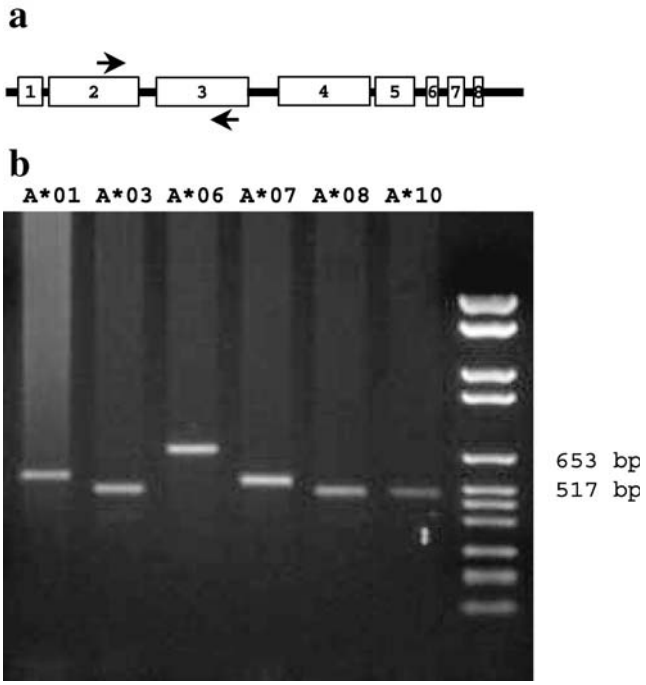

Fig. 2 The intron 2 of *Mane-A**06 possesses an unusual insertion. A schematic representation of the MHC-A gene organization is depicted in **a**. The sense and reverse primers used to amplify the intron 2 from *Mane-A**01, *Mane-A**03, *Mane-A**06, *Mane-A**07, *Mane-A**08, *Mane-A**10 are represented by *arrows*. In **b**, the amplified products were separated on 1.5% agarose gel. *Mane-A**06 specific products appear ~150 bp longer than amplicons obtained for the other alleles

The locus encoding *Mane-A**06 is highly prevalent in pig-tailed macaques and is oligomorphic

We next assessed the prevalence of *Mane-A**06 in a cohort of pig-tailed macaques. Site specific PCR amplifications for *Mane-A**06 were performed on pig-tailed macaque genomic DNA using the primers designed from sequences present in exons 2 and 3 for this allele. Co-amplification of a constant *Mane-DRB* sequence was used as an internal control of amplification as shown in Fig. 4. Genomic DNA samples from 63 pig-tailed macaques were tested for the presence of *Mane-A**06 (Table 1). Most of the animals (90%) were found to be positive. Sequence analysis performed on 28 (50%) of these positive samples revealed that the products were *Mane-A**06 related, but sequence variations were detected in both intron and exon sequences (Table 2 and data not shown). This result firmly establishes that this pig-tailed macaque locus encodes a family of *MHC-A* alleles related to *Mane-A**06. In a majority (69%) of the cases, the amplified product contained mixed base pairs at a few positions indicating that individual animals were carrying two different alleles (Table 1). Taken together, these results indicate that a majority of pig-tailed macaques possess a *MHC-A* locus encoding an oligomorphic family of alleles related to the *Mamu-A**05 allele

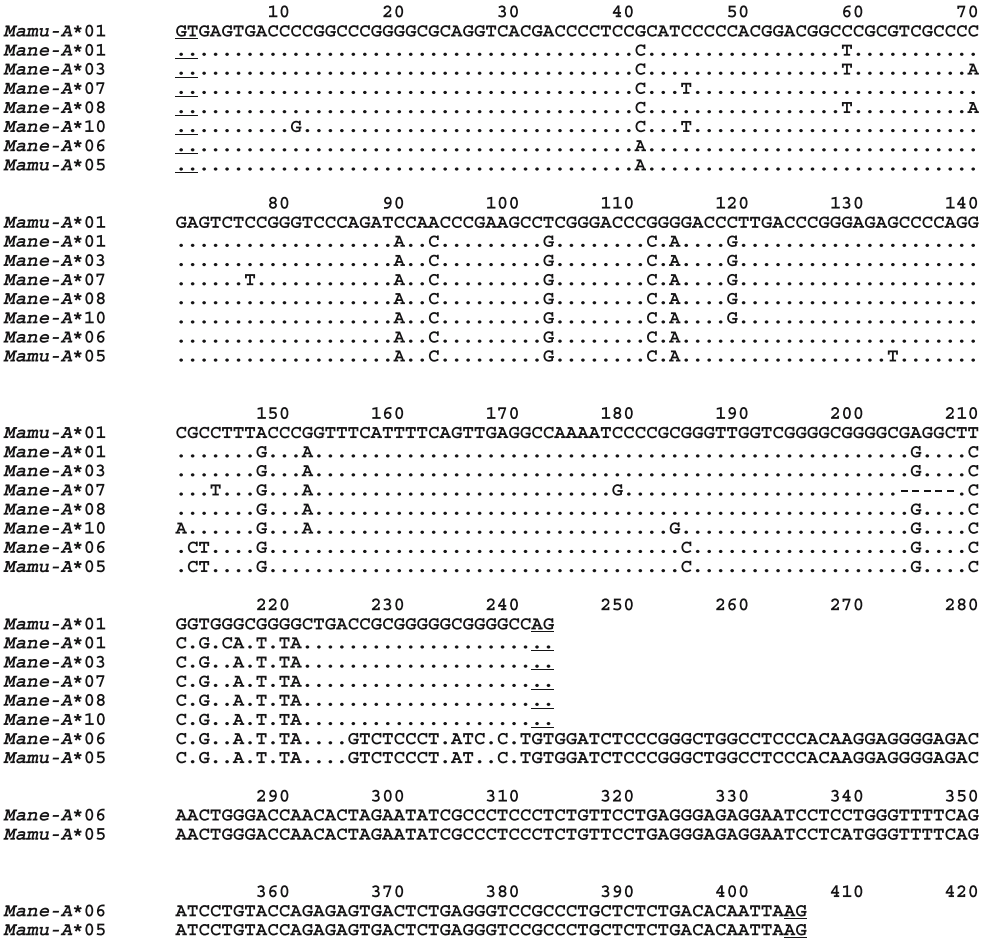

Fig. 3 Alignment of intron 2 sequence. The intron 2 sequences of six *Mane-A* alleles are aligned with that of *Mamu-A**05 and *Mamu-A**01. Compared to five *Mane-A* alleles, the intron 2 of *Mane-A**06 possess an alternative and expanded 3′ sequence, similar to *Mamu-A**05. The intron 2 sequences of *Mane-A**06 and *Mamu-A**05 differ by only three nucleotides at positions 133, 237, and 339. Donor and acceptor splice sites are *underlined*. The accession number of each sequence is indicated in "Materials and methods"

family from rhesus macaques. The failure to detect a *Mane-A**06 specific product in 10% of our cohort indicates that this *MHC-A* locus is not always present on the pig-tailed macaque chromosome. Therefore, several different haplotypes are present within the pig-tailed macaque population, some containing this *MHC-A* locus and others lacking it. This suggests that, similar to rhesus monkeys (Otting et al. 2005), pig-tailed macaques possess several MHC-A haplotypes, differing from one another by the number of *MHC-A* loci they carry.

The locus encoding *Mane-A**06/*Mamu-A**05 is highly prevalent in macaques but is absent from two African primate species

A similar analysis was performed on a cohort of 80 Indian rhesus macaques to assess the frequency of the *Mamu-A**05 family (Table 1). A majority (62%) of these animals tested positive using the *Mane-A**06 specific SSP-PCR assay. Sequence analysis performed on 13% of the positive samples confirmed that they were *Mamu-A**05 related and that a minority (23%) were derived from animals carrying two different *Mamu-A**05 alleles. To investigate further the presence of this particular *MHC-A* locus among non-human primate monkeys, we performed *Mane-A**06 SSP-PCR on genomic DNAs from cynomolgus macaques, African green monkeys (vervet), and Patas monkeys (Fig. 4). Thirteen cynomolgus macaques originating from Vietnam, China, the Philippines, Mauritius, or from unknown geographical regions were tested. A majority of these monkeys (85%) were positive, and only two animals, both of Chinese origin, tested negative by our *Mane-A**06 SSP-PCR. Sequence analysis performed on all positive samples confirmed that all were closely related to the *Mane-A**06 and *Mamu-A**05 orthologues (Table 1). Three cynomolgus macaques (27%) appeared to possess two different alleles at this locus. In two additional animals, one cynomolgus macaque originating from the Philippines and the other obtained from Mauritius, an identical 8 bp deletion was detected in exon 3 suggesting that the cynomolgus orthologue of the *Mane-A**06/*Mamu-A**05 locus might be non-functional in some animals. In contrast to these results, none of the two vervet African green monkeys or four Patas monkeys were positive by our *Mane-A**06 SSP-PCR. Based on this limited number of animals, the *MHC-A* locus encoding the *Mamu-A**05 and *Mane-A**06 family of alleles appears to be absent in two African non-human primate species and might only be specific to macaques. Another

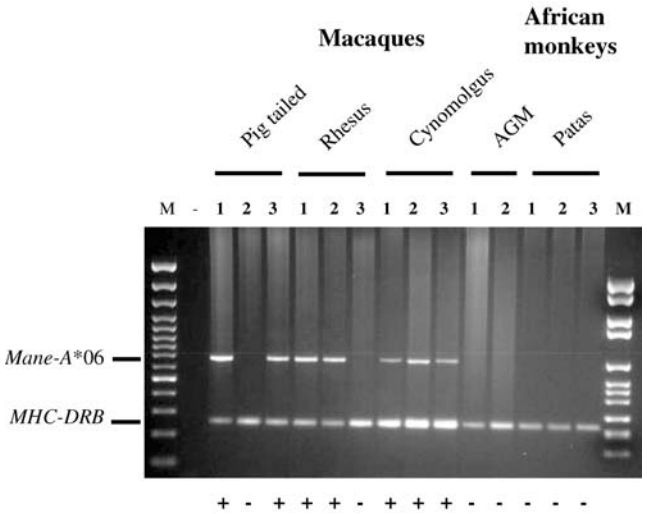

Fig. 4 The locus encoding the *Mane-A**06 allele family is present in the genome of three macaque species but not in two African non-human monkey species. *Mane-A**06 SSP-PCR amplifications are depicted for pig-tailed macaques (n=3), rhesus monkeys (n=3), cynomolgus macaques (n=3), African green monkeys (n=2), and Patas monkeys (n=3). A 246 bp MHC class II DRB specific product, used as internal control of amplification, was generated in all samples, except the negative control (H_2O; *lane labeled with a sign minus*), whereas an additional 679 bp product was amplified with *Mane-A**06 positive animals. The left DNA molecular weight marker is a 100 bp ladder having a bright band, a 500 bp (Generuler, Fermentas, Hanover, MD, USA)

possibility is that an orthologue of this locus is present in these African non-human primates on some but not all haplotypes and that the animals we tested were carrying haplotypes lacking this locus. Nonetheless, our results suggest that the *MHC-A* locus encoding the *Mamu-A**05 and *Mane-A**06 family of alleles was present in the common ancestor of the pig-tailed macaques, rhesus macaques, and cynomolgus monkeys at least 5.5 million year ago.

Table 1 Frequency of the *MHC-A* locus encoding the *Mamu-A**05/*Mane-A**06 allele families in several non-human primate species

Species	Number of animals	Positive (%)	Sequenced (%)	Heterozygous[a] (%)
Pig-tailed macaque	63	57 (90%)	29 (51%)	20 (69%)
Rhesus macaque	80	50 (62%)	13 (26%)	3 (23%)
Cynomolgus macaque	13	11 (85%)	11 (100%)	3 (27%)
African green monkey	2	0 (0%)	NA	NA
Patas monkey	4	0 (0%)	NA	NA

[a] Heterozygous animals were identified by the presence of mixed base pairs in the sequence of *Mane-A**06 SSP-PCR products.
NA Not applicable.

*Mane-A**06 alleles are oligomorphic in pig-tailed macaques

Sequence analysis revealed the presence of mixed base pairs in the *Mane-A**06 SSP-PCR products (Table 2). This observation suggested the presence of different *Mane-A**06 alleles in the pig-tailed macaque population. *Mane-A**06-related alleles were cloned from several individual pig-tailed macaques. Over the complete open reading frame (ORF), only 21 positions were found to be polymorphic, 9 (43%) of which were also polymorphic among *Mamu-A**05 alleles. The predicted protein sequence from *Mane-A**06 alleles identified four polymorphic sites in the leader peptide, two in the alpha 1 domain, four in the alpha 2 domain, two in the alpha 3 domain, two in the transmembrane domain, and two in the cytoplasmic tail (Fig. 5 and data not shown). One polymorphic site results in a leucine to isoleucine change at position 95, an amino acid known to be part of the F pocket of the peptide-binding site. This conservative mutation very likely has little effect on the peptide-binding characteristics of the Mane-A*06 allele family. Three additional polymorphic residues at positions 63, 167, and 171 of the mature MHC class I molecule affect amino acids that constitute the A pocket. This pocket interacts with the amino terminal residue of the peptide and could function as an anchor of the peptide as observed in the human HLA-A*26 or HLA-B*27 alleles (Griffin et al. 1997; Yamada et al. 1999). These polymorphisms could affect the peptide-binding properties and modulate the pool of peptides selected by the different Mane-A*06 alleles. However, the overall data suggest that Mane-A*06 and Mamu-A*05 alleles present overlapping peptide pools.

The *Mane-A**06 alleles are expressed at low level

Finally, the expression level of *Mane-A**06 alleles was analyzed by cloning full-length *Mane-A* transcripts from seven *Mane-A**06 positive pig-tailed macaques having different MHC genotypes. For each animal, 22 to 29 cDNA clones were isolated and the alleles they carried were identified (Fig. 6). One to four different *Mane-A* alleles were detected in each monkey. *Mane-A**01, *Mane-A**03, *Mane-A**10, or *Mane-A**18 transcripts were readily amplified from this panel of animals and comprised 48 to 100% of the transcripts detected per animal. RNA from other alleles, such as *Mane-A**0802 or *Mane-A**19, represented only 14 to 24% of the transcripts, a three- to fourfold lower level of transcription compared to the first group of *Mane-A* alleles. By comparison, *Mane-A**06 RNA transcripts were found at 10- or 20-fold lower frequency than the most abundant *MHC-A* allele transcripts. *Mane-A**06 transcripts represented a minor fraction (4 to 9%, 6 clones among 101 clones analyzed) of *Mane-A* transcripts in four of the cell

Table 2 Polymorphic sites in the intron 2 of the *MHC-A* locus encoding *Mane-A**06 and *Mamu-A**05 alleles

Position	Nucleotide	Variant	Pig-tailed (n=29)	Rhesus (n=13)	Cynomolgus (n=11)
45	C	T	Yes[a]	No	Yes
60	C	A	No	No	Yes
110–118	CCGAGACCC	Deletion	Yes	No	No
131	G	A	Yes	No	No
134	C	T	No	Yes	No
189	T	G	No	No	Yes
192	T	G	No	No	Yes
200	G	A	No	Yes	No
204–208	GGGGC	Deletion	No	No	Yes
205	G	A	No	No	Yes
237	C	G	Yes	Yes	Yes
283	C	A	Yes	No	Yes
302	A	G	No	Yes	No
303	T	G	No	No	Yes
317	T	G	Yes	No	Yes
339	C	A	No	Yes	Yes
341	G	C	Yes	Yes	Yes
353	C	G	Yes	No	No
364	G	C	Yes	Yes	Yes
387	G	C	Yes	No	No
389	T	A	Yes	No	No
390	C	G / A	Yes	No	No

[a] Yes indicates that the variant was detected in some animal from the macaque species.

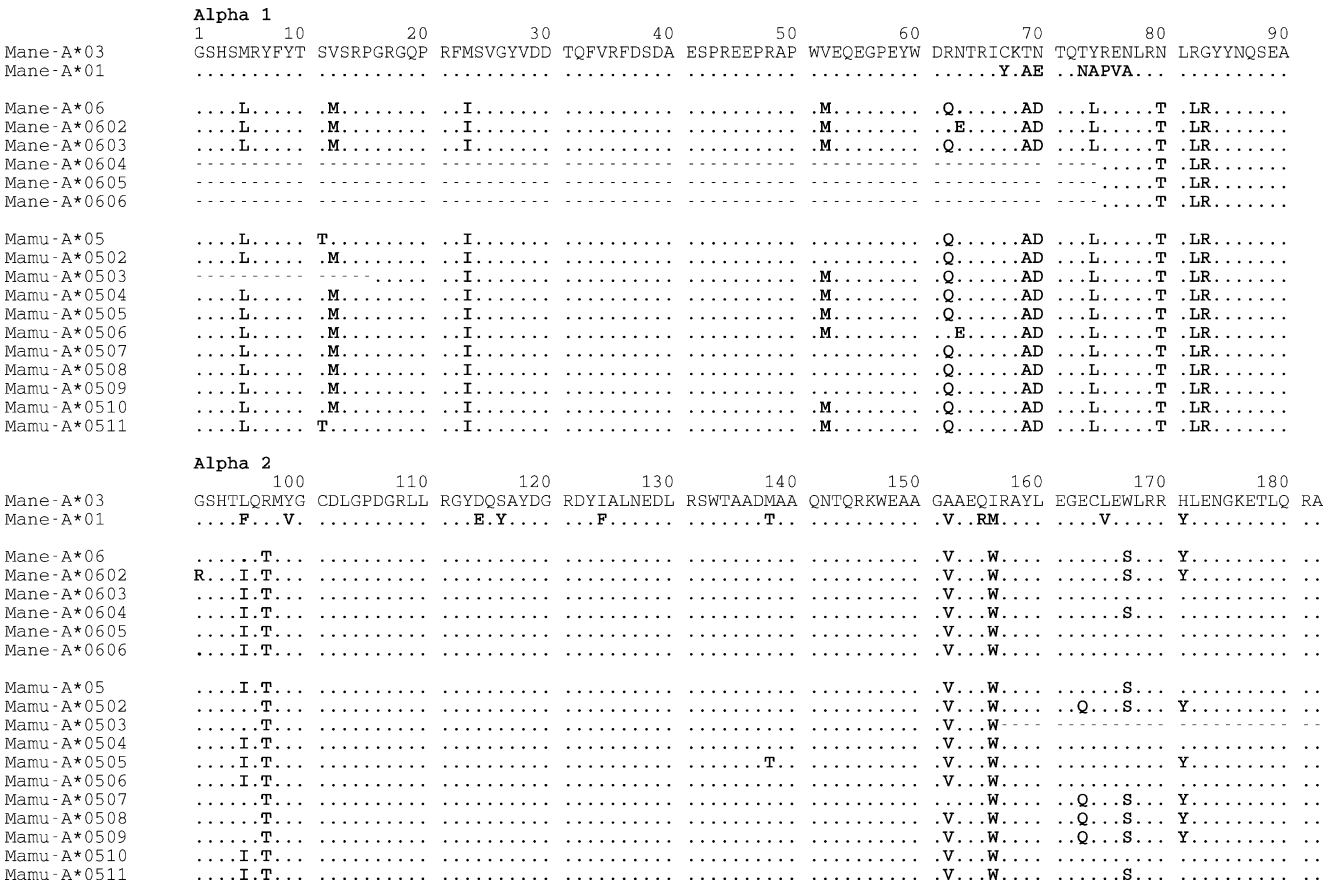

Fig. 5 Alignment of predicted protein sequences of Mane-A*06 alleles. The predicted alpha 1 and alpha 2 domain amino acid sequences of Mane-A*06 alleles are aligned with two unrelated Mane-A alleles (Mane-A*01 and Mane-A*03) and the 11 previously identified Mamu-A*05 alleles. Identity to Mane-A*03 sequence is indicated by *periods*. Gaps and stop codons are indicated by *dashes* and *asterisks*, respectively. Numbering follows the sequence of the mature Mane-A*03 protein

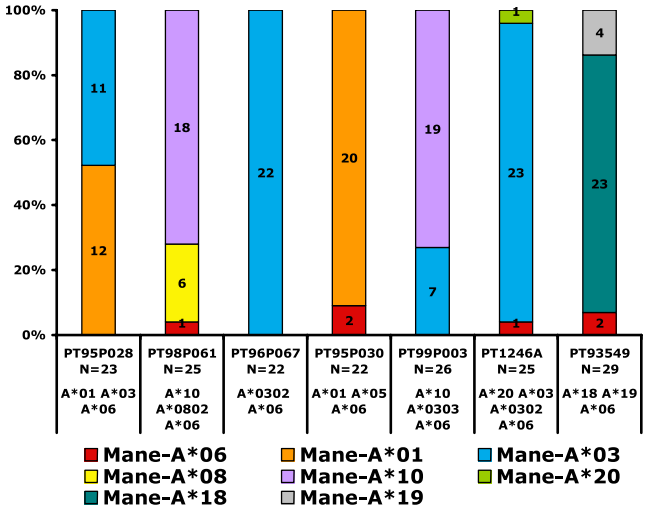

Fig. 6 *Mane-A**06 alleles are expressed at low levels in pig-tailed macaques. The allele distribution among *MHC-A* cDNAs obtained from seven *Mane-A**06 positive pig-tailed macaques is represented. The total number of clones analyzed is indicated *under* the animal identity number. For each animal, between 22 and 29 clones were analyzed and the number of clones for each allele is indicated in the *middle* of each column. Alleles *Mane-A**03, *Mane-A**0302, and *Mane-A**0303, differing by one and two nucleotides from one another, are all represented in *blue*. The different *Mane-A**06 alleles present in this panel of animals are represented in *red*

lines. In the three additional B-LCL, *Mane-A**06 full-length transcripts were not detected among 71 clones, although *Mane-A**06 transcript could be readily amplified by RT-PCR from the same B-LCL using *Mane-A**06 specific primers. Taken together, these results indicate that pig-tailed macaques differentially express their *Mane-A* alleles and that *Mane-A**06 alleles are among the least frequently transcribed.

Discussion

In this study, we have analyzed the presence of *Mane-A**06 in a cohort of 63 pig-tailed macaques. We found that *Mane-A**06 is a representative of a highly prevalent, oligomorphic *MHC-A* allele family in pig-tailed macaques that is orthologous to the *Mamu-A**05 allele family present in rhesus macaques. Cynomolgus macaques from four different geographical locations also possessed an orthologue of this gene. These observations suggest that the locus encoding these oligomorphic *MHC-A* alleles in pig-tailed, rhesus, and cynomolgus macaques was present in their common ancestor at least 5.5 million year ago (Morales and Melnick 1998; Tosi et al. 2000, 2003). Therefore, an orthologue of this locus is likely to be found in other Asian macaque species such as the Japanese macaque (*Macaca fuscata*) or the lion tailed macaque (*M. silenus*). We failed to detect a *Mane-A**06 orthologue in two African non-human primate species, namely, Patas monkeys and vervet African green monkeys. Furthermore, all *MHC-A* alleles described to date from two species of baboons (yellow and olive baboons) are phylogenetically related to the A1 group of *MHC-A* alleles, whereas the *Mane-A**06 and *Mamu-A**05 alleles cluster with the A2 group (Fig. 1; Lafont et al. 2003; Prilliman et al. 1996; Sidebottom et al. 2001). This suggests that baboons may also lack this specific locus. It is also possible that this locus is present on some MHC haplotype of African non-human primates but was not detected because our group of African monkeys was too small to reveal it. Further analyses on larger cohort of African non-human primates will be required to clarify this issue.

Haplotype analysis of rhesus macaques has previously shown that the *MHC-A* loci number can vary with two, three, or four *MHC-A* loci per haplotype (Otting et al. 2005). The locus encoding the oligomorphic *Mamu-A**05 allele family (locus 2) was described as not always being present on the rhesus chromosome (Otting et al. 2005). In this regard, the sequencing of the rhesus MHC region of two different haplotypes has revealed that one carries a *Mamu-A**05 allele (*Mamu-A**0504 in association with *Mamu-A**01; Kulski et al. 2004), whereas the other does not (*Mamu-A**04 and *Mamu-A**1403; Daza-Vamenta et al. 2004). We have found that a minority of animals in the three macaques species did not carry *Mane-A**06/*Mamu-A**05 alleles. It is therefore very likely that, similar to rhesus macaques, pig-tailed and cynomolgus macaques also possess different MHC haplotypes, with or without this specific *MHC-A* locus.

Our experiments have shown that an orthologue of this *MHC-A* locus was present in the genome of cynomolgus macaques. This result contrasts with three reports that used RT-PCR and reference strand conformational analysis (RSCA) to analyze the MHC class I alleles expressed by cynomolgus macaques originating from Vietnam, China, and Mauritius (Krebs et al. 2005; Wiseman et al. 2007) or on animals of unknown origin (Uda et al. 2004). Surprisingly, none of the 51 currently identified *Mafa-A* alleles in these studies are phylogenetically related to either *Mane-A**06 or *Mamu-A**05 allele families (data not shown). It is always possible that both studies were performed on animals lacking the locus encoding these allele families. However, this seems unlikely because we detected an orthologue of *Mane-A**06/*Mamu-A**05 in cynomolgus macaques originating from four different geographical locations including the three that were previously analyzed (Krebs et al. 2005; Wiseman et al. 2007). In particular, the locus was detected in Mauritian cynomolgus macaques, which exhibit limited genetic diversity of their mitochondrial DNA and MHC class I genes compared to cynomolgus macaques from other locations (Krebs et al. 2005;

Lawler et al. 1995). A more likely explanation is that *Mane-A**06 related alleles were not previously detected in cynomolgus macaques because their expression levels are quite low. In pig-tailed macaques, alleles of the *Mane-A**06 family are expressed at 10- to 20-fold lower levels than other *MHC-A* alleles. Sequence analysis of *Mane-A**06 SSP-PCR amplicons from 2 of 11 positive cynomolgus macaques also identified an 8 bp deletion within the coding sequence (exon 3), suggesting that this locus may have become a pseudogene, due to incapacitating mutations, in some cynomolgus monkeys. In rhesus macaques, the transcription levels of *Mamu-A**05 alleles are currently unknown. It should be stressed, however, that since their initial identification in 1995, *Mamu-A**05 alleles have not been associated with any particular epitopes, although these *MHC-A* alleles have been frequently detected (Boyson et al. 1996; Otting et al. 2005; Urvater et al. 2000a). For example in a study investigating Shigella-induced arthritis, 18 of 24 rhesus macaques (75%) infected with *Shigella flexneri* were positive for *Mamu-A**05 alleles (Urvater et al. 2000a).

Our observations may also be relevant in understanding the evolution of the MHC region in macaques. On the one hand, this locus is highly prevalent in the macaque population. Its high prevalence suggests that at some point in macaque evolution, this locus was under strong selective pressure to be retained in the monkey genome. On the other hand, our results show that either the current transcription level is low (in pig-tailed macaques and perhaps also in cynomolgus monkeys) or that the gene may be not functional (in cynomolgus macaques). These data suggest that the selective pressure to maintain this locus has diminished, or even disappeared, more recently, possibly reflecting its functional substitution by other MHC loci for presenting antigens or protection against contemporary pathogens. In this regard, it should be recalled that rhesus macaques possess up to 17 functional classical MHC class I genes on a single MHC haplotype (Daza-Vamenta et al. 2004; Kulski et al. 2004). This is a much higher number than that observed in most mammalian species. Humans, for instance, possess only three classical MHC class I genes per haplotype for protection against pathogens. The advantages and disadvantages of having a large numbers of MHC class I genes per individual has been discussed previously by others (Daza-Vamenta et al. 2004). Every additional MHC class I molecule possessing a different antigen presentation capability would result in a concomitant increase in the number of epitopes presented in an individual. As a consequence, additional T cell clones would be stimulated, giving the host the capacity to respond to a larger selection of antigens and pathogens. However, this increased capacity to present antigen would also apply to self-derived antigens and would result in the elimination, by negative selection, of a larger faction of the T cell repertoire due to its autoreactive properties. These opposing effects have probably contributed to the selection of a limited number of active MHC class I loci per individual, while conserving a large MHC diversity at the population level. In this respect, the large number of functional MHC class I loci present in rhesus macaques seems particularly surprising.

It seems quite possible that the regulation of MHC class I genes at the transcription level plays a significant role in countering the potential deleterious effect of having excessive numbers of functional MHC class I alleles. In this regard, our study shows that the transcription level of different *Mane-A* alleles is not identical in pig-tailed macaques. Some alleles appear to dominate the pool of MHC class I transcripts (*Mane-A**10 or *Mane-A**03), whereas others are less represented (*Mane-A**0802 or *Mane-A**19) or are even marginally expressed (alleles of the *Mane-A**06 family). Similar observations were previously reported for the *MHC-B* alleles of rhesus macaques (Daza-Vamenta et al. 2004; Otting et al. 2005). Studies in chickens have also shown that the two B loci present per haplotype are not equally expressed. One locus, B major, is transcribed at tenfold higher levels than the other, designated B minor, resulting in dominant protein production and peptide presentation by the former (Wallny et al. 2006). We currently have no information whether the differential pattern of transcription observed in our in vitro analysis occurs also in vivo in macaques and if MHC class I transcription is upregulated in a locus specific manner after immune stimulation. This could explain why the *Mane-A**06 allele family has been retained in the macaque genome, even though it appears to be marginally expressed in the in vitro assays described. Further analyses will be required to elucidate the role of *Mamu-A**05/*Mane-A**06 alleles in the adaptive immune responses to microbial infections.

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